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Effect of melatonin treatment on reproductive activity and immune response in Sarda sheep breed

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Abbreviation list

5-HT: serotonin hormone

5-HTP: 5-hydroxy-tryptophan

AAAD: aromatic acid decarboxylase

AANAT: arylalkylamine-N-acetyltransferase

AC: adenylate cyclase

ACTH: adenocorticotropic hormone

AR: adrenergic receptor

BCS: body condition score

cAMP: cyclic adenosine monophosphate

CAT: catalase

cGMP: cyclic guanosine monophosphate

CRE: cAMP- response element

CREB: cAMP responsive element binding proteins

CREM: cAMP responsive element modulator

DAG: diacylglycerol

DIML: distance in days from male introduction to lambing

E: estradiol hormone

GLM: general linear model

GnRH: gonadotropin releasing hormone

GPCR: G-protein-coupled-receptor

GPx: glutathione peroxidase

GSH: glutathione

HIOMT = **ASMT**: hydroxindole-O-methyltransferase

ICER: inducible cAMP early repressor

Ig: immunoglobulin

IL: interleukin

IP_3: inositol triphosphate

LH: luteinizing hormone

MBH: mediobasal hypothalamus

MEL: melatonin hormone

Mel1b = **MT2**: melatonin receptor type 2

ML1: high affinity melatonin receptor

ML2: low affinity melatonin receptor

MT1: melatonin receptor type 1

MTNR1A: MT1 gene receptor

NAS: N-acetyl serotonin

NE: noradrenaline

NK: natural killer cells

PAPC: pituitary adenylate cyclase

PCR: polymerase chain reaction

p-CREB = **p-CREB-ser^{133}**: activated CREB

PKC: protein kinase C

PT: *pars tuberalis* of adenohypophysis

QR2: quinone reductase

SCC: somatic cells count

SNC: suprachiasmatic nucleus

SNPs: single nucleotide polymorphisms

SOD: superoxide dismutase

STAR: steroid regulatory protein

TH: tyrosine hydroxylase

TM: transmembrane α-helices (domains) of MT1 receptor

TPH: tryptophan-hydroxylase

TRP: tryptophan

VIP: vasoactive intestinal peptide

Introduction

Pineal gland or epiphysis

The pineal gland, or epiphysis, is a neuro-endocrine gland that in all vertebrate originates from an evagination of the ceiling of the ventricle III, in its third caudal, in the posterior margin of the *corpus callosum* (Figure 1). This gland is unequal and lies on a median lumen, has a conical shape that resembles a pine cone (from which derives the Latin name pinealis), its size and shape are different in the various species.

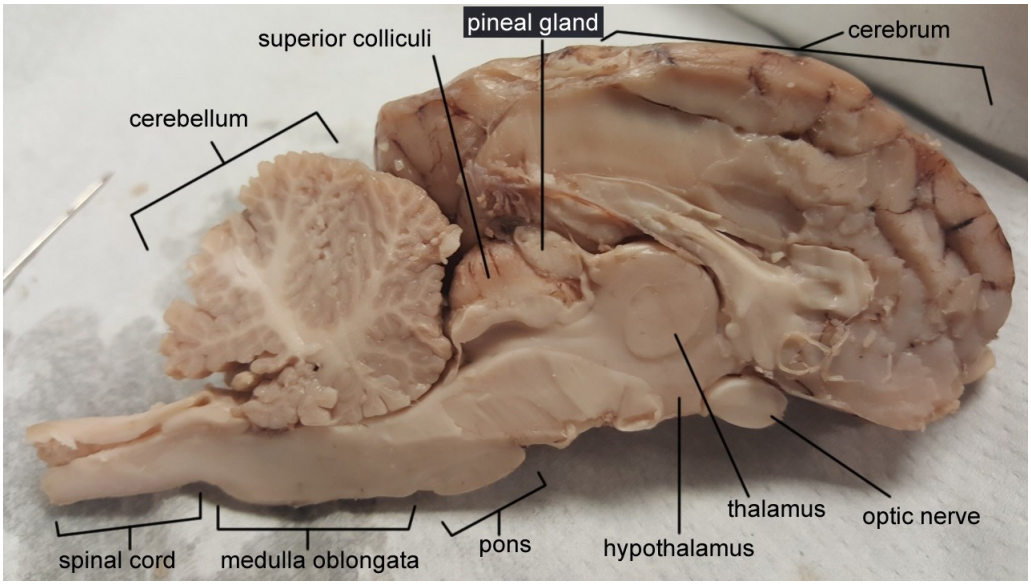

Figure 1. *Sheep brain dissection and location of the pineal gland (Human Anatomy and Physiology Lab, coureses.lumenlearning.com)*

The evolutionary history of the pineal gland is very long, it has undergone considerable variations during the evolution from

amphibians to mammals (Mano and Fukada, 2017).

In several animals such as amphibians and some fish, the pineal gland is called the "third eye" because same cells of it have photoreceptor characteristics, with particular photosensitivity and electrical activity. In simple vertebrates, such as lampreys, the gland is located on a peduncle near an orifice of the skull and has both endocrine and nervous properties; in these animals, since the epiphysis is tightly against the skin, it does not require interactions with eyes to record

day / night rhythm and is the main biological clock (Oksche and Vaupel–von Harnack, 1965).

In birds and reptiles the epiphysis, in addition to the photoreceptor function also performs the secretory function. In birds the pineal gland is located near the cranial vault, which is very thin and transparent to light, allowing its direct excitation. By coloring the skull of migratory sparrows with black ink, it was possible to highlight a close correlation with the migratory activity, in fact the black coloring of the skull resulted in the total loss of sense of orientation, as if the pineal functioned like a sort of internal compass (Gwinner, 1996).

The pineal gland is completely absent in crocodiles and mammals belonging to the order of toothless (anteaters, sloths and armadillos), while it is present in whales and elephants even if formed only by a few cells, in the human it has size of a pea.

In general, in mammals this gland is not directly photosensitive, although a nervous connection exists with the eyes which suggests a possible influence of light on its functions (Goldman, 1991). In these animals the structure of the epiphysis is a rather homogenous tissue containing pinealocytes (mono-, bi-, or tri-polarcells), few glial cells, phagocytic cells and neurons (Simonneaux and Ribelayga 2003). The pinealocytes, deriving from the neuroepithelial matrix layer, have lost the characteristic structure of the pineal photoreceptor cells of most of the lower vertebrates and have exclusively secretory function.

Being located outside the blood-brain barrier, it can come in contact with large blood plasma molecules, thanks also to the considerable size of the interstitial spaces. It is formed by a parenchyma which is constituted of cell cords separated by capillaries with fairly large perivascular spaces (Møller e Baeres, 2002). The pineal gland is the second most perfused organ of the organism, after the kidney, the blood supply is guaranteed through a dense network of vessels. The epiphyseal capillaries originate from the posterior choroid arteries branching out and first envelop the capsule of connective tissue, then penetrate in the parenchyma of the gland. Blood drainage of the gland is guaranteed by the dorsal passage of the large cerebral vein that allows a very rapid distribution of its secretion products to the target organs.

Transmission of the light signal is important to explain function of the

epiphysis. The light signal perceived by the photosensitive retinal cells is transformed into a nerve impulse, passing into the suprachiasmatic nucleus (SCN) and through the posterior hypothalamus and the sympathetic hypothalamic-spinal pathways, reaching the superior cervical ganglion. From the latter it branches off to post-ganglionic noradrenergic fibers that reach the epiphysis and regulate its secretory activity (Figure 2).

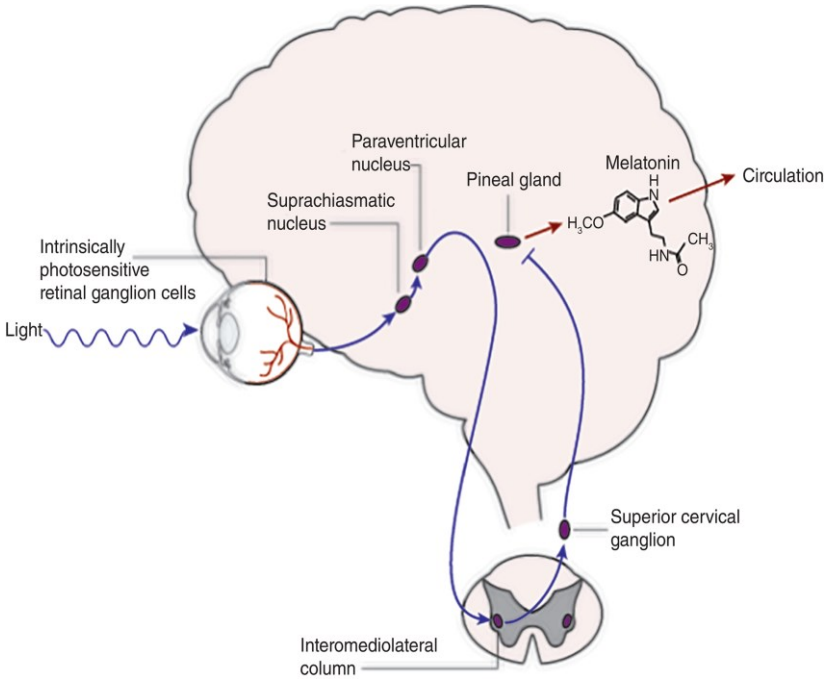

Figure 2. *Luminous signal pathway from eye to pineal gland (Ostrin, 2019)*

To enter epiphyseal cells, the nerve stimulus is converted into a chemical stimulus, through the linkage with specific receptors positioned on the pinealocytes membrane.

The epiphysis therefore acts as a photo-sensitive neuro-endocrine

transducer and thus translates external light stimuli into internal hormonal messages, so that the body can synchronize its functions as environmental conditions change (Simonneaux and Ribelayga 2003).

With the progress of experimentation and knowledge it has been possible to highlight how the innervation of the pineal gland is much more complex than what was initially thought. The pineal gland is innervated by nerve fibers of various origin. The main pathway consists of the retino-hypothalamus-pineal pathway, which ends with sympathetic input to the pineal parenchyma. The pineal gland also receives neural signals of central and parasympathetic origin (Figure 3) (Simonneaux and Ribelayga 2003).

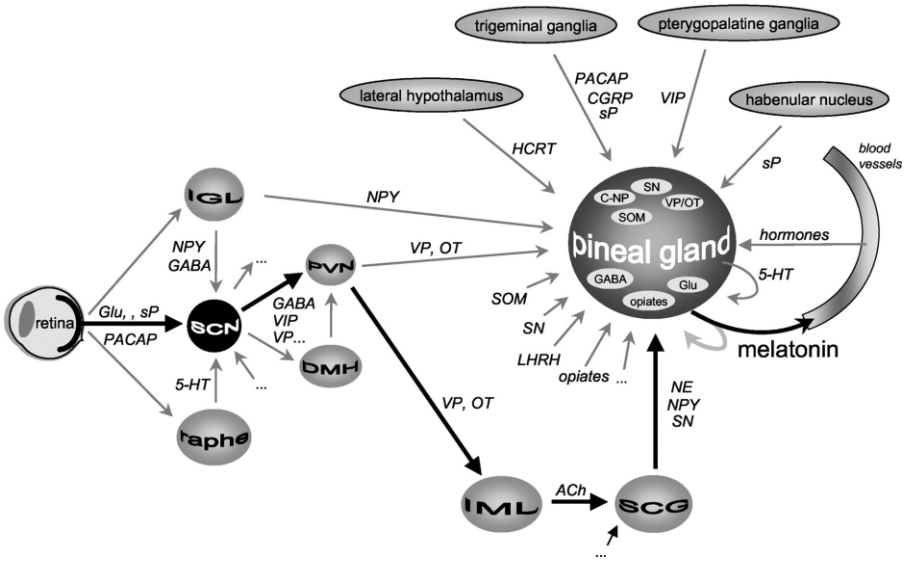

Figure 3. *Schematic presentation of the various neural, endocrine, paracrine inputs of the mammalian pineal gland (Simonneaux and Ribelayga 2003)*

The luminous signal perceived by the retina, results in signal perception of cells that contain the pigment named melanopsin, which allows the transmission of the aforementioned signal parallel to the visual stimuli perceived by the cones and rods (Hattar et al., 2002). Neuromediators of the retinal-hypothalamus tract are mostly glutamate and PACP (pituitary adenylate cyclase activating peptide) (Hannibal et al, 1997). In addition to the light signals other nerve signals from the thalamus and the raphe reach the suprachiasmatic nucleus (SCN). In many mammals, the nucleus acts as "biological clock" that presents an oscillatory activity which is synchronized circadianly by melatonin

and / or directly by the light signal (Lincoln et al. 2005). This pacemaker has an endogenous rhythm which is controlled by a series

of "clock" genes, the Per1, Per2 and Per3 called periodic genes, the Cry1 and Cry2 cryptochromic genes and the two, Clock and Bmal1 that encode some transcription factors. These genes act by activating or inhibiting the activity of the SCN (Reppert and Weaner, 2002). The function of these genes seems to be related to periodic processes of the organism, interacting with the signal coming from the epiphysis (Simonneaux et al., 2004).

The Paraventricular nuclei of the hypothalamus act as a link between the SCN and the epiphysis, in fact experimentaly induced lesions at this level have highlighted an important decrease in the melatonin secretion (Kalsbeek and Buijs, 2002). Finally, all the nerves, passing through the superior cervical ganglion innervate the epiphysis. The main innervation is of the noradrenergic type, but other fibers with different origins are also present. However, they all appear to play a role in regulating melatonin secretion (Simonneaux and Ribelayga, 2003).

Melatonin

The discovery of melatonin (MEL) dates back to 1958 and in the following year its chemical structure was revealed as N-acetyl-5-methoxytripamine (Lerner et al., 1958; 1959).

MEL is an endogenous synchronizer within circadian system, indeed

its most known activity is a hormonal transducer of photic/photoperiodic information. The pineal gland has two rhythms of secretion: a 24-h rhythm with a nocturnal peak and an annual rhythm closely dependent on seasonal variations in the photoperiod (Simonneaux and Ribelayga, 2003).

MEL is a molecule vastly present in nature, in fact, besides being produced by many invertebrates and vertebrates, it has also been found in bacteria, in unicellular eukaryotic organisms, in macro algae and in many parts of plants (Hardeland et al., 1996; 2003; 2015). In superior vertebrates its synthesis occurs to a greater or lesser extent in various parts of the body, such as in the retina, but especially in the gastrointestinal tract; as already mentioned above, the main producer is the pineal gland (Slominski et al., 2018).

Biochemically MEL is a product of amino acid tryptophan metabolism (Figure 4). Tryptophan (TRP) is an essential amino acid that once introduced with diet passes into the bloodstream and from there it is collected and transported in the pinealocytes with an active transport. Once inside, the tryptophan is converted in 5-hidroxy-TRP (5-HTP) in the pineal mitocondria by Trp-hydroxylase (TPH) (Lovenberg et al., 1967), 5-HTP is then converted into serotonin (5-HT) by the cytosolic enzyme aromatic amino acid decarboxylase (AAAD) (Lovenberg et al., 1962; Snyder and Axelrod, 1964). The gene that codes for Trp-hydroxylase has a higher nocturnal activity than in the day (Besançon

et al., 1996), unlike the AAAD activity which shows no difference in the 24 hours (King and Steinlechner, 1985). The nightly increase in TPH activity derives mainly from post-transcriptional/post-translational mechanisms (Sitaram and Lees, 1978, 1984; Ehret et al., 1991; Sun et al., 2002), this has been proven as it is more sensitive to the action of a protein synthesis inhibitor (cycloxy-oxide) than that of a transcription inhibitor (actinomycin D). AAAD is a non-specific enzyme of the pineal gland, in fact it is present in many tissues and is essential in the production of catecholamines.

