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Seasonality of reproduction in Awassi sheep

Ph.D. dissertation

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List of abbreviations

A	adenine
ACTH	adrenocorticotrop hormone
AI	artificial insemination
AL	autumn-lambing
ANOVA	analysis of variance
AUC	area under the curve
BC	body condition
BCS	body condition score
BHB	β-hydroxybutyrate
BW	body weight
C	cytosine
CL	corpus luteum
CLP	persistent corpus luteum
CV	coefficients of variation
d	day
DF	dominant follicle
E_2	17β-estradiol
eCG	equine chorionic gonadotropin
EGF	epidermal growth factor
ELISA	enzyme-linked immuno-sorbent assay
Fec	prolificacy genes in sheep (FecB, FecX etc.)
FGA	flourgeston acetate
FSH	follicle-stimulating hormone
G	guanine
GABA	gamma-aminobutyric acid
GH	growth hormone (syn. somatotrop hormone, STH)
glm	generalized linear model
GnRH	gonadotrop releasing hormone
GPG	syn. OvSynch protocol (GnRH- PGF2α-GnRH)
HTh	hypothalamus
IGF-I	insulin-like growth factor-I
IGFBP	IGF-I binding proteins (IGFBP-1 to 5)
IRMA	immunoradiometric assay
IU	international unit
Na-EDTA	ethylene-diamine tetraacetic acid, sodium salt
LD	long day photoperiodic treatment
LH	luteotrop hormone
LHRH	luteotrop hormone releasing hormone (syn. GnRH)
MAP	medroxyprogesterone acetate
MT	melatonin-treated
MT1	melatonin receptor 1a
NEB	negative energy balance
NEFA	non-esterified fatty acids (syn.: free fatty acids, FFA)
NS	statistically non-significant
OR	odds ratio
P4	progesterone
PAG	pregnancy associated glycoprotein
PGF2α	prostaglandin F2α
PMSG	pregnant mare serum gonadotropin (syn. eCG)
PP	postpartum
PUN	plasma urea nitrogen
$_{rb}$leptin	recombinant bovine leptin
$_{ro}$leptin	recombinant ovine leptin
RFLP	restriction fragment length polymorphism
RIA	radioimmuno assay (^{3}H-RIA, ^{125}I-RIA: ^{3}H- or ^{125}I-labelled version of this assay)
sCL	short luteal phase
SEM	standard error of the mean
SL	spring lambing
T	thymine
T3	triiodothyronine (3,3',5-triiodothyronine)
T4	thyroxine
TGF	transforming growth factor
TMR	total mixed ration

Summary

Dairy sheep products are typical seasonal goods due to the reproductive seasonality of the species. Simultaneously there is great economic interest to provide year-round continous milk supply for the dairy industry. However effective hormonal treatments are available to turn against physiological processes and achieve out-of-season breeding, there is increasing costumers' demand to turn towards more "clean and green" tools, and reduce hormonal interventions in food producing animals. Better understanding of reproductive physiology and increasing knowledge about the breeds used for milk production make possible to reach this target.

The aim of our research was to explore the seasonality of ovarian and testicular function, and the genetic interrelations of ovarian cyclicity with melatonin receptor 1a gene (MT1) polymorphism in Awassi sheep. We intended to characterize whether this breed and the inland population are capable of developing perennial ovarian cyclicity and out-of -season ovulation.

We showed that ovarian function of Awassi population is seasonal under temperate continental climate condition. In intensive production systems the lack of suckling together with the stimulatory photoperiodic signal brings the first postpartum ovulation of autumn-lambing dams forward, it may occur even before the completion of uterine involution, which may increase the risk of bacterial complications of involution (Exp 1). We proved that to delay the resumption of postpartal ovarian cyclicity during the stimulatory short-days, the implementation of additional artificial lightening is an adequate tool. At the same time the increasing photoperiodic signal has beneficial effect on milk production (Exp 2).

Investigation of melatonin receptor polymorphism in the given population showed that MT1 gene is polymorph at both the RsaI and MnlI restriction sites, with high incidence of the preferable R and M alleles. Ability of out-of-season cyclicity was expressed in mature animals having high plasma leptin level (indicating adequate energy stores). However, high milk production may negatively influence this capability. Our results suggest that the preferable allele (R at a significant level and M tendentiously) can enhance out-of-season cyclicity in dams with suboptimal metabolic condition related to low plasma insulin-like growth factor I (IGF-I) level. Nevertheless, these polymorphisms are not suitable for marker assisted selection because only the RsaI RFLP (restriction fragment length polymorphism) site showed significant effect and it was limited to a subgroup of the flock (Exp 3).

Following the monitoring of the seasonal traits of reproduction, we tested the efficacy of different hormonal treatments to improve out-of-season reproductive performance. Slow release melatonin implant inserted to ewes in February could not induce cyclic ovarian function; however the same treatment had beneficial effect when used in June. At the same time melatonin treatment influenced negatively plasma IGF-I level which may impair milk production in lactating dams. According to our results the OvSynch protocol as a possible alternative of long-term gestagen treatment for synchronisation for artificial insemination (AI) can only be effective when used near to the natural breeding season (Exp 4). The use of slow release, long acting melatonin implant in Awassi semen donor rams had obvious positive effect on the endocrine function of testicles in February, but at the same time this beneficial effect was not reflected in semen quality (Exp 5).

Apart of the new knowledge gained in the above described experiments, as practical application we worked out a series of suggestions and developed a new reproduction technology using natural or near-natural methods to optimize reproductive management and milk production of the investigated Awassi flock.

Összefoglalás

Hagyományos körülmények között a juhtejből készült termékek tipikusan szezonális cikkek, amelyek előállíthatóságát a faj szaporodóképességének évszakhoz kötött jellege a tavaszi, nyár eleji időszakra korlátozza. Ugyanakkor fontos piaci érdekek fűződnek az árutej-előállítás folyamatosságának biztosításához. Bár kiskérődzőkben a tenyészszezonon kívüli vemhesítés hagyományos eszközének számítanak a hormonkezeléseken alapuló ciklusindukciós technikák, egyre jelentősebbek azok a fogyasztói elvárások, melyek a "zöld és tiszta" állattenyésztési módszereket részesítik előnyben. Így élelmiszertermelő állományokban szerencsésebb tartózkodni a különböző hormonok tenyésztéstechnikai célú alkalmazásától. A szezonális ivari aktivitás élettani alapjainak pontosabb megértése és a tejhasznú fajtákkal kapcsolatos ismeretek összegyűjtése hozzásegíthet a természetközeli technológiák kialakításához.

Munkánk során a nemi működés szezonális jellemzőit kívántuk tanulmányozni egy hazai tejhasznosítású awassi populációban, valamint vizsgáltuk a szezonalitás mértékének genetikai vonatkozásait, a melatonin receptor 1a (MT1) gén polimorfizmussal fennálló kapcsolatát. További célunk volt feltérképezni, hogy alkalmas lehet-e a fajta a hagyományos tenyészszezonon kívül spontán tenyésztésre.

Kimutattuk, hogy az awassi juhállományi petefészek működése hazai körülmények között szezonális. Intenzív termelési rendszerben őszi ellésű anyákban az anya-bárány kapcsolat hiánya és a serkentő fotoperiódusos jel együttes hatása miatt az ellés utáni első ovuláció nagyon korán, többnyire még a méh involuciójának befejezése előtt következik be, ami növeli az involució bakteriális szövédményeinek esélyét (1. kísérlet). Igazoltuk, hogy az őszi ellési időszakban alkalmazott mesterséges kiegészítő megvilágítás alkalmas arra, hogy késleltesse az ellést követő első ovuláció idejét. Emellett a meghosszabbodott fotoperiódusos jel jótékonyan befolyásolja a tejtermelést is (2. kísérlet).

A melatonin receptor gén vizsgálata során kimutattuk, hogy a vizsgált állományban az MT1 gén mind az RsaI, mind az MnlI restrikciós emésztési helyeknek megfelelően polimorf, és az irodalmi adatok szerint kedvező hatású R és M allélek nagy arányban fordulnak elő. Tenyész-szezonon kívül ciklikus petefészek-működést legnagyobb arányban az idősebb, és magas plazmaleptin szinttel (megfelelő energiaraktárakkal) rendelkező anyák mutatnak. Ugyanakkor a nagy tejtermelés rontja ezt a képességet. Eredményeink szerint a szuboptimális metabolikus állapotban lévő (alacsony plazma inzulin-szerű növekedési faktor I (IGF-I) szinttel

jellemezhető) anyák esetében a kedvező MT1 alléleket hordozók nagyobb arányban mutatnak ciklusos petefészek működést a szezonon kívül (az R allél esetében szignifikáns, az M allél esetében tendencia szintű hatást igazoltunk). Ugyanakkor mivel ez a hatás csak az állatok egy csoportjában jelentkezett szignifikáns mértékben, az MT1 gén vizsgált mutációinak szelekciós markerként való alkalmazása nem javasolható (3. kísérlet).

A szezonális nemi működés nyomonkövetését követően különböző hormonkezelések hatékonyságát vizsgáltuk a tenyészszezonon kívüli időszakban. A februárban beültetett lassú kioldódású melatonin implantátum nem volt képes ciklust indukálni, míg júniusban alkalmazva jótékony hatása volt. A melatonin kezelés hatására ugyanakkor csökkent a plazma IGF-I szintje, ami laktáló állatokban csökkentheti a tejtermelést. Eredményeink alapján az OvSynch protokoll és azt követő fix idejű termékenyítés csak abban az esetben válthatja ki a hosszú tartamú gesztagénkezelést, ha a természetes tenyészszezonhoz közeli időben alkalmazzuk (4. kísérlet). Kosok esetében a februárban alkalmazott tartós melatonin kezelés bár egyértelműen pozitívan befolyásolta a here endokrin működését, nem javította a spermaminőséget (5. kísérlet).

A munkánk során nyert alapadatok felhasználásával kidolgoztunk egy természetes vagy természetközeli módszereken alapuló szaporodásbiológiai technológiai rendszert, melynek alkalmazásával intenzív tejhasznú awassi állományokban, hazai körülmények között folyamatos, magas szintű árutej-előállítás válik lehetővé.

1. Introduction

In traditional technologies, sheep' dairy products are typically seasonal goods. Due to the race's inherent seasonal reproduction activity, their manufacturing is restricted to early summer. However, great commercial interest is attached to the continuous milk production. In small ruminants, gestagen+eCG (equine chorionic gonadotropin) treatment is widely used for cycle induction to get out-of-season fertility. Although these protocols are permitted in dairy flocks, in view of the increasing consumer requirements, it is better to refrain from the use of sexual steroids in reproduction management of milked livestock. An alternative solution for the future is to try to form flocks, where most of the ewes are cyclic during the whole year – also out of the traditional autumn breeding season – allowing fertilization also in spring besides autumn. Based on researches concerning primarily different merino lines, we know that the ability of perennial cyclicity is genetically determined in sheep, and is in connection with melatonin receptor 1a (MT1) polymorphism, although the underlying mechanism is not known in details at the moment (Pelletier et al. 2000, Notter et al. 2003). At the same time numerous preconditions can influence the manifestation of this capacity: such as age, body condition (BC), and pheromone-exposure. In genotypes which are genetically capable of year-long ovarian cylicity, the use of long-known natural or near-natural breeding technologies (e.g. few days increased energy supplementation; flushing, pheromone exposure by introduction of vasectomized rams to ewes, photoperiodic and/or melatonin treatment) might gain new dimension, and contribute to clean, green and ethical animal breeding.

The growing demand for clean technologies in animal breeding led to the development of novel management tools also in dairy cow. Better understanding of the physiology of seasonality and the importance of the photoperiodic signal in its regulation led to the emergence of technologies which use long-day photoperiodic treatment to increase milk yield in dairy cow (Dahl et al. 2000). Later the positive link between long-day photoperiodic treatment and increased milk yield was also proven in goat (Mabjeesh et al. 2007). This drew our attention to a possible limitation of using melatonin implants for cycle induction in sheep. Treatments acting through the melatonin pathway to induce cycle in the short-day breeder sheep may negatively influence milk production in lactating dams.

Although seasonality of reproductive activity is less expressed in males compared to females rams also show year-round variation in sexual behaviour, testicular size, and quality of semen. To maximize the out-of-season reproductive performance not only ewes but also rams should be treated accordingly. Administration of melatonin in the non-breeding season was shown to

influence positively the hypothalamus-pituitary-gonadal axis in rams (Kennaway and Gilmore 1985, Lincoln and Ebling 1985, Lincoln and Kelly 1989, Bourla 1991, Chemineau et al., 1992) and the treatment was also able to increase ram effect (Rosa et al. 2000, Abecia et al. 2006a).

At the initiation of the studies the Awassi flock kept in Bakonszeg yielded more than half of the Hungarian sheep milk production. The investigated Awassi breed is a fat-tail sheep which originates from the dry, subtropical zone of the Middle East. It is exceedingly capable of economical milk production in great volume under inland circumstances. With adequate forage in the above mentioned intensive system the lactation is long, it lasts about 180-215 days (Gootwine and Pollot 2000). Milk production can reach at least 600-700 kg per lactation. The milk is concentrated, rich in protein (5.4-6.4%) and fat (6.8-7.4%), hence strongly convenient for industrial, mainly cheese, processing. In its homeland Improved Awassi flocks are kept indoors all year round and an accelerated lambing regime is practiced with several mating/insemination periods during the year (Epstein, 1985). Ewes are milked from the first day of lactation, and lambs are removed from the dam into an artificial rearing unit after birth. This type of milking regime is well known in dairy cows but unique in dairy sheep, as in most other cases lambs are left with their mothers allow suckling until weaning. Although Improved Awassi flocks are fertile following hormonal treatments year-round, in its homeland the reproductive season of the original Awassi population in the traditional extensive farming systems mostly depends on the availability of forages. Climatic and feeding conditions and also the daily and seasonal rhythm of daylight are completely different in the lowland area of the Carpathian basin than in the Middle East and the degree of seasonality of the inland population was not investigated before. The only reliable information at the beginning of our research was that the breeding season starts later than for merino sheep.

After reviewing the related literature, in the current thesis I wish to summarize our recent experiences regarding:

- the ovarian activity of autumn- and spring-lambing Awassi ewes following parturition,
- the effect of long-day photoperiodic treatment on the time of first postpartum ovulation in autumn-lambing ewes,
- the occurance of MnlI and RsaI polymorphism of MT1 gene in the investigated flock and its relation to out-of season cyclicity,
- the efficacy of gestagen and melatonin based cycle induction protocols in Awassi ewes,
- and the impact of melatonin treatments in rams.

2. Survey of litterature

2.1. Reproduction of ewes

2.1.1. The ovarian function, development and endocrine function of follicles, and seasonality of reproduction

The ewe is a seasonally polyestrous animal, following a seasonal pattern, i.e. alternating periods of anestrous and sexual activity. According to the current knowledge (Driancourt et al. 1985, Monniaux et al. 1997, McNatty et al. 1999, Migaud et al. 2002, Senger 2003; Hunter et al. 2004) in the beginning, only intraovarially located growth factors (transforming growth factor: TGF, epidermal growth factor: EGF, IGF-I) acting exclusively in a paracrine way, regulate differentiation of primary follicles to small antral follicles, which takes about 90-120 days in sheep. As soon as the follicles reached a diameter of about 2 mm, which are histologically already tertial (antral) follicles, they become sensitive to gonadotropes: their further development – not more than 5-7 days – is wavelike, basically induced by follicle stimulating hormone (FSH), and regulated by luteinizing hormone (LH). On basis of the wave-like growth of the follicles, dominant follicles (DF) develop with the ability for synthesis of gestagen-like and androgenic steroid precursor in the external (theca interna) cells with direct vascular connection, as well as for oestrogen (17ß-estradiol: E2) and inhibin production in the internal cell layers (granulose cells). LH-receptors appear on the granulose cells of the approximately 3.5 mm diameter follicles, respectively. The size of preovulatory follicles is about 6 mm. The subordinated follicles recruited at the same time as the DF, loose their endocrine activity and degenerate (atresia) without ovulation, in the same manner as those DF-s selected during the luteal phase in cyclic individuals, or out of the breeding season during the seasonal acyclicity. The endocrine mechanism regulating the final maturation is similar to that one observed in cattle (Driancourt 2001, Senger 2003, Hunter et al. 2004, reviewed by Huszenicza et al. 2003a, 2004). The pulse frequency of the pulsatile-oscillating basal LH secretion is of profound importance in sheep as well: one pulse in each 45-75 minutes is the predominant endocrine factor regulating the final maturation of DF. The regulatory role of LH pulsatility is completed by the intrafollicular effect of IGF-I as well as by the complex enzyme-related cascade cleaving the free bioactive form of IGF-I from its binding proteins (IGFBP) in the follicular fluid. This local mechanism plays a crucial role in selection of DF, divergent from the development of subordinated follicles from the same follicular cohort. The free bioactive IGF-I is the main activator of the aromatase enzyme system which determinates the ability of granulose cells to produce E2, although the insulin,

3,5,3'-triiodothyronin (T3) and leptin content of the follicular fluid which act intraovarially (playing a marked role in limiting the activity of the aromatise enzyme system which determinates the ability of the DF to produce E2 and in this way to limit the activity of the aromatizing enzyme system). The mentioned follicular fluid contents can be distinguished within different breeds and families (Thiery et al. 2002, Senger 2003, Hunter et al. 2004).

In sheep, the number of the follicular waves is generally 3-4 per cycle, but it was reported to vary between 2 and 5. As examination methods, e.g. endoscope, ultrasound etc. are fairly difficult to do in field practice, those statements are based on a limited investigations and therefore our current knowledge on the exact number of growth waves is not very reliable. In case of four follicular waves within one cycle, the between-ovulation interval (cycle length) is approximately 16 days; whereas depending on the follicular waves' number the time between two estruses may differ between 12-13 and 19-21 days (Monniaux et al. 1997, Adams 1999, Bartlewsky et al. 1999, Bister et al. 1999, Evans et al. 2000, Evans 2003).

2.1.2. Seasonal differences in the ovarian function

In most of the sheep breeds we can count with ovulation and subsequent formation of corpus luteum (CL) secreting progesterone (P4) (cyclic ovarian function) at times when the day length shortens, i.e. at the end of the summer, in autumn and at the beginning of the winter (breeding season) (Haresign et al. 1985, Roche et al. 1985, Senger 2003).

