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Variation in frequency of alleles in the *MTNR1A* gene with possible impact on ability of ewes to show oestrus out of season

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Variation i frekvens av alleler i *MTNR1A*-genen med påverkan på tackors möjlighet att visa brunst utanför ordinarie brunstsäsong

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Abstract

In Swedish sheep industry it is desired to have lambs slaughtered evenly distributed over the year. Swedish sheep are generally seasonal breeders with lambs born in the spring and slaughtered in the autumn. Some of the breeds, e.g. Swedish Finewool and Roslag sheep breeds have shown ability to lamb out of season. For increased knowledge on the possibilities to breed for out-of-season lambing in Swedish sheep breeds, the aim of this master thesis is to study the variation in frequency of two alleles in the *MTNR1A* gene in the three breeds; Gotland, Swedish Finewool, and Roslag sheep. This master thesis is divided into two parts. The first part is a literature review with emphasise on reproduction physiology, methods for heat synchronisation and induced out-of-season reproduction, selection for out-of-season reproduction and description of different methods for DNA analyses. Many of the investigated studies have shown a connection between out-of-season breeding and an increasing amount of C alleles in the polymorphic position C606T and G alleles in the polymorphic position G612A on the *MTNR1A* gene. Another study has also detected a relatively high frequency of the favourable *MTNR1A* alleles but on seasonal breeds. In the second part of the thesis the allele frequency and genotypes of nucleotide position 606 and 612 in the *MTNR1A* gene for three different Swedish sheep breeds were investigated. Blood was sampled from around 30 animals of each breed. DNA was extracted from the blood and the sequence containing both SNPs of the *MTNR1A* gene, was analysed. The three breeds had a similar allele frequency for both positions and the assumed seasonal Gotland ewes had a high frequency (0.87) of the G allele in position G612A. The results indicate that the SNPs investigated are not good markers to decide the Swedish breeds' ability for out-of-season breeding.

Sammanfattning

Inom den svenska fårnäringen finns en önskan om en jämnare fördelning av slaktade lamm över året. De svenskt fårraserna är i allmänhet säsongsbundna och de flesta lammen föds således på våren och slaktas på hösten. Raser som till exempel roslagsfår och det svenska finullsfåret har emellertid visat sig kunna lamma utanför den ordinarie lamningssäsongen. Syftet med detta examensarbete är att öka kunskapen kring svenska fårrasers möjlighet att lamma utanför den ordinarie lamningssäsongen genom att studera eventuell variation i frekvens av alleler i *MTNR1A*-genen hos gotlandsfår, svenska finullsfår samt roslagsfår. Arbetet är uppdelat i två delar. Den första delen är en litteraturstudie inriktad på reproduktionsfysiologi, metoder för synkroniserad brunst och inducerad reproduktion utanför brunstsäsong, selektion för reproduktion utanför brunstsäsongen samt en beskrivning av olika metoder för analys av DNA. Många av de granskade studierna indikerar ett samband mellan reproduktion utanför brunstsäsongen och en ökande mängd C-alleler på den variabla positionen C606T och G-alleler på den variabla positionen G612A i *MTNR1A*-genen. En av de granskade studierna har sett samma ökning av dessa alleler och på samma positioner men i detta fall inom säsongbundna raser. I den andra delen av examensarbetet undersöktes allel- och genotypfrekvenser för position 606 och 612 på *MTNR1A*-genen för de tre raserna. Blod togs från ca 30 djur av varje ras. DNA extraherades sedan från blodet och en sekvens innehållande de två polymorfa platserna på *MTNR1A*-genen analyserades. De tre raserna hade liknande allelfrekvenser vid de båda positionerna och de förmodat säsongsbundna gotlandstackorna hade en hög frekvens (0,87) av allelen G i position G612A. Resultaten tyder på att de undersökta polymorfa platserna inte är

några bra markörer för möjligheten att avgöra de svenska rasernas förmåga att reproducera utanför den ordinarie brunstsäsongen.

Introduction

In the end of the nineties, the meat industry in Sweden started to show an interest for lamb production in other seasons than in the autumn (Gates et al., 1998). Before this the slaughter houses froze a large amount of the lamb carcasses to secure the consumers demand for lamb meat during the year. The change was due to the high cost of storing, but also due to new methods for out-of-season breeding. Fresh lamb meat has become trendier in recent years, especially as an ecological and local product. The slaughter cooperatives give the sheep producers a premium price for lambs that are delivered in seasons when the demand is high, as for example in Easter. A contract between the producer and the slaughter house is often designed in beforehand. This contract states the number and in which week the lambs should be delivered. The lambs should also have a specific carcass classification and be of uniform size, otherwise the payment for the animals will be reduced (SLS, 2012). In Figure 1 the price, per kilogram (kg) lamb meat that the producer could get under various weeks is displayed. The weekly price notations for lamb meat were received from Swedish Slaughter animals & Service (SLS) (Czérna, 2012) which is a company for purchasing of Swedish animals to the slaughter cooperation Scan (SLS, 2012). Depending on these fluctuations in price over the year the interest for sheep breeds with year around lambing has increased. In this aspect, it is also important to stress that the feed cost in for example spring lamb production is much higher than for autumn lamb production due to the grazing period.

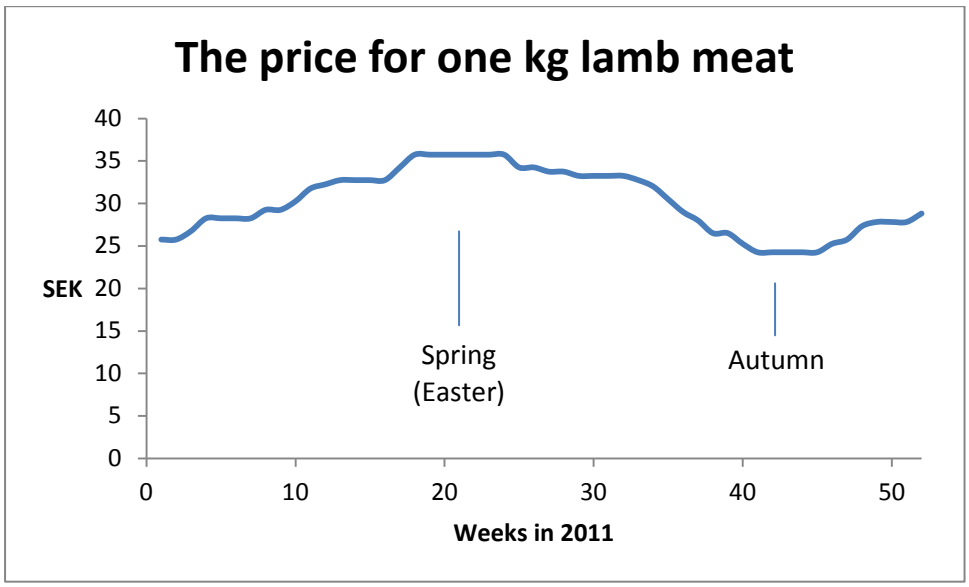

Figure 1. The price is calculated per kg lamb on a lamb with carcass weight between 16- 22.9 kg, class R and without any addition or reduction.

Sheep breeds differ in breeding patterns and oestrus expression, especially if they are in different climate zones (Hafez & Hafez, 2000). Sheep breeds in the temperate climate zones have a more seasonal reproduction compared to breeds in tropical zones. This is due to decreasing day length and the onset of an increasing amount of melatonin. In tropical zones the variation in day length

is less which enables year around breeding. This is also true when introducing breeds from temperate zones in tropical zones. However some of the breeds in Sweden, e.g. Swedish Finewool (Gates et al., 1998) and Roslag sheep breeds have shown ability to lamb out of season, whereas the Gotland ewes are known to be seasonal breeders (Näsholm & Eythorsdottir, 2011). Reppert (2004) has made a study that indicates variation in oestrus expression among and in different breeds depending on the genetic expression of high affinity melatonin receptors. In their study the melatonin receptor 1A gene (*MTNR1A*) has been shown to associate with the ability to lamb out of season.