The arylalkylamine-N-acetyltransferase (AANAT) considers the rate-limiting enzyme for the synthesis of MEL. It is an enzyme that catalyze the N-acetylation of 5-HT (producing N-acetyl serotonin, NAS), his gene is located on chromosome 11 in the mouse (Yoshimura et al.,1997) and present few interspecies differences in the gene sequence (Klein et al., 1997). His activity shows important diurnal/nocturnal variations, especially in the rat (Klein and Weller, 1970) and changes in its activity or structure cause changes in MEL production. In the pineal gland of the rat mRNA expression, proteins and AANAT activity are almost imperceptible during the day, while at night there is an evident increment (Borjigin et al., 1995; Klein et al., 1996; Roseboom et al., 1996; Gastel et al., 1998; Garidou et al., 2001). As for sheep, the higher nocturnal activity of AANAT with respect to the day, reflects the level of the AANAT protein in the pineal glands,

which in turn is partially controlled by the expression of the AANAT gene. This is demonstrated by a small but significant nocturnal increase of AANAT mRNA, this is proof that pre and post-translational mechanisms regulate the daily rhythm of AANAT activity (Coon et al., 1995). The main production of AANAT occurs in the pineal gland and in the retina, but this enzyme is also produced in the ovaries and testis, at very low levels. Melatonin can directly affect ovarian function. In humans, the concentration of melatonin in the preovulatory follicular fluid is significantly higher than in the peripheral serum (Brzezinski et al., 1987; Rönnberg et al., 1990; Nakamura et al., 2003).

Hydroxindole-O-Methyltransferase (HIOMT and now renamed ASMT) catalyzes the last step of the MEL synthesis process by transferring a methyl group from the S-adenosyl-methionine cofactor to the indole substrate (Baldessarini and Kopin, 1966). This enzyme also catalyzes the synthesis of other pineal 5-methoxyindoles (Figure 4) (Axelrod and Weissbach, 1961). While the enzymatic activity shows the nocturnal increase, the HIOMT gene expression is already high during the day but shows a 2-fold increase during the night (Gauer and Craft 1996; Ribelayga et al., 1999b). In vitro studies show that the nocturnal increase in pineal HIOMT activity results from noradrenergic-independent post-transcriptional mechanisms and not from nocturnal stimulation of HIOMT gene expression (Ribelayga et

al., 1997, 1999b). It is possible that the activity of HIOMT is a possible regulator of the amplitude of the night peak of melatonin (Simonneaux and Ribelayga, 2003). The nocturnal increase in AANAT and HIOMT is much more evident in rats than in sheep (Foulkes et al., 1997).

In sheep, like other species of mammals such as human (Arendt 1995), the nocturnal plasma MEL concentration has an important variability among individuals and it is clear, that this variability is under strong genetic control (Zarazaga et al., 1998).

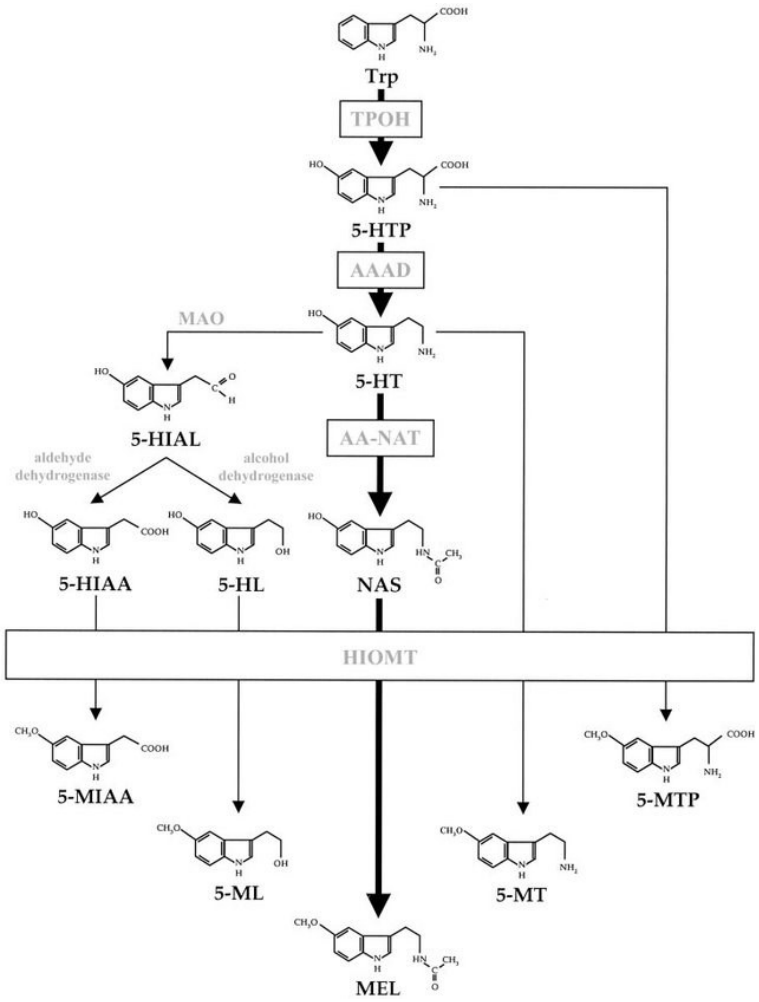

Figure 4. *Metabolism of indoleamines in the mammalian pineal gland, MEL is being the most important (Simonneaux and Ribelayga 2003)*

MEL is a particular hormone because, immediately after its production, it is released into the bloodstream by passive diffusion and into cerebrospinal fluid (Reiter, 1991). Therefore, once produced it is not stored like serotonin. This is possible thanks to the dense vascular system of the gland and the lipophilic structure of MEL, which allow melatonin to pass quickly through tissues, membranes and the blood-

brain barrier. The half-life of MEL is about 20 minutes (Gibbs and Vriend, 1981), and the catabolism occurs in the liver where it is converted into 6-hydroxymelatonin by cytochrome P450 (Skene et al., 2001) and then in the kidney, where it is converted in 6-sulphatoxymelatonin and excretes through the urine. Due to its short half-life, the concentration of circulating MEL exactly reflects the pineal synthesis, showing that the main producer is the pineal gland (Pèvet at al., 2017).

Noradrenergic regulation of MEL secretion

In rats, the main neurotransmitter implicated in the mechanism of MEL secretion is noradrenaline (NE). Numerous subtypes of adrenergic receptors (AR) are expressed in the pineal gland: β_1 (β1-AR) and α_1 (α1-AR) in the post-synaptic pineal membrane and in the pinealocyte membrane, α_2 (α2-AR) in the presynaptic terminals and in the membranes of the penealocytes. These ARs have been found and studied in rats (Roseboom et al., 1996), hamsters and sheep (Coon et al., 1995).

Both, density and mRNA expression of β-AR displays a circadian variation with highest levels at night time (Drijfhout et al., 1996).

The activation of β_1-AR alone causes an increase in cyclic adenosine monophosphate (cAMP) of about 10-fold, while the activation of α_1-

AR and β1-AR at the same time brings the maximum increase of the cAMP (Vanecek et al., 1985). Instead the activation of only α_1-AR is not able to increase cAMP levels. As for regualtion of cAMP, also the intracellular level of cyclic guanosine monophosphate (cGMP) reaches its maximum concentration when α-AR and β-AR are activated simultaneously (Chik and Ho, 1989).

The α-AR activation leads to an increase in the Ca^{2+} intracellular concentration and in the turnover of inositol triphosphate (IP_3), consequently it releases diacylglycerol (DAG) as the second messenger. The nocturnal increase of Ca^{2+} and much less DAG causes the activation of protein kinase C (PKC) which improves adenylate cyclase (AC) activity and consequently the synthesis of cAMP and MEL (Figure 5) (Ho et al., 1988b). It is known that the activation of PKC alone, without the activation of AC and β-AR, is not able to increase the intracellular levels of cAMP; this increase is essential to activate AANAT and consequently the MEL synthesis (Maronde et al., 1999b).

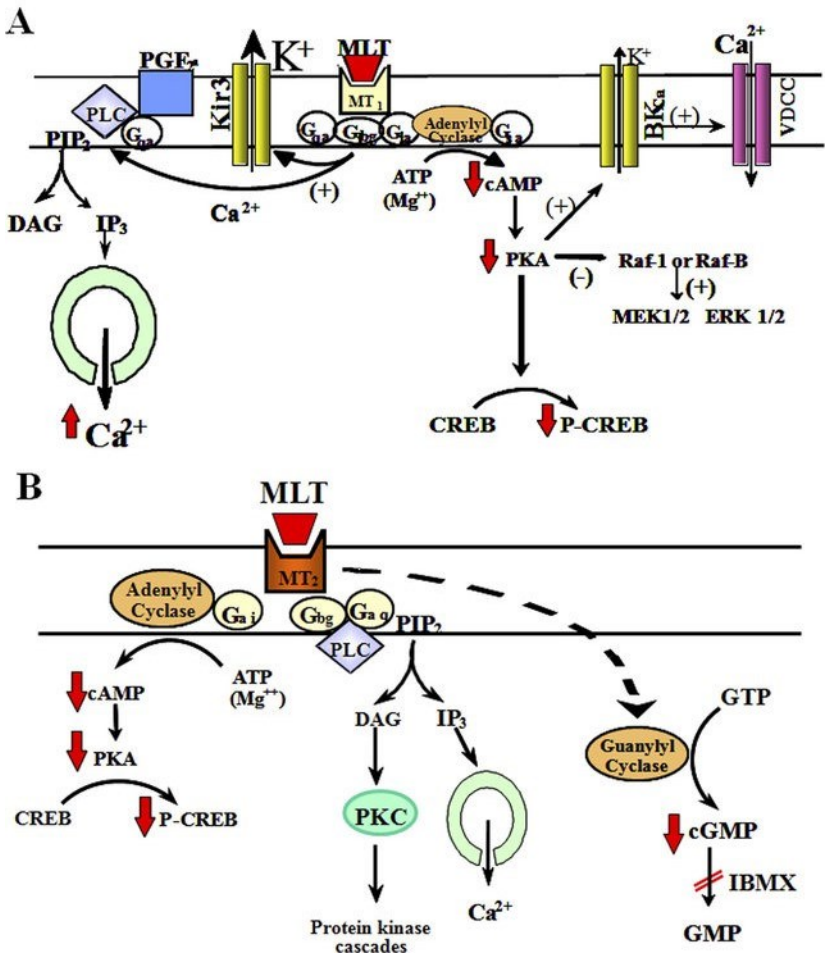

Figure 5.

MT1 and MT2 melatonin receptor signaling. A) MEL signals through activation of th e MT1 receptor via two parallel pathways; B) signaling pathways coupled to MT 2 melatonin receptor activation (Dubocovich et al., 2010)

AANAT regulation depend on a transcriptional and post-transcriptional control and generally the transcriptional phase has a circadian rhythm, this trend is much more evident in the rat than in other mammals (Saha et al., 2019).

In rats the transcriptional process is linked to the phosphorylation of CREB (cAMP Response Element Binding Proteins) on Ser^{133} and its activation, operated by PKA. Activated form p-CREB- Ser^{133} (pCREB) is a key element in the regulation of pineal gene expression, indeed pCREB improves the expression of genes that code for the enzymes responsible for MEL synthesis, which have putative cAMP-response element (CRE) sites in their promoter region such as AANAT (Baler et al., 1997; Burke et al., 1999) and HIOMT (Rodriguez et al., 1994). The cell transcription factor CREB acts in synergy or competes on CRE sites with cAMP responsive element modulator proteins (CREM). These proteins, in the pineal gland, are characterized by the fact of being strongly expressed in the form of a small transcription and, unlike the other members of this family, cMPA is able to induce its expression. The cAMP-induced transcription is strongly contrasted by inducible early repressor cAMP (ICER), (Stehle et al., 1993, 2001). ICER is a group of 4 proteins encoded by the CREM / ICER gene thanks to the presence of an internal promoter, P2, located in an intron of the CREM gene (Figure 6) (Mioduszewska et al., 2003).

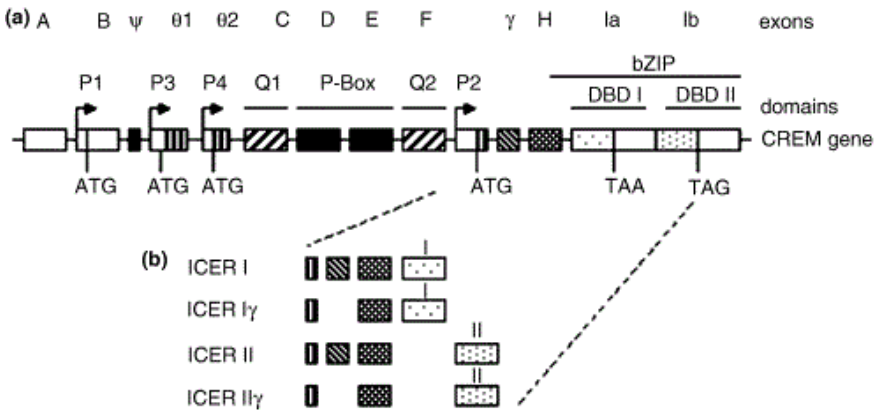

Figure 6. *Schematic representation of CREM / ICER gene (a) and isoform of ICER proteins (b). a): A–F, γ, H, Ia, Ib, exons; P1, P3, P4, CREM promoters; P2, ICER promoter; TAA and TAG, indication of stop codons; DBD I and II, DNA binding domains I and II; bZIP, basic leucine zipper modified. (Mioduszewska et al., 2003)*

Activation of the P2 promoter is due to activation of the transcription factor CREB (Molina et al., 1993). The ICER production is regulated by auto-negative feedback, in fact after 2-6 hours from the activation of CREB, ICER reaches a level sufficient to compete with CREB, consequently blocking its own transcription (Mioduszewska et al., 2003).

ICER acts as a strong repressor by inducing a down-regulation of the promoters of several genes that have in common a CRE sequence and their expression is induced by the intracellular increase of cAMP. The activity of the ICER consists therefore in antagonizing the pCREB that accumulates during the hours of darkness and decreases with the

increase of the ICER (which has a peak around midnight).

Therefore, the production of the key enzyme of MEL secretion, AANAT, may depend on the relationship between CREB / ICER (Stehle et al., 1993; Pfeffer et al., 1999).

Many studies have shown that nocturnal MEL secretion is managed by an increase in AANAT activity resulting from the accumulation of the same AANAT protein. However, there are interspecific differences on the accumulation and mechanism of action of AANAT. On this basis we can divide mammals into two groups: rodent species ("rat type"), in which increases AANAT gene expression and the synthesis of new AANAT molecules, and non-rodent species ("sheep type"), in which the AANAT mRNA shows high levels of day and night variation and the accumulation of AANAT proteins results from stabilization of the constantly translated protein (Figure 7). Many studies have shown that nocturnal MEL secretion is due to an increase in AANAT activity resulting from the accumulation of the same AANAT protein (Simonneaux and Ribelayga 2003).

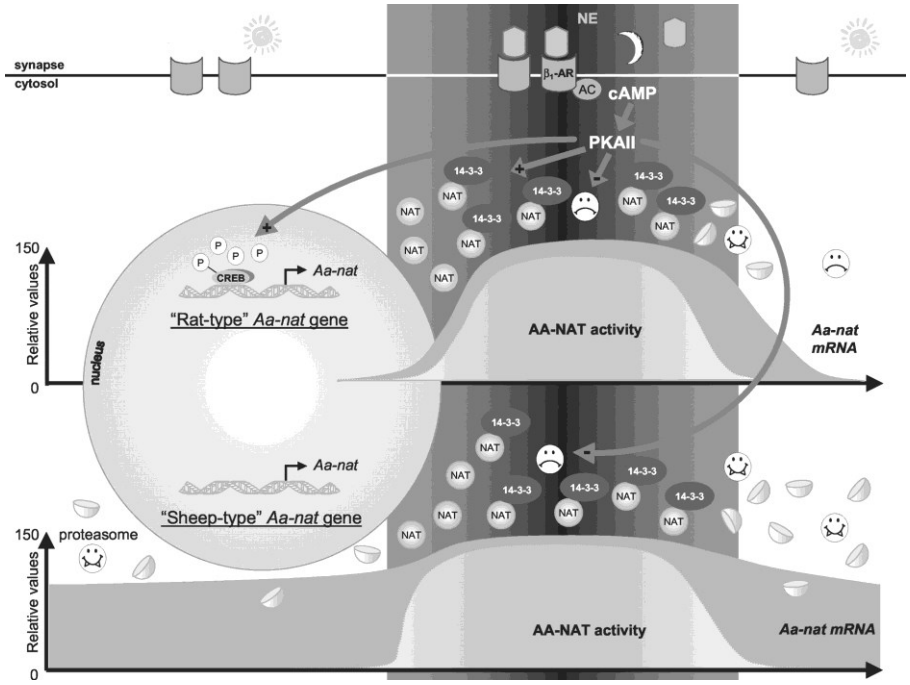

Figure 7. *Different mechanism of accumulation and action of AANAT in rodent and non-rodent species (Simonneaux and Ribelayga 2003)*

In non-rodent species, the production of AANAT is regulated only at the post-transcriptional level (Simonneaux and Ribelayga 2003). The increase in cyclic AMP levels (NE-induced), is essential to inhibit the breakdown of constantly synthesized AANAT proteins by proteasomal proteolysis, leading to an elevated enzyme activity. It is clear that the different mechanisms of MEL synthesis and secretion leads to a different speed in its release from the beginning of the dark, with a long delay (several hours) in rodent species and a very short delay (few minutes) in the non-rodent species (Stehle et al., 2001).