Although its turnover is probably slow, in most of the modern sheep breeds the wave-like pattern of gonadotroph-dependent follicular growth and maturation exists also out of the breeding season, resulting in the regular formation of E2-producing DF-s also during the anovulatory (syn. acyclic; sometimes also called, but not fully correct: anoestrous) period. At that time, however, certain neurons of the hypothalamus (HTh) located in the ventrolateral wall of the third ventricle (surge centre), which are responsible for the release of large amounts of gonadotrop releasing hormone (GnRH) triggering the preovulatory-like LH peak, loose their E2 sensitivity. Due to that lack of hypothalamic E2 sensitivity the effect of E2 synthesized by the DF is not powerful enough to provoke the preovulatory-like GnRH/LH release. Therefore no ovulation takes place and the DF becomes atretic. Using other terms, in sheep the physiological prerequisite of ovulation and cyclic ovarian function is the ability of the hypothalamic surge centre to react to the E2 production of DF-s with a massive GnRH/LH release (e.g. positive feedback), and this E2 sensitivity is restricted only on the breeding season. In the last 2 to 4 weeks of the acyclic period (transition period) the gonadotroph-sensitive phase of the follicular development and maturation is more rapid, the follicular

turnover is almost the same as within the breeding season. However, the E2-induced GnRH/LH responsiveness resulting in ovulation is still missing (Haresign et al. 1985, Webb et al. 1985, Adams et al. 1997, Thiery et al. 2002, Senger et al. 2003). Also the E2 sensitivity of hypothalamic regions responsible for oestrus behaviour is reduced out of the breeding season. In sheep and other ruminants this E2 sensitivity has not been fully restored yet at the time of the first ovulation (Haresign et al. 1985, Thiery et al. 2002): a several-day P4 rise produced by the first CL is needed for the complete return of receptivity resulting in that oestrous signs are not strongly connected to the first ovulation at the end of acyclic periods. So in sheep and goat the resumption of cyclic ovarian activity can only be proven by the CL-related increase in P4 levels following the first ovulation (Migaud et al. 2002, Thiery et al. 2002, Senger 2003; Notter and Cockett, 2005).

The ovarian function of animals not conceived by the end of the breeding season gets acyclic again. At the end of the breeding season the thyroid hormones act a crucial role in cessation of cyclicity in small ruminants; low thyroid hormone levels lead to elongated breeding season (Viguie et al. 1999).

The change between the length of light and dark hours (photoperiod), resulting in an effect on the ovaries, follows a pathway beginning with the eyes, the optical nerve via the body's "biological clock" (the suprachiasmatic nuclei of the hypothalamus, HTh) and the pineal gland secreting melatonin, known as the main signalling hormone. Both the melatonin synthesis and secretion in the pineal gland and the level of melatonin pattern in the peripheral blood follows the diminution of light intensity: with the reduction of light hours the part of the day characterized with elevated melatonin levels lengthens continuously, which en-hances the E2 sensitivity of GnRH-synthesizing surge centre in the HTh and also the release of gonadotrop hormones (LH and, in smaller amounts, also FSH) in the anterior pituitary gland in small ruminants. Some details of these mechanisms mediated by dopamin, serotonin, gamma-aminobutyric acid (GABA), β-endorfins and several other neurotransmitters (neu-ropeptid Y, proopiomelanocortin) have still been unknown (Lincoln and Wu 1991, Le Corre and Chemineau 1993, Scott and Clarke 1993, Lincoln and Baker 1995, Viguie et al. 1995a, 1995b, 1996, Xiong et al. 1997, Lincoln and Richardson 1998, Malpaux et al. 1993, 1997, 1998, Clarke et al. 2000, Notter and Chemineau 2001, Chemineau 2007, Thiery et al. 2002). Beside this, according to some opinion, melatonin may also act directly on the ovaries (Bister et al. 1999).

In connection with the variations in seasonality Notter and Chemineau (2001) detected differences in the plasma melatonin levels at night-time in certain crossbreed lines (50%

Dorset, 25% Rambouillet, 25% Finnish Landrace): in August the females selected for fertilization in autumn showed significantly lower plasma melatonin levels in the evening than those which were not selected on the basis of seasonality (the above mentioned quality was significantly hereditary: $h^2 = 0.43$; $P < 0.02$). The same study showed also differences in the prolactin levels during the night whereas the inheritance was not definitely shown.

2.1.3. The melatonin receptor and its polymorphism

The effect of melatonin is strongly connected to its specific receptors in the premammillary hypothalamus (Malpaux et al. 1998, Vanecek 1998, Malpaux 2006) by regulating the pulsatile release of GnRH (Viguie et al. 1995a, 1995b). By now, three different subtypes of melatonin receptors were found in mammals: Mel1a or MT1, Mel1b or MT2 and MT3 were cloned and described. Amongst those, at this time, a distinct meaning of different polymorphisms of MT1 is proven in small ruminants (Migaud et al. 2002). In the last decade the melatonin receptor gene 1a (MT1) polymorphism (notably the presence of the MnlI cleavage site and the presence of the RsaI cleavage site in exon II) was shown to be associated with the capability of out-of-season reproductive activity both in domestic sheep (Pelletier et al. 2000, Wright 2000, Notter et al. 2003, Mateescu 2009, Carcangiu 2009) and in mouflon (Carcangiu 2010). The connection is not likely to be direct, although there are no differences in the amino acid sequences in the mentioned parts of punctual mutations within the receptors. The idea of using the MT1 polymorphism as a genetic marker of ability for year-round ovarian cyclicity was present from beginning. But when comparing animals with different genotypes of MT1 the results were not always uniform. In a two-year French experiment with Île-de-France ewes, Hernandez et al. (2005) failed to observe any clear connection of MT1 genotype with the seasonal differences in daily prolactin and melatonin patterns in cyclic ovarian activity or in the growth of the animals' fleece. Recently Teyssier et al. (2011) failed to show any relationship between MT1 gene polymorphism and reproductive seasonality in Merinos d'Arles ewes. Taking account of results of various studies including those with contradictory findings currently we suppose that the MT1 receptor gene polymorphism may be a useful indicator of year-round ovarian cyclicity in some of the sheep breeds. However, the real manifestation of this genetic ability requires proper environmental (mainly feeding) conditions including the optimal body fat content (condition score; further details are given later).

In sheep there may be great breed-related differences in the length of the breeding season and the time of the first and last ovulations. Within breeds also the family and/or flock-

dependent variability may be obvious sometimes (Quirke and Hanrahan 1985; Webb and Gauld 1985, Notter 1992, 2005, Driancourt 2001). We can take into account that the length of the ovarian cyclicity is connected to the degree of domestication of various breeds as well as to the geographic-climatic conditions of the area where that breed was domesticated. In breeds of the temperate and cool continental climatic zones, such as the steppe-originating Merino (Rambouillet, Dorset), as well as the Finnish Landrace and the Russian Romanov breeds, the first ovulation takes place already in mid or late August, and the ovarian function of non-pregnant animals remains cyclic until the end of February, beginning of March. In some British breeds (Galway, Suffolk) the breeding season starts quiet late in mid September, but can last till the end of March or early April. In contrary, in Scottish Blackface the ovulation is possible only from mid October to late February. In May and June even the regular waves of follicular growth cease in this breed. The Awassi represents the fat tailed breeds of the subtropical arid zone in the Middle East. The first ovulation of its breeding season is usually seen from late August to mid September in the Israeli flocks (Notter 1992) but the onset of ovarian cyclicity may vary within a wide range both in its homeland population and the flocks imported to the USA (Notter personal communication 2005). The real seasonal characteristics of Awassi ewes kept under the temperate continental climate of Central-East Europe are still unknown. The Racka is one of the ancient breeds of the Carpathian basin existing in white, black and brownish (Gyimesi) variants. According to our earlier experiences based on P4 determination in three blood samples of 50-50 individuals taken 7 days apart almost all the well-fed ewes of this three genetic variants had already cyclic ovarian activity in late August, and those animals not conceived meanwhile remained cyclic till the end of January (Becskei, Kulcsár and Huszenicza unpublished data). Mouflon, one of the ancestors domesticated sheep, shows cyclic ovarian function only between October and early to mid January, and after early March ovulation cannot be induced in them with gestagen + eCG (syn. pregnant mare serum gonadotrophin, PMSG) administration, either (Lázár, Kulcsár and Huszenicza unpublished data).

The great variations in seasonality of cyclic ovarian function in different breeds can be related to the MT1 polymorphism. Up to now, however, this supposed connection between some MT1 gene alleles and the ability of breeds for year-round ovulation has been proved by studies mainly on older animals (Notter and Cockett 2005). The triglyceride content of subcutaneous and visceral fat depots usually increases simultaneously with the aging of animals. So it may be possible that although the relation with the age of animals is obvious but the explanation may lie in the fact that also the body condition improves with ageing

(Bocquier et al. 1998, Chilliard et al. 2005, Faulconnier et al. 2001). Notter et al. (2003) showed that older animals are more likely to cycle beyond season. Selection for out-of-season oestrus did not result in the elevation of out-of-season cyclicity in first parity animals, however in the forthcoming years they expressed this ability. However, this hypothesis needs further improvement in studied on wider range of species and farming systems with particular attention to the role of lipid depot localized in the hump-like fat tail of certain breeds. On the other hand, according to our knowledge there is no proven connection between the MT1 polymorphism and seasonality in goats (Migaud et al. 2002).

2.1.4. Nutritional aspects in seasonality of ovarian function

Beside the breed and genotype the nutrition of animals and in this respect their body condition is a fact of great importance as it determines the time of the first ovulation and therefore the beginning of the breeding season, respectively the length of the anovulatory/acyclic period. To achieve that the surge centre of the HTh restores its sensitivity to E2, and so its ability to trigger the first preovulatory LH peak and ovulation, the triglyceride content of subcutaneous and visceral fat depots (e.g. the body condition of the ewe) has to be above a certain threshold. Body condition score (BCS) was shown to have a significant effect on seasonality. Forcada et al. (2006) showed that ewes maintained on a diet resulting in 2.8 BCS had significantly longer reproductive season compared to the 2.3 BCS group. The same tendencies were seen in horses where higher BCS resulted in less expressed seasonality (Fitzgerald and McManus, 2000).

This permissive form of regulation is supposed to interact with the endocrine consequences of changes in daylight furthermore with breed-related (and perhaps with other unspecified genetic) factors (Robinson 1990, Downing and Scaramuzzi 1991, Dunn and Moss 1992, Adams et al. 1997, Senger 2003, Hunter et al. 2004, Martin et al. 2004). Breeds from the Northern and Western European areas need generally a larger fat depot than breeds originating from the steppe or the arid subtropical zones (Robinson 1990).

Weak animals and especially long lasting deterioration of the body condition causes a delay of six to eight weeks of transition and strongly affects the time of the first ovulation within a flock. In very severe cases ovarian function may remain acyclic throughout the whole year. However, the European way of husbandry and feeding does not give a real chance for that (Robinson 1990). By optimizing the energy intake of medium to poor conditioned animals 2 to 3 weeks before the beginning of the breeding season (“flushing”) first ovulation will take place earlier and the deviation within the flock will be decreased (Robinson 1990,

Downing and Scaramuzzi 1991, Dunn and Moss 1992, Senger 2003, Hunter et al. 2004, Martin et al. 2004). Compared to the meaning of the energy intake on inducing ovulation the effect of the protein supply is only of secondary importance (Teleni et al. 1989a, 1989b, Senger 2003). The energy balance of an animal has a strong influence on glucose maintenance and / or glucose metabolism of the GnRH synthesizing neurons; furthermore, it works via the intrafollicular availability of IGF-I, insulin, leptin and thyroid hormones (Adams et al. 1997, Huszenicza et al. 2003c, Hunter et al. 2004, Martin et al. 2004, Munoz-Gutierrez et al. 2004).

Leptin and insulin-like growth factor I (IGF-I) are probably the most important players of the signaling pathways between energy balance and the hypothalamus-pituitary-gonadal axis too.

Leptin as a signaling protein released from the white adipose tissue plays an important role in long-term regulation of food intake and reproduction (Bokori 2000, Schneider 2004, Chilliard et al. 2005, Zieba et al. 2005). Age and body fatness are strongly related to each other, and a threshold level of fat stores is needed to reach puberty (Chilliard et al. 2005). Physiological processes leading to ovulation are very similar at the time of puberty and at the beginning of the transition period. Rate of synthesis in the white adipose tissue and actual plasma level of leptin are in positive relationship with triglyceride content of adipocytes and thus body fatness (long term regulation). Leptin gene expression is also regulated in the short term by the actual energy balance of the organism (Delavaud et al. 2007). Zieba et al. (2007) presents *in vitro* evidence that the secretion of melatonin from the ovine pineal gland is stimulated by leptin during short days (decreasing photoperiod). Thus, the adequate leptin levels may enhance optimal melatonin signal to increase GnRH pulsatility. Furthermore, for a given body fatness level, plasma leptin and adipose tissue leptin gene expression were decreased by short day length which could increase the sensitivity of reproduction to a critical plasma leptin threshold (Bocquier et al. 1998).

Another key element of the neuroendocrine pathways connecting metabolic status to reproductive events is IGF-I (Breier 1999, Renaville et al. 2002). A threshold level is needed to enable follicular growth and maturation (Spicer et al. 1993, 1996; Spicer 2001). On the other hand in sheep IGF-I takes part in the regulation of mammogenesis and galactopoiesis too. High milk yield increases energy expenditure and leads to impaired energy balance, which may delay the onset of ovarian cyclicity following lambing.

These findings draw attention to the fact that when judging the utility of genetic markers, ex. MT1 polymorhism, for less expressed seasonality one must be aware that the phenotypic appearance is highly dependent on the physiological status of the individual animal.

2.1.5. The "ram effect"

The first ovulation marking the beginning of the breeding season can be triggered if an intact or vasectomized (teaser) ram is present ("ram effect"). The stimulating effect of the ram results from certain species- and gender-specific odorous substances called pheromones. In some breeds (Merino, Rambouillet, Dorset, Finnish Landrace, Romanov) sometimes the pheromone effect induces ovulation out of the breeding season only through chemosensory cues (Rekwot et al. 2001, Knights et al. 2002, Evans et al. 2004, Martin et al. 2004, Notter and Cockett 2005).

2.1.6. Ovarian activity in the postpartum period

As seen in the bovine (Huszenicza et al. 2002, 2003a, 2003b, 2003c, 2004; Kátai et al. 2003), the first postpartum (PP) follicular growth in sheep occurs very early after lambing and is followed by regular wave-like pattern of follicular growth regardless of the season of lambing, the plane of nutrition and the presence or absence of suckling. However, the time of the first ovulation is determined by the season and the presence of a nursing lamb (Mandiki et al. 1993, Bartlewski et al. 1999, Vincent et al. 2000, Huszenicza et al. 2003a) and also the nutritional influence may be of practical importance (Robinson 1990, Dunn and Moss 1992).

The individuals conceived in the autumn's breeding period lamb in the end of the winter - beginning of spring. This lambing is followed by an approximately 3-month-long lactation during which the ewes usually suckle their offspring and are weaned only at the end of this period. Despite the relatively rapid onset of the wave-like follicular growth pattern in spring-lambing ewes the first PP ovulation usually occurs only in the subsequent breeding season (at the end of August, beginning of September) because of the consequences of long-lasting suckling and intensive dam-offspring bound combined with the effect of seasonality (Lincoln and Richardson 1998, Bartlewski et al. 1999, Hunter et al. 2004). However, depending on the breed and nutritional status of animals there are flocks where a variable number of spring-lambing ewes may ovulate 60-70 days after lambing (Senger 2003). Reproductive technologies based on autumn breeding - spring lambing are robust, ewes can tolerate easily also certain degree of a temporary feed restriction in a dry summer (Dunn and Moss 1992, Robinson 1990). However, this management system is not really cost effective mainly not for intensive dairy flocks.

In those ewes which become pregnant out of the breeding season (after oestrus induction) and lamb in the autumn period in case of nursing the onset of ovarian cyclicity is similar to that one in suckling beef cows: the first ovulation may take place 35-45 days after lambing. So their re-conception is possible at the end of the same autumn period (Mandiki et

al. 1993, Bartlewski et al. 1999, Vincent et al. 2000, Senger 2003, Hunter et al. 2004). Any forms of the improper energy supply of dams, however, can significantly delay the re-sumption of cyclicity. In a more severe case the ewes can ovulate only in the late August, early September of the next year (Robinson 1990, Dunn and Moss 1992) that results in significant financial losses.

In intensive dairy flocks the year-round milk production provides advantages on the market and the continuous use of the expensive milking machinery is one of the major prerequisites of the financial success. These arguments justify lambing periods twice a year both in the late winter - early spring and also in the autumn season. So the common effect of milk production and seasonality on the time of the first postpartum ovulation can be of extreme importance under these conditions. According to our recent experiences in our intensive milk producing Awassi population (Faigl et al. 2011) the above mentioned principles in PP resumption of cyclic ovarian function can serve only as guidelines. In intensive dairy flocks the newborn lambs are weaned immediately or after the colostral period and fed with milk replacers thereafter. Therefore the consequences of suckling and the continuous dam-offspring bound are not present allowing the very rapid resumption of ovarian cyclicity first of all in the autumn-lambing ewes. This phenomenon might provide a chance for prostaglandin F2α (PGFα) based synchronization techniques in management of reproduction. At the same time, however, these ewes may ovulate before the completion of PP uterine involution that may also be a risk factor. As seen in dairy cows (Lewis 2004, Sheldon and Dobson 2004) the early PP formation of the first CL and the related increase in plasma P4 may predispose the animals for uterine bacterial complications (mucopurulent-purulent forms of endometritis, perhaps pyometra). On the other hand in Awassi ewes the daily milk yield as well as the fat and protein content of the milk may be very high (>2.5 kg/day, >7.0 % and >6.0 %, respectively) and the lactation is longer than 180-200 days that are completely unusual in other sheep breeds. The metabolic consequences of high milk production and the relatively stressful conditions of dairy units have been reported to depress the reproductive performance of dairy cows (Huszenicza et al. 2002, 2003a, 2003b, 2003c, 2004; Kátai et al. 2003, Chilliard et al. 2005). Consequently, these extremities in milk production of dairy ewes and their interaction with other management-specific factors (two lambing seasons per year, lambing in maternity barn, early weaning, and so on) are supposed to (i) influence the onset of cyclicity and the course of uterine involution in the early weeks of lactation, (ii) interfere with the efficacy of oestrous induction / synchronization techniques administered during the last weeks of lactation, and/or (iii) depress the fertility of late-lactating animals. However, further

studies are required to reveal and understand the real metabolic and reproductive characteristics of dairy ewes.

2.1.7. Prolificacy (ovulation rate, twin pregnancy, embryonic and/or early foetal mortality)

In ewes the twinning rate (prolificacy) is an important indicator of reproductive performance based on a breed-related genetic ability for double (or multiple) ovulation and is under the complex influence of nutritional factors, body fat content and climatic conditions (Robinson 1990, Dunn and Moss 1992, Senger 2003, Davis 2004). It has major importance in meat-producing breeds.