This master thesis includes a literature study with description of the genetic and physiological background for reproduction and out-of-season breeding in sheep. The aim was to study any variation in frequency of the mutations C606T and G612A on the *MTNR1A* gene in the Gotland, Swedish Finewool, and Roslag sheep breeds. Our hypothesis was that alleles for C606T and G612A of the *MTNR1A* gene are associated with the ability for the Swedish Finewool and the Roslag sheep to lamb out of season, whereas other alleles are associated with the seasonality in reproduction shown by the Gotland sheep.

Literature review

Reproduction physiology in sheep

Spring is the most optimal time for animals in temperate climate zones to deliver their progeny (Noakes et al., 2001). Ewes have a gestation length of approximately 150 days, and in for example several Swedish breeds the ewes come into heat in late autumn. This means that they are seasonally polycyclic (Hafez & Hafez, 2000) and can come in heat several times during a shorter period of time as long as the environment remains favourable. The hormonal process behind heat is regulated by for example environmental factors like decreased day length which increases the production of melatonin from the epiphysis (*Corpus pineale* or pineal gland). The right amount of circulating melatonin will initiate follicular growth and ovulation. Other factors that affect the time of heat are the age of the ewe, the breed and the ewe's nutritional state.

One strategy used to increase the number of eggs during ovulation is to provide supplementary food for ewes in the breeding period. This is called flushing (Eggertsen, 2007). Henderson (1990) however, argues that the best results are obtained if the ewes have an average body score or a good body condition. Descriptions of a good body condition in sheep can be found in most sheep rearing manuals or books. Starvation or lack of required amounts of feed before or during the gestation period usually lowers the number of lambs per ewe. The same applies if the ewes are over-fed and get fat. Hafez and Hafez (2000) write that supplementary feeding with lupines can increase ovulation rate in Merino sheep. Another important thing to consider in sexually active ewes is that they should not be rushed or stressed (Söderquist, 2007). Therefore, it is favourable if the shearing of sheep occurs before fertilisation. It could also be necessary to give the ewes an addition of vitamins and minerals. In Sweden, the amount of selenium in the forage, for example, is poor and lack of this micronutrient in the ewe can result in week or dead lambs. Therefore it is essential to give a small extra amount of selenium to pregnant ewes in Sweden.

According to Noakes et al. (2001) ewes reach puberty at the age of six to fifteen months. In the study by Hafez and Hafez (2000) it was mentioned that the age of the first heat takes place when the ewe is five to seven months old. The variation in puberty age is likely caused by a number of

factors. The complex process of sexual matureness includes cyclic activity, ovulation, and seasonality as well as heat symptoms.

According to Henderson (1990), it is difficult to detect if a ewe is in oestrus as the oestrus signs in ewes can be very subtle. With the help of a ram oestrus detection is much easier and the rams also can induce synchronised oestrus. The ram could either be fertile or a so-called teaser ram, which is sterilised (Evans and Maxwell, 1987). Thus he has intact testicles and pheromones can be secreted. If the ewe is in oestrus, she walks up to the ram to be covered. There is often competition between the ewes. This can cause the young ewes to avoid showing oestrus signs (Henderson, 1990).

The length of the heat cycle varies from 14 to 19 days, and the oestrus, which is the period when the mating takes place, is approximately two days long. About 30-36 hours after the onset of the heat or oestrus, ovulation of follicles takes place. The number of follicles that are ovulated is affected by age and breed (Hafez & Hafez, 2000).

In the autumn, shorter day length will increase the amount of melatonin that is secreted into the blood circulation. The increasing melatonin levels will stimulate hypothalamus to produce increased amounts of gonadotropin releasing hormone (GnRH) (Malpaux et al., 1999). GnRH is a hormone that stimulates the pituitary gland to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (SLU, 2009). Follicular growth in the ovaries is stimulated by FSH. The follicles are vesicular-like structures containing an oocyte. The follicle produces oestrogen (more as the follicle grows) that prepares the uterus for implantation and causes the heat symptoms. When the follicle has reached its maximum size, it will burst and the oocyte (the egg containing female germ cells) will travel into the uterine tube where fertilisation takes place. FSH and LH then cause the remaining parts of the follicle to transform into the corpus luteum (CL). CL produces progesterone which is a gestation conservation hormone that prevents the ewe to come into heat and prevents embryotic abort. During the rest of the pregnancy the CL will be active. At partum (lambing), CL undergoes luteolysis, and the ewe can yet again become cyclic (SLU, 2009).

Methods for induced out-of-season reproduction and heat synchronisation

According to (Gates et al., 1998) the demand for lamb meat in other season than in the autumn has increased the interest for reliable methods in production systems with concentrated lambing all year around. Ovulation rate, litter size and lambing percentage of the ewes are traits of economic importance and are lower outside the normal breeding season. Therefore many different methods for induced out-of-season reproduction and heat synchronisation have been developed.

Light treatment

One out-of-season method described by Chemineau et al. (1992) is light treatment. In light treatment programs, the animals are tricked to believe that there is a specific season. This is made by reducing or increasing the length of time that they are exposed to light. When the day length is around eight hours the animals start to show that they are sexually active and when the day length is around 16 hours or more they are not sexually active. Chemineau et al. (1992) has displayed that the day length and the sexual activity of animals can be altered with light

treatments and reproduction can be induced when it normally wouldn't take place. It has also been revealed that when altering the day length to short days the animals will be sexually active around 60-80 days but will then stop even though the light program for short days still is running. The same is true when altering the day length to long days and this phenomenon is named refractoriness and occurs around day 120. If the season for mating should be changed by light programs the programs have to run for about 60 days and alter between long and short days. This is due to that the response to photoperiod is reliant on the earlier photoperiod that the animals were exposed to. To be able to induce short days the animals has to be exposed to 60 long days. Long day do not necessarily have to be 16 hours of light, it is sufficient to have seven hours of light and then one more hour of light 16 hours after the dawn. In sheep the pineal gland secretes more melatonin when it is dark in relation to when it is light. Therefore the ewes can be tricked to believe that the day length is short either by light treatment or with melatonin treatment. Melatonin treatment can either be done by oral or intramuscular administration of melatonin to the ewe every afternoon or by subcutaneous insertion of a melatonin implant. The implant can work inhibitory by removing photoperiodic information or by providing the ewe with short day signals. The method used by Chemineau et al. (1992) was implants with short day signals.

The problem with light treatments is that the animals have to be kept in light isolated building so that the programs are not disturbed by the sunlight. To rear animals like sheep this way is not that preferable. Furthermore, the association for Swedish sheep breeder do not use hormonal treatment in sheep production (Söderquist, 2007). The effect of the programs can work on a satisfied way for rams in Artificial Insemination (AI) stations but are according to Chemineau et al. (1992) much harder to use on seasonal ewes. Melatonin can be used to elongate the breeding season for these ewes by one or two months. Thus, light and melatonin treatments were not enough to induce year around breeding in seasonally ewes from Northern Europe sheep breeds, examined in that article. However Cameron et al. (2010) was capable to construct a photoperiodic program that allowed Canadian Rideau Arcott ewes to lamb three times in two years. The long days (LD) in this experiment consisted of 16 hours of light and the short days (SD) of eight hours. The ewes were kept in a light and isolated building. The artificial light came from lamps with an average light intensity of 35 lx for LD and 15 lx for SD. The regime consisted of alternating four months period of LD and SD. Melatonin were not used in this experiment and one of the goals was to show that there are other ways than hormone treatment to get a functional year around breeding.