MEL and seasonality (circannual / cyrcadian rhythms)

The seasonality of the animals was born as an adaptation to climatic conditions, which allows reproductive success and the transmission of genes to the next generation (Gerlach and Aurich, 2000). In nature the possibility of survival of the descent is mainly linked to the availability of food and water at the time of birth, so the best time to give birth is spring. In order to guarantee birth in spring, each species, based on the duration of gestation, presents different breeding seasons. This allows to divide the species in two groups: "long-day breeders", such as horses and hamsters, that cycle when days get longer (spring) and are in anestrus in fall and winter; and "short-day breeders", such as sheep, that cycle when the length of daylight shortens (fall) and are in anestrus in spring and summer (Figure 8) *(Karsch et al., 1984)*.

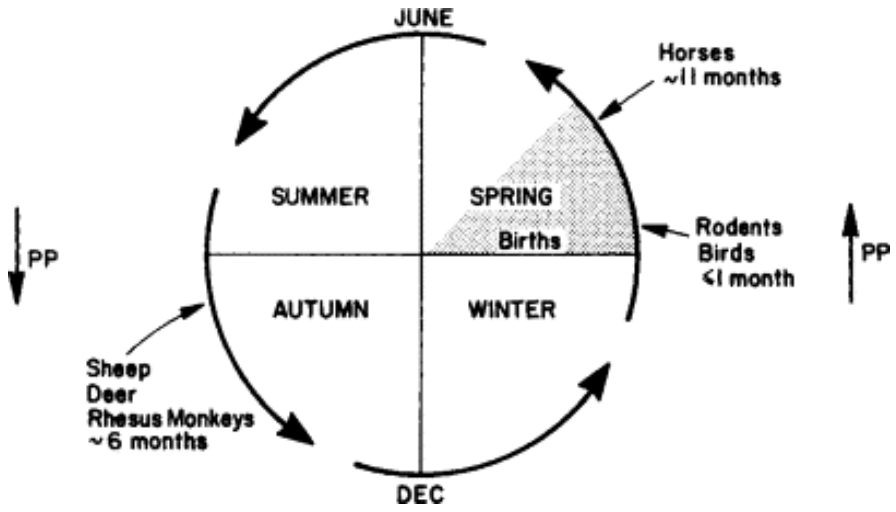

Figure 8. *Schematic presentation of breeding seasonality in "long-day breeders" and "short-day breeders", PP = photoperiod (Karsch et al., 1984)*

It is not entirely known what is the mechanism by which MEL acts to regulate seasonal functions and how the organism is able to understand information to regulate breeding season.

The pineal gland is a neuroendocrine transducer that informs mammals about photoperiodic changes allowing them to adapt their physical state to the same annual changes, a clear example of this adaptation is the activation / inactivation of the reproductive axis as a response to photoperiodic changes. It is MEL that through the binding with the high affinity MT1 receptor, located in the suprachiasmatic nucleus (SCN), performs its chronobiotic effect. MEL is not the only endocrine output of the circadian clock, indeed other hormones, such as corticosterone, play an important role, but MEL is one of the most

stable and measurable that directly gives a fair and reliable estimate of how the clock works (Pevet et al., 2017).

Several MEL target organs have been identified, each of which is linked to specific physiological functions. The highest concentration of MEL receptors is in the *pars tuberalis* (PT), where MEL stimulates the secretion of prolactin (Hazlerigg et al., 2001 for review), also at the level of premammylar hypothalamus MEL controls reproductive seasonality (Malpaux et al., 1998).

However, it is not clear which aspect of the MEL secretory model (phase, duration, amplitude, or total quantity) is the most important for the control of its biological functions (Figure 9).

Duration Hypothesis: the observations of the MEL secretion model showed that the night MEL peak duration reflects the night length (Rollag and Niswender, 1976) and that the peak duration is inversely proportional to the photoperiod (Foulkes et al., 1997). The duration changes of this peak, during the year, are the signals perceived by the reproductive neuroendocrine axis (Bittman and Karsch, 1984). This hypothesis has been demonstrated by studies conducted on different species, in which the administration of MEL during long days extends the endogenous peak of indolamine and consequently advances the reproductive activity, simulating a typical secretory rhythm of short days (Klein et al., 2002).

Coincidence hypothesis: MEL can complete its physiological effects when its secretion coincides with a phase of sensitivity of the target organs (Simonneaux e Ribelayga, 2003).

Amplitude hypothesis: the signal perceived by the organism would be the amplitude of the nocturnal peak of MEL.

The last theory appears the last possible, since multiple factors such as temperature, humidity and food availability can influence the amplitude of secretion, consequently modifying the metabolism of the hormone (Goridou-Boof et al., 2005).

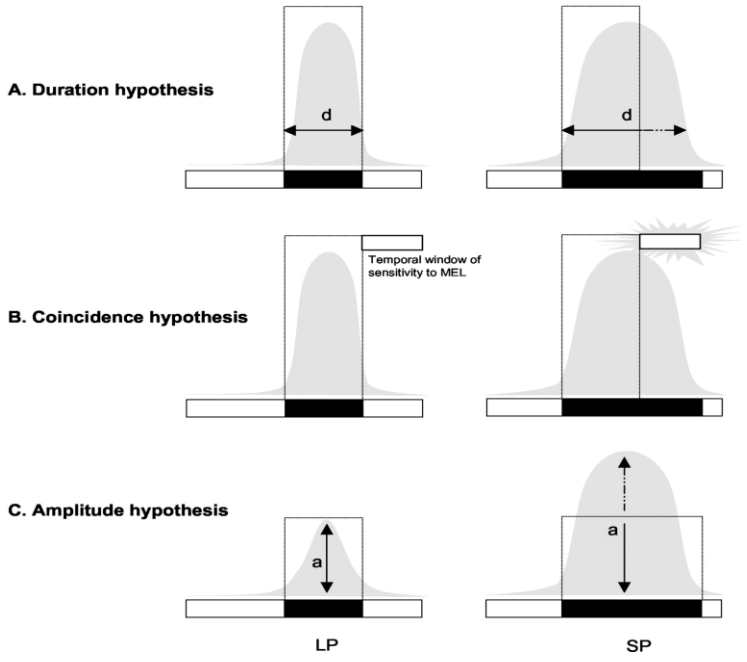

Figure 9. *Schematic presentation of different theoretical models that explain how the photoperiodic MEL signal is decoded by the target organs (Simonneaux e Ribelayga, 2003)*

MEL receptors (MEL-R)

The MEL distribution in the body is very rapid thanks to its small size, lipophilic nature and an active transport mechanism (Finocchiaro and Glikin, 1998), which allows it to act independently of its receptors. The use of radio-ligand 2[125]iodomelatonin allowed the detection of high-affinity MEL receptors in different vertebrate species (Vakkuri et al., 1984).

This allowed the localization of the receptors in multiple tissues and it was thus possible to highlight a great variability both in the number and in their localization in the various species (Pévet 2003).

Several subtypes of mammalian MEL receptors have been cloned and pharmacologically characterized: one of high affinity (ML1) and one of low affinity (ML2), (Dubocovich, 1995; Reppert and Weaver, 1995b; Vanecek, 1988a).

Further three high-affinity receptors were discovered: Mel1a (later renamed MT1) and Mel 1b (later renamed MT2) are present in all vertebrates, the first mainly in the brain, the second mainly in the retina (Reppert et al.,1994; 1995a), the third called Mel 1c is present in birds (Reppert et al., 1995b). The high-affinity receptors MT1 and MT2 are members of the seven-transmembrane G-protein coupled receptor family. Whereas the low-affinity ML2 receptors (renamed to MT3 or Gpr50) use quinone reductase (QR2) as a second messenger

(Nosjean et al., 2000), and its function is not yet completely known in mammals (Dufourny et al., 2008; Hazlerigg and Loudon, 2008).

The binding of MEL to the MT1 receptor causes different effects, many of which depend on cAMP inhibition, through G proteins and through increases in cytosolic calcium through Gq11 (Brydon et al., 1994). The binding of MEL to MT2 receptors in addition to determining the inhibition of cAMP also causes inhibition of cGMP (Petit et al., 1999).

The molecular structure of MT1 is composed by 350 amino acids and presents homology of 60% with the 362 amino acid MT2 (Reppert et al., 1996). Both, MT1 and MT2 receptors show the characteristic structure of the G-protein-coupled receptor (GPCR) with 7-transmembrane (TMI-TMVII) α-helices, linked by 3 alternating intracellular (ic1-ic3) and 3 extracellular (ec1-ec3) domains (Figure 10) (reviews Reppert 1997; Dubocovich et al., 2003).

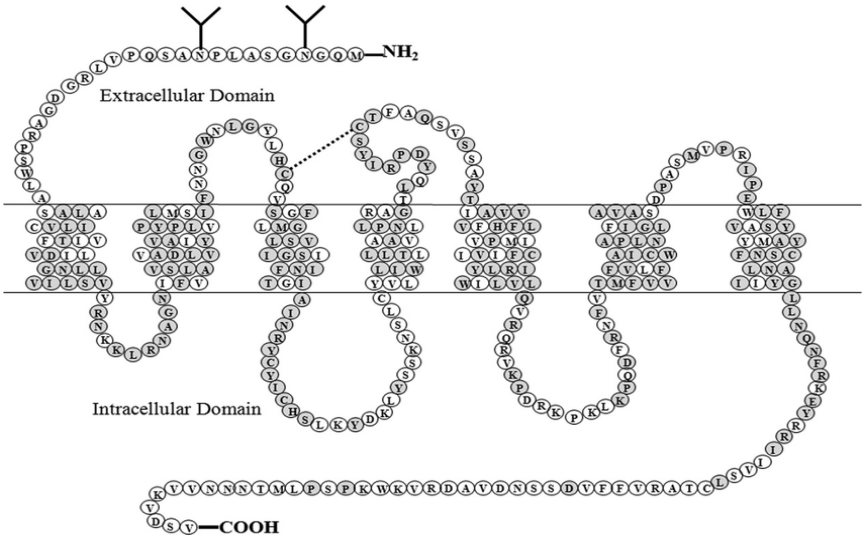

Figure 10. *Topology of the MT 1 receptor showing amino acids conserved in the MT 2 receptor. The gray circles indicate identical amino acids in the MT 1 and MT 2 receptors. The two glycosylation sites on the MT 1 receptor are indicated (Y) in the N terminal (Dubocovich et al., 2010)*

It was established that the presence of glycin in position 20 of TM VI, valine in position 4 of TM IV, histidine in position 7 of TM IV, serotonin in positions 8 and 12 of TM III is essential for binding with MEL (Gubitz e Reppert, 2000; Conway et al., 2000; 2001). This is because the binding of MEL with specific domains and / or amino acids defines its ability to connect, activate and modulate the receptor itself (Witt-Enderby et al., 2003). Also for MT2 any amino acid alteration in a protein can influence the interaction between the receptor and its ligand, for instance the presence of cysteine113 and cysteine190 in the second and third extracellular loop are important for

the overall structure because they are providing a disulfide linkage between these two residues (Xiao et al., 2007).

The distribution of MEL receptors is more limited in mammals and is species specific. In sheep MT1 receptors are localizzed in the hypothalamus including the area of suprachiasmatic nucleus (SCN), the site of a biological clock, adrenal glands, kidneys, B and T lymphocytes. The MT2 receptors are found at different sites in the brain, moreover in retina and small intestine. In other organs such as arteries, heart, liver, lungs and skin, both receptors are found simultaneously (Masana et al., 2002; Naji et al.,2004; Pozo et al., 1997; Richter et al., 2008; Sallinenet al., 2005; Slominski et al., 2007; Ting et al., 1999). Melatonin binding sites have been discovered in granulosa cells of human preovulatory follicles (Yie et al., 1995; Niles et al., 1999), in porcine cumulus and granulosa cells (Kang et al., 2009), in bovine oocytes and cumulus cells (El-Raey et al., 2011). Tian et al. (2017) found for the first time that the MT1 and MT2 receptors were not expressed only in sheep granulosa cells, but also in cumulus and oocyte cells.

The genes that code the MT1 and MT2 receptors are found in two different chromosomes and show similar structure. The genes structure is composed of two exons divided by a large intron, of more than 8 kb, the exon I encoding for the first cytoplasmic loop, the TM domain and the N-terminal extracellular domain, the exon II encoding for the other

parts of receptors (Reppert et al., 1996).

In sheep, the MT1 receptor has been cloned by Reppert et al. (1994) and mapped on chromosome 26 (Messer et al., 1997). Inside the MT1 gene, which encodes the corresponding receptor, two polymorphic sites (SNPs) have been identified through the use of restriction enzymes. The effect of the presence of these single nucleotide polymorphisms (SNPs) has been studied to assess the influence of this gene on the seasonality of reproductive activity (Messer et al., 1997). In fact, the studies conducted by Pelletier et al (2000) showed a correlation between the expression of reproductive seasonality and the alleles of the MT1 gene receptor (MTNR1A). This relationship was also highlighted in sheep bred in North America and this peculiarity was used by Notter et al. (2005) as a genetic marker in selection to improve reproductive activity in sheep. The candidate gene as a mediator of seasonality reproductive in sheep is therefore the MT1 and particular action is due to the structure and polymorphism of exon 2 of MTNR1A gene (Carcangiu et al., 2009; Martínez-Royo et al., 2012). This gene has been studied in different sheep breeds (Martínez-Royo et al., 2012; Meena et al., 2013; Mura et al., 2014; Luridiana et al., 2015; He et al., 2019) and specific genotypes have been associated with seasonal reproductive activity (Carcangiu et al., 2009; Saxena et al., 2015; He et al., 2019). In previous studies conducted in different sheep breeds, was found that the reproductive competence out of the normal

season is influenced by the presence of C allele at position g.17355458 and of a G allele at position g.17355452 of the MTNR1A gene exon II (according to the latest genome version Oar Ramb_v1.0). In the Rasa Aragonesa sheep breed, however only the T allele at position g.17355458 resulted in association with a greater percentage of oestrous cyclic ewes between January and August (Martínez-Royo et al., 2012). This demonstrates that the effect of these polymorphisms could be different based on several environmental and genetic factors.

There is no relationship between the different genotypes found in MT2 and reproductive seasonality (Xiao et al., 2007).

MT3 has not yet been isolated in the sheep, or in other ungulates but has been found in liver, kidney, brain adipose brown tissue, skeletal muscle, lung, intestine, testis and spleen of hamster, mouse, dog and monkey (Nosejean et al., 2001). Furthermore, this receptor would also be involved in the regulation of the intraocular pressure in rabbit (Pintor et al., 2001) and in the inflammatory response in rat (Lotufo et al., 2001).

Roles of MEL

Antioxidant / oxidant effects

MEL shows antioxidant-oxidant effects, but its mechanisms of action are not yet completely known. MEL is known to be a scavenger of free

radicals with a direct effect and also increasing the efficiency of mitochondrial electron transport and promoting the activity of antioxidant enzymes (Tomás-Zapico and Coto-Montes, 2005).

Free radicals are unstable atoms that can damage cells, causing illness and aging, are linked to aging and a host of diseases. These agents include reactive oxygen species (ROS) that react and cause a molecular destruction called oxidative damage (Pierrefiche et al., 1995). ROS are responsible for the destruction of macromolecules such as proteins, DNA and lipids, leading to cell death through apoptosis (Simonneaux and Ribelayga 2003). ROS are normally formed during cellular metabolic processes, therefore the cells have developed several enzymatic and non-enzymatic antioxidant mechanisms to reduce oxidative damage. Among the enzymatic mechanisms are known: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). The non-enzymatic mechanisms including: vitamin E (a-tocopherol), vitamin C (ascorbate), glutathione (GSH), β-carotene and MEL. These act directly, remove ROS due to their ability to donate electrons, thus neutralizing the potential toxicity of ROS (Tomás-Zapico and Coto-Montes, 2005).