The disposition to double or multiple ovulations is an important genetically determined breed characteristic that can be easily examined with DNA-based techniques of molecular biology nowadays (Davis 2004). In breeds belonging to the Merino group the most important genetic factor of prolificacy is the dominantly inherited, autosomal Booroola (FecB) gene, which has an additive effect on ovulation rate. One copy of this gene increases the ovulation rate by about 1.5 and two copies by about 3.0 (Montgomery et al. 2001, Mulsant et al. 2001, Davis 2004). Other genes that increase the ovulation rate are restricted to occur only in certain breeds or populations (FecX2 gene in Coopworth ewes in New Zealand; Lacaune gene in the French Lacaune meat sheep population) and/or cause sterility in homozygous carrier females (FecX gene in Romney sheep; FecGH gene in Cambridge and Belclare breeds). So, compared to that of FecB gene, their practical importance is limited in Central-East Europe (Davis 2004).

In flocks the proportion of polyovulation depends also on the nutritional state and in a smaller percent on the current energy balance of animals. With the method of “flushing” the ovulation rate can be elevated by 20 % (Robinson 1990; Downing and Scaramuzzi 1991; Dunn and Moss 1992, Hunter et al. 2004, Martin et al. 2004). Following sweet lupin (*Lupinus albus*) seed feeding the ovulation rate increases in higher percent than with the use of other isocaloric/isonitrogen feed ingredients. The advantages of the lupin seed feeding are evident already 4-6 days later. This effect is multifactorial: beside the stimulation of the glucose metabolism in the GnRH-producing neurons, it activates gradually the aromatization in the gonadotroph-sensitive granulose cells of the antral follicles and increases the intrafollicular IGF-I concentration as well as its biological availability (Munoz-Gutiérrez et al. 2002, 2004, Hunter et al. 2004, Martin et al. 2004). According to the above mentioned facts, flushing technology with lupin seed may be suitable to bring forward the first ovulation at the beginning of breeding season and to increase the ovulation rate.

Another possibility is to trigger multiple ovulations with pharmacological methods, e.g. administration of eCG with FSH-like activity (McNeilly et al. 1985, Scott and Clarke 1993, Bister et al. 1999) or as a more recent alternative, with immunisation against either oestrogen precursors produced by the theca interna cells of the follicles or inhibin (Wilkins 1997, Ptaszynska 2002; Senger, 2003). After eCG treatment the ovulation rate depends, amongst others, on the pretreatment with gestagen (Bister et al. 1999). In Europe currently there are no commercially available preparations for active immunization against precursors of estrogens and / or inhibin. However, their introduction on the market does not depend on scientific facts but more on economical policy.

As shown in experiments in cattle (Gong 2002) the daily treatment with growth hormone (GH) throughout one oestrous cycle causes an increased production of IGF-I in the liver. Consequently, the high level of IGF-I in the peripheral blood – and also in the follicular fluid of antral follicles – provides an ideal microenvironment for more than one members of a cohort of follicles to emerge, develop and reach the stage of dominance. So at the end more than one DF-s per follicular wave is available at the time of the preovulatory LH peak and will ovulate later. The same mechanism works perfectly also in ewes (Bister et al. 1999). However, according to the actual food-safety regulations the GH treatment is strictly forbidden in Europe. So we set aside from its further specification.

It is important to mention that in sheep an unknown percentage (7-46 %?!) of twin pregnancies is reduced to a single pregnancy or lost completely (Ptaszynska 2002, Senger 2003, Martin et al. 2004).

According to our current understanding in ewes embryonic and/or early foetal mortality may occur almost exclusively in the early CL-dependent phase (in the first 50 days) of gestation mainly due to an obvious, sometimes critical P4 decrease between days 14-20 and 50. After day 50 the placenta starts to produce sufficient quantity of P4 (Edqvist and Forsberg 1997, Senger 2003; Martin et al. 2004). Nutritional and/or pharmacological methods themselves are not able to increase the chance of embryonic survival (Wilkins 1997). Beside twins and the heat stress also the shortage of energy supply and the energy- and perhaps protein-overfeeding have been proven to trigger the interruption of pregnancy during the first 50 days. In dairy cows overfed with rumen-degradable protein sources the massive intraruminal production of ammonia and the subsequent increase of plasma urea may be detrimental for the embryo survival (Butler et al. 1996, Butler 1998). Similar losses may occur sometimes also in alfalfa-fed dairy ewes (Kulcsár et al. 2005). The shortage of energy supply can reduce the secretory capacity of endometrium. The paradoxical effect of too

abundant energy intake is explained by the increased P4 metabolism in the liver. Due to this latter reason it is essential to know that "flushing" used to induce the first ovulation of the breeding season and/or stimulate multiple ovulations shall be stopped at the proper time within some days after ovulation/conception (Robinson 1990, Stewart 1990, Dunn and Moss 1992, Senger 2003, Martin et al. 2004, Kleemann and Walker 2005a, 2005b). However, in the practice our currently available methods are not sufficient enough to identify the dams affected by embryonic/early foetal mortality and to reveal its current cause. So, both the real incidence of and the underlying factors leading to this form of losses remain usually undefined under flock conditions.

2.1.8. Other nutritional effects upon reproduction

The actual feeding may also influence the incidence of pregnancy toxicosis (ketosis), the quantity and quality of colostrum, the viability of the newborn lambs and the sperm production in rams (Robinson 1990, Downing and Scaramuzzi 1991, Dunn and Moss 1992, Martin et al. 2004). However, this complex issue cannot be discussed entirely within the frame of this paper and passes over the subject of the current thesis.

2.1.9. Pharmacological methods used to manipulate the ovarian function

In management of ovine reproduction there are two main forms of pharmacological methods used to manipulate the ovarian function (Ptaszynska 2002, Senger 2003): (a) the induction of cyclicity in acyclic animals, furthermore (b) the synchronisation of oestrous and ovulation in animals with cyclic ovarian activity.

Induction of ovarian cyclicity in seasonally acyclic ewes

Ovulation with subsequent cyclic ovarian activity can be induced out of the breeding season in the spring to early summer period (pregnancies for autumn lambing) or before the beginning of the breeding season in mid to late August (shortening the transition period). The methods that are used are more or less independent from the season whereas the results are better in August than in April to June (Ptaszynska 2002, Senger 2003). The optimal body condition is an important prerequisite of the success (Robinson 1990). The ovarian response to these treatment procedures can be enhanced by a preceding 4 days to 3 weeks energy supplementation ("flushing") and/or the exposure of males ("ram effect" related to pheromone exposure) (Knights et al. 2002, Ptaszynska 2002, Senger 2003, Evans et al. 2004, Martin et al. 2004, Notter and Cockett 2005).

The 10 to 14-day-long gestagen treatment combined with administration of 400-600 IU eCG at the time of gestagen removal is the most common method in the everyday practice which has been used successfully on thousands of animals for many years. As an active ingredient both the natural P4 and its synthetic analogues (medroxyprogesterone acetate: MAP; cronolone syn. flourgeston acetate: FGA and others) can be administered as an intravaginal pessary, sponge or as a subcutaneous implant (Alifakiotis 1985, McNeilly et al. 1985, Boscos et al. 2002, Ptaszynska 2002). During the 10 to 14-day-long gestagen treatment the pulse frequency of the oscillating basal LH release is suppressed. So the final maturation of DF-s is blocked, the most of them become atretic. Injection of FSH-like eCG at the time of gestagen removal induces a simultaneous recruitment of a new cohort of follicles. Soon after gestagen removal the LH pulsatility accelerates which increases the E2 production and maturation of one or two selected DF-s meanwhile. Additionally, the P4 (-like) priming enhances the E2 sensitivity of hypothalamic surge centre resulting in ovulation at about the 48^{th} to 60^{th} hour (Senger et al. 2003). During the FGA treatment a slight decrease in ACTH (adrenocorticotrop hormone) and cortisol levels, increasing tendency of plasma leptin and weaker ACTH-induced cortisol responses were observed. After removal these clinically negligible alterations disappeared completely (Kulcsár et al. 2005). Beside their low cost and general acceptance the main advantage of these gestagen-based methods is that the animals can be mated or inseminated at a fixed time, e.g. 48 and 60 hours (2x) or about 55 hours (1x) after removal. The only disadvantageous aspect of their use is that each animal must be caught and handled twice i.e. at the time of administration and removal of the gestagen source.

Although in some studies about half of the treated ewes ovulated and became pregnant due to the lack of P4 priming the GnRH treatment is usually considered not powerful enough to induce ovarian cyclicity out of the breeding season in sheep (Ptaszynska 2002, Senger 2003).

Photoperiodic treatments and the use of melatonin

The melatonin treatment is a recent alternative of gestagen-based methods. It can be administered as a subcutaneous implant releasing the ingredient from the vehicle continuously for approximately 8 weeks. By the end of this 8-week period while elevated melatonin levels (additional to the circadian changes of this hormone) are seen, the pulsatile pattern of GnRH and LH secretion has been modified: both the pulse frequency and concentration range of the

basal LH secretion increases (Lincoln et al. 1982; Arendt 1995). First ovulations are expected to occur about five to six weeks after the melatonin insertion and the ovarian function becomes cyclic thereafter. Due to the biodegradable feature of its vehicle there is no need of implant removal. However, ovulations and estruses are only induced but not synchronized within the flock: so an additional synchronization procedure is needed for the fixed-time insemination (Roche et al. 1985, Thimonier and Ortavant 1985, Malpaux et al. 1998, Bister et al. 1999, Thiery et al. 2002, Senger 2003, Deletang 2004). Melatonin implants are mostly administered around the time of the summer solstice (mid June) to trigger the first ovulation at the beginning of the breeding season (Rondon et al. 1996, Ptaszinska 2002, Deletang 2004, Gomez et al. 2006, Abecia et al. 2006b), and application in early summer usually fails to induce cyclicity due to the decreased sensitivity to melatonin in that phase of the year, - the phenomenon called "photorefractoriness" (Bittman and Karsch 1984, Chemineau et al. 1996a, 1996b). In Mediterranean breeds, however, good ovarian response was observed also after a late winter to early spring implant insertion (Deletang 2004). Melatonin can be used alone, or combined with other hormonal treatments, eventually after an artificial light (photoperiodic) treatment. In several studies concerning ewes of different breeds, melatonin administration has been found to advance the onset of reproductive activity (Arendt et al. 1983, Karsch et al. 1984, English et al. 1986); furthermore melatonin-treated ewes appeared to have better oocyte quality, greater ovulation and conception rates (Stellflug et al. 1988, Kouimtzis et al. 1989, Tsiligianni et al. 2009).

Further studies are needed to reveal (i) the real efficacy of melatonin treatment in breeds of steppe origin kept under Central-East European conditions, (ii) its interaction with lactation and MT1 genotype as well as (iii) its supposed direct effect on ovarian structures and embryonic cells.

Synchronisation of oestrous and ovulation in cyclic animals

In cyclic animals the oestrous and ovulation can be synchronized with the same gestagen-based methods as used for induction of ovulation and cyclicity although the dose of certain gestagens (FGA) may be higher in than out of the breeding season. The luteolytic dose of PGF2α might be an alternative of gestagens that seems to be very attractive from food safety considerations. Its disadvantage is, however, that in sheep the luteolytic dose of PGF2α is relatively high and therefore expensive (Pteszynska 2002, Senger 2003). The administration of PGF2α may damage the wave-like dynamics of the follicular growth and the CL

development causing great differences in the time of oestrous and ovulation (Barrett et al. 2002). Furthermore, its administration is completely ineffective in acyclic individuals (Pteszynska 2002, Senger 2003). The latter argument is of importance because nowadays there is no available diagnostic method applicable in the everyday practice of the ovine reproduction that can provide reliable information concerning the presence or absence of ovarian cyclicity and/or the current stage of the cycle.

In dairy cows the combined administration of GnRH-PGF2α-GnRH (OvSynch or GPG protocol) is more and more popular. During the 9-12 weeks of lactation this treatment is expected to induce ovulation and cyclicity in acyclic individuals and synchronize the ovarian activity in cyclic cows. Although its effect is more reliable in the latter (already cyclic) animals fixed time insemination is possible in both cases (Pteszynska 2002, Senger 2003, Gábor et al. 2004). The food safety consideration of this procedure is excellent. So its administration seems to be promising first of all in dairy flocks. The reason for not having too much experience in sheep is the high price of products furthermore the fact that in ewes there might be remarkable individual variations in dynamics and per-cycle number of waves of follicle growth. Greek experiments tested the success of this method on 2.5-4.0 year old ewes with average body condition (n=28) in the middle of the biological breeding season (Deligiannis et al. 2005). According to the species-specific dynamics of the follicular growth the first treatment with GnRH (day 0.; resulting in ovulation and development of CL or intrafollicular luteinization) was followed by induction of luteolysis by PGF2α on day 5 and 48 h later a second dose of GnRH was administered. The ewes were inseminated with fresh diluted semen (intrauterine deposition, through laparoscopy) 16-20 hours after the second GnRH. Half of the animals became pregnant. Despite of these relatively promising preliminary results further studies are required to demonstrate the reliability of this method in sheep. We are sceptic concerning its applicability as a single technique out of the breeding season. However, the species- (and breed?) specific version of OvSynch may represent some value in reproductive management of dairy flocks after a preceding melatonin administration.

2.2. Reproduction of rams

2.2.1. Seasonality of reproduction in rams

To maximize the efficacy of commercial sheep flock during the non-breeding season good quality sperm production is elementary, especially in systems where artificial insemination (AI) is used. Similarly to ewes the most important regulatory factor of reproductive

seasonality is the photoperiodic signal in males as well. At the same time reproductive seasonality of rams is in general less expressed relative to ewes. Seasonal changes out-of the breeding season are reflected in smaller size of testicles, decreasing concentration and poorer quality of semen, decreased LH pulsatility and decreased basal testosterone level (Ortavant et al. 1985, Olster and Foster 1988, Senger 2003). Intrestingly prepubertal rams seem to be less sensitive to the inhibitory long-day photoperiod, and under natural light regime reach puberty before the older rams of the same flock enter the breeding season (Olster and Foster 1986, 1988; Claypool and Foster 1990).

2.2.2. Melatonin and photoperiodic treatments in rams

Similarly to ewes long-lasting melatonin treatment was shown to influence several parameters of reproductive activity in ram as well. In rams, the administration of melatonin under long daylight period, induces an increase of luteotrop hormone (LH) and follicle stimulating hormone (FSH) concentrations as well as a reduction of prolactin level in blood plasma; it has also been demonstrated that melatonin increases mean testosterone concentration (Kennaway and Gilmore 1985, Lincoln et al. 1985, Lincoln and Kelly 1989, Bourla 1991, Chemineau et al. 1992). Melatonin treatment increases ram effect, which may synchronize estrus of dams and lead to more expressed estrous behavior, resulting finally in better reproductive results (Rosa et al. 2000).

Although numerous studies and reviews present data connected with the effect of melatonin treatment and different light programs applied in rams, no data are available concerning Awassi rams kept in the temperate continental zone of Europe and used in artificial insemination program.

3. Aims of the studies

Among other breeds of dairy sheep, there are some imported flocks of Improved Awassi in Hungary. The investigated population was kept in an intensive management system, and at the beginning of our research more than half of the Hungarian sheep milk production was yielded by the examined flock. The climate and feeding in the lowland area of Carpatian basin are completely different than that in the Middle East, and also the daily and seasonal rhythm of daylight is fully disagree. Our aim was to investigate the reproductive seasonality of the dairy Awassi sheep in Hungary and its relation to the melatonin receptor 1a (MT1) polymorphism. Secondly we wished to test the value of hormonal and non-hormonal tools to alter seasonal variations. To have an insight on these caracteristics, we aimed to perform the following experiments:

1a) Following up of ovarian activity in the postpartum period, to determine the time of resumption of cyclicity and to identify the factors influencing it (season, age, parity, milk yield, metabolic status, as a consequence of the possible presence of negative energy balance in lactating dams: lipid mobilization, hyperketonaemia).

1b) On the basis of data collected in the first experiment, in the second trial we tried to use one of the identified factors (daylength/light exposure) to modify the length of postpartum acyclicity.

2) Determination of the proportion of dams showing out-of-season ovarian cyclicity and to recognize the factors affecting this capability (age, parity, body weight, metabolic status, milk yield and possible impact of MT1 polymorphism).

3) To compare the definite efficacy of different cycle induction/synchronization protocols at different time points of the year. With special attention to the efficiency of long-lasting melatonin treatment and the so-called Ovsynch protocol.

4) To evaluate the effect of long-term melatonin treatment applied during non-breeding season on semen characteristics and endocrine function of testicles in Awassi rams used as semen donors in artificial insemination programs.

5) Finally by synthetizing the knowledge acquired through the above experiments we intended to work out a recommendation of reproductive management system (know-how) for intensive dairy Awassi flocks with focusing on the biological speciality of the given breed under continental weather.

4. Materials and methods

4.1. Animal housing and management

Description of the common housing, management and nutrition systems of the experiments are given here, while specifications on the circumstances of the given study are detailed under the trial (Sections 5.1., 5.2., 5.3 and 5.4.). All studies were performed in accordance with the rules and under the permission and control of the state veterinary service. All of them were carried out following the guidelines given by the code of ethics of the Szent István University, Faculty of Veterinary Sciences Animal Welfare Board.

Experiments were conducted on a lactating but non suckling commercial Awassi flock in Bakonszeg, Hungary (latitude: 47º11', longitude: 21º26'). Rams of Experiment 4 were housed at the AI center of the farm in open-sided barns. Ewes were kept in intensive system, housed in opened barns and fed with total mixed ratio (TMR) according to their actual milk production. The diet was formulated to provide net energy, metabolisable protein, minerals and vitamins at National Research Council (1985) recommended levels. TMR was offered twice a day under the trial. Ewes had ad libitum access to water, hay and trace mineral salt blocks. Lambs were weaned within 24 hours after birth and were fed with milk replacers. Dams were machine milked twice daily.

4.2. General methods

4.2.1. Following up the ovarian activity

Ovarian activity of lactating dams was monitored by individual milk progesterone profiles. Milk samples were collected at the beginning of morning milking into plastic tubes containing potassium-bicromate as preservative. Samples were stored at +4ºC until assaying.

In case of Experiment 3 in first parity ewes and non-lactating dams gestagen metabolites were assayed from feces. Samples were collected from the rectum, put into polypropylene bags and stored at -20˚C until further procedure.

4.2.2. Body condition scoring

Body condition was scored at each time by the same person with the method used in merino breeds (Thompson and Meyer 1994). We used a 5 scale range and took account with palpation of subcutanous fat thickness at the area of backbone just behind the rib cage. Unfortunately this system did not take into consideration the fat depots of the fat tail.