Heat synchronisation

As mentioned above, slaughter organisations prefer if all the lambs in a herd can be delivered at the same time. It is also easier for the sheep breeder if all the ewes give birth to their lambs in more or less the same time. In his book, Henderson (1990) explained two options for oestrus synchronisation. One was the use of teaser rams and the other the use of hormone-treated vaginal tampons.

In a sterilised or vasectomised ram a part of each vas deferens is removed so that no sperm can mix with semen. The teaser ram continues to produce sex hormones, and is equally willing to defray as before the surgery (Evans and Maxwell, 1987). The sex hormone pheromone is secreted from the rams wool wax and allows the ewes to come into oestrus. Within half an hour

after the ram has been introduced to the ewes, they begin to produce the hormones that activate ovulation. However, it is required that the ewes have not noticed or felt the smell of a ram in at least four to six weeks. It is also important that the ram should not be introduced too early before the ewes are naturally sexually active. The ewes will ovulate within a few days after the ram is introduced but they will not show any heat signs and therefore most of the ewes will not be mated by the ram at this time. Seventeen days after this "silent oestrus" or about three weeks after the introduction of the ram, most of the ewes are in oestrus again and will show heat signs. A few ewes even have "silent oestrus" twice. The second "silent oestrus" takes place one week after the first one and this means that these ewes will show the usual signs of heat four weeks after the introduction of the ram. Instead of using a standard ram or a teaser ram, a castrated ram treated with testosterone can be used (Henderson, 1990).

Use of hormone-treated vaginal tampons usually gives inferior results than oestrus synchronisation with teaser ram (Söderquist, 2007). When vaginal tampons impregnated with synthetic progesterone are applied to the ewe and progesterone are induced through the vaginal walls in to the bloodstream (Henderson, 1990). This stops the ewe to come into oestrus until the tampon is removed. If tampons from all ewes in a herd are collected simultaneously a synchronised oestrus will be obtained. The tampons are operational as long as they are used on ewes that are in the normal mating season. Outside the mating season "pregnant mare serum gonadotropin" (PMSG) or “equine chorionic gonadotropin” (eCG) as it usually is named today can be given to the ewe when the tampon is removed. This is one of the most used methods today, where hormonal treatment is accepted for out-of-season breeding (Cameron et al., 2010). Roy et al. (1999) suggested that eCG might be a risk factor for infertility in ewes after AI and Viñoles et al. (2001) have noticed growth of ovarian follicular cysts and subsequently low pregnancy rates after progestagen treatments. Tsiligianni et al. (2007) argued that the synchronisation of a sheep herd provides an abbreviated lambing season. Experiments made in Hungary showed that Chios ewes can be synchronised by using 1.5 mg of dexamethasone 21-isonicotinate (DEX) on day 146 post insemination.

Selection for out-of-season reproduction

There is genetic variation between breeds in the length of the sexual season. In some sheep breeds ability to breed out of season has been shown. Dorset, Merino, Rambouillet (Hafez & Hafez, 2000) and to some extent Swedish Finewool (Gates et al., 1998) are examples of such breeds. By altering gene frequencies the fertility period for ewes can be extended and oestrus behaviour can appear in the spring (Lewis et al., 1996). This is beneficial because it gives the opportunity for more frequent lambing’s and thereby more lambs. One example of this is the photoperiodic program used by Cameron et al. (2010) that resulted in three lambing times in two years. This can be named an accelerated lambing system and in Cornell University, Ithaca, NY, the STAR accelerated system have been developed to get up to five lambings in three year (Lewis et al., 1996). This program does not require the same confinements of animals or the same amount of labour and energy as light and hormone treatments. In this system the year is divided into five 73-day periods with breeding in each of the periods. The sheep breed used in Cornell’s STAR System was Dorset. Earlier studies showed that accelerated lambing systems with Dorset (Iniguez et al., 1986), Dorset crosses (Notter and Copenhaven, 1980) and Finnish land race (Walton and Roberson, 1974) gave lambing rates of 1.21; 1.27 and 1.6 respectively. Unfortunately, only a few of the ewes in the STAR system were able to lamb throughout the year

and the average lambing frequency was only one lambing per year. This was partly due to very low fertility in June for Dorset ewes of all ages. The other systems had their out-of-season mating between mid-January to late April. According to Lewis et al. (1996) the STAR system might have been improved if mating in June would be avoided. Another thing mentioned by the author, that could have improved the STAR system was if the Dorset ewes were selected for year around fertility.

According to Al-Shorepy and Notter (1997) selection for fertility in a fall-lambing sheep flock suggest that selection can be used to improve fertility in year around breeding flocks. Notter et al. (2011) reported that selection on autumn-lambing ability not only can result in ewes with shortened seasonal anoestrus but also lesser selectivity to long day photoperiods. This implies (Notter, 2012) that ewes can be genetically improved for out-of-season lambing and thereby accelerated lambing schemes. Ewe lambs that are born in autumn should therefore be genetically superior to ewe lambs born in spring for out-of-season breeding. The problem with this is that autumn ewe lambs are not more than seven month of age when it is time for the next out-of-season breeding in April. This gives low fertility even though they are genetically enhanced (Notter and Cockett 2005). One way to solve this dilemma, according to Notter (2012) is to detect ewes that have the ability to lamb in the autumn and to select on their daughters that are lambed the following April (spring). This enhance bot the genetic merit and the timing of the replacement ewes. The enhancement can be further increased if the dam is mated with a ram that has high breeding values. Another way to improve the genetic progress for out-of-season breeding is to use ewe lambs that are crosses between the sheep breed of choice and a breed that is adapted for out-of-season breeding.

With a well-planned and relevant breeding program genetic progress can be achieved. Important though is that the genetic potential is matched to the environment and management. It is also favourable to optimise the reproductive efficiency rather than to try to maximise it (Notter, 2012). In Romney sheep, a gene on the X chromosome called the Inverdale gene ($FexX^1$) has been found to influence the ovulation rate (Davis et al., 1991). This is a single gene with a large effect on a specific trait, a so called major gene (Simm, 1998). Heterozygotes for this gene ($FexX^1/FexX^+$) has a higher ovulation rate than non-carriers ($FexX^+/FexX^+$) (Davis et al., 1991). However the homozygotes for the gene ($FexX^1/FexX^1$) are sterile (McLeod et al., 1997). By analysing concentration of gonadotrophins in the blood plasma from ewe lambs, the infertile homozygotes can be identified and excluded from breeding. This shows that it is important to validate what impact breeding on major genes may have on the offspring.

Quantitative trait loci (QTL)

Estimates of heritability for fertility in spring lambing has been low. This is not surprising due to that most reproductive traits usually have low heritability ranging from 0.05 to 0.15 and thereby also can be hard to improve by selection within breeds (Notter, 2012). These traits are affected by many genes and are therefore said to be quantitative traits (Simm, 1998). They are also affected by many environmental factors (Mateescu and Thonney, 2010). There are different loci that effect quantitative traits and these are called Quantitative trait loci (QTL). With the help of markers for QTL, selection response could increase markedly (Notter and Cockett, 2005). Eight so called “clock genes” involved in circadian interaction have been identified in mammals (Reppert and Weaver, 2001). According to Notter and Cockett (2005) these genes may also be

useful as QTL influencing the circannual or seasonal reproduction. Mateescu and Thonney (2010) found candidate QTLs that affect aseasonal reproduction in seven of the sheep chromosomes. This gives hope for future understanding of aseasonal reproduction and proves that this trait is associated with multiple chromosomes.