Anticancer effects

MEL functions as a "smart killer" because it drives anti-apoptotic

processes in normal cells and promotes pro-apoptotic signals in cancer cells (Bizzarri et al., 2013). MEL plays an important role as a non-toxic, apoptotic, oncostatic, angiogenic, differentiating and antiproliferative factor in solid and liquid tumors (Di Bella et al., 2013). It is clear that MEL cannot be used alone in tumor therapy, but it can help to amplify the cytostatic and cytotoxic effects of other conventional drugs (Bizzarri et al., 2013).

Immunomodulatoy effects

In the 1926 Berman was the first to indicate the possible link between the pineal glands and the immune system. Subsequently, several studies have shown that different cells and tissues of the immune system (like thymus and spleen, B cells) containing or / and produce MEL, express AANAT and HIOMT mRNAs (Carrillo-Vico et al., 2005). A bidirectional link between the immune system and the pineal gland is demonstrated by the fact that cytokine signaling affecting pinealocytes has resulted in a transient inhibition of melatonin synthesis and shifting MEL production to circulating macrophages (Da Silveira Cruz-Machado et al., 2010, Fernandes et al., 2006, Markus et al., 2013, Markus and Ferreira, 2011). These systems also share a common language through messengers synthesized in both systems, such as acetylcholine, adrenocorticotropic hormone (ACTH),

endorphin, vasoactive intestinal peptide (VIP), somatostatin and growth hormones (Blalock et al., 2007). The role of MEL as coordinator and modulator of the immune system is demonstrated also by a seasonal variation of immune functions in many mammals (Nelson, 2004). In fact, MEL monitors diurnal and seasonal rhythms of leukocyte proliferation (Drazen et al., 2001), cytokine production (Scheff et al., 2010) and naturals killer (NK) cell activity in mammalian bone marrow cells (Matsumoto et al., 2001).

MEL plays a dual role as an immunostimulant and an immunosuppressant, depending on the different situations. It acts as an immunostimulant in basal or immunosuppressive conditions, bringing a pre-activated state for a more effective early immune response. Contrary, it acts as an anti-inflammatory molecule (affecting the activity of lipoxygenase), in conditions of chronic or exacerbated immune response, such as septic shock (Figure 11) (Carrillo-Vico et al., 2013).

MEL acts in the immune system with an autocrine, endocrine and paracrine mechanism. It has effect on the modulation of pro-inflammatory enzymes, controls the production of inflammatory mediators (cytokines and leukotrienes) and participates in the regulation of leukocyte lifetime by interfering with apoptotic processes (Herman et al., 2015).

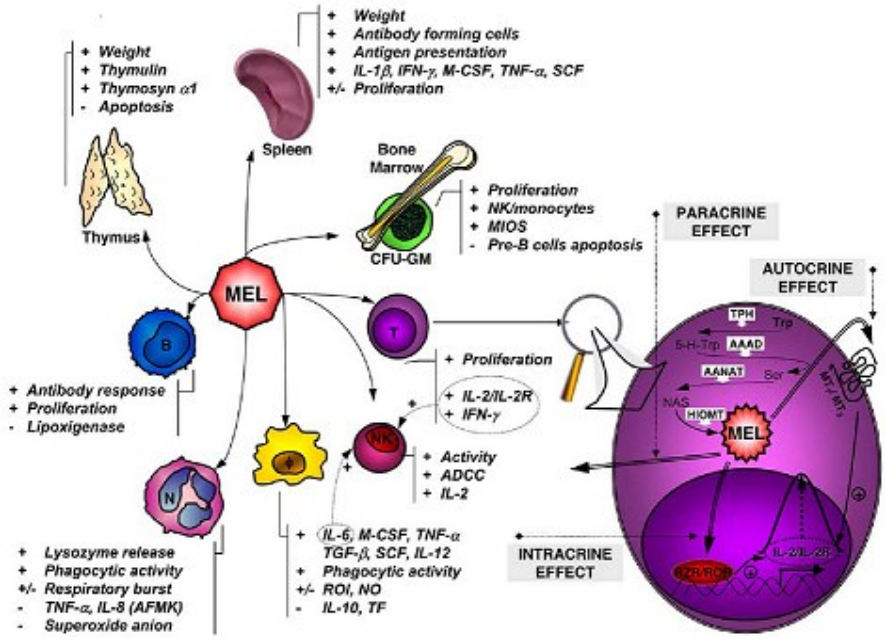

Figure 11. *Different pathways of action of MEL in the immune system (Carrillo-Vico et al., 2005)*

A bidirectional pathway between the pineal gland and the immune system is known but the effect of inflammatory feedback on the functions of the gland is not yet completely known. Immune mediators such as cytokines, prostaglandins and histamine have easy access to the brain and the pineal gland, in fact they enter these during the immune response phase (Fabris, 1994). It has also been shown that antigenic stimulation (Markowska et al., 2005), histamine (Zawilska et al., 1997), cytokines (Achumnarnkul et al., 1990; Mucha et al., 1994) and prostaglandins (Voisin et al., 1993) could influence the secretory activity of the pineal gland. This influence has also been demonstrated

in sheep where treatment with IL-1β has been shown to suppress NE stimulated melatonin secretion (Herman et al., 2015).

A study shows that in goats MEL implants have improved the quality of raw milk by reducing somatic cells count (SCC) via decreasing udder oxidative stress (Yang, 2017). Also in cows, the subcutaneous injection of melatonin greatly reduces SCC in animals with subclinical mastitis, thanks to its antioxidant, anti-inflammatory and immuno-potentiation properties. In cows with mastitis, MEL works by reducing cortisol levels and increasing levels of IgG, IgM, lymphocytes and neutrophils (Yang, 2017).

Reports showed the high nightly levels of MEL in cows with low-milk SCC (Asher et al., 2015).

Reproductive effects

In temperate climates, most sheep breeds have a seasonal breeding model with a breeding season in the fall. Seasonality is stronger in primitive breeds (Hafez, 1952).

The role of MEL as a regulator of reproductive physiology is demonstrated by the synthesis and presence of this indolamine in different sites of ovaries and testes. Also in the reproductive tract MEL acts by means of the endocine, paracrine and autocrine mechanisms (Acuña−Castroviejo et al., 2014), but it is not yet fully known how the

hormone works. It is clear that MEL guarantees the quality of the oocyte and the sperm (Acuña−Castroviejo et al., 2014), moreover it was show that it is inhibiting the normal aging processes of the reproductive system in rat (Wolden-Hanson et al., 2000). Even in sheep, MEL plays its anti-aging role, indeed it can improve the functionality of the reproductive system in older animals (Forcada et al., 2007).

In the reproductive tract MEL acts through the hypothalamic neuronal systems to regulate the frequency of the GnRH pulse, this mechanism is necessary to raise the frequency secretion of LH, finally to activate the breeding period (Figure 12) (Malpaux et al., 1997).

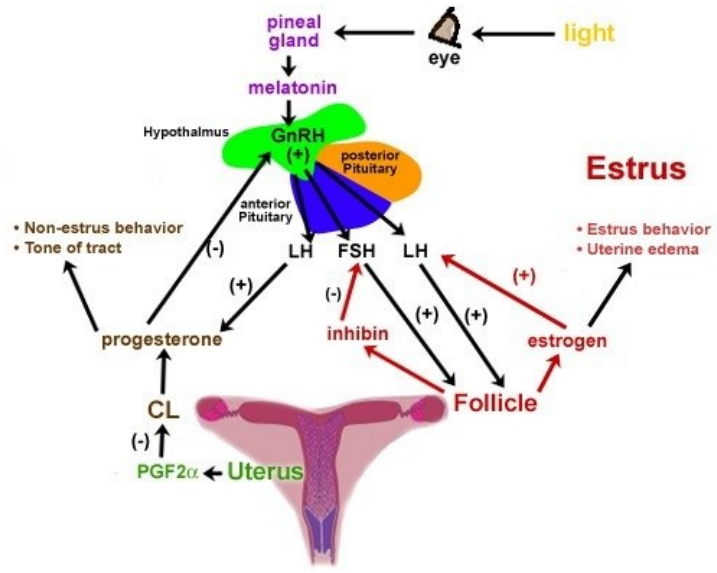

Figure 12. *Melatonin action to regulate reproductive activity (adapted from Hormone Manipulation, ansci.wisc.edu)*

A study showed that only a MEL micro-implant placed in the mediobasal hypothalamus (MBH) was able to stimulate the secretion of LH (Malpaux et al., 1993). Although the higher concentration of MEL bindings is expressed in the *pars tuberalis* (PT) of the adenohypophysis, the placement of MEL micro-implant in this area does not modify the secretion of LH (Malpaux et al., 1995). Whereas, the micro-implants located in the MBH or in the third ventricle are able to stimulate the release of LH (Malpaux et al., 1993; 1994; 1995). Previous studies have shown that PT participates in transductions of MEL effects on seasonal change in prolactin secretion. This is demonstrated by the fact that the placement of microimplants in the PT and MBH lead to the same suppression of prolactin levels (Malpaux et al., 1995). Therefore, MEL acts directly on the pituitary gland, it may be in the PT, to control prolactin secretion (Lincoln et al., 1994). In contrast, MEL does not act directly on GnRH neurons to control the release of the hormone, in fact its action could be mediated by a complex system of interneurons. In this system the dopaminergic terminals of the median eminence play a key role (Malpaux et al., 1995). To act its strong negative feedback on GnRH secretion, in prepubescent animals and during anoestrus, estradiol (E) uses dopamine as a transducer, more precisely, the hypothalamic nucleus A15 mediates the inhibitory effects of E (Thiéry et al., 1989). Furthermore, causes an increase in tyrosine hydroxylase (TH), a

limiting step enzyme of catecholamine synthesis, (Gayrard et al., 1994).

Exposure to short days causes a decrease in dopaminergic activity in the median eminence due to both the reduction in the amount of dopamine (Thiéry et al., 1991) and the activity of the TH (Viguié et al., 1996). Similarly, MEL implants are able to increase LH secretion, reducing TH activity in the median eminence (Viguié et al., 1997). The inhibitory action of TH is evidenced by the fact that this enzyme has a higher concentration, in adult animals, during the anaestrus period (Viguié et al., 1997). Furthermore, the administration of a TH antagonist during long-days causes a pulsatile increase of LH and consequently the reactivation of reproductive activity (Tillet et al., 1997). It is therefore possible that the inhibition exercised by this enzyme is at the basis of the period preceding puberty.

It remains to be clarified what are the factors that actually trigger the onset of puberty, and in particular the concrete role of MEL. This could be essential to reduce the non-productive periods in farms.

Over the years, numerous studies have been carried out using both the artificial photoperiod and exogenous MEL to improve the reproductive activity in sheep (Kennaway et al., 1984; Poulton et al., 1987) and goat (Tamanini et al., 1985; Prandi et al., 1987; Deveson et al., 1992b). To understand if and how melatonin was implicated in the mechanism of

induction of puberty in the female, experiments that included pinealectomy in ewe lambs subjected to natural photoperiod were conducted (Seamark et al., 1981). Surprisingly, these studies, conducted on Merino sheep breed, showed no abnormal seasonality despite the absence of epiphyses. This result was attributed to the possible insensitivity to the photoperiod of the Merinos, sheep with a relatively long annual fertile season and a practically indistinct anoestral period. A subsequent study, however, conducted on crossbreed of Merino X Dorset showed a considerable delay in reaching puberty following pinealectomy (Kennaway et al., 1985). In light of this, it was assumed that breed differences existed in the modulation of the photoperiod and that exogenous melatonin could, in receptive breeds, regulate the beginning of reproductive activity.

The first experimental research included the administration of 2-3 mg of MEL in sheep in anoestrus, showed that was able to induce the anticipation of the reproductive season (Kennaway et al., 1982; Arendt et al., 1983). It was discovered that in order to obtain these results, the administration had to be continuous and of a duration longer than 45-50 days, since administration on alternate days seemed to have no effect (O'Callaghan et al., 1991). However, given the low practicality of daily administration, slow-release ruminal boluses and implants have been studied that are able to maintain high blood concentrations of MEL for long periods (Arendt et al., 1988; Poulton et al., 1988).

The commercial use of MEL implants in sheep is currently authorized in several European countries, such as Italy, the United Kingdom, France, Greece, Portugal and Spain. At the Mediterranean latitude, subcutaneous implants are the most used to anticipate the sexual season of the sheep. Normally, MEL treatments are accompanied by the union of sheep with males, since this triggers the "male effect" that causes the increase in LH tonic secretion and therefore the change in the state of the ovary (Martin et al., 1986; Chanvallon et al., 2011). Although chronic exposure to implants causes some damaging effects on reproductive parameters in adult Blackface (Jordan et al., 1990), their medium-term influence, associated with the male effect, in Mediterranean breeds remains to be clarified. In sheep, MEL implants can be used to control reproductive activity even if there is no uniformity in the results obtained in the various breeds (Haresign et al., 1990; Carcangiu et al., 1993, Abecia et al., 2011a). In goats, the experiences with MEL are much lower and the results obtained have not always been satisfactory (Prandi et al., 1987; Deveson et al., 1992; Abecia et al., 2011b).

In ewe lambs the administration of MEL has not shown consistent effects on the onset of reproductive activity (Kennaway et al., 1984; Foster and Olster, 1985; Sawalha et al., Mura et al., 2010; Abecia et al. 2016; Luridiana et al 2016).

Several studies on exogenous MEL treatments conducted on adult

sheep, had the aim to evaluate the involvement of MEL on the speed of ovarian response to the male effect (Abecia et al., 2006) or on the viability and shelf life of embryos in artificial insemination practices (Forcada et al., 2006). In both studies, MEL showed a clear influence in the improvement of the aforementioned activities.

Aim

Melatonin is a hormone that has several functions in the body, and all are of considerable importance. In fact, in addition to regulating the circadian rhythm by synchronizing the physiological activities within 24 hours, influences the reproductive seasonality and the immune response. Most of these actions are performed through specific receptors present at numerous sites in the animal body.

Concerning to the control of reproductive seasonality, although much is known about the effects of melatonin, it remains to be explained whether some physiological states of the animal may influence the advance of reproductive activity in sheep. Sometimes, melatonin treatment has reproductive responses that are not as expected, and this is often due to the fact that animals are not suitable for reproduction at that time. Therefore, understanding which factors can cause this response is very important for optimization of reproduction in sheep.

In order to obtain a maximum reproductive efficiency in sheep, it is also possible to intervene by improving the male effect. In fact, managing this method in a rational way could lead to an improvement in the reproductive efficiency of sheep with a greater income for sheep farms.

Finally, understanding how the polymorphisms of the melatonin

receptor gene (MTNR1A) can influence reproductive resumption in sheep could provide important data that could be used in genetic selection of sheep.

Therefore, the proposal of this thesis was to evaluate the different actions of melatonin in five different objectives that can be formulated as follows:

1) to evaluate the effect of melatonin implanted

a) in different periods of the year,

b) in different lactation periods;

2) to evaluate the influence of melatonin treatment in ewes and male replacement on reproductive activity;

3) to evaluate the effect of melatonin treatment in male on the onset of puberty in Sarda ewe lambs;

4) to evaluate the influence of the different MTNR1A genotypes on reproductive activity;

5) to evaluate the influence of the melatonin treatment on milk production and mammary immune system.

Material and Methods

Aim 1a (MEL treatment in different periods of the year)

Animal and experimental design

This study was conducted on 3200 Sarda sheep breed from 16 farms, located in the same area of the Northern Sardinia (40°N). The animals were reared in the same conditions on all the farms. During the day the animals grazed on leguminous and gramineous grasses and during milking they also received 300 g of commercial concentrate feed per animal every day (20.4% of raw protein and 12.5 MJ ME / kg of DM). During the night the sheep were penned and received hay (11.1% raw protein and 7.2 ME / kg DM) and water *ad libitum*.

For the research, 200 clinically healthy sheep were chosen from each farm, for a total of 3200 sheep. The females included in the study were lactating, 3-6 years old, with body condition score (BCS) between 2.5 and 4.0 and with a single lamb born between 1st November and 10th December.