4.2.3. Blood sample processing

Blood samples were collected before the morning feeding from the jugular vein in Experiment 1, 2 and 3. In Experiment 4 blood samples for determination of basal hormone levels of rams were collected following sperm collection, and later according to the timing of the challenge test. Coat activated tubes were used for serum and heparinised tubes for plasma collection. All samples were centrifuged within 60 minutes. Separated plasma was divided into five equal parts for different hormone assays, serum was used for β-OH-butyrate and non-esterified fatty acid measurement. Samples were stored at -20ºC until further procedure.

4.2.4. Laboratory procedures

Hormone and plasma metabolite analysis used in several studies is detailed here while some procedures special for an experiment are completed at the given section. All of the endocrine assay systems used in these studies were validated previously for ovine plasma: the binding pattern of serially diluted plasma or milk samples (with known high quantity of the hormone analyzed) was parallel to that of the standard curves; the recovery rates of added known quantity of hormones to standard ovine plasma samples (n=6 in each case) varied between 95 and 106%. Using triplicates of quality control samples with known (low, intermediate and high) quantity of the analyzed hormone, the reproducibility (intra- and inter-assay coefficient of variation, CV%) of each assay run was checked continuously. The results were accepted only if the actually measured concentrations of quality control samples were within the 95% confidence limit.

4.2.4.1. Progesterone, progesterone metabolite, testosterone and pregnancy associated glycoprotein (PAG) determination

Progesterone content of skimmed milk was determined similarly to our previous works with a locally developed microplate enzyme-linked immuno-sorbent assay (ELISA) (Nagy et al. 1998, Huszenicza et al. 1998; Taponen et al. 2002), within 14 days after collection. According to our former experiments in the same population, the threshold P4 level for active corpus luteum was determined at 4 nmol/L (Márton et al. 2009). Samples were assayed in triplicates. Three different ranges of quality controls (low, medium, high) were used. Interassay CV was 13.7%, 10.2% and 6.1% for low, medium and high controls respectively. Intraassay CV was <10%. Sensitivity: 0.53 ± 0.036 nmol/L (mean ± SEM).

For assaying ***progesterone metabolites***, steroids were extracted from feces similarly as descriebed in ferret by Proháczik et al. (2011). The progesterone metabolite concentrations were quantified in triplicate with the same ELISA system as used for milk samples. Cross-reactivity with different gestagen metabolites was the following: 5β-pregnan-3,20-dione:

100%; 11α-OH-progesterone: 20%; 5α-progesterone-3,20-dione: 15.6%; 17α-OH-progesterone: 3.6%; pregnenolone: 1.8%; 11β-OH-progesterone: 1.6% (Siklódi et al. 1995).

Plasma ***testosterone*** was assayed with a ^{3}H-radioimmuno assay method (Csernus 1981), adapted and validated for small ruminant (sheep, goat, roebuck) plasma and serum (Leitold et al. 2004) (minimal detectable concentration: 1.84-1.98 nmol/L, interassay CV 3.22%-15.5%, intraassay CV: <10%)

Ovine ***PAG*** concentration was measured from plasma samples with a homologous radioimmunoassay method according to Vandaele et al. (2005).

4.2.4.2. Metabolic hormones and blood metabolites

Insulin level, as free insulin, was determined with a commercialy available ^{125}I-immunoradiometric assay (IRMA) kit validated for sheep (BI-Insulin IRMA kit; CIS Bio International Ltd – Subsidiary of Schering S.A., Gif-Sur-Yvette, France; Sensitivity: 0.86 pmol/L; intra- and interassay CV: 1.3-5.6 % and <8.5 %).

Insulin-like growth factor I concentration was measured with an ^{125}I-IRMA method (DSL-5600 Active IGF-I Coated-Tube IRMA Kit; Diagnostic Systems Laboratories Inc., Webster, Texas, USA) developed for human samples, which was adapted and validated for this purpose with a small modification. Following a preceding extraction of IGF-I with an ethanolic HCl solution and neutralisation of the solution, the extract was incubated at +4ºC for 12 hours (instead of 3 hours at room temperature). Further analysis was performed according to the manufacturers' prescription. (Sensitivity: 0.13 nmol/L; intra- and interassay CV: 3.5-6.9 % and <7.0 %).

Plasma ***leptin*** concentration was quantified by a modified version of the ruminant-specific, homologous, double-antibody, non-equilibrium ^{125}I-radioimmuno assay (RIA) of Delavaud et al. (2000, 2002). The current adaptation of this assay was based on the use of an anti-ovine leptin antibody yielded by Delavaud et al. (2000) in rabbit. Instead of recombinant ovine leptin ($_{ro}$leptin), however, in this version a commercially available form of recombinant bovine leptin ($_{rb}$leptin) was used for radioiodination, as well as for preparing the standards. Furthermore the bound and free ligands were separated by a magnetisable immunosorbent suspension, rather than applying a specific anti-rabbit second antibody. The $_{rb}$leptin was labeled with ^{125}I by Chloramine T method (Hunter and Greenwood 1962), modified by Kulcsár (2007). During the assay procedure: 100 µL horse serum was added to all the standards, as well as to the B0 and NSB tubes to obtain a similar protein matrix as in samples. As samples and quality control tubes (n=3; with 0.058±0.004, 0.241±0.008 and 0.577±0.023

nmol/L levels, as regular quality control samples with “low”, “medium” and “high” leptin content in each assay run), triplicate aliquots of 100 µl plasma were used. Bound and free ligands were then separated by adding 500 µl ice-cold magnetisable immunosorbent suspension. Tubes were incubated for 30 minutes at room temperature. After centrifugation (3600 g, 30 min., + 4˚C) all the tubes were put in a magnetic separator, and the supernatant was decanted. The remaining radioactivity on the microparticules was quantified by gamma counter. Sensitivity of this assay was 0.032 nmol/L. Inter- or intraassay coefficients of variation (CV) were 12.15, 5.61 and 6.13%, or 10.06, 4.57 and 5.28% in ranges of quality control samples with “low”, “medium” and “high” leptin content, respectively.

Thyroxin level was determined with ^{125}I-RIA. The thyroxine (T4) kit (^{125}I-T4 CT-spec. RIA, Institute of Isotopes Co. Ltd., Budapest, Hungary) was originally developed for human use, and was slightly modified for assaying animal samples (Huszenicza et al. 2000, Kulcsár et al. 2006). ***β-OH-butyrate*** (BHB), ***non-esterified fatty acid*** (NEFA) and ***plasma urea nitrogen*** (PUN) levels were analyzed with commercially available enzymatic kits (NEFA and BHB: Randox Laboratories Ltd, Ardmore, UK ; PUN: Diagnosticum Ltd., Budapest, Hungary).

4.2.4.3. Determining RFLP of MT1 gene

Blood samples for PCR reaction were collected into vacuum tubes containing ethylene-diamine tetraacetic acid sodium salt (Na-EDTA) as anticoagulant. For PCR reaction DNA was extracted from blood using a salting out procedure (Zsolnai and Orbán 1999). Primers (Applied Biosystems, USA), PCR conditions for amplification of exon II of the MT1 gene and the restriction conditions for each MT1 RFLP (restriction fragment length polymorphism) test (RsaI and MnlI) were as described by Messer et al. (1997). Products of restriction digestion were resolved by electrophoresis on a 2.5% Metaphor Agarose gel (Cambrex Bio Science, Rockland, ME, USA), in parallel with a 100 bp DNA marker (Fermentas, Finnland). Samples were genotyped according to the length of the digested PCR products. The allele was called “R” and “M” when the PCR product was cut, and called “r” and “m” if it was not cut by the restriction enzymes RsaI and MnlI, respectively (Figures 4.2.3.1. and 4.2.3.2.).

Allel names were given as follows:

RsaI: 290 base = r allele [presence of a thymine (T) at position 606 of the sequence]

267 base = R allele [presence of a cytosine (C) at position 606 of the sequence]

MnlI: 303 base = m allele [presence of a adenine (A) at position 612 of the sequence]

236 base = M allele [presence of a guanine (G) at position 612 of the sequence]

Figure 4.2.3.1. Polymorphism of the cleavage site RsaI: first lane L is a molecular ladder 100 bp; lane RR and rR are the genotypes according to the substitution

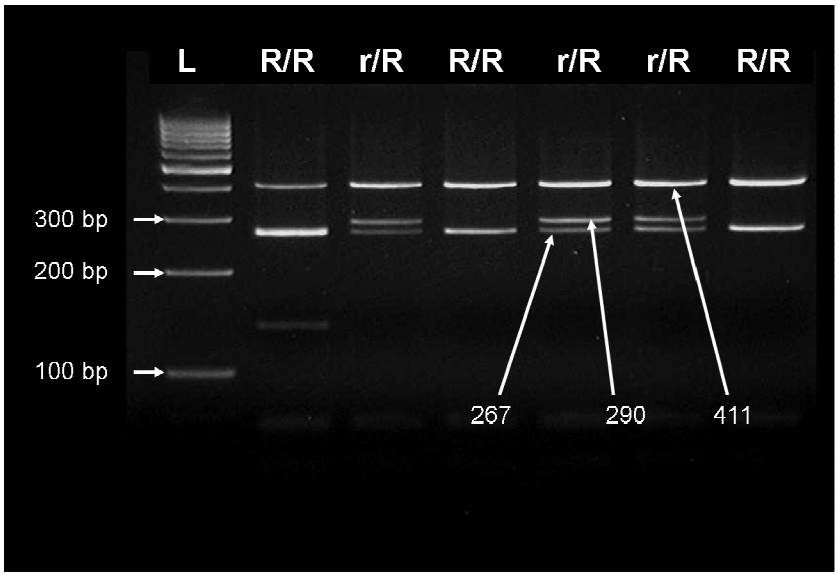
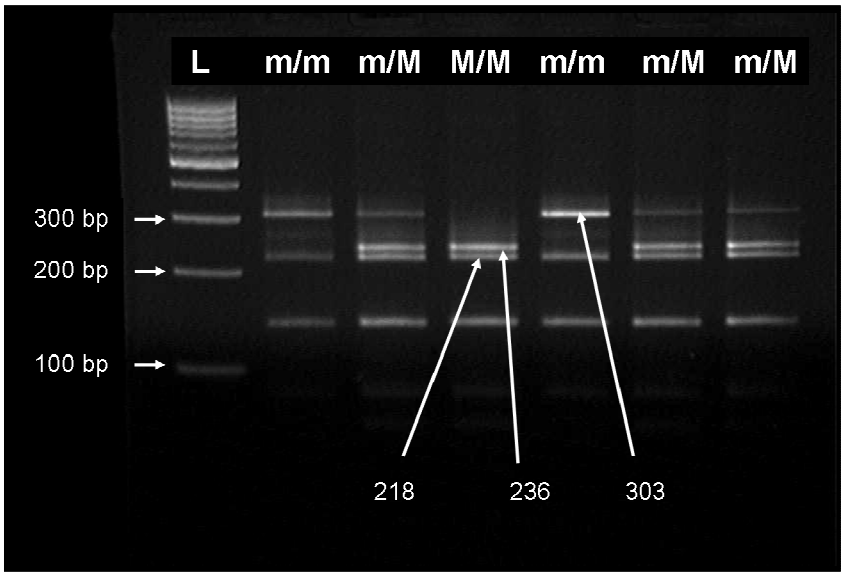


Figure 4.2.3.2. Polymorphism of the cleavage site MnlI: first lane L is a molecular ladder 100 bp; lane mm, mM and MM are the genotypes according to the substitution.



5. Results and discussion

5.1. Effect of season and photoperiod on the time of first postpartum ovulation in Awassi ewes (Exp 1 and 2)

Experiment 1: Effect of season

In the first experiment (Exp. 1) 1-11 parity autumn-lambing (AL; n_{AL}= 37; lambed between the end of September and mid October) and spring-lambing (SL; n_{SL}= 41; conceived following cycle induction and lambed between the end of February and end of March) ewes were involved. Autumn lambing dams were kept together with rams, thus natural mating was possible. Contrary to the autumn lambing group spring lambing animals were separated from rams with fence, therefore although pheromone effect was similar to the AL group, but mating and early conception was not possible in SL ewes during the experimental period.

Periparturient energetic status was monitored by bodyweight and body condition scoring 1 week before lambing and again on weeks 1, 2 and 5 postpartum. On the same days blood samples were collected and assayed for some metabolites (BHB, NEFA) and metabolic hormones (insulin, IGF-I, thyroxin). Postpartum ovarian activity was monitored by measuring milk P4 level trice weekly as described above. Data on milk production and reproduction were followed up until the day of conception or the end of lactation. Day of conception was calculated retrospectively from the subsequent lambing dates (lambing date minus 150 days).

Statistical analysis

Statistical analysis was performed using Statistica 9.0 software (StatSoft Inc., Tulsa, USA). Data are shown as means ± the standard error of mean (±SEM). Two means were compared with Student's t-test. Proportion of twin lambing was collated with chi-square test.

Day of first postpartum ovulation and postovulatory ovarian activity were estimated by means of individual progesterone profiles (Figures 5.1.3 and 5.1.5). Day of first postpartum ovulation was evaluated in animals which did not dry off before the first ovulation. Luteal activity was defined as elevated P4 (≥4 nmol/L) values in at least two consecutive milk samples. Day of first postpartum ovulation was expressed as the day when the last low progesterone value was measured before the appearence of corpus luteum. Comparison of ovulation day and length of lactation was made by survival analysis. Difference between two survival curves when the sample size of any of the groups was below 30 (ex. the day of the first postpartum ovulation in both experiments and lactational length in the second

experiment) is given using Cox's F test (Cox 1964, Cox and Oakes 1984). Length of lactation in the first experiment is compared by Kaplan-Meier cumulative proportion surviving by group. In the second experiment, where the observation period was shorter than the duration of lactation, data of animals still in milk at the end of trial was marked as censored (Harrington and Fleming 1982).

In most of the cases P values higher than 0.05 are not shown, they are signaled as non significant (NS).

Results of Experiment 1

Milk production, body condition, and metabolic status

Age and proportion of twin lambing was similar in AL and SL group (age: AL 4.7 vs. SL 5.1 years; proportion of twin lambing 8%; NS). Daily milk yield varied between 1.0-2.5 L on the 2nd – 3rd week postpartum with no significant differences between groups. In AL group length of lactation was significantly shorter as compared to SL ewes (P=0.008; Figure 5.1.1.). All SL ewes dried off until d 150, however maximum lactation length in the AL group was close to 250 days. Nine AL and 7 SL dams dried off before d 40; these profiles were too short for further analysis, thus these animals were not involved in the evaluation of first postpartum ovulation. Predominantly, SL animals lactated at least for 70-80 days, most of them for more than 100 days. Average lactation period was 77 days in AL vs. 124 days in SL ewes (P=0.002).

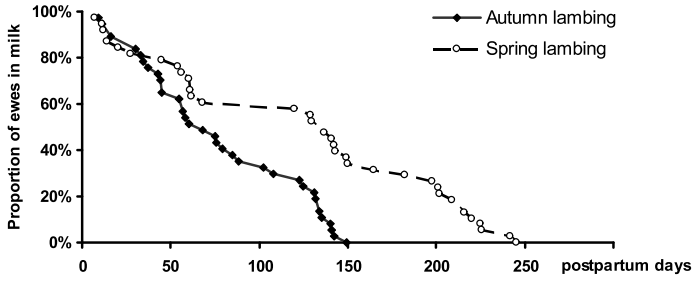


Figure 5.1.1. Length of lactation in autumn lambing and spring lambing dams. (P=0.008 Kaplan-Meier cumulative proportion surviving by group) (Exp.1)

By the end of gestational period BCS varied between 3-5 points independently of season (mean: 3.89±0.18). Body weight ranged between 58 and 92 kg. During the first week postpartum BCS dropped slightly and body weight decreased by 7-11 kg, however these

changes were not significant. No further change was seen in BCS or weight during the forthcoming period.

Metabolic parameters remained within the normal physiological range throughout the experiment (Table 5.1.1.), we did not see any sign of energetic imbalance or elevation of BHB level indicating subclinical ketosis (e.g. elevated BHB level was defined as BHB ≥1.60 mmol/L or ≥1.20 mmol/L in two consecutive samples; Henze et al., 1998). NEFA and BHB values were slightly higher on week -1 compared to the postpartum period, but this difference was statistically not significant. From week 1 to 5 postpartum the above mentioned parameters remained unchanged. No seasonal differences were seen in BHB and NEFA levels.

Table 5.1.1. Non-esterified fatty acid (NEFA) and β-OH-butyrate (BHB) levels in the periparturient period (mean ± SEM) (Exp.1)

	Week -1	Week +1	Week +2	Week +5
Autumn lambing				
NEFA (mmol/L)	0.50 ± 0.08	0.39 ± 0.04	0.29 ± 0.05	0.14 ± 0.02
BHB (mmol/L)	0.55 ± 0.05	0.35 ± 0.02	0.34 ± 0.03	0.44 ± 0.05
Spring lambing				
NEFA (mmol/L)	0.51 ± 0.09	0.30 ± 0.04	0.23 ± 0.02	0.24 ± 0.03
BHB (mmol/L)	0.60 ±0.05	0.45 ±0.03	0.46 ±0.04	0.48 ±0.05

Compared to Holstein Friesian cattle (Nikolić et al. 2003, Lucy et al. 2009) T4, insulin and IGF-I levels were high by the end of gestation (Figure 5.1.2). At the same time despite similar feeding, seasonal variation in these hormone levels was found. Thyroxin, insulin and IGF-I concentrations were significantly lower in AL group compared to SL. In both seasons T4 levels increased gradually following parturition during the experimental period. In SL group insulin and IGF-I decreased significantly in the same period. In AL dams IGF-I remained unchanged following parturition and insulin showed slight and fluctuating elevation. T4 and IGF-I values were constantly higher in SL compared to AL, at the same time seasonal differences in insulin concentration became moderate during the first 2 weeks of lactation and equalized by week 5 postpartum.