Melatonin receptor 1a (MTNR1A)

Additional to seasonality in reproduction (circannual rhythms), melatonin also modulates the circadian rhythms (Cassone, 1990). Circadian rhythms (from Latin *Circa*, approximately, and *dies*, day) are physiological processes with a frequency of about 24 hours (Campbell and Reece, 2008). These rhythms are not controlled by any known environmental variable but can be set by environmental signals like day length. According to Reppert (1994) melatonin is secreted by the pineal gland and exerts its circadian effects in the hypothalamic suprachiasmatic nucleus (SCN). The reproductive or circannual effect of melatonin is exerted through high affinity receptors in the hypophyseal pars tuberalis (PT). The high affinity melatonin receptors MT1, former Mel1a are so far the only identified receptors that regulates seasonally breeding in sheep and other non-human mammals like pig and cattle. These receptors are G-protein-linked receptors which signal by inhibition of cyclic AMP (Barrett, 2003). G-protein-linked receptors are the largest assembly of cell-surface receptors and can respond to a huge variety of extracellular signal molecules. The receptors consist of a single polypeptide chain that passes through the cells lipid bilayer seven times and when a signal molecule binds the receptor activates a G protein located inside the cell. There are different G proteins which have the ability to regulate second-messenger molecules like ions, enzymes and cyclic AMP. By affecting the activity of adenylyn cyclase, which synthesise cyclic AMP from ATP, the concentration of cyclic AMP inside the cell will change and thereby mediate different cell responses depending on the type of target cell and extracellular signal (Alberts, 2004). The gene for MT1, melatonin receptor 1A gene (*MTNR1A*), consists of two exons divided by an approximately 8 000 base pair (bp) long intron (Carcangiu et al., 2009). The first exon (exon I) codes for the first intracellular loop while the second exon (exon II) codes for the rest of the receptor.

Amplifications of most of the exon II have been carried out in PCR reactions with primers that start at position 285-304 and ends at position 1108-1089 of the ovine *MTNR1A* gene which has the GenBank number: U14109 (Carcangiu et al., 2009). The sequence is an 1149 bp long mRNA locus. Restriction enzyme cleaves the PCR products at specific nucleotide sites. After enzyme digestion the product are resolved by electrophoresis in parallel with a DNA marker and then subjected to genotyping. The two restriction enzymes used by Messer et al. (1997), Notter et al. (2003), Chu et al. (2006), Mateescu et al. (2009) and Carcangiu et al. (2009) were *Ras*1 and *Mnl*1. *Ras*1 cleaves the DNA at the restriction site “GTAC” and *Mnl*1 at restriction site “CCTC”. Carcangiu et al. (2009) found four different cleavage sites on the PCR product for *Ras*1 and seven different cleavage sites for *Mnl*1. When examining the exon II of the *MTNR1A* gene for different sheep with the restriction enzymes *Ras*1 some individuals had a restriction site in position 606 and some didn’t. The same occurred for *Mnl*1 on position 612 with. Individuals that had a cytosine (C) in position 606 were cleaved by *Ras*1 and subsequently called allele “C”. Individuals with thymine (T) in the same position wouldn’t be cleaved at this position and therefore could be notified to have the allele “T”. Individuals that had a guanine (G) in position 612 were cleaved by *Mnl*1 and were said to have the allele “+”. Individuals with adenine “A” in the same position wouldn’t be cleaved at this position and therefore could be notified to have the

allele "-". Due to that the different animals would have different base pair lengths depending on cleavage or not the allelic frequency and the genotype frequencies could be calculated after that the PCR products had been in electrophoresis and then analysed (Carcangiu et al., 2009). These two marker sites C606T and G612A do not generate any changes in the makeup of amino acids and are thereby silent or non-coding markers. Barrett et al. (1997) found eight mutations on the MT1 receptor of which the three mutations in position 893, 1121 and 1129 led to a change in amino acid. The mutation in position 893 occurs in the third extracellular loop and the mutation in position 1121 and 1129 occurs in the carboxy-terminal tail. None of these three mutations has been shown to cause any changes in the MT1 receptor. According to Barrett et al. (1997) changes in the carboxy-terminal tail could possibly cause alterations in the functionality of the receptor but this could not be examined due to lack of information for the MT1 receptor. Pelletier et al. (2000) found one additional mutation in position 706 that causes an amino acid substitution of valine to isoleucine in the fifth transmembrane domain. This substitution causes a change in the relationship between the receptor and one of the melatonin binding parameters but further studies are required to establish the actually effect of this change (Pelletier et al., 2000).

According to Carcangiu et al. (2009) the genotypes for position 606 and 612 for each of the Sarda ewes in their study could be decided by the method described above. This information was matched with the ewes' recorded lambing frequency in the period September-December (out of season) and in the period January-April (normal lambing season). The ewes with genotype C/C had a higher rate of lambings out of season than the ewes with genotype T/T and the heterozygote ewes C/T had similar rates of lambings out of season and normal lambing season. The ewes with genotype +/+ had a higher rate of lambings out of season than the ewes with genotype -/- and the heterozygote ewes +/- had similar rates of lambings in out of season and normal lambing season. This confirmed that C/C and +/+ genotypes affected seasonality reproduction in the Sarda ewes. Pelletier et al. (2000) report that -/- genotypes in Merino d'Arles ewes are connected to inactivity of the ovary in spring fertility. Notter et al. (2003) showed that crossbreed ewes (50% Dorset, 25% Rambouillet and 25% Finnsheep) with at least one + allele had greater spring fertility. Mateescu et al. (2009) have been able to prove that Dorset and Dorset crossed ewes (3/4 Dorset 1/4 East Friesian) with at least one + allele also had greater spring fertility. Small Tail Hansheep (Chu et al., 2003) has also shown an association between the *MTNR1A* gene and seasonal reproduction. Different studies on different sheep breeds do not agree on the out-of-season association for the C allele.

Hernandez et al. (2005) found that the *MTNR1A* gene had no association for prolonged breeding season in the seasonal Ile-de-France breed. Notter and Cockett (2005) also reported that different breeds, considered to be seasonal could have a relatively high frequency of the favourable *MTNR1A* gene. Notter (2008) explained that this limits the *MTNR1A* gene as a breed specific marker and Notter (2012) reported that there are not yet any comparable major genes for out-of-season breeding in sheep found today. However Mateescu et al. (2009) emphasises the importance of the *MTNR1A* gene as a potential out-of-season DNA marker for populations where the effect of the *MTNR1A* gene is known. Notter and Cockett (2005) and Notter (2008) also emphasise the importance of the expanding knowledge in the genomic control of circannual and circadian rhythms. There have been discoveries of an array of candidate genes that can have potential influences in seasonal breeding. Screening large population of ewes with favourable or

uncommon seasonal breeding for functional sequence variants in these candidate genes might give rise to the discovery of major genes affecting year around breeding across all sheep breeds.

Description of different analysis methods

DNA extraction

Extraction of DNA from blood samples can according to eNotes (2012-09-07) be carried out in a wide range of ways depending on the source and size of the sample. The first step is lysis, or breakdown, of the sample cells thus allows the nucleic acids to be released from the nucleus. The samples can be lysed with the help of salt solutions containing detergents or enzyme-digesting proteins like proteinase K and sometimes both. Second the DNA is isolated in a way that optimizing the DNA quality and yield for the specific sample. One way to isolate DNA is to use a high salt concentration to bring down the nucleic acid and thereby forming a salt of nucleic acid. By adding alcohol and centrifuge the mixed DNA can be recovered. Another way is to let the DNA bind to magnetic particles and separate the DNA from the sample with the help of magnetic rods. A third method uses columns containing silica or ion exchange based matrices which binds the DNA while the columns are washed with salt solutions that remove the unwanted materials (eNotes, 2012-09-07).

Real time PCR

Studies of different mutations can be carried out by DNA amplification in an ordinary PCR followed by enzyme digestion and electrophoresis. The result after these steps are different restriction fragment length polymorphism (RFLP) that opens on to the ability to detect the genotypes for specific individuals. For this thesis these three different steps could be reduced to one by using Applied Biosystems® StepOnePlus™ real time PCR instead of an ordinary PCR. The biggest difference between ordinary PCR and real time PCR is that the real time PCR instruments can monitor the steps during amplification and deliver analysed result right away without the need of different analysing instruments after amplification (Logan et al., 2009). The real time PCR genotyping process can be divided into two steps: thermal cycling and endpoint detection.