Treatment and registration data

On each farm, the selected animals were randomly divided into 2 groups (M and C), each containing 100 sheep. During the study the controls and the treated ewes were kept together all the time. The sheep of group M received a melatonin implant (18 mg) (Melovine,

CEVA Animal Health, Agrate Brianza, Milan) in the left retro-auricular area; group C served as a control and was not treated. In the first 4 randomly selected farms, the melatonin implants were implanted on 10th of February, in the second 4 farms on 10th of March, in the third 4 on 10th of April and in the last 4 farms on 10th of May. 35 days after the treatment of the ewes, adult rams (male/female ratio 1/20) were introduced into the groups for 45 days in all the studied farms. One week before the sheep, the rams were treated with 3 MEL implants in the left retroauricolar area. Sheep and rams were kept separate for three months before the rams were introduced. Starting from 45 days up to 90 days from the introduction of rams, gestation was diagnosed by transdominal ultrasound examination through the Tringa Esaote equipment (Esaote Europe BV, Maastricht, Netherlands) equipped with a 5 multi-frequency linear probe, 5 - 7.5 MHz. The date of the lambing and the number of newborn lambs of each ewe were recorded from 150 days to 190 days after the introduction of the rams.

Statistical Analysis

All statistical analysis was executed using the computing environment R (Version 3.6.1. R Core Team 2019). A logit-link Hierarchical Linear Model (HLM) suitable for binomial (lambing / not lambing) data was used to analyse the fertility rate of different treatment time. Variables considered were treatment and treatment time. To analyse the effect of treatment time on period in days from male introduction to lambing a

HLM procedure according to the following model was performed.

$$Y_{ijk} = \mu + T_m(Pe)_{ij} + \varepsilon_{ijk}$$

Where Y_{ijk} is the period in days from male introduction to lambing, μ is the overall mean, T_m is the fixed effect of treatment, Pe is the nested time effect within treatment, and ε_{ijk} is the error effect. The same model was used to analyse the litter size. A P value < 0.05 was considered statistically significant. Multiple comparisons of the means were performed using Tukey's method.

Aim 1b (MEL treatment in different lactation periods)

Animal and experimental design

For this study two farms located in Northern Sardinia (between 39 and 40°N) were chosen. The animals were raised under the same conditions on the two farms and kept under natural photoperiod since birth. Food management included grazing leguminous and gramineous grasses during the day and the daily administration of 300 g per sheep of commercial concentrate feed (20.4% raw protein and 12.5 MJ ME/kg DM) during milking. In each farm (identified as F1 and F2) 120 lactating ewes were chosen and then divided into four groups: group 1 with 30 ewes lambed between 20th of October and 20th of November; group 2 with 30 ewes lambed between 1st and 30th of December; group 3 with 30 ewes lambed between 1st and 30th of

January and group 4 with 30 ewes lambed between 1st and 28th of February. All animals included in the study were between 3 and 6 years old, with a body condition score (BCS) between 3.0 and 4.0 and were kept separated from the rest of the flock

Treatment and registration data

Each group of 30 ewes was divided into two subgroups (M and C) of 15 ewes each. On the 1st of April all the ewes of subgroups M received a melatonin implant (18 mg) (Melovine, CEVA Animal Health, Agrate Brianza, Milan) in the left retro-auricular area; groups C served as a control and were not treated. In each group (M and C), 35 days after the treatment of the ewes, two adult rams of proven fertility were introduced and were kept for 70 days. Every week, from 45 days after the introduction of rams up to 45 days after the removal of rams, pregnancy diagnosing was performed. Pregnancy diagnosis was performed by transabdominal ultrasound examination through the Tringa Esaote equipment (Esaote Europe BV, Maastricht, Netherlands) equipped with 5-7.5 MHz multi-frequency linear probe. The date of the lambing and the number of newborn lambs of each ewe were recorded until 220 days after the introduction of the rams.

Statistical Analysis

R statistical software, (Version 3.6.1. R Core Team 2019) was used to perform the statistical analysis. A General Linear Model (GLM)

procedure was carry out to analyze the effect of lambing period and treatment on the litter size and on the distance in days from male introduction to lambing. To confront percentages of lambed ewes within each lambing period chi-square test was used. A P value <0.05 was considered statistically significant.

Aim 2 (MEL treatment and male replacement)

Animal and experimental design

For this study a farm located in the northern Sardinia (approximately 40°N) was chosen. All the animals raised on the farm (about 1000) have been subjected to natural photoperiod since birth. During the day the animals grazed on legumes and gramineous grasses and during milking they also received 300 g of commercial concentrate feed per animal every day (20.4% of raw protein and 12.5 MJ ME / kg of DM). During the night sheep were penned and received hay (11.1% raw protein and 7.2 ME / kg DM) and water *ad libitum*. The chosen sheep were between 3 and 5 years old, had a BCS between 2.5 and 4.0 and had lambed between 20th October and 1st December. In addition, the chosen ewes were at least at third lambing. The first and second lambing ewes were excluded from the study because in the Sarda sheep breed the first lambing generally occurs between January and April, followed by a large milk production. This leads to a poor

reproductive function in the following two months and a delay in the lambing date in their second year (Carcangiu et al., 2012).

Treatment and registration data

The 400 animals chosen were divided into 4 groups with 100 sheep each. In group M the sheep were treated with one MEL implant and males were not replaced; in the MR group the sheep were treated with MEL implant and a weekly replacement of the males was performed. Group C served as a control and were not treated and males were not replaced; in the CR group the sheep was not treated but a weekly replacement of the males were performed. From the formation of the groups and during the study period, the different groups were kept separately from each other. On 20th of March the ewes of groups M and MR received a melatonin implant (18 mg) (Melovine, CEVA Animal Health, Agrate Brianza, Milan) in the left retro-auricular area. On 24th of April 5 adult rams with proven sexual experience were introduced into each group to induce male effect and remained in cohabitation with ewes for 40 days. Males were not treated with MEL implant. To stimulate the ram effect, the sheep were previously isolated from the males for at least 6 months, the rams were kept at a distance of 10 km, so that the auditory and olfactory stimuli coming from them could not reach the ewes. At the ram introduction every male has been provided with harnesses to paint the back of ewes for recording of mating, so detecting estrous ewes. Every day the number

of sheeps marked by rams were recorded and the color of the marking were changed weekly, Furthermore, the sexual behavior of rams was evaluated for the first five days after their introduction into the groups to assess whether rams were able to perform sexual activity. Recoded were anogenital sniffing, nudging and mounting attempts of the ewes. Starting from 45 days up to 90 days from the introduction of rams, gestation was diagnosed by transdominal ultrasound examination through the Tringa Esaote equipment (Esaote Europe BV, Maastricht, Netherlands) equipped with a 5.0-7.5 MHz multi-frequency linear probe. The date of the lambing and the number of newborn lambs of each ewe were recorded from 21st of September to 1st of November. On the basis of the recorded data, for each group it was established: the fertility rate (as percentage of lambed ewes), the distance in days from male introduction to lambing (DIML), and the litter size (number of newborn lambs per ewe).

Statistical Analysis

The analysis of collated data was performed using the statistical software R (Version 3.6.1. R Core Team 2019). A P value < 0.05 was considered statistically significant. The fertility rate was scan using the Fisher-Freeman-Halton exact probability test for multiple group comparisons. The distance in days from male introduction to lambing (DIML) and litter size, expressed as mean values ± standard deviation (SD), were normally distributed ($P > 0.05$) (Shapiro–Wilk normality

test). Analysis of Variance was performed to analyze the effects of melatonin treatment (T) and male replacement (M) on reproductive activity (DIML or litter size). The BCS and age of animals did not show statistical significance and thus, were not included in the model. The following linear model was used for all dependent variables:

$$Y_{jkm} = \mu + T_j + M_k + T_jM_k + e_{jkm}$$

where Y was the variable measured (DIML or fertility or litter size), μ was the overall mean, Tj was the effect of the melatonin treatment, Mk was the effect of the male replacement, TjMk was the interplay of melatonin treatment (M) and male replacement (R) and ejkm was the error effect.

Multiple comparisons of the mean average were carryed out using Tukey's method. To compare the number of ewes lambing through all the groups a chi-square (χ^2) test was used.

Aim 3 (influence of MEL treatment in male)

Animal and experimental design

For this research a farm located in the north of Sardinia was chosen. In this farm about 1.000 animals were reared, from which 200 ewe lambs were chosen. The enrolled ewe lambs were born in November and had a body weight of about 30 kg. Furthermore, on the same farm, 28

males of proven fertility were chosen, with an average age of 4.5 years. The animals were kept in the same conditions and under natural photoperiod since birth.

Treatment and registration data

On 25th May, 14 rams were treated with 3 melatonin implants each. On 20th June, the chosen ewe lambs were divided into four groups M, MS, C, CS, each of 25 animals. On 1st of July two rams were introduced into each ewe lamb group. In the M and MS groups treated males were introduced, while in the C and CS groups the untreated males were introduced. In addition, a weekly male replacement was performed in the MS and CS groups. The males were removed 40 days later. From 150 to 190 days after the introduction of the males, the date of lambing and the number of newborn lambs were recorded.

Statistical Analysis

The analysis was carried out by statistical software R (Version 3.6.1. R Core Team 2019). A P value < 0.05 was considered statistically significant. The fertility rate was examined using the Fisher-Freeman-Halton exact probability test for multiple group comparisons. The distance in days from male introduction to lambing (DIML) and litter size, expressed as mean values $\pm$ standard deviation (SD), were normally distributed ($P > 0.05$) (Shapiro–Wilk normality test). Analysis of Variance was performed to analyze the effects of

melatonin treatment (T) and male replacement (M) on reproductive activity (DIML or litter size). The following linear model was used for all dependent variables:

$$Y_{jkm} = \mu + T_j + M_k + T_jM_k + e_{jkm}$$

where Y was the variable measured (DIML or fertility or litter size), μ was the overall mean, Tj was the effect of the melatonin treatment, Mk was the effect of the male replacement, TjMk was the interaction of melatonin treatment (M) and male replacement (R) and ejkm was the error effect. Multiple comparisons of means were carryed out using Tukey's method. To compare the number of ewes lambing across all the groups a chi-square (χ^2) test were used.

Aim 4 (influence of different MTNR1A genotypes)

Animal and experimental design

For this study 8 farms were selected, all located in the north of Sardinia (on the 40°N). Animals were reared with similar nutritional and management regimes and kept under natural photoperiod since birth in all the farms. Animals grazed on leguminous and gramineous grasses during the day and during milking they also received 300 g of commercial concentrate feed per animal every day (20.4% of raw protein and 12.5 MJ ME / kg of DM). In each farm (identified F1-F8) about 400 ewes were genotyped in position g.17355452 G > A of exon

II of the MTNR1A gene. In each farm, 150 sheep were selected (50 for each genotype A / A, A / G, G / G). All animals included in the study were between 3 and 5 years old and, were multiparous (at their third parturition) and lambed between 20th of October and 1st of December. The chosen animals were kept away from the rest of the flock. In each group of 150 sheep, eight adult rams (3 to 6 years old) of proven fertility (male/female ratio 1/20) were introduced to the sheep at different times (see table 1). After 70 days the rams were removed.

Table 1: *Time of ram introduction to ewes on eight farms*

Time Period	Data of male introduction	Farms
T1	25 March	F1
	25 March	F2
T2	15 April	F3
	15 April	F4
T3	5 May	F5
	5 May	F6
T4	1 June	F7
	1 June	F8

Before the introduction of rams, sheep and rams were kept separate for 90 days. Every week, from 45 days after male joining with ewes up to 45 days after the removal of rams, gestation diagnosing was done. Pregnancy diagnosis was performed by transabdominal ultrasound examination through the Tringa Esaote equipment (Esaote Europe BV,

Maastricht, Netherlands) equipped with 5-7.5 MHz multi-frequency linear probe. The date of the lambing and the number of newborn lambs of each ewe were recorded until 220 days after rams introduction to ewes.

Blood sampling and primers sequences

DNA analysis was performed from the whole blood of each sheep, to identify the different allelic variant g.17355452 G > A of the Oar_rambouillet_v1.0 sequence of the MTNR1A gene. A 10 ml blood sample were collected from the jugular vein of each ewe using vacuum tubes with EDTA as an anticoagulant (BD Vacutainer Systems, Plymouth, UK). Genomic DNA was extracted from whole blood samples using a genomic DNA extraction kit (NucleoSpin® Blood, Macherey-Nagel, Germany) and then kept at −20 °C until use. 150 ng of genomic DNA were subjected to polymerase chain reaction (PCR) using specific primers synthetized by Sigma Genosys Ltd. (Pampisford, Cambs, United Kingdom) according to Messer et al. (1997). The primers corresponded to positions 285 to 304 (sense primer 5'– TGT GTT TGT GGT GAG CCT GG – 3') and 1108 to 1089 (antisense primer: 5'– ATG GAG AGG GTT TGC GTT TA – 3') of the sequence by Reppert et al. (1994) (GenBank accession number U14109).

DNA amplification and genotyping

The PCR reaction was carried out in 50µL of total volume. The composition of the PCR mix is as follows: 10 × PCR Buffer (50 mM /l KCl, 10 mM / L Tris–HCl (pH 8.0), 0.1% Triton X-100) 5 µL, 1.5 mM MgCl2 3 µl, 0.2 mM of each dNTP 8 µl, 10 pM / L of each primer, 1 µl of 100 – 150 ng ovine genomic DNA and 5U Taq DNA polymerase (HotMaster Taq DNA Polymerase, Eppendorf AG, Germany).

Mastercycler® Gradient (Eppendorf AG, Germany) was used to perform the PCR. The PCR protocol is shown in table 2.

Table 2: *PCR protocol*

Denaturation	***Denaturation***	***Annealing***	***Extension***	***Final Extension***	***Cycles***
94°C 5 min.	94°C 1 min.	62°C 1 min.	72°C 1 min.	72°C 10 min.	35

The PCR products were separated by electrophoresis on 2% agarose gel (GellyPhor, Euroclone, UK), in parallel with 100 bp DNA marker (Invitrogen, Carlsbad, USA).

All PCR products were subjected to restriction enzyme analysis with the MnlI endonuclease (New England England Biolabs, USA). The MnlI enzyme cut the sequence at the succession of bases 5'-CCTC(n)7^- 3. The digestion reaction was carried out in 30µL final volume, containing: 20µL of PCR product, BSA (100 µg / mL) 0.3 µL, Buffer 1 × (10 mM Tris–HCl, 50 mM NaCl, 10 mM MgCl2, 1 mM

Dithiothreitol, pH 7.9) 3 μL. The digestion process was performed by incubating the mix in thermostatic bath at 37°C for 2 hours, and then carrying out enzymatic deactivation at 65°C for 20 minutes. These products of digestion were performed by electrophoresis on a 4% agarose gel (GellyPhor, Euroclone, UK), in parallel with a 50 bp DNA marker (Invitrogen, Carlsbad, USA).

Sequencing

To determine if the variant g.17355452 G > A of the Oar_rambouillet_v1.0 sequence (GenBank assembly accession number: GCA_002742125.1) was associated with other nucleotide substitutions, different PCR products were sequenced, for each genotype. 300 amplified sequencing were performed using the Applied Biosystems DNA Analyzer 3730 (Perkin-Elmer Applied Biosystems, USA). So, the sequences were aligned and compared with the GenBank ovine sequence U14109 and GCA_002742125.1, to confirm the correspondence of the known nucleotide changes. Sequence analysis is important because it can allow us to highlight other possible substitutions. The sequences obtained were compared with those deposited in the database through the BLAST program (National Centre for Biotechnology Information: https://blast.ncbi.nlm.nih. gov/Blast.cgi). Furthermore, the CLUSTALW program (http://www.genome.jp/tools-bin/clustalw) was used to align the sequences.