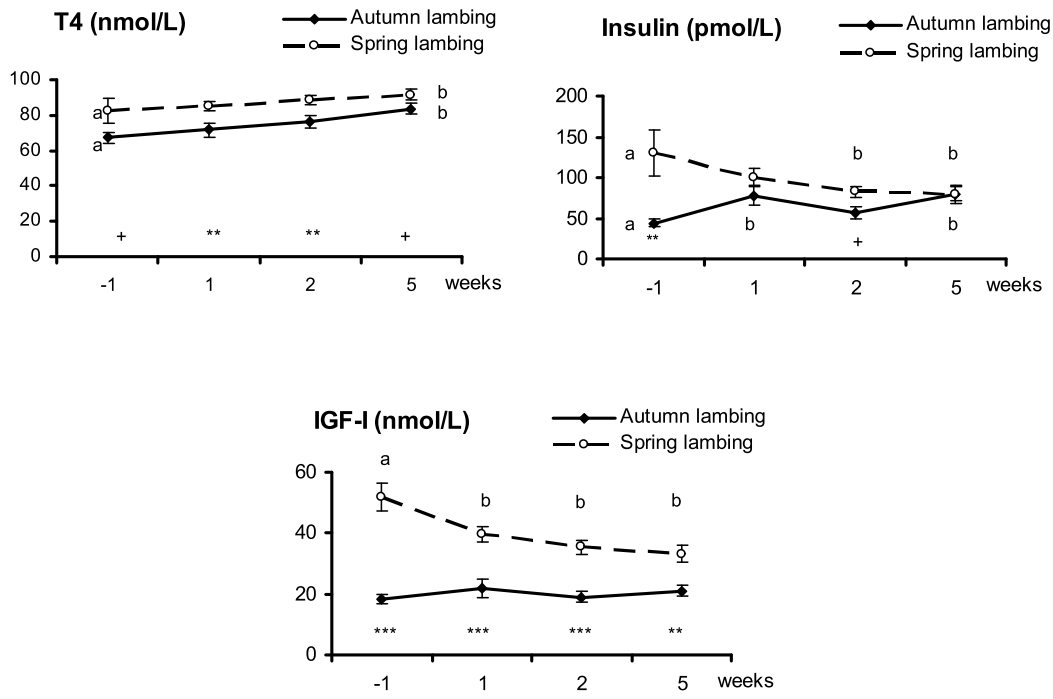


Figure 5.1.2. Thyroxin, insulin and insulin-like growth factor I levels in the periparturient period (Exp. 1) Note: differences between AL and SL marked as $^{+}P<0.1$; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. Differences between different sampling days: $^{a\text{-}b}P<0.05$.

Postpartum ovarian activity

In the AL group following up of ovarian activity was possible in 27 ewes which had complete set of samples. Cyclic ovarian activity resumed in all cases before day 56 postpartum. Great proportion of ewes (n=24=89%) ovulated before day 35; two of them before d 9 and six further animals between d 10-19, i.e. these ewes ovulated from the 1st or 2nd postpartum follicular wave. Animal "A" of Figure 5.1.3. is a characteristic example for the above described P4 pattern, where first ovulation occurs around day 10-11, it is followed by a sCL and 4 regular luteal phases. In January around day 100 postpartum the animal became acyclic.

Following the first ovulation luteal phase was shorter than 10 days in 11 dams (41%). When short luteal phase (sCL; <10 days) appeared following the first ovulation we considered it as a physiological event. During the autumn season altogether 11 ewes conceived from rams following the 1st to 4th ovulation (41%; 1, 6, 3 and 1 ewes following the 1st, 2nd, 3rd and 4th ovulation, respectively), and most of them dried off soon after, similarly to animal "B" of Figure 5.1.3. where first ovulation happened between day 15-16 postpartum, it was followed by a luteal phase with normal length but reduced progesterone production. Two normal luteal

phases come after. Finally the animal conceived from the 3rd ovulation and dried off 17-20 days later.

Three animals in the AL group which got pregnant around days 46, 54 and 63 postpartum lactated for more than 100 days, just as like animal “C” of Figure 5.1.3. First postpartum ovulation showed up around d 22, it was followed by a sCL and 3 physiological CL phases. The animal got pregnant from the 4th ovulation and lactated permanently.

Animals which did not conceive (n=16) lactated for a longer period. Seven among them became acyclic by January-February (Figure 5.1.3., animal A). Irregular progesterone profiles were frequent among ewes which did not conceive. Animal “D” of Figure 5.1.3. ovulates around d 16-17, first ovulation is followed by a luteal phase with normal length but reduced P4 secretion. Three subsequent normal luteal phases can be detected, but following the 4th ovulation CL persistency for 30 days is shown followed by 3 sCL phases. Subsequent presence of numerous CLP and sCL phases may refeer to inflammatory process of the endometrium (pyometra / endometritis) (Huszenicza et al., 1999; Földi et al., 2006). In the AL group persistent corpus luteum (CLP) was seen in 5, and short-lived corpus luteum (sCL) following the 2nd or later ovulation was found in 8 cases. In 4 animals both irregularities were present at the same time. Thus irregular P4 profiles were seen in 9 dams: in 33% of AL group, in 56% of non-conceiving ones!

Evaluation of progesterone profile was possible in 37 SL ewes. In contrast to AL dams only 9 ewes (9/37 = 24%) of SL group ovulated before day 35, between d 26 and 35. Another 8 dams (22%) ovulated during the 6^{th} to 10^{th} week of lactation. Characteristic progesterone profiles of those dams can be presented through animal “E” of figure 5.1.4. where first ovulation is seen on day 48-49 postpartum, it is followed by a sCL considered to be physiological following 1st ovulation. The consequent 2 normal luteal phases reach lower P4 values (4.0 – 8.0 nmol/L), and the interovulation interval is tipically longer compared to AL group. Presence of CL was proven in 30 animals (81%) before d 116 (Figure 5.1.5.). However three of the above mentioned cyclic ewes became acyclic by May. At the same time no CLP or irregular sCL were present in SL group. The other 7 ewes (19%) did not ovulate during summer, and remained acyclic throughout the experimental period as presented by animal “F” of Figure 5.1.4.

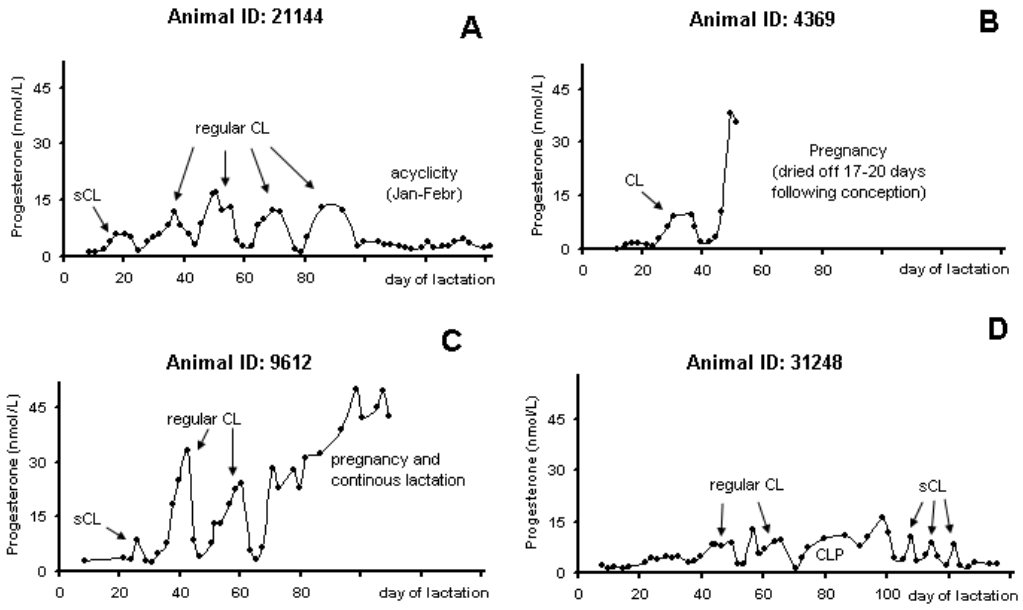


Figure 5.1.3. Some examples for progesterone profiles of autumn lambing (AL) dams (Exp. 1)

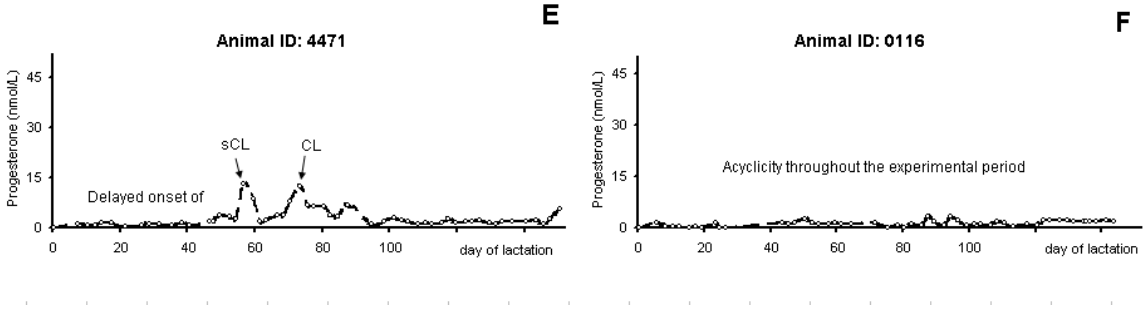


Figure 5.1.4. Some examples for progesterone profiles of spring lambing (SL) dams (Exp. 1)

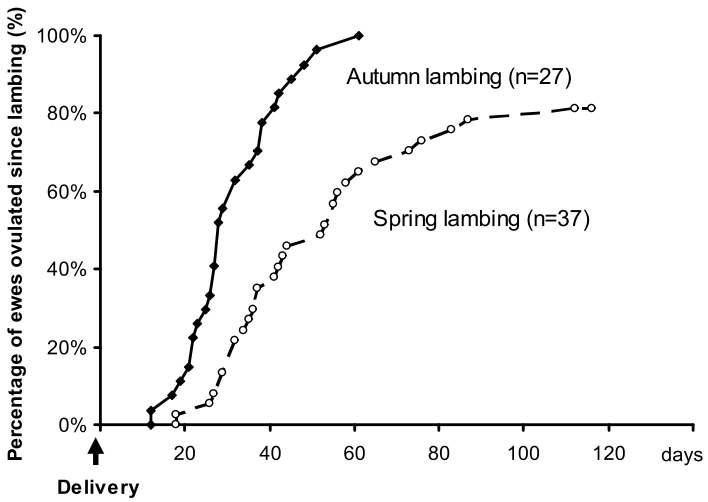


Figure 5.1.5. Proportion of animals ovulated since lambing in autumn lambing (AL) and spring lambing (SL) dams (Cox's F-test P<0.00001) (Exp. 1)

Relationship between body condition, metabolic status and ovarian activity

We studied correlation between changes of BCS and ovarian activity. In AL group comparing the periparturient decrease in BCS (ΔBCS) and the time of first postpartum ovulation, we found that in the animals ovulating before d 35 postpartum decrease in BCS was not significant ($BCS_{week\ -1}$ = 3.7±0.2; $BCS_{week\ +1}$ = 3.3±0.2; P=0.16); however those animals which ovulated later (after d 35 postpartum) were in 'supraoptimal' condition before parturition, and BCS loss was more expressed, ΔBCS was close to 1 point ($BCS_{week\ -1}$ = 4.3±0.3; $BCS_{week\ +1}$ = 3.4±0.2; P=0.033). Despite the above mentioned findings in BCS we did not find any statistically significant interaction between ovarian activity and plasma metabolites or metabolic hormones. In SL group the low number of dams ovulating before d 35 did not allow similar evaluation of data.

Experiment 2: Effect of additional light exposure

Exp. 2 was conducted one year later to Exp. 1 on the same commercial farm. Autumn lambing, 2-7 parity dams were involved (n=48). Some days before the expected dates of lambing (on the 11th September) animals were allotted into two treatment groups with regard to similar distribution of age and previous milk production. Dams were physically separated from rams but as they were kept in the same barns, so pheromone effect could not be excluded. Long-day photoperiodic treatment group (LD group, n=23) was exposed to additional artificial light from sunset till midnight (100 watt tungsten lamps positioned 2 meters above the head of animals ensuring 40 lux light intensity at the level of their eyes). Ration of light to dark hours was approximately 16:8 hours. Control group (n=25) received no treatment and experienced natural photoperiod. Milking, feeding and other management interventions were carried out during natural light hours in both groups.

Ovarian activity was followed up from the end of colostrum period until 20th November.

Periparturient status of metabolites and metabolic hormones was monitored as described in Exp. 1 with the modification that energetic status was not followed up on week 2 postpartum and besides the parameters analyzed in Exp. 1 plasma urea nitrogen was also assayed. The experimental period (collection of reproductive and lactational data) ended up on the 19th December.

Statistical methods used for evaluation of data were similar to Exp. 1.

Results of Experiment 2.

Between 10-20 days following parturition daily milk production of ewes ranged from 1.0 to 2.5 L in both groups. Smaller proportion of LD treated animals tended to dry off during the examination period compared to Controls (P=0.061). More than 60% of the LD ewes were still in milk on day 100 PP, contrary to the Control group where only 36% remained lactating at that time (Figure 5.1.6.).

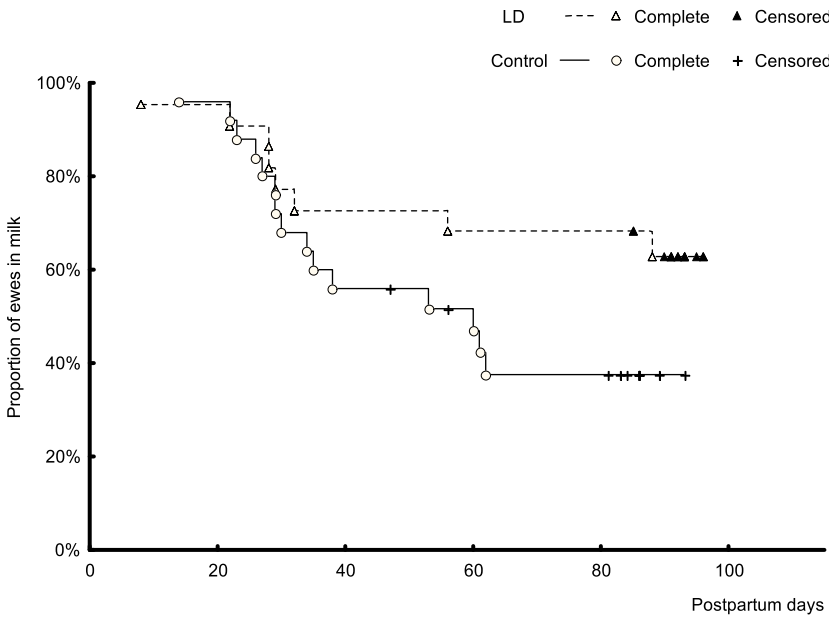


Figure 5.1.6. Proportion of ewes in milk in long-day photoperiod treated (LD) and control dams. (P=0.061 Kaplan-Meier cumulative proportion surviving by group) (Exp. 2)

Table 5.1.2. Plasma metabolites and metabolic hormones in the periparturient period (Exp. 2)

Weeks post-partum	BHB (nmol/L)	NEFA (nmol/L)	PUN (mmol/L)	Insulin (pmol/L)
Long day photoperiod (n=23)				
-1	0.28±0.06	0.27±0.036	4.52±0.29	52.24±5.56
+1	0.32±0.05	0.33±0.040	5.17±0.40	82.61±11.00
+5	0.24±0.02	0.19±0.047	5.27±0.27	60.70±9.62
Control (n=25)				
-1	0.26±0.04	0.24±0.04	4.23±0.24	54.60±4.90
+1	0.34±0.07	0.33±0.04	4.92±0.27	97.28±16.14
+5	0.23±0.02	0.19±0.02	5.43±0.23	56.21±5.94

Note: no significant differences were found between groups

Plasma metabolites and metabolic hormones were in the physiological range in all cases. We found no differences between treatment groups (Table 5.1.2.). Plasma IGF-I and T4 levels were similar in LD and Control animals (Figure 5.1.7.) and these hormone levels also

resembled the autumn lambing values of Experiment 1. First ovulation delayed in LD animals compared to Controls (P=0.047) (Figure 5.1.8.)

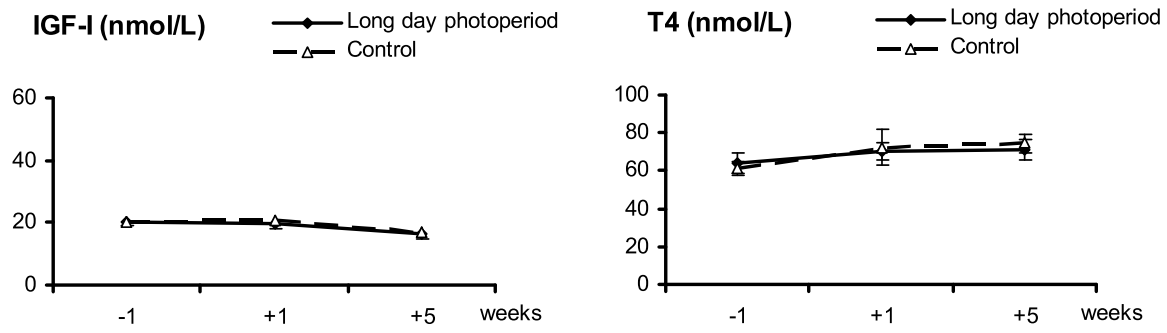


Figure 5.1.7. IGF-I and thyroxin levels in Long-day photoperiod treated and Control groups (Exp.2)

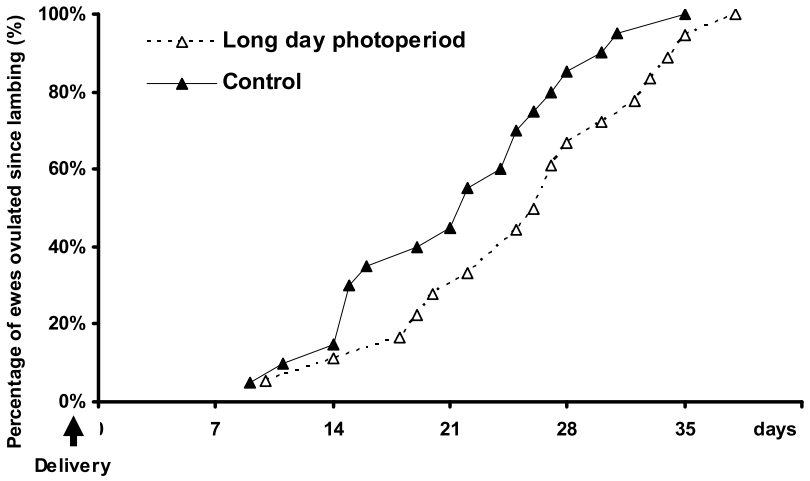


Figure 5.1.8. Proportion of animals ovulated since lambing in LD (n=18) and Control (n=20) groups (average day of first postpartum ovulation: 25.7±1.7 vs. 21.5±1.6 days; survival analysis with Cox's F-test P=0.047) (Exp. 2)

Discussion of Experiment 1 and 2

Our aim was to study the factors influencing ovarian activity of lactating but non suckling ewes kept in commercial flocks of the East (dry and hot) part of the Carpatian basin in the early postpartum period. In dairy cattle the most causative agent influencing ovarian reactivation is the postpartum negative energy balance (NEB) which delays the time of first ovulation through inhibition of LH pulse frequency and low levels of blood glucose, insulin and IGF-I that collectively restrain dominant follicles from estrogen production (Butler 2000). Contrary to dairy cattle in the present study mobilization of fat depots was never seen, or only in the last days of pregnancy before parturition. We found slight elevation of NEFA and BHB levels in the last prepartum week, but all metabolites remained in the physiological range. In

contrast with the trends seen in dairy cattle, in the first weeks of lactation severe energy deficit or subclinical ketosis in Awassi ewe is unlikely probably partly due to the compensatory effect of the easily mobilised fat depots from the fat tail.