There are many different real time PCR platforms and thereby many different varieties of digestion and analysis methods (Logan et al., 2009). In order to amplify DNA the real time PCR also have to be equipped with a type of thermal cycler. The thermal cycler heats the samples enabling the denaturation of DNA molecules and analyses of melting curves (Logan et al., 2009). DNA denaturation or DNA melting is when the double-stranded DNA unwinds and separates into single-stranded strands due to breaking of the hydrogen bonding that exist between the bases. Because cytosine / guanine (G-C) base pairs have three hydrogen bonds and adenosine / thymine A-T base pairs only have two the melting temperature are higher if the DNA has a higher amount of G-C then A-T base pairs. If one base pare differs between two DNA sequences the melting curve for the different DNA will vary suggesting that there is a single nuclear polymorphism (SNP). Another way to detect a SNP is, as for this case to use primers and probes that binds or anneals to the DNA.

In order for endpoint detection the real time PCR has to be equipped with an integrated fluorimeter that can detect and monitor the level of fluorescence created during the PCR process

(Applied Biosystems®, 2010a). The instruments also have to be equipped with a light source that can cause excitation of the fluorophore in the reaction vessel and fluorescent emission detectors that can receive the emitted light from the samples.

Amplification of DNA in a PCR requires that the DNA strands are denaturised enabling the separation of the DNA molecule into single-strands (Applied Biosystems®, 2010a). This is performed by heating the DNA to around 95^{o}C. After denaturation the temperature is decreased to around 50-60^{o}C which in turn enables two different primers and probes, which are specific designed oligonucleotides to anneal or hybridise to a specific placer on the target DNA strands (Nicholas, 2010). The primers are around 20 base pairs long and come in pairs with one forward primer, also called sense primer and one reverse primer, also called antisense primer. The forward primer anneals to the 3' end of the antisense DNA strand which goes from 3' to 5'. The reverse or antisense primer anneals to the 3' end of the sense DNA strand which goes from 5' to 3'. Mutation detection in a real time PCR can be carried out with the help of two allele specific probes. The probes are like the primers, specific designed oligonucleotides but often a bit shorter. Attached to the primers there are a dye and a quencher. VIC® dye is designed for allele one and FAM™ dye is designed for allele two. The probe with the VIC® dye is therefore designed anneal if allele one is present and the probe with the FAM™ dye if allele two is present.

If the probe anneals to the right place (match) and then are cleaved (hydrolysed) by a taq polymerase the dye will create a fluorescent signal (Applied Biosystems®, 2010a). The dye won't create this signal if the probe is intact (unhydrolysed). This is due to that the dye, which is linked to the 5' end of the probe, cannot create its fluorescent signal as long as it is close to the non-fluorescent quencher that is located on the 3' end of the probe. When the probe is hydrolysed the dye gets separated from the quencher and the signal from the dye can be detected by the emission detectors. If the probe attaches to the loci with the wrong allele (mismatch) the taq polymerase won't hydrolyse it but instead push it away, see Figure 2. The probes used in this experiment were TaqMan® minor groove binder (MGB). Due to that the melting temperature is lower, for shorter sequences then for longer sequences the probes has to be modified. Minor groove binders improve the binding for primers and thereby increasing the melting temperature.

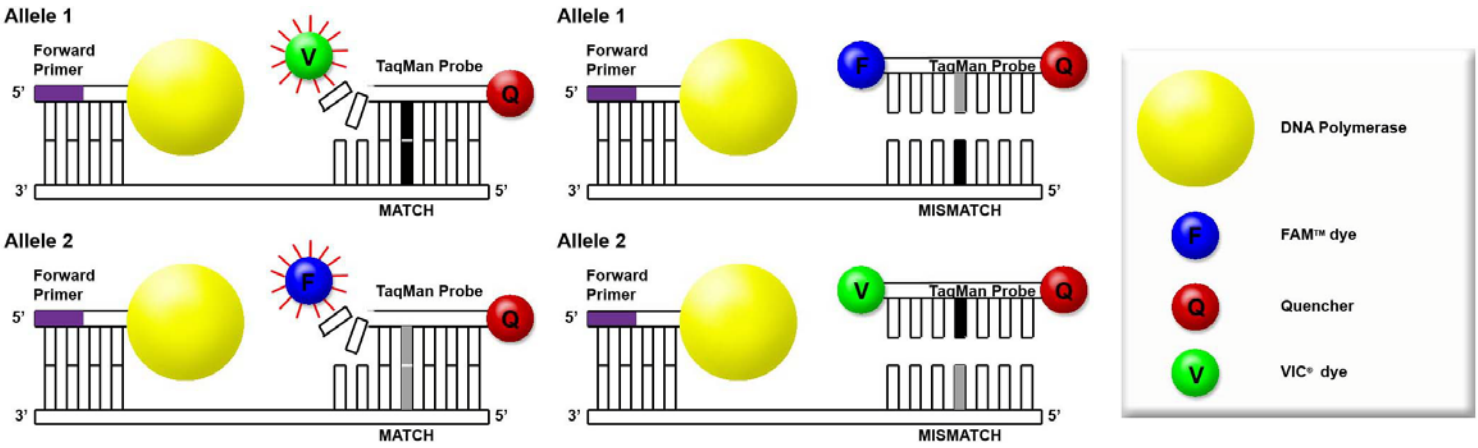

Figure 2. If there is a base pairs match the dye will be hydrolysed from the probe and create a signal. If there is a mismatch between the base pairs the probe will be punched away. Based upon Applied Biosystems® (2010a).

When the primers and probes have attached to the DNA strand the temperature is increased to about 70°C and the enzyme DNA polymerase starts to replicate the DNA between the primers, greatly amplifying the amounts of DNA (Nicholas, 2010). This phase is called extension. When the first PCR machines were made the available DNA polymerases were denaturised in higher temperature and therefore new DNA polymerase had to be added after each thermo cycle. New PCR technics were evolved and in the 1990s easier and faster amplifications could be conducted thanks to a DNA polymerase called Taq polymerase. Taq polymerase is thermo stable polymerase produced by bacterium *Thermus aquatic* which lives in hot springs. Taq polymerase survives the heat created by the thermo cycler and enables faster amplification in closed tube due to that Taq polymerase only has to be added to the samples once before running the PCR (Applied Biosystems®, 2010a). The taq polymerase used for this experiment was AmpliTaq Gold®. When the replication of the DNA sequence is finished the temperature rises again and a new amplification cycles with denaturation, annealing and extension can be initiated. Totally around 40 cycles are run.

DNA-sequencing

One important tool for sampling information about the DNA and determining the order of the nucleotide bases is DNA-sequencing (Nicholas, 2010). One of the methods for DNA-sequencing today is based on the chain-terminator method developed by Frederick Sanger in the 70-tis. By dividing the DNA molecule into single stands of DNA and applying a primer, DNA polymerase and deoxynucleotides triphosphates (dNTPs) a strand-elongation starts. But if dideoxynucleotide triphosphates, which lack a 3'-OH group essential for bond formation between nucleotides, are added to the reaction the elongation will be stopped. The four different dideoxynucleotides (ddATP, ddGTP, ddCTP, and ddTTP) will supplement the deoxynucleotides randomly which results in fragments of varying length. By creating fragments in fore different reaction using only one of the four dideoxynucleotides for each reaction and then sort the fragments with the help of gel electrophoresis all the bases of the temple DNA can be determined.