Statistical Analysis

Allele and genotype frequencies were definited by direct counting of the observed genotypes. The chi-square test was used to determine Hardy-Weinberg equilibrium of the mutation (Genepop 4.2). The R statistical software (Version 3.6.1. R Core Team 2019). was used to perform the statistical analysis. The farm effect was not included in the model as the farms were all located within 20km each other, therefore, there were similar climatic conditions and animals had the same veterinary, feed and reproductive farm management. The data for the climatic variables (humidity, environmental temperature), and for the animals (number of lambing and the lambing period, BCS and age) were statistically assessed and there were no differences detected. A general linear model (GLM) procedure was performed to analyze the effect of treatment period and genotype on the litter size and on the distance in days from rams introduction to lambing, based on the following model:

$$Y_{ilmn} = \mu + S_i + G_l + P_m + (GlP_m) + e_{ilmn}$$

where Yilmn is the variable measured (pregnancy rate or duration of time in days from joining rams with ewes to lambing), μ is the overall mean, Si is the random effect of the sire, Gl is the fixed effect of the genotype, Pm is the fixed effect of period, (GlPm) is the interaction between Gl and Pm, and eilmn is the error effect. To confront

percentages of ewes lambing with each genotype and each time period, a chi-square test was used. A *P* value <0.05 was considered statistically significant when comparing the sequences.

Aim 5 (influence of the MEL treatment on milk production and mammary immune system)

Animal and experimental design

For this study, 200 ewes from a farm located in the countryside of Sassari were chosen. These ewes lambed between November 25 and December 30 and were 3 to 5 years old. The sheep were raised under natural photoperiod since birth.

Treatment and registration data

On 20th March, the sheep were divided into two groups (M and C) of 100 animals each, and the M group was treated with an 18 mg melatonin implant in the left retro-auricular region. Group C received no treatment. For each animal, for 5 months, monthly, starting from February 20th, the quantity of milk produced was measured and a sample of milk was taken to analyze the percentage of fat, protein, lactase and the number of SCC. The daily milk yield (kg / day) was calculated as the sum of morning and afternoon milking yields. Milk fat, protein and lactose were analyzed using a Milkoscan FT 6000 FOSS milk analyzer (Foss Electric A / S, Denmark), according to FIL-

IDF recommendations (ISO 9622:2013; ISO-IDF, 2013). SCC was determined with a Fossomatic 5000 FOSS somatic cell counter according to ISO 13366 / IDF148 (2006). SCC was logarithmically transformed to normalize the distribution: SCC to SCS, as proposed by Ali and Shook (1980).

Statistical Analysis

The R statistical software (Version 3.6.1. R Core Team 2019) was used to perform the statistical analysis. A general linear model (GLM) procedure was performed to analyze the effect of treatment on the milk yield, fat, protein, lactose and SCC.

Results

1a (MEL treatment in different periods of the year)

Number of sheeps with ultrasonographically diagnosed pregnancy varies from number of sheeps which lamb by about 3%. Melatonin treatment significantly increased fertility rates in all treatment periods compared to the control groups ($P < 0.05$). The highest fertility rate was observed in sheep treated in April and May compared to sheep treated in February and March ($P < 0.05$) (Table 3). The average time in days from the male introduction to the lamb was shorter in the treated animals than in the controls, in all the experimental periods (Table 3). Melatonin treatment was not able to influence the litter size in all the observed periods, so there was no difference between the treated and the control groups.

Table 3: *Fertility rate, mean litter size and mean distance in days from male introduction to lambing (DIML) in the treated (M) and control (C) ewes in four period*

Time	M	C		M	C	M	C	
	Fertilty rate	Fertility rate		Litter size	Litter size	DIML	DIML	
10th February	68%a	61%a	*	1.29	1.18	164.69	175.57	*
10th March	75%a	66%a	*	1.20	1.20	165.66	174.77	*
10th April	81%b	72%b	*	1.18	1.17	165.62	175.07	*
10th May	83%b	76%b	*	1.19	1.22	165.90	175.02	*

*Means within a column with different lowercase are significantly different ($P < 0.05$); In group comparison means within a row with * differ significantly for $P < 0.05$*

Based on the daily observation of lambing tendency, the treated ewes lambing about 10 days earlier compare to controls. On the 160th and 170th day after the male introduction, in all four treatment periods the treated group showed a higher number of lambed ewes compared to the control ($P < 0.01$). On the 180th and 190th day after the male introduction, the treated ewes had a higher number of lambed ewes compared to the controls ($P < 0.05$). In this case the difference between the treated and the control group was lower than in the first 20 days of lambing. In all four treatment periods, the lambing peak was recorded on day 170 in the treated sheep and on day 180 from the male introduction in the controls (Table 4).

Table 4: *Total number of lambed ewes from 150th to 190th day after male introduction (every 10days) in the treated (M) and control (C) Sarda sheep breed, in the four observed periods (total ewes n =3200)*

	150-160 DIML			161-170 DIML			171-180 DIML			181-190 DIML		
	M	**C**		**M**	**C**		**M**	**C**		**M**	**C**	
10th February	83	17	**	213	57	**	253	177	*	272	244	*
10th March	77	21	**	230	68	**	276	201	*	300	264	*
10th April	83	22	**	246	75	**	217	226	*	324	288	*
10th May	83	20	**	248	78	**	302	231	*	332	304	*

*DIML = distance in days between male introduction and lambing; In group comparison means within a row with * or ** differ significantly at $P < 0.05$ and at $P < 0.01$ respectively*

Aim 1b (MEL treatment in different lactating period)

Greater fertility ($P < 0.01$) and less distance in days from male introduction to lambing ($P < 0.01$) were recorded in treated animals, compared to controls (Table 5). Furthermore, the treated animals lambed of 10 days earlier compared to controls.

Table 5: *Fertility rate, mean ± SD distance in days from male introduction to lambing and litter size according to Groups (M or C) in Sarda sheep breed*

Group	Subgroup	Animals	Fertility rate (%)	DIML (days)	Litter size
1	**M**	30	85 B	174.1±15.1 A	1.18
	C	30	73 A	185.4±18.3 B	1.21
2	**M**	30	87 B	178.6±16.2 A	1.19
	C	30	72 A	187.9±15.4 B	1.25
3	**M**	30	70 B	188.4±18.9 A	1.22
	C	30	61 A	196.5±14.8 B	1.24
4	**M**	30	52 B	194.2±17.5 A	1.19
	C	30	40 A	203.6±18.1 B	1.14

DIML = distance in days between male introduction and lambing; The statistically significant differences between treated and control subgroups are related within the group to which they belong; A, B ($P < 0.01$)

Moreover, the best reproductive performance was found in animals that lambed in October-November and December compared to the other groups. The lambing peak of the animals in the 1M and 2M subgroups is between 160 and 170 days after the male introduction, while in the control animals the peak is recoded between 170 and 180 days.

The lambing peak of the animals in the 3M subgroup is between 180-190 days and in the 4M subgroup between 190-200 days after the male introduction. Therefore, the MEL treatment allowed an early response to the ram effect and consequently a more compact period of mating and delivery, compared to untreated animals.

MEL treatment was not able to influence the litter size.

2 (MEL treatment in ewes and male replacement)

In general, the fertility rate is higher in the animals of the treated groups than in the untreated groups. More specifically, the highest fertility rate was recorded in the MR group and the lowest in group C ($P < 0.01$) (Table 6). Furthermore, the fertility rate is better in the M and MR groups than in the C and CR groups ($P < 0.01$). As for the comparison between the animals of the treated groups, those of the MR group show higher fertility than those of the M group ($P < 0.01$). The same situation occured in the untreated groups in which the animals of the CR group show a higher fertility compared to the animals of the C group ($P < 0.01$).

In the comparison between the different groups, the lowest average interval from the introduction of the rams to the lambing was found in the MR group and the highest in the C group ($P < 0.05$). Furthermore, the M and MR groups show a lower average interval from the male introduction to the lambing compared to the C and CR group ($P < 0.05$). Furthermore, the MR group showed a lower interval from the introduction of the rams to the lambing compared to the M group ($P < 0.05$).; the same tendency was found comparing the CR group with the

C group, where the C group showed a higher value ($P < 0.05$) (Table 6). All rams were able to engage in sexual activity, as evidenced by an observation of their sexual behaviour and the recording of ewes with the mark of rams. A difference of about 3% was recorded between mated and pregnant ewes, the same average difference was also recorded between ewes diagnosed pregnant and the lambed ones (Table 6).

In the M and MR groups the mating peak was recorded about 20 days after the introduction of the rams in the groups. While in the CR group the peak was delayed to 30 days after the introduction of the rams and in group C 40 days after the introduction of the rams. Obviously, the same trend was obtained of the date of lambing, in fact the lambing peak of the M and MR groups was recorded at 170 days after the male introduction. While the lambing peak of the CR and C groups was recorded at 180 and 190 days respectively after the male introduction. At the 170th day after the introduction of the rams, the number of ewes that had lambed was more than double in the MR and in M group compared to that of the C and CR group ($P < 0.01$) (Figure 13).

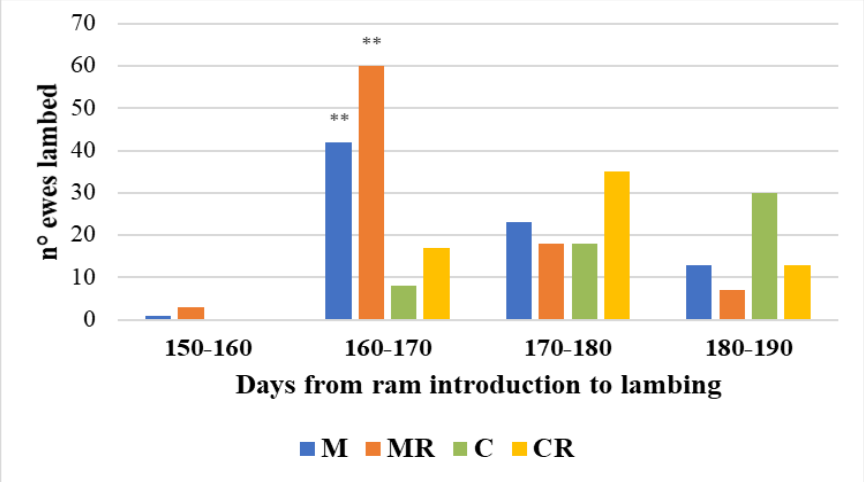

Figure 13. *Graphical presentation of the number of lambed ewes measured at 10 days intervals from 150 to 190 days after male introduction to the four groups. M=melatonin treatment; MR=melatonin treatment and male replacement every week; C=without melatonin treatment; CR=without melatonin treatment and male replacement every week;*

***P < 0.01*

Both, the treatment with MEL implant and the male replacement were not able to influence the litter size (Table 6).

Table 6: *Total, mated, pregnant, lambed and empty ewes, mean ± SD distance in days from male introduction to lambing and mean litter size among the groups*

Group	Total	Mated	Pregnant	Lambed	Empty	DIML	Litter size
M	100	84C	81C	79C	21	171.1 ± 8.2b	1.22
MR	100	92D	90D	88D	12	166.7 ± 5.6a	1.18
C	100	62A	59A	56A	44	178.8 ±8.6d	1.14
CR	100	71B	69B	65B	35	174.4 ± 6.9c	1.20

DIML: distance in days from male introduction to lambing; M: melatonin treatment without male replacement; MR: melatonin treatment and male replacement every week; C: without melatonin treatment and without male replacement; CR: without melatonin treatment and with male replacement every week. Different capital letters in columns differ significantly for $P < 0.01$; Different lowercase letters in columns differ significantly for $P < 0.05$.

3 (influence of MEL treatment in male)

In general, the MS group showed higher fertility than the other groups ($P < 0.01$) (Table 7). The fertility of the MS and CS group was greater than that of the M and C groups, respectively ($P < 0.05$) (Table 7). As for the comparison between the groups in which ewe lambs mated with treated males, those in the MS group show higher fertility than those in group M ($P < 0.05$) (Table 7). The same situation occured in groups in which ewe lambs mate with untreated males, animals of the CS group

show higher fertility than animals in group C ($P < 0.01$) (Table 7). Regarding the interval in days from the introduction of rams to lambing, this was lower in the M and MS groups compared to the C and CS groups ($P < 0.05$). The lowest mean range was found in the MS group and the highest in the C group ($P < 0.05$).

Furthermore, the MS group showed a lower interval from the introduction of the rams to the lamb compared to the M group ($P < 0.05$). The same tendency is showed by comparing the CS group with the C group, in which the C group showed a higher value than CS group ($P < 0.05$) (Table 7).

Table 7: *Fetility rate, mean litter size and mean distance in days from male introduction to lambing of each group of ewe lambs*

Groups	Fetility (%)	Litter size	DIML
M	62C	1,10	177,6b
MS	86D	1,12	174,2a
C	52A	1,08	182,3c
CS	66B	1,12	180,0d

DIML: distance in days from male introduction to lambing; Different capital letters in column differ significantly for P < 0.05; Different lowercase letters in columns differ significantly for P < 0.01.

The interval in days from birth to first lambing was lower in the M and MS groups compared to the other two groups ($P < 0.05$).

4 (influence of different MTNR1A genotypes)

The allelic and genotypic frequency presented no difference in the sheep of the eight farms included in the study. The average of these are shown in the Table 8. The most frequent allele was G (0.68) in position g.17355452 of the latest version of the genome Oar_rambouillet_v1.0 (GenBank access number GCA_002742125.1) and, consequently, G / G was the most frequent genotype (53%). The population was not in Hardy-Weinberg balance due to the small number of heterozygotes ($P < 0.05$).

Table 8: *Genotype and allele frequencies of the MTNR1A gene allelic variant in ewes on the eight farms (n=1200 ewes)*

Allelic variant	**g.17355452 G > A**		
Genotypes	**A / A**	**A / G**	**G / G**
Genotype frequency	0.14	0.33	0.53
Alleles	**A**	**G**	
Alleles frequency	0.32	0.68	

DNA sequencing confirmed the presence of the polymorphic site in all samples. The position of the single nucleotide polymorphism (SNP) reported in this study refers to the Oar_rambouillet_v1.0 genome version (GenBank access number GCA_002742125.1).

The alignment of the sequences with that present in GenBank indicated that there was a total number of eight SNPs: six of these were silent

(g.17355611 T > G, g.17355458 C > T, g.17355452 A > G, g.17355281 G > A, g.17355263 G > A, g.17355173 T > C) and two with an amino acid change (g.17355358 G > A causing p.Val220Ile, g.17355171 C > A causing Ala282Asp substitution in the amino acid sequence (Table 9). The g.17355452 A > G variation was always associated with g.17355358 G > A.

Table 9: *Nucleotide and amino acid changes within the MTNR1A gene exon II in Sarda ewes (n=300 sequenced ewes)*

SNP positions	Nucleotide Changeb	Codon changec	Amino acid changed
g.17355611	T > G	AC**T**/AC**G**	None: Thr135Thr
g.17355458	C > T	TA**C**/TA**T**	None: Tyr186Tyr
g.17355452	A > G	CC**A**/CC**G**	None: Pro188Pro
g.17355358	G > A	**G**TC/**A**TC	Val 220Ile
g.17355281	G > A	CT**G**/CT**A**	None: Leu245Leu
g.17355263	G > A	AG**G**/AG**A**	None: Arg251Arg
g.17355173	C > T	CC**C**/CC**T**	None: Pro281Pro
g.17355171	C > A	G**C**C/G**A**C	Ala282Asp

a: According to the latest genome version Oar_rambouillet_v1.0;

b: Sequence is in a reverse orientation on the Oar_rambouillet_v1.0 genome version, so that nucleotide substitution appears in the reverse form compared to the present study;

c: Nucleotide changes within codons are in bold;

d: According to NCBI Reference Sequence: Chr 26 (NC_040277.1).

A difference of about 3% was recorded between the ewes diagnosed pregnant and the ewes that lambed. Considering g.17355452 G > A SNP, at T1 and T2 the pregnancy rate in ewes with G / G or A / G genotypes was higher compared to sheep with A / A ($P < 0.01$) and to sheep in T3 and T4 ($P < 0.05$) (Table 10). More specifically, ewes with a G / G and A / A genotype, shown a difference in the lambing rate ($P < 0.05$) between the T1-T2 and T3-T4 periods, while the ewes with

A / G genotype shown a similar lambing rate in the different periods (T1-T4).

Table 10: *Lambing rate in the four periods (T1 to T4) based on genotypes at position g.17355452 G > A (n=1200 ewes)*

		Lambing rate			
Time	**Genotypes**	**G / G**	**A / G**	**A / A**	**P**
T1		88%b	78%	60% a	< 0.01
T2		92% b	80%	65% a	< 0.01
T3		85%a	81%	70% b	< 0.01
T4		85% a	81%	74% b	< 0.05

Date of ram joining with ewes - T1: 25 March; T2: 15 April; T3: 5 May: T4: 1 June;Different lower case letters in columns differ P < 0.05; P value refers to the significance within the row.