Periparturient levels of IGF-I, thyroid hormone, and surprisingly insulin as well, were strongly influenced by season, although the seasonal difference seen in insulin levels was eliminated shortly after parturition. Fluctuation of thyroid hormone levels is well known in sheep with highest levels in spring and lowest values during the autumn-winter period (Wallace 1979). In the 2nd experiment thyroxin level of autumn-lambing dams was similar to those of the previous year irrespectively of the light regime, thus regarding the role of the external factors guiding seasonal hormonal variations we assume that other factors e.g. temperature may override photoperiodic changes in the case of thyroid hormones.

Photoperiodic variation of IGF-I level was intensively studied in dairy cow in the last decade, and it was shown that long-day treatments increase while short-day treatments lower plasma IGF-I values (Dahl et al. 2000, 2002). The present study (Exp. 1) showed elevated IGF-I levels during the periparturient period in spring compared to autumn-lambing animals. Mabjeesh et al. (2007) found in goat that plasma IGF-I levels may increase in light-treated animals compared to non-treated ones before parturition. This is consisted with our findings in Awassi sheep where slow release melatonin implant used for cycle induction negatively affected peripherial IGF-I level (unpublished data). In contrast to the above mentioned findings in the present study (Exp. 2) IGF-I levels were similar in long-day treated and control groups. Light-treatment protocols used to influence reproductive performance are the most effective, when used in the photosensible period (16-18 hours following sunrise in sheep), and out of the photorefractoriness (Chemineau et al., 1996, 2008). The efficacy of the light programs when used to increase milk production in dairy cow is the best when started on the day of parturition (Dahl et al. 2000). In Exp. 2 additional lightening started few days before parturition, and by the end of the experiment the duration of the light period was less than 18 hours per day. This may raise the possibility that the treatment we used could not have maximal biological impact. On the other hand it can also be assumed that apart from the proportion of light to dark hours other environmental factors (such as temperature) can also be responsible for the seasonal differences seen in plasma IGF-I levels. Further investigation is needed to clear the importance of the different signaling mechanisms leading to seasonal IGF variations.

Prepartum insulin level was higher in spring-lambing than in autumn-lambing ewes. Insulin level is known to be related to short term changes in energy metabolism, therefore we suppose

that the difference found is more contributed to the actual carbohydrate and fiber composition, namely fermentable organic matter content of the TMR than representing seasonal environmental differences.

Despite the adequate feeding lactation period in AL group was significantly shorter compared to SL animals: approximately one forth of the animals dried off before the 7th week of lactation. Furthermore those animals which became pregnant finished their lactation soon after conception (in the 3rd to 5th week of gestation period). In the second experiment additional lighting had beneficial effect on the length of lactation. Long-day photoperiodic treatments were shown to increase daily milk production in heifers (Dahl et al. 2000) and goats (Mabjeesh et al. 2007) and extend lactation in goats (Mabjeesh et al. 2007). Extension of lactation period has great financial impact in dairy small ruminants, thus all the technical tools increasing the number of milking days has to be considered. According to our findings which are in good agreement whith the above cited literature data, additional lighting for AL animals can be beneficial in intensive dairy sheep farming. However rams should be removed from the flock as too early reconception is disadvantageous.

Seasonal difference of postpartum ovarian reactivation was the main target of our research. We hypothesized that the lack of mother-lamb interaction may bring forward the first postpartum ovulation. At the same time we could not exclude the possible impact of negative energy balance due to the high energy demand of milk production. Finally as described above severe energy deficit or subclinical ketosis seems unlikely in the studied population. Obvious seasonal difference in the time of ovarian reactivation was found. In spring lambing animals the first ovulation delayed compared to their autumn-lambing flock mates and in more than half of them the postpartum anovulation overlapped seasonal anoestrous, first ovulation happened only by the end of August. The prerequisite of regular follicular growth and maturation is the adequate LH pulsatility. In small ruminants the initiation of the reproductive season at the end of summer is triggered by longer daily melatonin signals which act in the mediobasal hypothalamus to modulate pulsatile luteotrop hormone releasing hormone (LHRH) secretion. Microimplants of melatonin in the hypothalamus of females and males under inhibitory conditions for reproduction, stimulate gonadotropic activity (Thiery et al. 2002). Our findings support the above facts as extended light phase was able to delay the resumption of ovarian cyclicity in Exp. 2. Apart from the direct melatonin action other factors may also be involved in the seasonal regulation of ovarian quiescence. One of the suspected mediators is plasma thyroxin level. Although seasonal thyroxin level fluctuation is obvious, it does not seem to affect directly the resumption of postpartum ovarian activity as in the second

trial in contempt of the different time of ovarian reactivation, similar T4 values were found in both long-day treated and control dams. This is consistent with the study of Gifford et al. (2007) where lower thyroxin levels due to propylthiouracil treatment did not affect postpartum acyclicity in spring lambing ewes.

As lambs were weaned soon after birth, the absence of suckling and mother-lamb interaction along with the stimulatory photoperiod lead to very early resumption of ovarian cyclicity in autumn lambing ewes. More than 90% of AL dams became cyclic before the completion of uterine involution. In traditional technologies lambs are kept together with their mother and suckle for at least 60-70 days, thus ovulation occur not earlier than day 35-45, when uterine involution is already completed. Early ovulation and subsequent elevation of progesterone level have a local, immunosuppresive effect on the endometrium, and thus may increase the risk of bacterial complication of uterine involution. This phenomenon is well known in dairy cattle and has direct clinical importance (Huszenicza et al. 1999, Földi et al. 2006). In the present study early ovulation may lead to more frequent bacterial complications (endometritis, pyometra), but in ewes the most cases of these disorders are invisible under farm conditions. Nevertheless as great proportion of the cycling ewes kept together with rams did not conceive, we can assume their presence. Another indirect indication of subclinical endometritis can be the high prevalence of irregular (sCL, CLP) progesterone profiles in AL dams. The LD treatment, as was used in Exp. 2. was capable to slightly retard first ovulation which may lower the incidence of complications during involution.

Due to seasonal differences, despite the lack of suckling, the first ovulation in spring lambing animals occured only after the completion of uterine involution, not earlier than 5-8 weeks postpartum. In cyclic spring lambing dams progesterone profiles referred to slower follicular growth and less intense luteal activity. Though these lower hormone levels do not have direct implication in the farm management from practical point of view.

In all groups we found occurence of ovulation before the 20th day postpartum which presumably means ovulation from the first follicular wave. Apart from the effect of season and early weaning, socio-sexual stimuli (ex. pheromone effect of rams, Senger 2003) can be responsible for this phenomenon.

Conclusion

It seems that in a given animal-keeping technology, beside the suspected genetic background, the most important regulatory factor of the resumption of reproductive activity is the photoperiod.

In autumn-lambing dams first postpartum ovulation may happen before the completion of uterine involution which increases the risk of uterine infections. In autumn-lambing dams additional artificial light (approximately 16 hours light) may delay the time of first postpartum ovulation, and thus may be a suitable tool to avoid the unwanted premature ovulation. At the same time long-day photoperiodic treatment may also prevent early drying-off of autumn-lambing animals. Additional studies are needed to optimize the intensity and timing of light-treatment.

5.2. Relationship between seasonality of reproduction, milk production, metabolic hormone levels and MT1 receptor gene polymorphism in Awassi ewes (Exp 3)

Experimental design

First to tenth parity Awassi dams were involved in the study between the age of 15 month and 10.5 years on the day of delivery (n=395). Lambing date varied from 6th February to 20th April. Ovarian activity was monitored by measuring milk progesterone level three times 7 days apart between 10-12 weeks postpartum between middle of May and end of June. Animals were judged cyclic if the milk progesterone level was higher than 4 nmol/L in at least one of the samples were taken.

Monthly records of test milking were collected from each animal during the first 3 month of lactation including daily milk yield, milk fat and milk protein content. To evaluate the current metabolic status blood samples were collected on the 10th week postpartum (between days 70-77 postpartum) and plasma level of two metabolic hormones, leptin and insulin-like growth factor I (IGF-I), were measured. Blood samples were collected from each ewe for MT1 genotyping on the same day. Samples were stored at -20ºC until processing. Body weight of dams was recorded on the day of blood sampling.

Laboratory procedures

Description of hormone assays and RFLP method can be found in Section 4.2.4.

Statistical analysis

Data were subjected to allelic frequency analysis using Genepop 4.0 software (Raymond and Rousset 1995, Rousset 2008). Melatonin receptor haplotype frequencies were estimated by EH software (Xie and Ott 1993). Comparison of genotype or haplotype frequencies, and the association between MT1 genotypes and reproductive activity was evaluated by chi-square test.

To explore how season, age, body weight, milk yield, leptin, IGF-I levels, and genotype are linked to cyclicity, logistic regression was applied by the generalized linear model (glm) function in R 2.12.2. All two-way interactions among these variables, but no higher order ones were included in the model. To accommodate to potential non-linear relationships, age, IGF-I and leptin variables were transformed into categorical ones. We used the following age groups: ≤3, 3–4, 4–5, 5–7, >7 years. For leptin and IGF-I we created 3 categories using the 1/3- and 2/3-quantiles as cut points. This resulted in the following classes: 0.195-0.267,

0.267-0.297, 0.297-0.480 nmol/L for leptin level, and 9.0-11.4, 11.4-12.7, 12.7-19.7 nmol/L for IGF-I level. We repeated the analysis dividing the ranges of the variables into four categories (the results were very similar, not reported). Date of sampling was strongly linked to lambing date (data were collected 70 days after delivery) and was distributed during a 2.5 month period. Thus to explore the effect of season dates were transformed to a linear scale according to the sequential number of the given day within the year (1st of January = 1; 2nd of January = 2 etc.)

To check the effect of genotype on cyclicity in groups by metabolic status, leptin and IGF-I ranges were split at their median values and in each of the resulting 4 groups dependence of cyclicity on genotype were tested by Fisher's exact test.

Statistical significance was accepted at $p<0.05$ throughout.

Results

Distribution of alleles and genotypes

Distribution of various MnII and RsaI alleles and genotypes for the two RFLP sites are shown in Table 5.2.1. The examined population was not in Hardy-Weinberg equilibrium for either of the restriction sites ($P<0.001$ and $P=0.04$ for RsaI and MnlI respectively). The genotype frequencies of the two single nucleotide polymorphisms sites were considerably different. Haplotype frequencies were estimated both under the hypothesis of independent segregation and association among loci, the difference were not significant (Table 5.2.2).

Table 5.2.1. Distribution of various MnlI and RsaI alleles and genotypes for the two RFLP sites in the examined Awassi sheep population (n=395)

	Allele frequency		Genotype frequency		
	m	M	mm	Mm	MM
MnlI	0.45	0.55	0.26	0.38	0.36
	r	R	rr	Rr	RR
RsaI	0.17	0.83	0.04	0.26	0.70

Table 5.2.2. Haplotype frequencies under the hypothesis of independent segregation and association among loci in the examined Awassi sheep population (n = 395).

Allele at MnlI	Allele at RsaI	Haplotype Frequency Independent	Association allowed
m	r	0.077	0.038
m	R	0.371	0.410
M	r	0.094	0.133
M	R	0.458	0.419

Effect of genotype on out-of-season cyclicity

Table 5.2.3 shows the distribution of the frequency of the m/M and r/R allele combinations. As it is showed there were only a few r/r animals (altogether 17 our of 395) and the r/r combinations with the MnlI alleles were particularly rare, animals having r/r genotype were excluded from this analysis. Although higher number of R/R ewes showed cyclic ovarian function and higher proportion of M/M ewes for MnlI genotype was cycling, the effect of the genotypes was not significant (Figure 5.2.1).

Table 5.2.3. Number of animals with different allele combinations

	rr	rR	RR
mm	1	10	90
mM	0	80	72
MM	16	11	115

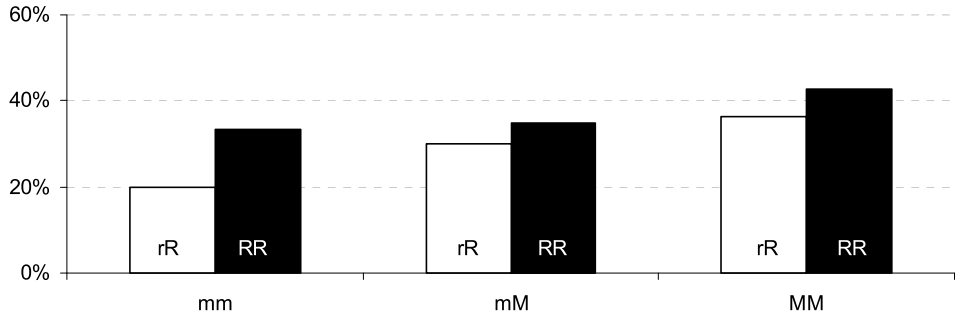


Figure 5.2.1. Proportion of out-of-season cycling Awassi ewes within each MnlI genotype group

Relationships between season, body weight, milk yield, age, genotype or metabolic hormone levels and out-of-season cyclicity

Logistic regression analysis resulted in no significant relationship with the genetic variables, age and BW. Also, no interaction terms proved to be significant. The final model, after exclusion of the non-significant terms, contained season, milk yield, leptin, and IGF-I. Regression coefficients, standard errors, p-values, and the derived odds ratios (OR) are shown in Table 5.2.4. The fit of model to data was acceptable (residual deviance = 381.2 on 378 degrees of freedom).

Table 5.2.4. Relationship between leptin, IGF-I level, milk-yiled or season and out-of-season cyclicity in Awassi sheep (n=395). OR: odds ratio

Factor/Category	Regression coefficient	SE	P-value	OR
leptin ≤ 0.267 nmol/L (reference category)				
leptin 0.267-0.297 nmol/L	1.667	0.357	<0.0001	4.67
leptin > 0.297 nmol/L	1.929	0.362	<0.0001	5.33
IGF-I ≤ 11.4 nmol/L (reference category)				
IGF-I 11.4-12.7 nmol/L	2.02	0.356	<0.0001	5.66
IGF-I > 12.7 nmol/L	1.48	0.361	<0.0001	4.07
Milk yield	-0.673	0.205	0.001	
Season	-0.018	0.009	0.048	

The highest proportion of cyclic ewes occurred among those with high leptin level (OR compared to those with low leptin level is 5.33; P<0.0001); and those with medium IGF-I level (OR compared to those with low IGF-I level is 5.66; P<0.0001). Daily milk yield and season were negatively correlated with cyclicity (P=0.001 for milk yield and P=0.048 for season).

Figure 5.2.2. displays more details about how the proportion of cyclic ewes is related to leptin and IGF-I levels. Here too, cut points are quantiles of the variables (1/6, 2/6, etc.). As it is presented on the graph highest proportion of out-of-season cycling ewes (marked with light grey colour) can be found in the areas related to high leptin, and moderate IGF-I levels.

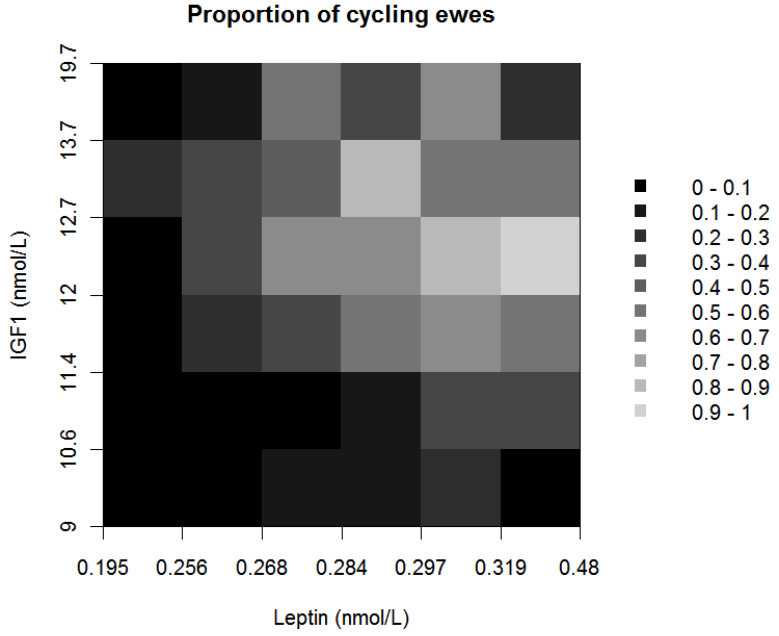


Figure 5.2.2. Proportion of cycling ewes by leptin and IGF-I level. To balance the number of cases in the cells as much as possible, quantiles of the variables are used as cut points.

Table 5.2.5 summarizes milking data in the 3rd month of lactation (month closest to hormone level determination) in groups by IGF-I level that were used in the logistic regression model. Results show that milk yield and milk fat related to high IGF-I levels is significantly elevated compared to animals with medium plasma IGF-I. Those two factors (milk yiled and IGF-I) act in the opposite way in terms of cyclicity.

Table 5.2.5. Test milking results from the third month of lactation in Awassi dams according to their IGF-I levels (mean±SEM).

	Milk yield (L/day)	Milk fat (%)	Milk protein (%)	Milk fat (g/day)	Milk protein (g/day)
IGF-I ≤ 11.4 nmol/L	1,29±0.06	4,32±0,22	4,27±0.11	58,81±3.92	55,50±2.79
IGF-I 11.4-12.7 nmol/L	1,16±0.05[a]	4,22±0.22	4,26±0.11	52,70±3.85[a]	49,76±2.66[a]
IGF-I > 12.7 nmol/L	1,32±0.06[b]	4,65±0.21	4,40±0.08	64,04±4.19[b]	57,57±2.85[b]

[ab] Pairwise comparison: means in the same column with different superscripts differ significantly at P < 0.05 level.