The technique of choice today permits the sequencing to be made in one reaction instead of four and is detected on a capillary electrophoresis instrument (Hanrahan, 2009). This analysis technique uses, as the chain-terminator method four dideoxynucleotides that terminates the elongation but in this case each of the four dideoxynucleotide groups is labelled with a specific fluorescent dye enabling the determination of the four bases A, G, C and T. This is also called dye-terminator sequencing. In general the capillary electrophoresis instruments consist of narrow-bore capillaries, two buffer reservoirs, a high-voltage supply, an introduction device for the samples and a detection scheme. After fragmentation the DNA fragments consist of different amounts of base pairs and therefore have a difference in ion migration velocities. This results in a difference in mobility between the fragments and thereby also a separation. The high electric field pulls the negatively charged DNA fragments through the detector (Applied Biosystems®, 2010b). The smaller fragments reaches the detector before the larger fragments due to that the smaller fragments migrate faster. In the capillary electrophoresis machine, Applied Biosystems® 3500xL Dx Genetic Analyzer which was used in this thesis laser are used to excitate the dye attached to the DNA fragments. The fluorescent lights are then detected by the instrument optics and signals are transferred to the computer software which will generate an electropherogram (figure 3). The electropherogram illustrates which colour that is detected for each sequence, starting from the smallest sequence to the largest, and witch base that is connected to that colour.

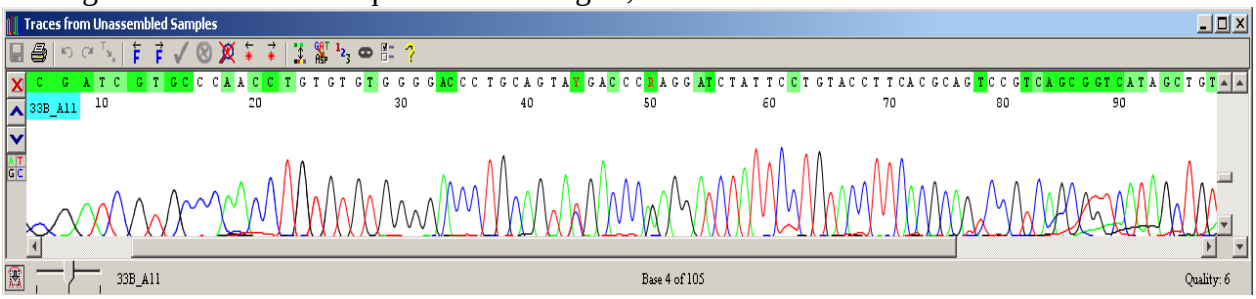

Figure 3. Screen shot of an electropherogram inside the program CodonCode aligner.

This study

Materials and Methods

Blood and data collection

The collected material for this thesis was blood samples as well as exterior and fertility data from sheep. The animals were 28 ewes from the Gotland sheep breed, 30 ewes from Swedish Finewool sheep breed and 32 ewes of the Roslag sheep breed in for each breed two different herds. The blood was sampled in 4 ml BD Vacutainers (plus blood collection tubes) and the sampling was made by persons authorised by the ethical board for animal research in Sweden. The animal experiment was authorised by this board in beforehand.

The exterior data collected were measurements of the nose length, the length between the ears, withers height, back length and tail length as could be seen in Figure 4. The chest girth was also measured on each of the ewes. The fertility data collected were the ewe's date of birth, how many lambing's she had so far and the amount of lambs per lambing. Also the SE number and the parity of the ewes were registered. All the ewes, except 15 ewes of the Gotland breed from one of the herds had lambed at least once.

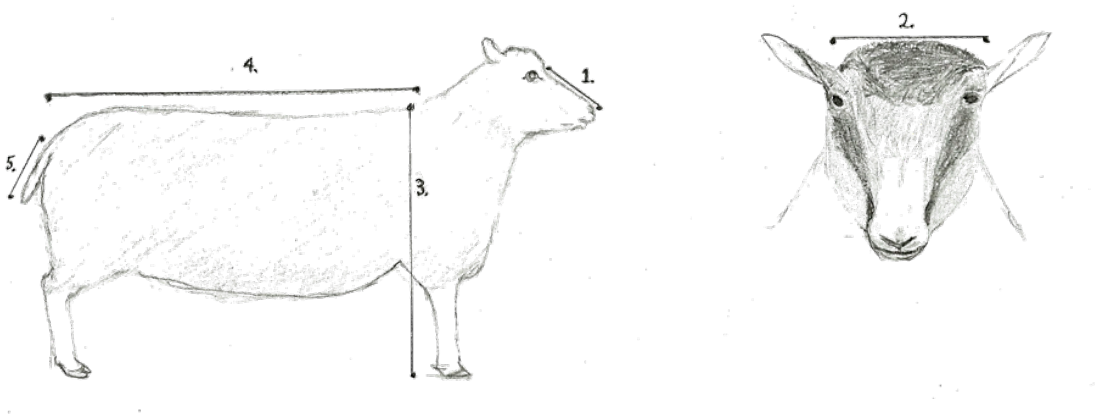

Figure 4. Illustration of measurements done in the study. 1. The nose length 2. The length between the ears 3. The withers height 4. Back length 5. The tail length (Illustration by Alexander Falk 2012).

DNA extraction

Extraction of DNA from each of the ewes collected blood sample was carried out using the QIAsymphony® robot (Qiagen®, Hilden, Germany). This machine purifies the DNA with the help of proteinase K and magnetic particles. Sample tubes suitable for the QIAsymphony® were filled with 1.8 ml blood. The Vacutainers were gently agitated around before blood was moved to the sample tubes. After this the QIAsymphony® machine was prepared with all necessary equipment including the DNA Midi Kit, the numbered sample tubes and a rack with numbered elution tubes for the extracted DNA. Before loading the reagent cartridge the container with magnetic particle were removed from the cartridge frame and run in a vortex vigorously and thoroughly before placed in the cartridge frame. After around two hours the extraction was finished and the tubes with 200 µl of DNA stored in the freezer.

The next step was to analyse the concentration and pureness of the samples with the help of NanoDrop 8000 Spectrophotometer. The DNA concentration was determined by absorbance measurement at 260 nm and calculated in nanograms per microliter (Qiagen®, 2010). The purity of the sample was calculated by subtracting the absorbance at 260 nm and 280 nm by the absorbance at 320 nm and then locking at the ratio: $(A_{260}-A_{320})/(A_{280}-A_{320})$. The ratio should be around 1.7-1.9. The DNA concentrations were approximately 80 (ng/µl) for more or less all the samples and the pureness of most of the samples had an ideal A_{260}/A_{280} ratio. The samples were then diluted to an approximate concentration of 40 (ng/µl) with a volume of 30 µl.

DNA-sequencing

The SNPs were located at position 606 and 612 on the Melatonin receptor 1a (*MTNR1A*). Position 606 is polymorphic C/T, giving the possible genotypes C/C, C/T and T/T. Position 612 is polymorphic G/A giving the possible genotypes G/G, G/A and A/A.

Eight of the Swedish DNA samples were first tested in a PCR run together with 15 Italian samples. The Italian samples were from Sarda ewes, genotyped for C606T and G612A on *MTNR1A* by Carcangiu et al. (2009). Due to problems with probes annealing over both of the

two *MTNR1A* mutations this method were abandoned in favour for DNA-sequencing. By sequencing the area with the C606T and G612A mutations the genotype for each ewe could be established. The DNA-sequencing was carried out with Applied Biosystems® 3500xL Genetic Analyser. The sequencing primers were designed with the help of the National Center for Biotechnology Information (NCBI) and their software Primer3. The primers were designed to have a M13-tale on its 5' end. The sequence of the different primers and tales can be seen below, M13-tale written in italic. The PCR-product was around 100 base pairs long with the mutation sites in the middle.