The average duration (expressed in days) from the introduction of rams was shorter in animals with G / G and A / G than in animals with an A / A genotype ($P < 0.01$) (Table 11).

Table 11: *Duration of time in days from ram joining with ewes to lambing in the four periods (T1 to T4) based on genotypes at position g.17355452 G > A (n=1200 ewes)*

		Duration in days from ram joining with ewes to lambing			
Time	**Genotypes**	**G / G**	**A /G**	**A / A**	**P**
T1		177.6 ± 14.8	179.5 ± 14.2	193.3 ± 14.3	< 0.01
T2		176.1 ± 14.5	178.9 ± 14.0	192.1 ± 15.1	< 0.01
T3		175.7 ± 14.2	178.8 ± 14.3	189.0 ± 15.6	< 0.01
T4		174.8 ± 13.5	178.6 ± 14.6	189.7 ± 15.9	< 0.01

Date of ram joining with ewes - T1: 25 March; T2: 15 April; T3: 5 May: T4: 1 June

Regarding the litter size, both genotypes and dates of ram introduction

(T1-T4) have shown no influence. The average total litter size was 1.20, 1.18 and 1.15 respectively for the G / G, A / G and A / A genotype, therefore quite similar across the three different genotypes.

The tendency of lambing in ewes with different genotypes and through the different periods is presented in Figure 15. In all periods (T1 to T4), ewes with G / G or A / G genotypes showed an earlier lambing compared to sheep with genotype A / A. At T1 and T2 periods, the greatest number of ewes with the G / G genotype, lambed between 160 and 170 days after joining of rams with the ewes. In the same periods, the greatest number of ewes with the G / A genotype, lambed between 170 and 180 days after joining of rams with ewes. Finally, the greatest number of ewes with the A / A genotype, lambed between 190 and 200 days after ram introduction. During T3 and T4 periods the greatest number of ewes with the genotypes G / G and G / A lambed between 160 and 170 days after joining of rams with ewes, while the greatest number of ewes with the genotype A / A lambed between 180 and 190 days after ram introduction.

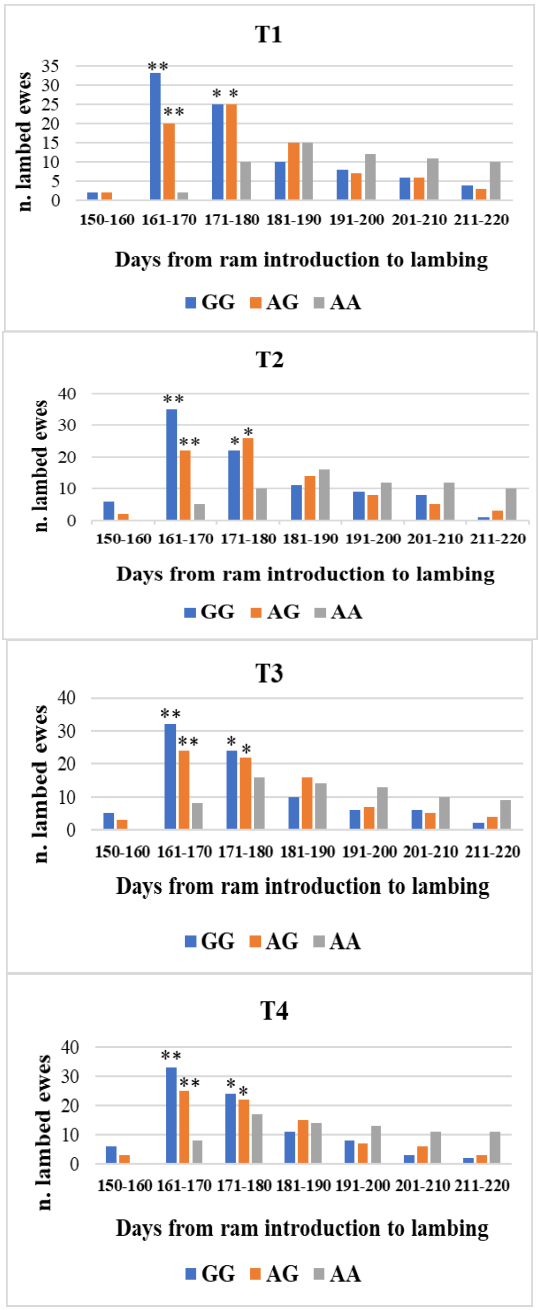

Figure 15. *Presentation of the number of ewes that lambed in 10-day intervals from 150 to 220 days after rams introduction in the four periods, according to genotype position g.17355452 G > A; T1=male joining with ewes on March 25; T2=male joining with ewes on April 15; T3= male joining with ewes on May 5; T4= male joining with ewes on June 1; **P < 0.01; *P < 0.05*

5 (influence of the MEL treatment on milk production and mammary the immune system)

Milk production showed a similar trend in both treated and control groups, with an increase in the 3rd and 4th sampling compared to the others (Table 12; 13). MEL treatment did not influence milk composition. The concentrations of fat, protein and lactose were similar in the two groups through the different samplings (Table 12; 13). SCC was constantly increasing in the control group ($P < 0.05$) from the 1st to the 5th sampling (Table 12). In contrast, in the treated group, SCC showed a remarkable reduction ($P < 0.01$) from the 1st to the 5th sampling (Table 13).

Table 12: *Average milk yield, milk composition and SCC of the C group ewes, in the 5 samplings*

Sampling	Milk yield (g)	Fat (g/100ml)	Protein (g/100ml)	Lactose (g/100ml)	SCC (x103/ml)
1st	1603,01	6,41	5,04	6,29	92,97a
2nd	1726,24	6,32	5,01	6,37	100,99a
3rd	1780,59	6,21	5,01	6,35	107,00b
4th	1789,00	6,35	5,04	6,47	115,68b
5th	1734,53	6,53	5,00	6,35	118,59b

SCC: somatic cells count. Different lower case letters in columns differ $P < 0.05$.

Table 13: *Average milk yield, milk composition and SCC of the M group ewes, in the 5 samplings*

Sampling	Milk yield (g)	Fat (g/100ml)	Protein (g/100ml)	Lactose (g/100ml)	SCC (x103/ml)
1st	1600,03	6,46	5,07	6,38	98,74B
2nd	1732,09	6,32	5,14	6,31	96,84B
3rd	1827,48	6,24	5,11	6,33	78,55A
4th	1897,10	6,38	5,21	6,41	69,71A
5th	1723,84	6,47	5,34	6,38	58,13A

SCC: somatic cells count. Different capital letters in columns differ P < 0.01.

Discussion

1a (MEL treatment in different periods of the year)

In the first phase of my thesis I have studied response of different parameters in sheep to melatonin treatment. More precisely, I evaluated the response when the treatment was performed at different times of the year and at different lactation periods.

The results showed that MEL is able to advance reproductive activity in a dairy sheep. These data are in agreement with other previous studies on the Sarda sheep breed (Carcangiu et al., 2012; Luridiana et al., 2015; Mura et al., 2017) that have shown how melatonin has improved reproductive efficiency in this sheep breed. The results are also in agreement with the results of other studies conducted on different sheep breeds raised in the Mediterranean basin (Chemineau et al., 1991-1996; Haresign, 1992; Staples et al., 1992; Papachristoforou et al., 2007).

In particular, as regard the treatment in different period of the year, experiments performed mainly from late April to September, are not completely comparable to this experiment (Haresign et al., 1990; Forcada et al., 1995; Scott et al., 2009). On the other hand, the data indicate that the treatment time influences the reproductive response;

in fact, the fertility rate was higher in animals treated with MEL implant in April and May (early spring) than in animals implanted between February and March (late winter). This result is not in agreement with the results of Abecia et al. (2007) obtained in three sheep breeds in Spain, where the fertility of the Rasa Aragonesa sheep did not improve when MEL was implanted in January and February (winter) compared to April and May (spring), it increased fertility in the Assaf breed, but this did not happen when it was administered in April (spring). In the same experiment, the MEL implantation in the Merino breed in February (winter) and April (spring) led to an improvement in fertility rate but not in January (winter) and May (spring) (Abecia et al., 2007). These results are different probably due to the different sensitivity to photoperiod in the different sheep breeds. In fact, after a short-day period, a photo-refractory period occurs in different sheep breeds, corresponding to the profound anestrous which can have an individual length in the different sheep breeds (Chemineau et al., 2010). Therefore, the breed differences in response to MEL and male effect will have an impact on reproductive efficacy. However, the present results show that in the four treatment times, the reproductive response was different, in fact the treatment performed in spring was more effective than those performed in winter.

The beginning of the breeding season is a consequence of numerous

long days the sheep were exposed to (Malpaux et al., 1989) and it is possible that this phenomenon influenced on Sarda sheep to respond to a short-day treatment like that derived from MEL implants (O'Callaghan et al., 1994; Malpaux et al., 1997). Sweeney et al. (1997) hypothesized that a sheep is able to restart its reproductive activity in response to 35 long days (photo-refraction period) followed by short days or MEL treatments at any stage from the winter solstice to that of summer. To confirm this, the first treatment with melatonin was performed on 10th February in this study, thus about 50 days after the winter solstice. Therefore, it is plausible that the sheep were already in a late phase of their period of photorefractoriness and that the progressive increase in the fertility rate in the other three doses was due to a greater sensitivity of the reproductive system to MEL.

The differences in fertility rates between treated and control sheep are primarily due to the direct effect of MEL on the ovary (Yie et al., 1995) and also due to its effect on the secretion of gonadotropin at the hypothalamic pituitary level (Viguié et al., 1995). Furthermore, in present experiment a difference was established between sheep treated with MEL and those not treated in lambing periods. For all four treatment times, the treated ewes showed fewer days after introducing the rams to lambing than the controls. This could be explained that treated animals had a faster response to the male effect than the

controls. The treated sheep mated within the first 17 days after the introduction of the males and among these the percentage of cyclic sheep was higher compared to the sheep of the untreated group. The MEL implantation was able to stimulate the restart of reproductive activity also according to the results of Abecia et al. (2006). The animals treated with MEL showed reproductive activity about a week before the untreated ones. Indeed, between 160 and 170 days after the introduction of the males, the number of the lambed ewes was higher in the treated group than in control group. In contrast, between 180 and 190 days after male introduction, the number of lambing was higher in the control group than in the treated group. This tendency is probably linked to the carry-over effect, which occurs when treated and control animals are kept jointly (Abecia et al., 2006). When the sheep treated with melatonin and the untreated ones are kept together, the start of the reproductive activity is anticipated in the control sheep compared to the isolated controls (Kennaway et al., 1987). In addition, the introduction of oestrus sheep, exposed to controlled photoperiod, in anestrous flock leads to a beginning of reproductive activity, indicating that female-female social interactions can influence the timing of reproductive transitions in sheep (O'Callaghan et al., 1994). Establishing the number of sheeps in the flock to be treated to achieve the female-female effect on the beginning of reproductive activity

could be important for farmers, since the treatment of a part of the flock is enough to improve the reproductive efficiency of the whole flock. It is clear, that in the present research, treated ewes had an advance of the start of lactation compared to control ewes, and this translates into an economic advantage for farmers. In fact, the treated ewes had on average 10 days less time between male introduction and lambing compare to control ewes. Regarding the litter size, the same value was observed in treated and control ewes in all four treatment times of this study, as established also in previous studies on the Sarda sheep breed (Carcangiu et al., 2012; Luridiana et al., 2015). The data of this study did not agree with those of Abecia et al. (2002) and Scott et al. (2009), which found an improvement in the litter size in treated ewes. Furthermore, Abecia et al. (2007) using MEL, found an increase in litter size only in the Rasa Aragonesa breed, but not in the Assaf breed. Some other studies have also not found on effect of MEL treatment on the litter size (Rajkumar et al., 1989; Schoeman and Botha, 1995; Gates et al., 1998). Therefore, since Sarda and Assaf are both dairy sheep, we can assume that the absence of the effect of melatonin on the litter size could be due to the genetic characteristics of these breeds.

1b (MEL treatment in different lactating period)

As regard the MEL treatment at different lactating periods, the results have shown that the treatment was effective in advancing the reproductive resumption in all groups of ewes. In fact, the treated animals responded earlier to the ram effect, thus showing a more compacted mating and lambing periods, compared to untreated animals. Furthermore, MEL treatment caused a significant improvement in fertility rate showing a greater number of lambed ewes compared to the control subgroups. It is assumed that MEL stimulates secretion of GnRH and LH, and even directly the ovary, which improved the fertility rate in treated ewes (Abecia et al., 2006; Tamura et al., 2009). MEL receptors are present in different ovary structures in which this hormone produces a greater number of ovulatory follicles, less atresia and is involved in improving oocyte maturation and luteal function (Yie et al., 1995; El -Raey et al., 2011; Tian et al., 2017). Furthermore, another role of MEL could be to protect oocytes from free radicals damage, thus favoring the growth of follicles (Tamura et al., 2014). The best reproductive performance was recorded in ewes that lambed between October and December compared to the other groups. The lowest fertility rate was observed in ewes lambed in January and February, both treated and controls, which is certainly

related to the metabolic-hormonal state typical of lactation. These ewes were in a phase of high milk production and the body's resources were used for milk secretion (Carcangiu et al., 2012).

As confirmation of what has already been observed in the previous research, the litter size did not show variations between the groups. In previous studies we have confirmed that treatment time is able to influence the response to melatonin treatment. In this study we wanted to evaluate how the melatonin treatment can improve the response to male effect.

This experiment confirmed effects of the MEL treatment shown in previous studies. Ewes treated with MEL responded more quickly to the male introduction than the untreated ones. As a result, mating was advanced and consequently the lambing period was more concentrated in the groups of treated ewes than untreated. The results of Abecia (2006) are consistent with those of the present study. It can be concluded that MEL certainly plays a role in the transition from an anoestrus phase to full reproductive activity, with its effects on the hypothalamus, pituitary gland and ovary. The effect of MEL on the hypothalamic-pituitary axis could be mediated by the Kisspeptin protein, which participates in the reproductive seasonality command (Smith 2012). In the arcuate nucleus of the hypothalamus, kisspeptin

mRNA expression is higher during the reproductive period than in the anestrus season, consequently there is an increase in GnRH secretion during the reproductive season (Chalivoix et al., 2010; Beltramo et al., 2018).

Furthermore, in this study it was found that fertility rate was higher (about 10%) in the groups with male weekly replacement compared to those in which the males were not replaced.

These results are consistent with those of other authors who have shown that male replacement every 15 days leads to a better reproductive response (Hawken et al., 2009a). This factor, as reported by Hawken et al. (2009b), could be due to the novelty effect triggered by the presentation of new males in the ewe group. Another important consideration is that male replacement has greatly improved the effect of MEL on fertility. For this reason, it can be assumed that MEL has favored the transition to a less profound anoestus and that male substitution has further strengthened the start of reproductive activity. Today it is known that the male effect is more effective in superficial anoestrus, which is the transition period between the deep anoestrus and the reproductive season, if compared to the anoestrus period (Chanvallon et al., 2011). The male effect causes in the anoestrus ewe increase in the secretion of LH and estrogens which bring a positive

feedback for the preovulatory peak of GnRH and LH (Martin et al., 1986; Chanvallon et al., 2011). The high level of estrogen secretion is associated with increase in acute steroid regulation (STAR) in granulosa cells (Fabre-Nys et al., 2015). In conclusion, it can be stated that the best reproductive performance was recorded in the group where the sheep were treated and in which the males were replaced, compared to the other groups. Simultaneous action of all these factors is most probably the cause of this. Therefore, the male replacement reinforces the male effect in stimulating the resumption of reproductive activity in sheep. Finally, this technique, combined with the use of the MEL implant, would certainly improve the reproductive efficiency in commercial Sarda sheep farms.

2 (MEL treatment in ewes and male replacement) and 3 (influence of MEL treatment in male)

The next phase of the research was to evaluate how the treatment with MEL in males, in addition to the male effect, can influence reproductive activity in sheep.