Relationships between genotype and out-of-season cyclicity in subgroups by metabolic hormone levels

Effect of genotype was tested in each subgroup separately (Figure 5.2.3). Both for leptin and IGF-I, a level above median group resulted in higher proportion of out-of-season cyclicity (62%). Effect of the R allele was found to be significant in only one of the subgroups, the one characterized by high leptin, low IFG-I level. Here the proportion of cyclic animals was 14.3% (3/21) and 49.0% (25/51) for r/R and R/R genotypes, respectively (r/r did not occur in this subgroup), though the beneficial effect of R allele could be shown.

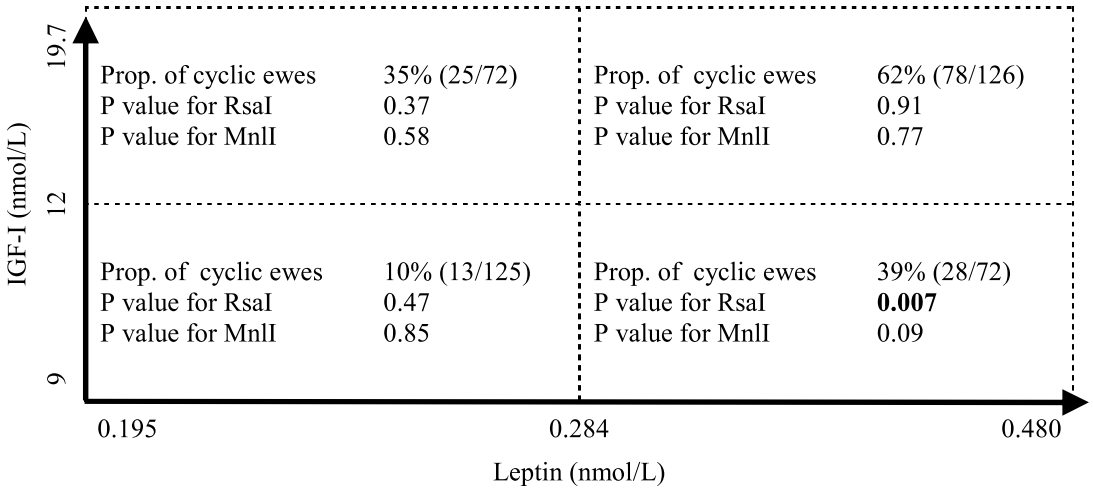


Figure 5.2.3. Proportion of dams showing out-of-season ovarian cyclicity, and of its relationship with the MnlI and RsaI polymorphism of the MT1 gene on it in the leptin x IGF-I matrix.

Discussion

Distribution of alleles and genotypes and influence of genotype on out-of-season cyclicity

Our results show that in the examined Awassi population both RsaI and MnlI restriction sites are polymorphic. Homozygous (MM, RR and mm, rr) and heterozygous (Mm, Rr) combinations did occur for both cleavage sites in our study, however heterozygous combinations were underrepresented for both the RsaI and MnlI restriction site. Concerning haplotypes, according to our data random recombination of the two loci seems unlikely, in agreement with the findings of Notter et al. (2003) in the Virginia OOS flock and Mateescu et al. (2009) in Dorset ewes.

Testing the relationship between genotype and out-of-season cyclic ovarian function in all the 395 dams did not result in a significant effect of MT1 polymorphism, though proportion of cyclic animals bearing the same MnlI genotype was higher when it was combined with RR than rR, and ewes having MM genotype were more likely to cycle than Mm or mm animals (Figure 5.2.1.). Significant association was found between these alleles (R and M, respectively) and lower seasonal reproductive activity by different research groups (Pelletier et al. 2000, Notter et al. 2003, Carcangiu 2009 and 2010). Contrary to these findings, other research groups failed to show any significant effect of the MT1 polymorphisms on seasonality in Île-de-France (Hernandez et al 2005) and in Merinos d'Arles (Teyssier et al 2011). These contradictory finding are not surprising if we take into account that the single nucleotide polymorphisms (SNPs) studied do not cause any amino acid changes and are thus unlikely to be functionally responsible for the phenotypic differences. Recombination might occur between the SNPs genotyped and the casual mutation, which can be either within the MT1 gene or a closely linked gene, resulting in linkage disequilibrium between the examined SNPs and the casual mutation in some breeds. Furthermore the MT1 gene is only one of several genes regulating seasonality, and it is not known how many other genes influence this phenotype or how significant their impacts are (see also Chemineau et al. 2010).

There are several other factors beside the genetic background which affect seasonality, it is widely accepted that the most important limiting factors of out-of-season cyclicity are age and metabolic status, furthermore adequate socio-sexual stimuli may enhance ovarian activity (Notter and Cockett 2005). Therefore the manifestation of the beneficial effect of the genetic component may be limited to a part of the flock. To test this hypothesis, first we explored how milk yield, leptin and IGF-I levels were related to cyclicity, and in a second step we examined of the putative relationship between genotype and this capability in different metabolic status.

Relationships between season, milk yield, leptin or IGF-I and out-of-season cyclicity

When we stratified animals according to leptin and IGF-I levels, we found that higher leptin was linked to higher proportion of cyclicity, whereas for IGF-I an optimal medium-range zone was found where the percentage of cycling ewes reached its maximum (Table 5.2.4 and Figure 5.2.2). Animals with high milk yield were less likely to cycle out of the breeding season. At the same time these parameters overwhelmed the potential effect of different polymorphisms when they were present in the model. Lambing date was spread during a 2.5 months long time interval and sampling dates varied also in the same range accordingly (between mid-April and the end of June). Date of sampling (season) significantly interfered with cyclicity; animals were less likely to cycle at the end of the experimental period. We attribute this phenomenon to the normal physiological response to the increasing, inhibitory photoperiod.

Body weight had no significant effect on cyclicity therefore it was not involved in the final model. Individual variations in the frame of animals were high in the examined flock and thus bodyweight is not well correlated with body condition changes in this set of ewes. In the present study feeding was adjusted to actual milk production and blood sample collection was performed in each animal at the time of morning milking. As short-term effects were possibly minimized by this feeding regime and sampling method, plasma leptin level can be considered a reasonable indicator of body fatness (Delevaud et al., 2007). Our results concerning leptin level and cyclicity were in good accordance with the previously described widely accepted theory (Atti et al. 2004) that high body fat store allows for more intensive reproductive activity.

We expected that proportion of cyclicity will gradually increase with IGF-I level. Contrary to our expectations, although both medium and high IGF-I animals performed better compared to low IGF-I dams, OR was highest in medium IGF-I group (OR=5.66) while high IGF-I level group was inferior to this (OR=4.07). We compared test milking records of animals with different IGF-I levels. Milk records of the third month, which was the closest date to the blood sampling period proved that high IGF-I level was coupled to significantly higher daily milk yield, daily milk fat and daily milk protein production than medium circulating IGF-I (Table 5.2.5). Thus proportion of cyclicity may be lower because of the higher energy expenditure in the high IGF-I group which is also coherent with the negative correlation found between milk yield and cyclicity. Unfortunately plasma metabolites related to energy

balance (glucose, non-esterified fatty acid and β-hydroxy butyrate) were not measured which does not allow for deeper discussion.

Relationships between genotype and out-of-season cyclicity in animals with different metabolic hormone levels

To evaluate the relationship between genotype and cyclicity in animals with different metabolic status the range of leptin and IGF-I was devided into classes and the relation between genotype and cyclicity in the combinations of these groups was investigated. The sample size and the distribution of allele combinations did not allow a finer categorization but splitting the hormone level ranges at their median values, resulted in four subgroups. Relying on former experiences from Notter et al. (2003) we expected the strongest genetic effect in the subgroup with the highest proportion of cycling animals (above median leptin - above median IGF-I). Surprisingly however, the maximum effect of MT1 polymorphism was seen at below median IGF-I and above median leptin levels, thus in a "suboptimal" metabolic situation (Figure 5.2.3). Proportion of cyclicity is minimal in the low leptin – low IGF-I condition, making it difficult to show statistical significance in this group. It is widely accepted that weak metabolic status practically inhibits out-of-season cyclicity, while optimal hormone levels are the main permissive elements. According to our findings genetic merit had the greatest impact in animals with nearly optimal metabolic status where modified photoperiodic sensitivity may increase the chance to the onset of cyclicity.

Conclusions

Melatonin receptor 1a gene was found to be polymorphic at both the RsaI and MnlI restriction sites, with high incidence of R and M alleles in the examined Awassi flock. The ability of out-of-season cyclicity is expressed in mature animals having high plasma leptin level (indicating adequate energy stores). However, high milk production may negatively influence this behavior. Our results suggest that the preferable allele (R at a significant level and M tendentiously) can enhance out-of-season cyclicity in dams with suboptimal metabolic condition related to low plasma IGF-I. Nevertheless, these polymorphisms are not suitable for marker assisted selection because only the RsaI RFLP site showed significant effect and it was limited to a subgroup of the flock. However, beside the melatonin receptor, several other biologically active components (e.g. hormones, proteins) were suggested and/or proved to play a crucial role in the neuroendocrin control of seasonal reproductive activity in sheep, of which genetic background could be interesting to investigate in our experimental flock.

Melatonin-based induction of ovarian cyclicity in intensive dairy Awassi flocks (Exp 4)

Experimental desing and treatments

In the first phase of the study 85 autumn lambing, in the second phase 115 spring lambing dams and young ewes were involved.

Phase 1 and 2 started in 10th February and 22nd June respectively (day 0 of the experiment, Figure 5.3.1). Animals were allotted into one of the following 3 treatment groups in each phase with respect to similar distribution of age. Cyclic ovarian function was induced in Group Gest (Control) according to the protocol used previously on the farm: 14 days-long gestagen sponge treatment (introduced on day 56 of the experiment removal on day 70; Chrono-Gest sponge, Intervet, Angers, France) followed by 600 IU eCG injection at removal (Folligon inj., Intervet, Angers, France). In the Mel+Gest group slow-release melatonin implant (18 mg, Melovin®, CEVA, Libourne, France) was administered subcutaneously on day 0 of the experiment and later animals were synchronized as decriebed above with gestagen+eCG combination. Mel+GPG dams were implanted with melatonin on day 0 similarly to Mel+Gest group, however synchronization was performed by the so-called Ovsynch protocol used in cows (day 63: GnRH (Cystoreline inj.®, CEVA, Libourne, France)⇒ ovulation/intrafollicular luteinization, day 70: $PGF_{2\alpha}$ (Enzaprost-25 inj., CEVA, Liburne, France)⇒ luteolysis, day 72: GnRH ⇒ ovulation).

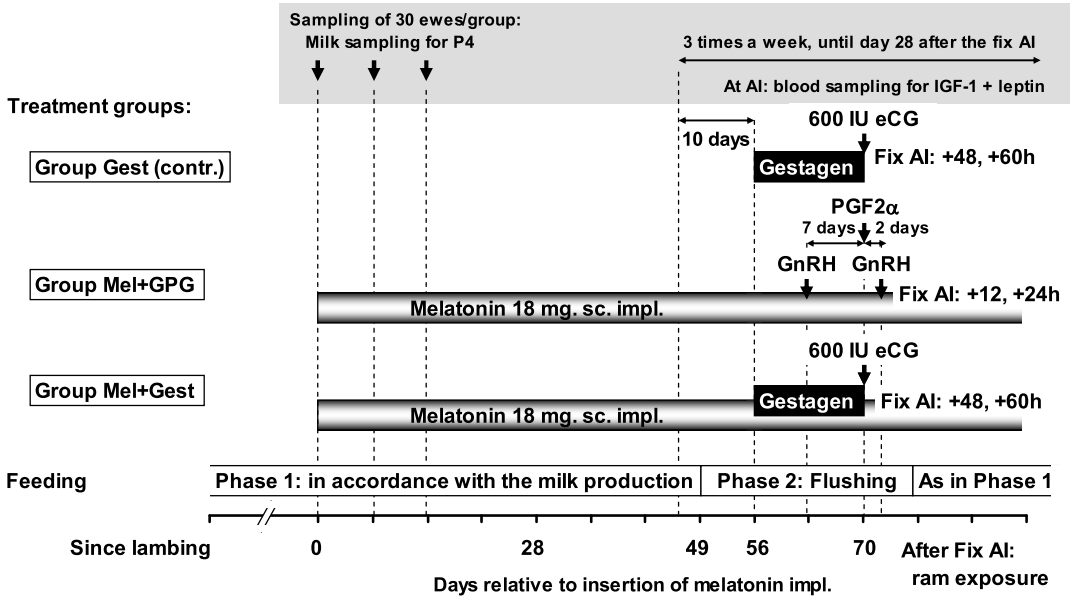


Figure 5.3.1. Experimental design (Exp 4.)

Breeding technology

Dams were inseminated twice with fresh diluted semen. Gest and Mel+Gest groups were inseminated 48 and 60 hours following sponge removal, Mel+GPG ewes were inseminated 12 and 24 hours following the second GnRH injection (fix timed AI). Ewes were introduced to rams 14 days later in harems.

Monitoring the resumption of the ovarian cyclicity

On day 0, 7 and 14 of the experiment, milk samples were collected from lactating dams, and fecal samples from young or dry ewes. From day 46 of the experiment to day 28 following AI (during 52 days) milk samples were collected three times a week from lactating ewes while blood samples were taken twice weekly from the others. Progesteron level of milk and blood samples and gestagen metabolite level of feces was assayed at each time points. Following up of the individual progesterone profile made possible to determine:

- the proportion of cyclic and acyclic ewes on day 0 of the experiment (before melatonin treatment),
- the proportion of cyclic and acyclic ewes between day 46 and 56,
- to follow up of ovarian activity during and following synchronization.

Pregnancy diagnosis

Pregnancy associated glycoprotein level was determined 28 and 60 days following AI from blood samples. Pregnancy diagnosis was confirmed by progesterone and PAG levels. Day of conception was calculated relative to lambing date (lambing date -150 days).

Determination of plasma IGF-I and thyroxin levels

IGF-I and thyroxin levels were determined from blood samples collected weekly between week 6 and 14 of the experiment from non-suckling dams.

Statistical analysis

Proportion of cyclic or acyclic and pregnant or non-pregnant ewes was compared by Chi-square test. Two means were compared by Student's t-test by using R-statistics (R 2.12.1).

Results and discussion

Autumn-lambing ewes

According to the detected progesterone values 39% of ewes showed cyclic ovarian function, but only 6% remained active until the end of April. There was no difference between groups in this respect. Following cycle induction/synchronization those receiving gestagen+eCG

treatment with or without melatonin ovulated in higher proportion compared to those getting long-term melatonin implant followed by Ovsynch protocol (Gest: 96% vs Mel+Gest: 95% vs Mel+GPG: 45%; P=0.040 concerning the effect of treatment). This suggests that melatonin treatment in February was too early and was not able to override photorefractoriness, thus was unable to induce cycle (Chemineau et al. 1996a). In Gest and Mel+Gest groups 14% of ewes conceived from timed AI, however only 3% of Mel+GPG animals got pregnant. Rams fertilized within 1 month 10% of Gest group, 5% of Mel+Gest group and 3% of Mel+GPG (NS). At the same time 31-43% of ewes conceived several months later, at the end of summer, beginning of autumn, and 38-62% remained opened for more than 220 days (Table 5.3.1). Conception rate was obviously influenced negatively by the high proportion (54%) of young ewes. According to literature data average age of puberty in Awassi ewes is 7.5 months (4-15 months) (Talafha and Abadneh 2011) which supports our teory. Our previous research in the same flock in accordance with literature data suggests that proportion of out-of-season cycling ewes is depending on age, older dams are more likely to cycle (Avdi et al. 2003, Faigl et al. 2006).

Table 5.3.1. Reproductive performance of autumn-lambing ewes

	Gest	Mel+Gest	Mel+GPG	Chi^2
Cyclic in June	33%	25%	31%	0.887
Cyclic between day 45-56 of exp.	4%	5%	9%	0.669
Ovulated following synchronization	96%	95%	45%	0.040
Pregnant altogether	55%	62%	37%	0.411
Conceived from fix AI	14%	14%	3%	0.327
Conceived from ram	10%	5%	3%	0.487
Conceived in the next breeding season	31%	43%	31%	0.709

Spring-lambing ewes

In June 4% of ewes were cycling. Proportion of cyclic ewes between day 45-56 became higher in Mel+Gest and Mel+GPG groups compared to control, but this difference was statistically not significant (19% Gest vs. 44% Mel+Gest vs. 47% Mel+GPG; NS). High proportion of dams ovulated following synchronization: 100% in both Gest and Mel+Gest groups, and 88% in Mel+GPG (NS). At the same time only 24% (Gest), 22% (Mel+Gest) and 5% (Mel+GPG) conceived from timed AI (NS). Rams fertilized 65% of ewes during the same breeding season. 8-27% of dams remained opened for more than 150 days following synchronization (NS) (Table 5.3.2).

Table 5.3.2. Reproductive data of spring-lambing ewes

	Gest	Mel+Gest	Mel+GPG	Chi^2
Cyclic in June	3%	6%	3%	0.792
Cyclic between day 45-56 of exp.	19%	44%	47%	0.109
Ovulated following synchronization	100%	100%	88%	0.833
Pregnant altogether	92%	86%	73%	0.657
Conceived from fix AI	24%	22%	5%	0.104
Conceived from ram	65%	65%	65%	1.000
Conceived in the next breeding season	3%	0%	3%	

Comparison of day of conception by survival analyses revailed no differences between groups neither in Autumn, nor in Spring lambing dams (Figure 5.3.2, Autumn-lambing P=0.361; Spring-lambing P=0.131).