- Forward primer sequence (incl. M13 forward tail):

  5'- *TGTAAAACGACGGCCAGT*TCGTGGCGATCGTGCCCAAC-3'

- Reverse primer sequence (incl. M13 reverse tail):

  5'- *CAGGAAACAGCTATGACC*GCTGACGGACTGCGTGAAGGT-3'

The sequencing were carried out with BigDye® direct cycle sequencing kit by following the instructions in the BigDye® direct cycle sequencing kit protocol (Life Technologies™, 2011). The workflow was divided into four different steps:

1. Performing PCR amplification
2. Performing cycle sequencing
3. Purifying the sequencing products
4. Performing capillary electrophoresis

First one test plate with 16 forward and 16 reverse samples was prepared for PCR. The DNA samples were mixed with PCR master mix and deionised water together with both reverse- and forward primers with M13 tail. The PCR amplification was made in a 9700 thermal cycler with a few changes from the original protocol (Bergström, 2012).

Table 1. Run protocol for PCR reaction

Stage	Temp	Time
Hold	95°C	15
Cycle (35)	95°C	1
	58°C	1
	72°C	1
Hold	72°C	7
Hold	4°C	∞

After PCR 4 µl of product were taken from seven samples and an electrophoresis was carried out on 0.3% agarose gel with an AmpliSize™ 50-2000 bp Ladder. This was made to test the DNA template quality (Figure 5).

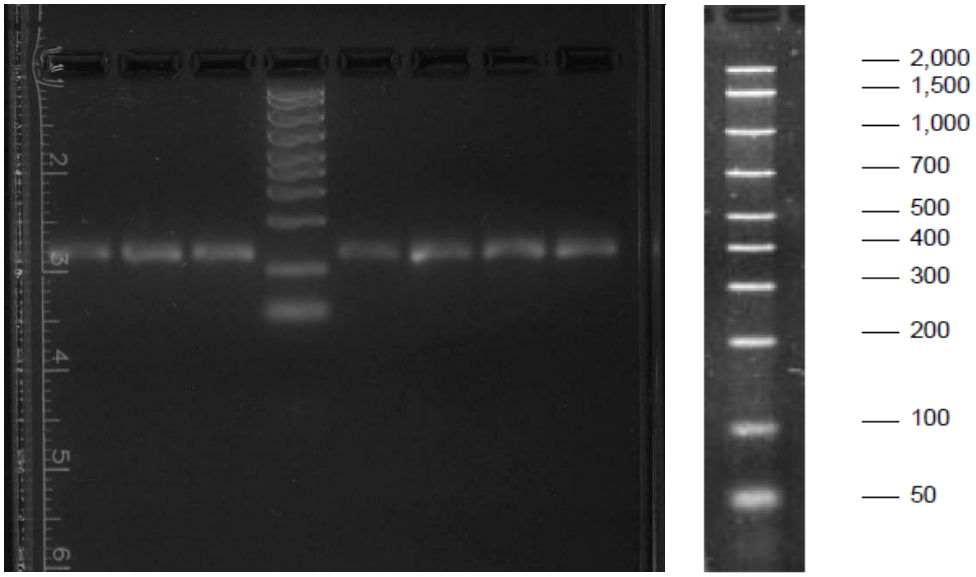

Figure 5. A photo of DNA fragments separation on agarose gel and there approximated base pair size.

Preparation for cycle sequencing consisted of mixing BigDye® direct sequencing master mix with BigDye® direct M13 forward primers and adding this to the samples that were being forward sequenced. The "reverse" samples went through same presider but with BigDye® direct M13 reverse primers instead. The cycle sequence was performed in a 9700 thermal cycler according to the protocol. Then the samples were purified with the help of BigDye® XTerminator®. After mixing SAM™ solution and XTerminator® solution the plate were vortexed at 2000 rpm for 20 minutes in an IKA MS3 digital vortex and then spun in a swinging-bucket centrifuge at 1000 x g for two minutes. The last step was to analyse the templates with the

help of capillary electrophoresis carried out in Applied Biosystems® 3500xL Dx Genetic Analyzer. The run condition was set for BigDye® terminator v3.1 Kit with POP-7™ polymer and 24 capillaries. Most of the test samples gave good readings and it was decided to prepare and analyse the DNA from all the 88 Swedish ewes plus eight of the Italian controls. It is recommended to sequence both strands to confirm the sequence. The PCR were performed the same way as the test plate for both the forward- and reverse plate. The cycle sequencing forward mix, containing the BigDye® direct M13 forward primers were added to the forward plate and the cycle sequencing reverse mix containing the BigDye® direct M13 reverse primers were added to the reverse plate before running the plate in the thermal cycler. After the purification some of the wells in the forward plate had a high amount of beads from the XTerminator®. Therefore 12 µl of template were pipetted out of the purified plate and into another plate. This plate was then run in the capillary electrophoresis with standard run conditions.

The results from the DNA sequencing were analysed with the help of the software Codon Code Aligner and the results from the forward sequencing were compared to the results from the reverse sequence. The program was also used to determine if there were any other polymorphisms in the sequenced region. The data was analysed to find the alleles and genotype for all ewes on location C606T and G612A (Table 2). The allele frequency and percentage of genotypes per breed were then calculated and also presented in Table 2. A Chi-square test was conducted to test if the allele frequency in each of the breeds were in Hardy Weinberg equilibrium. Then two sided Fisher exact tests were made to determine if there were any difference between the breeds in allele frequency or genotype in location C606T and G612A on *MTNR1A*. The Fisher exact tests were made in the statistical software R. The result tables for these tests are presented in appendix A.

Results

The electrophoresis confirmed that the PCR products were of expected size. With the run condition set to standard all the samples got analysed results for both there forward and reverse sequence in the Applied Biosystems® 3500xL Dx Genetic Analyzer. After controlling that the forward and reverse sequence for each individual matched for the SNPs of interest the sequenced were checked through fore other SNPs. No new sequences variants were to be found. The result displayed in Table 2 indicates that all the three breeds have a high amount of the genotypes assumed to inherit out-of-season breeding.

Table 2. Number of alleles and genotypes for the three different sheep breeds on location C606T and G612A of the *MTNR1A* gene. In brackets frequency (*f*) of alleles and percentage of genotypes

	Allele # (*f*)				**Genotype # (%)**					
	C606T		G612A		C606T			G612A		
	C	T	G	A	CC	C/T	TT	GG	G/A	AA
Roslag sheep	30 (0.44)	38 (0.56)	48 (0.71)	20 (0.29)	8 (24%)	14 (41%)	12 (35%)	16 (47%)	16 (47%)	2 (6%)
Gotland sheep	16 (0.31)	36 (0.69)	45 (0.87)	7 (0.13)	3 (12%)	10 (38%)	13 (50%)	19 (73%)	7 (27%)	0 (0%)
Swedish Finewool sheep	16 (0.29)	40 (0.71)	46 (0.82)	10 (0.18)	1 (4%)	14 (50%)	13 (46%)	18 (64%)	10 (36%)	0 (0%)

The results from the Chi-square tests did not show any significant deviation from Hardy Weinberg equilibrium in any of the breeds (Table A1, Appendix A). Thereby all breeds were in Hardy Weinberg equilibrium. There were a significant difference in allele frequency for position 612 between the Roslag sheep and Gotland sheep breed (p-value of 0.048). With significance level of 0.05 no other difference between the breeds could be demined (Appendix A).

Discussion

The Gotland ewes had the highest quantity of homozygotes for the genotype GG but are said to be the most seasonal breeding among the three breeds that were tested in this thesis (Näsholm & Eythorsdottir, 2011). Even though, as explained by Mateescu et al. (2009) the gene has to be validated for the specific breeds breeding behaviour the results given in this thesis indicate that G612A are not a good marker for out-of-season breeding in Gotland ewes. The same conclusion was made by Hernandez et al. (2005) for the Ile-deFrance ewes. They did not notify any difference in the dates and duration of the breeding season between Ile-de-France ewes with GG or AA genotype at position G612A on the *MTNR1A* gene. Hernandez et al. (2005) suggests a lack of relationship between the polymorphism and reproductive seasonality for Ile-de-France ewes and implicate that other genes has to be investigated for further knowledge about the genetic variability of seasonal functions. Even the results given from genotypes at position C606T indicate that this locus cannot be used as a good marker for out-of-season breeding due to the similarity in allele frequency between the Swedish breeds. In Gotland and Ile-deFrance ewes there could be a recombination resulting in high frequency of the targeted markers even though the breed is seasonal. Mateescu et al. (2009) indicates the importance of the *MTNR1A* gene as a potential out-of-season DNA marker for populations where the effect of the *MTNR1A* gene is known. In their study which was conducted on 91 Dorset and 25 ¼-East Friesian x ¾- Dorset ewes with phenotypic records the gene frequencies for C and T alleles on position 606 were 0.35

and 0.65 respectively. The gene frequencies for G and A alleles on position 612 were 0.64 and 0.36 respectively.