The treatment of males and their weekly replacement has been shown to anticipate puberty in Sardinian sheep lambs. The male substitution has shown to be a valid method to stimulate the beginning of

reproductive activity in lambs. Evidently the "novelty effect", determined by male replacement every week, and the MEL treatment of males are able to stimulate the hypothalamus-pituitary-ovary axis in an effective way to determine a better fertility of the ewe lambs.

Like ewes, also males show a period of decline in reproductive activity at the end of winter with a decrease in semen quality and libido. In male treatment with MEL, performed after long-day sensitization improves their reproductive performance. This is demonstrated by numerous experiments carried out in different sheep breeds, in fact an increase in testosterone and an improvement in male libido have been observed after treatment with MEL (Abecia et al., 2011; Rosa & Bryant, 2003). The introduction of MEL-treated males in the flock showed a marked improvement in the reproductive response of the sheep with an increase in their fertility (Abecia et al., 2016b). Furthermore, Abecia et al. (2016a) reported an advance of the puberty of Rasa Aragonesa lambs when subjected to males sensitized with increasing photoperiodic and MEL treatments. The results of our research are in agreement with the aforementioned results, indeed an advance of puberty was observed in groups of ewe lambs in which males treated with MEL were introduced compared to controls. Presumably, these MEL-treated animals were able to further stimulate the reproductive activity of ewe lambs because they were more

sexually active. In fact, we should also consider that the males were introduced on 1 July and therefore have been widely sensitized to long photoperiods, which seems to be the essential requirement to obtain the effect of MEL, as reported by Abecia et al. (2017). However, in the present research the best results on reproductive activity of ewe lambs were obtained when MEL treatment in males was combined with weekly male replacement. Evidently, the inclusion of new sexually active males has created an additional male effect, also called "novelty of the effect", which has further stimulated the reproductive activity in ewe lambs with an advance of puberty (Hawken et al., 2009).

This confirms what was already shown in the previous study of this thesis in which the substitution of males guaranteed a stimulus to the reproductive recovery of the adult sheep. Results of this study demonstrate that the ewe lambs subjected only to the substitution of the males showed an advance of puberty equal to that found in the group of ewe lambs subjected to males treated with the MEL only. All this confirms that treatment with MEL in males is a valid method to improve their reproductive performance. Furthermore, treatment of males with MEL combined with their weekly replacement is a valid method to stimulate the start of reproductive activity in lambs.

4 (influence of different MTNR1A genotypes)

After confirming the role of MEL treatment and the male effect on reproductive activity, we wanted to evaluate the influence of different MTNR1A genotypes.

In this study we analyzed the sequence of exon II of the MTNR1A gene. The genotypic and allelic frequencies of the analyzed locus were in agreement with those previously reported by studies conducted in the same breed (Carcangiu et al., 2009b; Luridiana et al., 2015a). In position g.17355452 G > A of the sequence of exon II of the MTNR1A gene, the Sarda sheep breed had a relatively higher frequency of the mutant G allele. This is similar to other European sheep breeds (Messer et al., 1997; Mateescu et al., 2009). In contrast, in *Ovis gmelini musimon* (a wild sheep) allele A was mainly present (Carcangiu et al., 2010). Comparing the frequency of the G allele in domestic sheep breeds, there was a lower percentage in our study compared to Rasa Aragonesa and other sub-temperate and subtropical sheep breeds such as Magra, Marwari, Chokla, Malpura, Patanwadi, Sandyno and Niligiri (Martínez-Royo et al., 2012; Saxena et al., 2014; 2015a, 2015b). The results of the present study indicate that the reproductive response to the male introduction of adult ewes of Sarda breed is influenced by the polymorphism in g.17355452 G > A. In fact,

the ewes with the G / G or A / G genotypes showed a higher lambing rate and a shorter duration in days between ram introduction to lambing compared to those with the genotype A / A, in all the periods in which the evaluations occurred. The result is in agreement with those of previous studies conducted on different sheep breeds, in which the G / G genotype at the same locus was associated with breeding and conception out of season and with a shorter interval between the first and the second lambing (Chu et al., 2006; Mateescu et al., 2009; Carcangiu et al., 2012). Moreover, the Sarda ewe with the G / G genotype showed a shorter anoetrus period in spring after melatonin administration (Mura et al., 2017). The current results indicate that in the Sarda breed, the presence of only one G allele influences the reproductive response to the male union with the ewes and the results are in agreement with those previously reported for the same and other sheep breeds (Mateescu et al., 2009; Mura et al., 2014). In other European sheep breeds, the same allelic variant as evaluated in the present study had no effect on reproductive performance (Hernandez et al., 2005; Martínez-Royo et al., 2012). This difference may be due to breed or the effects of environmental factors. In fact, in many studies the fertility of the ewes was recorded after the union of the males with the ewes, while in the study by Hernandez et al. (2005) reproductive activity was assessed only by recording progesterone

concentrations and associating them with whether ewes were or were not pregnant. Therefore, the lack of male effect could have influenced the results of the aforementioned studies. In the present study, the increased lambing rate in sheep with the G / G or A / G genotypes confirmed the hypothesis of a lower photoperiod sensitivity of the sheep with these two genotypes. It is possible that this lower sensitivity resulted in a shorter period of anoetrus, which caused a better response to the union of rams and ewes, compared to sheep with the A / A genotype, which could have longer periods of anoestrus. The different hypothalamic sensitivity to photoperiodic stimuli could be the basis for the different reproductive responses between the three genotypes. The difference in the fertility rates of the sheep with the A / A genotype among the ewes joined with rams in the T1-T2 and T3-T4 periods could be due to the gradual transition from anostrous to the reproductive season, with a consequent higher hypothalamic sensitivity to the estradiol (E2) positive feedback signal, as proposed by Fabre-Nys et al. (2015) or a reduction in the negative feedback to estradiol (Karsch et al., 1984). Furthermore, from the tendency of lambing, it can be assumed that animals with different genotypes have different anoestrus duration. This is supported by the fact that ewes with genptypes G / G and A / G lambed before the ewes with the genotype A / A, which also confirms the most rapid response to the union with

the males due to shorter periods of anoestrus. This effect, however, is difficult to explain because this allelic variant is not associated with a change in the amino acid that influences the transdomains and therefore should not be involved in the functionality of the receptor. The eight mutations detected in exon II of the MTNR1A gene (g.17355611 T > G, g.17355458 C > T, g.17355452 A > G, g.17355281 G > A, g.17355263 G > A, g.17355663 T > C, g.17355358 G > A, g.17355171 C > A) were the same as those found in previous studies in Sarda (Carcangiu et al., 2009b) and in other sheep breeds (Pelletier et al., 2000; Reppert et al., 1994). Moreover, in the studies of Reppert et al., (1994) and Pelletier et al., (2000) the same genetic trait and two other mutations have been detected, in position g.426 C > T and g.555 G > A of the melatonin receptor sequence with GenBank access number U14109 corresponding to position g.15099671 C > T and g.15099575 G > A of the version of the genome Oar4.0: NW_014639035.1, respectively. None of these allelic variants leads to changes in amino acids. Instead, Saxena et al. (2014) reported another mutation in the Indian Chokla sheep breed, at position g.931 G > C of the sequence U14109, corresponding to g.15099166 of the sequence NW_014639035.1, which also determines a substitution of amino acids (p.Ala295Pro). These differences are probably due to the different evolutionary paths of the breeds, based on the focus of the

selection for the different products (milk, meat or wool). In our study, and in agreement with the study by Saxena et al. (2015b), the variation g.17355452 A > G has always been associated with g.17355358 G > A, which determines a variation amino acids in the position p.Val220Ile (reference sequence NCBI: Chr 26 NC_040277.1). These data are very significant because of the relationship between these variations that may explain the effect on reproductive seasonality of the polymorphism g.17355452 G > A. Although nucleotide substitution g. 17355358 G > A leads to a change in the amino acids in the protein sequence, it is not part of the transmembrane domain of the MTRN1A gene and this is in agreement with that found by Barrett et al. (2003), so there should be no changes in receptor functionality. However, the position of this amino acid change in the protein is close to histidine in positions 211 and 195 of the amino acid sequence (NP_001009725.1), which are involved in MEL signal transduction (Conway et al., 1997; Kokkola et al., 1998). This amino acid change could be the cause of a change in the steric conformation of the amino acid chain with a consequent alteration of the signal (Trecherel et al., 2010). The substitution of the amino acid p.Val220Ile influenced the inhibition of adenylate cyclase, thus proposing a possible modification in the transmission of the MEL signal in this variant (Trecherel et al., 2010) and therefore the differences shown in the reproductive

functions between ewes with genotypes G / G, A / G and A / A (Pelletier et al., 2000). It is clear that studies on the second messengers involved in the activation of the melatonin receptor are needed to elucidate this hypothesis (Kokkola et al., 1998; Brydon et al., 1999). In a study by Calvo et al. (2018) conducted in the Rasa Aragonesa sheep, it was assumed that the SNP in position g.15099004 C > T could be the causative mutation for the effects on the reproductive seasonality traits, since there is an arginine-cysteine substitution in the amino acid sequence at position 349. In our study with Sarda sheep, this variation has not been identified, but this research indicates interesting prospects for further investigation. Moreover, the data of the present study indicate that in ewes with G / G or A / G genotypes, the replacement of males with ewes induced a higher stimulus on the resumption of reproductive activity when the replacement was performed at T1 and T2 compared to T3 and T4. This was surprising, since the reproductive response in ewes with the G / G and A / G genotypes was expected to be increased from March to June. Instead, the effect was better when the number of ewes that were in anoestrus was higher (March and April), rather than when the time that the initiation of the oestrous cyclic functions was approaching at the end of the anoestrous period when induction of oestrous cyclic functions is usually simpler to induce (May and June). It is assumed that the effect of the G allele is

greater when the length of the day is shorter, confirming that ewes with one or both G alleles are less sensitive to photoperiod inhibition of reproductive functions. The lambing data in our study were variable when there was an evaluation of the different genotypes and periods. In fact, the ewes with the G / G or A / G genotypes had the lamb peak before the ewes with the genotype A / A for all the periods in which the evaluations occurred. Obviously, the tendency of the lambing rate is associated with the mating tendency, so that the ewes with the G / G and A / G genotypes had a greater response to the union of males with ewes. In different studies, an investigation on the origin of variability in the reproductive response to the union of males with ewes was conducted. A study by Chanvallon et al. (2011) reported that at the end of the anoestrus season there was a better response to the male introduction to the ewes. Instead, in our study, ewes with the G / G and A / G genotypes were not influenced by photoperiodic repression that in all the examined periods and better response of the ewes to the union with males could be due to a number of positive factors, such as increased estradiol secretion along with higher amounts of acute steroid regulatory protein (STAR) in granulosa cells Fabre-Nys et al. (2015). Further studies are needed to clarify whether the G allele may be associated with better secretion of E2 and higher amounts of STAR than the A allele.

5 (influence of the MEL treatment on milk production and mammary immune system)

In recent years, a number of studies have been conducted on the influence of MEL on the quantity and quality of milk and on the regulation of immune activity. The double effect of MEL, direct and indirect, on milk secretion is known in several species. In fact, the administration of MEL in cows showed a reduction in milk production and in the concentration of lactose but did not affect the other milk constituents. Moreover, in cows, goats and sheep, milk secretion is greater during long photoperiods compare to short photoperiods (Morrissey et al., 2008; Garcia-Hernandez et al., 2007; Peters et al., 1981). Prolactin was the initial candidate responsible for the galactopoietic effects of the photoperiod. During long days circulating concentrations of prolactin increase in different species, including cattle (Tucker et al., 1984). On the contrary, in short days, high MEL levels cause a reduction in circulating prolactin (Sanchez-Barcelo et al., 1991). Considering the effects of prolactin on milk secretion and the inhibitory effect of MEL on prolactin, the mechanism that determines this decrease in milk production is probably explained. However, in our research carried out during the long days there was no decrease in daily milk production or changes in milk composition. This

suggests that perhaps MEL too low dose was used to influence prolactin secretion. However, the dose we use is the normally used on to stimulate reproduction and therefore able to mimic short days in sheep. In previous experiments in the Sarda sheep breed the use of a prolactin antagonist, despite having resulted in almost total reduction of prolactin, did not cause a decrease in milk production (Floris et al., 1990). Therefore, further research is needed to clarify, at least in the Sarda sheep breed, the effect of MEL on milk secretion.

The effect of MEL on the immune system of human and other laboratory animals is well known (Carrillo-Vico et al., 2013). However, the effect of MEL administration on udder health is not elucidated yet. Udder pathologies such as mastitis are the main determining factor in the reduction of milk production in dairy animals (Yang et al., 2017). The main problem is related to subclinical mastitis since they are asymptomatic and difficult to diagnose but still cause a quantitative and qualitative decrease in milk production. One of the parameters that offers considerable help in the diagnosis of these diseases are the somatic cells present in the milk. The increase in the cells count is a sign of udder health problems. In cows and goats, the administration of MEL has led to a decrease in the number of somatic cells in milk, suggesting that this molecule stimulates the immune system by improving udder health (Yang et al., 2017).

In sheep this is the first study that examines the effects of MEL administration on the udder. The results obtained are in agreement with what was observed in cows and goats with a substantial decrease in the number of milk SCC in the three consecutive samples analyzed after the treatment. This effect could be due to the antioxidant action of MEL, which protects cell against free radicals damage (Yang et al., 2017).

Clinical and suclinical mastitis cause a decrease in quantity of milk produced, a decline in milk quality and an increase in somatic cells count. Furthermore, in cows with subclinical mastitis, the administration of MEL also causes a decrease in blood cortisol compared to healthy one controls (Yang et al., 2017). This well-known hormone has an important role in inflammation leading to an immunodepression with a decrease in the number of lymphocytes and acidophilic leukocytes in the blood. Therefore, the decrease of cortisol levels could have affected the immune response favoring the improvement of the health status of the mammary gland (Lecchi et al., 2016; Malinowski and Gajewski, 2010). Therefore, MEL treatment in cows led to a decreasing of the SCC in milk and improved milk quality (Zang et al., 2017). The effect of MEL deserves a further study because it could notably improvement not only health of the mammary gland but of the entire animal. In fact, the administration of foods rich

in this molecule could give considerable advantages, for example reducing the use of antibiotics. *Zea mays* supplementation in goat with regular diet for 40 days significantly increased the endogenous MEL blood level, proliferative response of peripheral blood mononuclear cells and antioxidative enzymes activity with total antioxidant capacity of the plasma. An increase in circulatory IL-2 and IL-6 level with declined TNF-α, malondialdehyde and nitric oxide was noted with elevated endogenous MEL concentration (Singh and Haldar, 2017). Therefore, the use of these foods could be used in order to increase the functionality of the immune system and the antioxidant capacity of the organism.

Conclusions

The present thesis has provided several important results, which certainly improve the knowledge of the mechanisms by which melatonin acts and its use. Furthermore, the provided data can be used to improve management of reproductive seasonality and animal health.

The data confirmed that melatonin is able to advance reproductive recovery in Sarda sheep breed in spring. Furthermore, the most suitable time of a year to perform the MEL treatment in order to obtain the best reproductive response has been identified. Treatment with subcutaneous melatonin implants in April and May guarantees the best response to the male effect in sheep.

It has been established how long after lambing is the best reproductive response to treatment with melatonin in sheep. The obtained data indicate that melatonin treatment should be performed 3 or 4 months after lambing, in order to obtain optimal reproductive results.

Management of rams has shown to have a remarkable importance in optimizing the reproductive activity of the Sarda sheep breed. A weekly substitution of the males in the flock guarantees a better reproductive efficiency both in the adult ewes and in the ewe lambs. Furthermore, the treatment with melatonin in males has shown to improve their reproductive activity and consequently the reproductive

response of sheep to the male effect.

Another interesting conclusion is that treatment with melatonin in lactating sheep does not change the quantity and quality of milk. This result is comforting since melatonin has shown to influence the quality and quantity of milk in other dairy species.

Finally, the administration of melatonin has shown to influence the number of somatic cells in milk. The treated sheep showed a decrease of somatic cells count in milk over a month, suggesting that this molecule can preserve health of the udder.

In conclusion, this thesis has provided many results directly applicable in Sarda sheep farms in order to improve reproductive efficiency. Furthermore, it has shown an interesting additional use of melatonin for preserving animal health. Investigating the effects of melatonin on immunity, and perhaps administering foods with high contents of this molecule, is certainly very important and could provide important improvement of animal health and production.

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