To study the influence of various alleles on individual ewes' phenotypic records on their ability for out-of-season reproduction are needed. With observations of out-of-season breeding for the Swedish Finewool and the Roslag breeds it is possible that position 606 and 612 of the *MTNR1A* gene could be used as silent markers for this trait on individual ewes. This could also be the situation for individual Gotland ewes. Due to the short length of the analysed sequence it was not possible to investigate other SNPs of the *MTNR1A* gene that have been detected by several authors (e.g. Barrett et al., 1997; Pelletier at al., 2000; Chu et al., 2006). Notter and Cockett (2005) and Notter (2008) also emphasised the importance of the expanding knowledge in the genomic control of circannual and circadian rhythms, which makes DNA-sequencing over the whole *MTNR1A* gene of great interest for the Gotland, the Swedish Finewool, and the Roslag sheep breeds. With this knowledge all the mutations on the *MTNR1A* gene could be compared between seasonal and non-seasonal breeds and give a better understanding of which SNPs that are involved in out-of-season breeding. Mutations that differ in the comparison between breeds would be of great interest especially if they make a change in protein build-up.

Conclusion

The results from this master thesis indicate that the SNPs investigated are not good markers for the possibility to decide the Swedish breeds' ability for out-of-season breeding. More studies covering the whole of the *MTNR1A* gene are needed to investigate if the individuals in the Swedish breeds have the same association between different genotypes at C606T and G612A and other mutations on *MTNR1A*. This could give a better understanding of which SNPs that are involved in the connection between the *MTNR1A* gene and seasonal breeding in sheep.

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Appendix A

Table A1. Chi-square test for Hardy Weinberger equilibrium

Chi-square (X^2)	**C606T**				**G612A**			
	CC	CT	TT	**Tot**	GG	GA	AA	**Tot**
Roslag sheep	0.29	0.46	0.18	**0.92**	0.05	0.25	0.30	**0.60**
Gotland sheep	0.12	0.10	0.02	**0.25**	0.01	0.15	0.47	**0.63**
Swedish Finewool sheep	0.72	0.58	0.12	**1.42**	0.04	0.39	0.89	**0.32**

Degrees of freedom: 3-2=1

Chi-square if probability is 0.05: 3.84

Table A2:1. Chi-square contingency table with genotype at position C606T for all breeds, observed data

	CC	**CT**	**TT**	
Roslag sheep	8	14	12	34
Gotland sheep	3	10	13	26
Swedish Finewool sheep	1	14	13	28
	12	38	38	88

Table A2:2. Chi-square contingency table with genotype at position C606T for all breeds, expected data

	CC	**CT**	**TT**	
Roslag sheep	4.64	14.68	14.68	34
Gotland sheep	3.55	11.23	11.23	26
Swedish Finewool sheep	3.82	12.09	12.09	28
	12	38	38	88

P-value = 0.153

Table A3:1. Chi-square contingency table with allele frequency at position C606T for all breeds, observed data

	C	T	
Roslag sheep	30	38	68
Gotland sheep	16	36	52
Swedish Finewool sheep	16	40	56
	62	114	176

Table A3:2. Chi-square contingency table with allele frequency at position C606T for all breeds, expected data

	C	T	
Roslag sheep	23.95	44.05	68
Gotland sheep	18.32	33.68	52
Swedish Finewool sheep	19.73	36.27	56
	62	114	176

P-value = 0.219

Table A4:1. Chi-square contingency table with allele frequency at position C606T for two breeds, observed data

	C	T	
Roslag sheep	30	38	68
Gotland sheep	16	36	52
	46	74	120

Table A4:2. Chi-square contingency table with allele frequency at position C606T for two breeds, expected data

	C	T	
Roslag sheep	26.07	41.93	68
Gotland sheep	19.93	32.07	52
	46	74	120

P-value = 0.185

Table A5:1. Chi-square contingency table with allele frequency at position C606T for two breeds, observed data

	C	T	
Roslag sheep	30	38	68
Swedish Finewool sheep	16	40	56
	46	78	124

Table A5:2. Chi-square contingency table with allele frequency at position C606T for two breeds, expected data

	C	T	
Roslag sheep	25.23	42.77	68
Swedish Finewool sheep	20.77	35.23	56
	46	78	124

P-value = 0.093

Table A6:1. Chi-square contingency table with allele frequency at position C606T for two breeds, observed data

	C	T	
Gotland sheep	16	36	52
Swedish Finewool sheep	16	40	56
	32	76	108

Table A6:2. Chi-square contingency table with allele frequency at position C606T for two breeds, expected data

	C	T	
Gotland sheep	15.40	36.59	52
Swedish Finewool sheep	16.59	39.41	56
	32	76	108

P-value = 0.836

Table A7:1. Chi-square contingency table with genotype at position G612A for all breeds, observed data

	GG	GA	AA	
Roslag sheep	16	16	2	34
Gotland sheep	19	7	0	26
Swedish Finewool sheep	18	10	0	28
	53	33	2	88

Table A7:2. Chi-square contingency table with genotype at position G612A for all breeds, expected data

	GG	GA	AA	
Roslag sheep	20.48	12.75	0.77	34
Gotland sheep	15.66	9.75	0.59	26
Swedish Finewool sheep	16.86	10.5	0.64	28
	53	33	2	88

P-value = 0.169

Table A8:1. Chi-square contingency table with allele frequency at position G612A for all breeds, observed data

	G	A	
Roslag sheep	48	20	68
Gotland sheep	45	7	52
Swedish Finewool sheep	46	10	56
	139	37	176

Table A8:2. Chi-square contingency table with allele frequency at position G612A for all breeds, expected data

	G	A	
Roslag sheep	53.70	14.30	68
Gotland sheep	41.07	10.93	52
Swedish Finewool sheep	44.23	11.77	56
	139	37	176

P-value = 0.083

Table A9:1. Chi-square contingency table with allele frequency at position G612A for two breeds, observed data

	G	A	
Roslag sheep	48	20	68
Gotland sheep	45	7	52
	93	27	120

Table A9:2. Chi-square contingency table with allele frequency at position G612A for two breeds, expected data

	G	A	
Roslag sheep	52.7	15.3	68
Gotland sheep	40.3	11.7	52
	93	27	120

P-value = 0.048

Table A10:1. Chi-square contingency table with allele frequency at position G612A for two breeds, observed data

	G	A	
Roslag sheep	48	20	68
Swedish Finewool sheep	46	10	56
	94	30	124

Table A10:2. Chi-square contingency table with allele frequency at position G612A for two breeds, expected data

	G	A	
Roslag sheep	51.55	16.45	68
Swedish Finewool sheep	42.45	13.55	56
	94	30	124

P-value = 0.147

Table A11:1. Chi-square contingency table with allele frequency at position G612A for two breeds, observed data

	G	A	
Gotland sheep	45	7	52
Swedish Finewool sheep	46	10	56
	91	17	108

Table A11:2. Chi-square contingency table with allele frequency at position G612A for two breeds, expected data

	G	A	
Gotland sheep	43.81	8.19	52
Swedish Finewool sheep	47.19	8.81	56
	91	17	108

P-value = 0.603