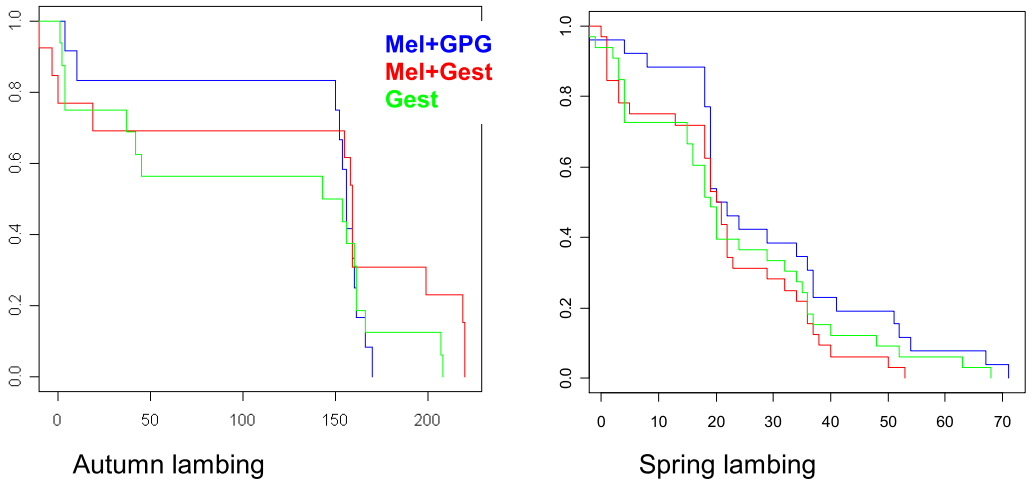


Figure 5.3.2. Day of conception in autumn and spring-lambing ewes (survival analysis)- Autumn lambing P=0.361; Spring lambing P=0.131

Plasma thyroxin and IGF-I levels

As the success of melatonin treatment was far away from our expectations, it raised the possibility that the given production lot of the drug had no effect or that the dose suggested by the producer was not adequate to have a biological effect in the relatively large Awassi ewes. To exclude this possibility we assayed blood samples collected from the autumn lambing animals between day 46 of the experiment until day 28 following AI (during 52 days) for thyroxin and IGF-I levels. Those two hormones have well-known seasonal pattern according to our previous results (Faigl et al. 2011) and literature data. In accordance with literature data plasma IGF-I levels of first-parity ewes was significantly higher in all groups compared to

non-lacting older dams (P=0.001-0.095), thus we evaluated the IGF-I results of the different age groups separately. Between week 6 and 10 after the insertion of melatonin implants, Mel+Gest and Mel+GPG animals had lower plasma IGF-I levels in all age-groups compared to the Gest group (P=0.005-0.100). These differences were equalized by the end of the experimental period (Figure 5.3.3). Plasma thyroxin was not affected by melatonin treatment or age (data not shown).

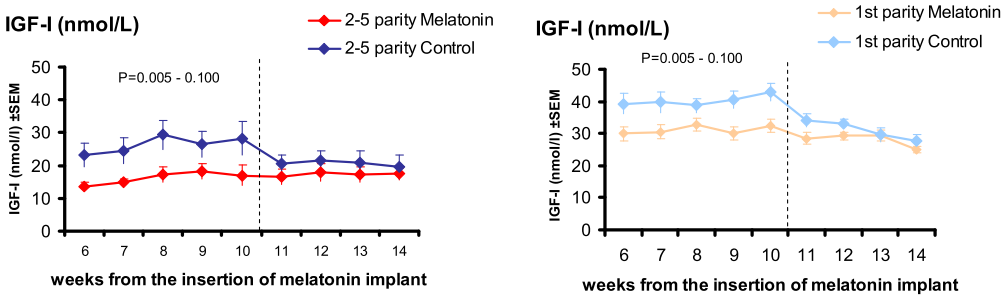


Figure 5.3.3. Plasma IGF-I level in first parity (n=50) and non-lactating (n=37) autumn lambing ewes from day 46 of melatonin treatment until day 28 post AI (for a 52 day long period). Comparison of Mel+Gest and Mel+GPG group vs Control. Samples were collected twice a week (note: according to the producer's description plasma melatonin level stays elevated for 10 weeks after insertion of the implant).

The above results proved biological effect of melatonin treatment, which indeed was not able to induce ovulation in acyclic animals at the beginning of spring. Long-lasting melatonin administration depressed plasma IGF-I level between day 45 and 70 following treatment. This raises the possibility that melatonin implants used for cycle induction in intensive dairy flocks during lactation may negatively influence milk production. This is in accordance with our previous findings in the same flock connected to the effect of photoperiodic treatments on lactation legth and milk yield (Faigl et al. 2011). Simultaneously we found no difference in thyroxin level, which suggests that the seasonal change in thyroxin level is also influenced by other factors than photoperiod (ex. temperature).

Conclusions

Our results suggests that reproductive activity of Awassi is seasonal under continental climate, and breeding season is shorter than in its homeland, at the near-east where it lasts from April until September (Zarkawi 1997). Melatonin implant used in February was not able to induce cycle, however it had beneficial effect in June. At the same time through depression of IGF-I level, melatonin treatment may negatively influence milk yield and the length of lactation. GPG protocol for synchronization can only be effective when used near to the natural breeding season and in ewes at least 2 years of age.

Testicular function and semen characteristics of Awassi rams treated with melatonin out of the breeding season (Exp 5)

Animals

Sixteen 4-8 year old Awassi rams of a commercial dairy farm were used. Animals were housed in opened sheds. Eight of them were used for AI and trained for mounting and spontaneous ejaculation. The others were previously mated naturally.

Experimental design

Animals were allotted into two treatment groups (melatonin treated: MT group and control: Control group) with regard to similar distribution of age. On 23rd of February (day 0) rams in MT group (n=8) were implanted with 54 mg melatonin (3 pieces of Melovin® implant, CEVA Libourne, France) according to the producer's instructions. Melovin® implant is a long acting, slow release device resulting in approximately 3 month long elevated plasma melatonin level. On day 0, 49 and 71 exocrine and endocrine function of testicles were evaluated. Animals in the control group received no treatment (n=8).

Following up of sperm production

Only half of the animals were trained for mounting and ejaculating into artificial vagina, so from 4 rams in each group semen was collected with electroejaculation (IMC, France). The quantitative and qualitative indexes of the spermatological parameters of semen gained by electroejaculation are well known for showing great standard deviations, the data obtained from those rams were excluded from the final evaluation. The following spermatological parameters were evaluated: concentration (Improved Neubauer cell counting chamber), morphology (Spermac™ staining, Beernem, Belgium), total motility and fast and slow forward motility (Medealab™ CASA System, Erlangen, Germany) of spermatozoa. Motility analysis with CASA was carried out at the concentration of 0.8-1.2 X 10^8 /mL after dilution of the semen sample with PBS.

Testing the endocrine function of testicles

To monitor the endocrine function of testicles, basal and GnRH-induced plasma testosterone levels of all animals were measured on the days of sperm collection (d0, d47 and d71).

Stimulation was performed by giving 0.008 mg buserelin, a GnRH agonist iv. (Receptal inj.®, Intervet, Angers, France). Blood was collected before injection and again 15 minutes apart during 90 minutes.

Statistical analysis

Statistical analysis was performed by using R 2.2.1 (R-project, freely available). Spermatological parameters were compared by ANOVA (analysis of variance) test. GnRH-induced testosterone response was evaluated by comparing the modified area under the curve (modified AUC), meaning total AUC – (t0 value x 90 min). Basal testosterone and IGF-I level was evaluated by Student's t-test.

Results

Spermatology

Melatonin treated and Control group did not differ regarding the total volume of collected semen (range 0.5 – 1.2 mL), number of motile spermatozoa and percentage of cells showing fast and slow forward motility (Figure 5.4.1).

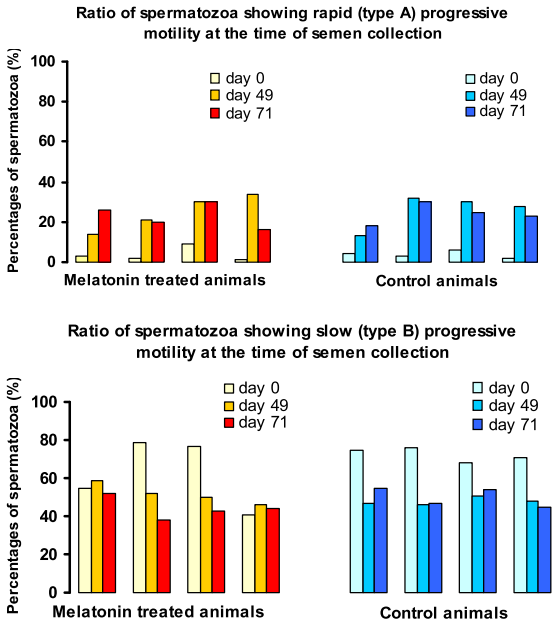


Figure 5.4.1. Ratio of spermatozoa showing rapid (type A) and slow (Type B) progressive motility at the time of semen collection

During treatment period, in both group, percentage of fast forward motile sperm increased ($p < 0.001$), and - at the same time - slow forward motility decreased ($p < 0.05$). Concentration of spermatozoa did not differ according to treatment, but we found elevated concentration in both group according to time ($p < 0.01$) (Figure 5.4.2).

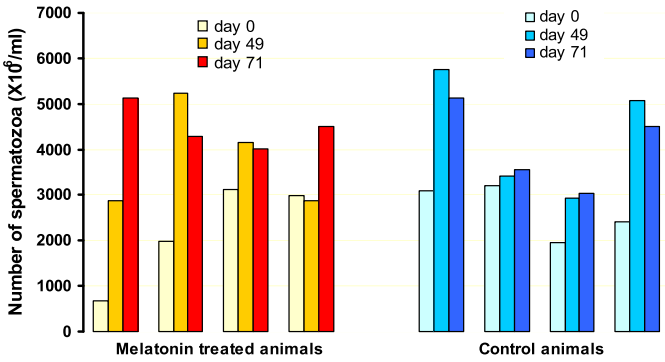


Figure 5.4.2. Concentration of spermatozoa in Melatonin treated and Control animals.

We found no difference between groups regarding the proportion of spermatozoa with normal or abnormal morphology (data not shown).

Hormone results

On d0 testosterone level was similar in both groups (MT: 4.48 ± 1.20 nmol/L; Control: 3.99 ±1.04 nmol/L). However, basal testosterone level became significantly higher in melatonin treated animals 49 and 71 days later (MT vs. Control 6.83 ± 1.86 nmol/L vs. 3.61 ± 0.50 nmol/L on d49 p=0.023; 6.18 ± 1.06 nmol/L vs. 2.92 ± 0.67 nmol/L on d71 p=0.021 respectively) (Figure 5.4.4). GnRH-induced testosterone response (modified AUC) remained unchanged throughout the experimental period (Figure 5.4.3).

At the same time plasma IGF-I level remained unchanged and did not differ between groups.

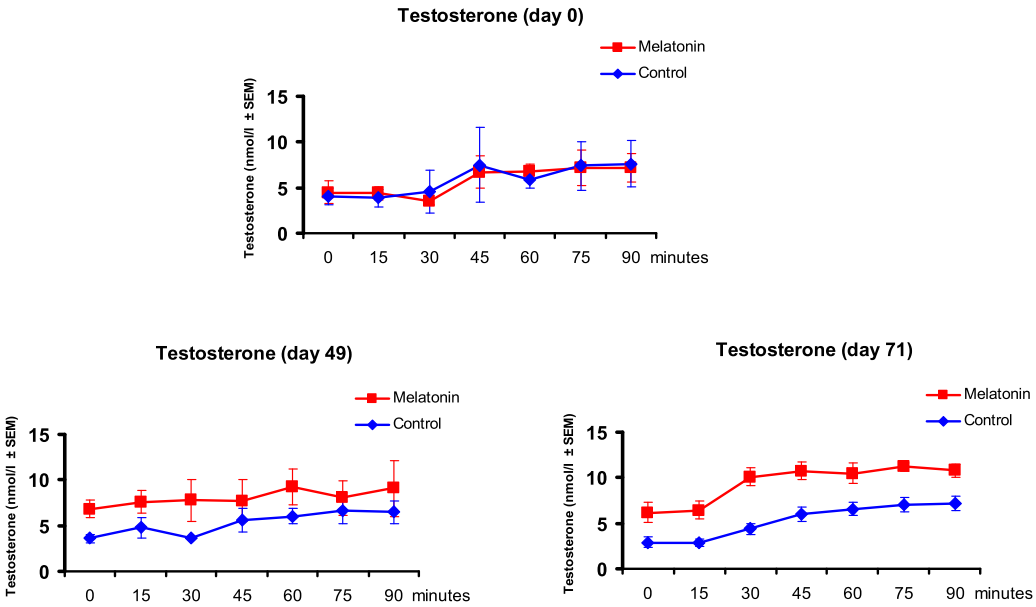


Figure 5.4.3. Basal testosterone level and GnRH-induced testosterone response in Melatonin treated and Control group

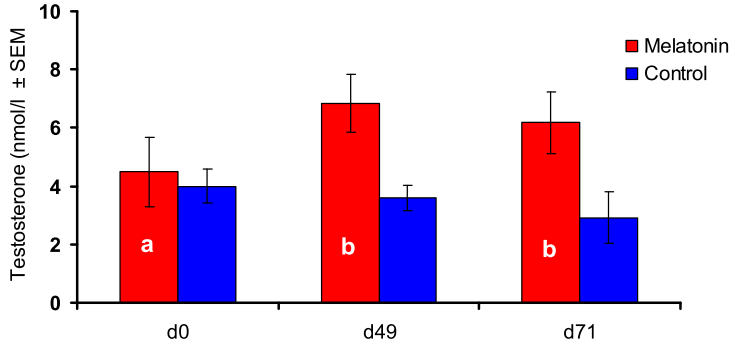


Figure 5.4.4. Basal testosterone level measured at the time of semen collection in Melatonin treated and Control group Columns indicated with different letters a vs. b are significantly different from each other

Discussion

Seasonal variance of reproductive activity is regulated by hormonal changes. The endocrine status is influenced by environmental impacts among which, in small ruminants, the most important one is the photoperiod. Photoperiodic changes are translated to endogenous signal by means of melatonin production of the pineal gland. In short day breeders, longer dark period and elevated plasma melatonin level indicates the beginning of breeding season. Melatonin triggers GnRH secretion in the mediobasal hypothalamus and thus leads to increased FSH and LH secretion which results in elevated testosterone response (Pelletier and Ortavant 1975a, 1975b; Chemineau et al. 1992; Kokolis et al. 2000; Ramadan et al. 2009; Tsantarliotou et al. 2008). These observations led to the development of long acting (slow release) melatonin devices which are able to stimulate reproductive activity before the natural breeding season. Numerous studies demonstrated beneficial effect of melatonin treatment in rams and bucks on sexual behavior, libido, ram-effect, size of testicles, sperm characteristics and production, sperm quality indicated by cryosurvival, acrosin activity, activity of plasminogen activator and inhibitor of spermatozoa and plasmin inhibition which might indicate changes in fertilizing ability of spermatozoa (Lindsay et al. 1984; Lincoln 1994; Rosa et al. 2000; Kaya et al. 2001; Casao et al. 2010; Ramadan et al. 2009). But most of these works were performed under mediterranean latitudes with less seasonal photoperiodic circumstances.

Efficacy of photoperiodic treatment depends on breed and weather (Chemineau et al., 2004), suggesting that melatonin treatment may also be influenced by these factors. We know from former experience that Awassi ewes became seasonal under Hungarian weather conditions, which raised the question whether males would respond to melatonin treatment started after

the end of breading-season with increasing day length, thus risking photo refractoriness. Although quality of ejaculate is one the most important factors regarding the success of fertilization, semen characteristics are influenced by many external factors like: experience of ram, feeding, stress, training, lameness etc. (Schatten and Constantinescu, 2007). Endocrine parameters are much more robust and easier to handle from experimental point of view.

In our study, no treatment-related difference was found in semen quality which is in contrast with paper of Chemineau et al. (1998), but in agreement with data of Fitzgerald and Stellflug (1991). They found that sperm concentration measured from weekly ejaculates of melatonin treated rams ranged from 1.5-4.5 X 10^9 cells per milliliter and was not affected by treatment. Our observation on spermatozoa concentration is in agreement with it, since we also found no difference in spermatozoa concentration between MT and Control group, although, we obtained increased sperm number in both groups over the time. However given the small number of rams examined we refrain to drew general conclusions concerning the effect of melatonin treatment on sperm production of rams. Sperm motility was evaluated by CASA and we detected that the proportion of spermatozoa showing fast forward motility increased in both treatment groups according to time (day 0 vs. day 49/71; $P<0.001$), and at the same time slow forward motility decreased (day 0 vs. day 49/71; $P<0.05$). Our possible explanation to these above mentioned phenomenon, namely increasing concentration and total motility in both group, is that they are due to the experience of weekly mounting/semen collection which itself could stimulate sperm production.

Casao et al. (2010) studied the effect of melatonin implants administered during non-breeding season in Rasa Aragonesa rams on sperm motility characteristics evaluated by CASA and in vitro fertilizing ability measured by zona pellucida binding assay. Similarly to our observation, they also found that melatonin increased the percentage of progressive motile spermatozoa, particularly during 46-75 days after melatonin implantation. Beside the changes in motility they observed significantly higher number of spermatozoa attached per oocyte in the group of oocytes inseminated with spermatozoa collected from melatonin treated rams compared to oocytes incubated with sperm from control rams. They concluded that melatonin treatment applied during non-breeding season modifies sperm motility parameters and seems to improve the fertilizing ability.

Basal testosterone level was significantly higher in MT group on d49 and d71 which reflects the expected effect of long-term melatonin treatment, in accordance with previous studies (Pelletier and Ortavant 1975a; Pelletier and Ortavant 1975b; Kennaway and Gilmore 1985; Lincoln and Ebling 1985; Kaya et al. 2001; Ramadan et al. 2009; Tsantarlioton et al. 2008).

The novelty of the results of our experiment, apart of the breed and weather, is the testosterone challenge. Our results indicate that GnRH-induced testosterone response was maintained in melatonin-treated animals.

Apart from sexual steroids we also measured plasma IGF-I levels. It was shown in female dairy goat that plasma IGF-I level is depending on actual photoperiod (Mabjeesh et al., 2007). As the signaling pathway of photoperiodic changes involves melatonin, one possible interpretation of this finding is that there is a negative connection between plasma melatonin and IGF-I level. The above mentioned theory was proven in Awassi ewes in one of our former experiments where melatonin treated dams showed significantly lower plasma IGF-I levels compared to their non-treated flock mates between days 45-70 of the treatment (unpublished data). Regarding rams we found no difference in IGF-I levels between the treatment groups. One possible explanation is that number of animals in treatment groups was not high enough to demonstrate significant differences. Another possible hypothesis is that another external factor overregulated the effect of melatonin. Such factors can be body condition, nutrition level etc. Our third hypothesis is that IGF-I regulation is different in the two sexes and possibly sexual steroids have determining role in this regulation system.

Conclusion

The use of slow release, long acting melatonin implant in Awassi semen donor rams had positive effect on the endocrine function of testicles in February, but at the same time this beneficial effect was not reflected in semen quality. Plasma IGF-I level was not influenced by melatonin treatment. Further investigation is needed to dissolve this contradiction between exocrine and endocrine findings concerning testicles and also to study the interaction between melatonin and growth hormone - IGF-I axis in small ruminants.

6. Overview of the new scientific results

The below results are thought to represent remarkable novelty value:

1. In non-suckling autumn-lambing Awassi dams first postpartum ovulation happens very early (89% before day 35 PP), even before the completion of uterine involution which increases the risk of uterine infections. Short luteal phases and persistent corpora lutea are frequent (irregular progesterone profiles altogether 33%) in autumn-lambing dams and a remarquable proportion (41% of the autumn-lambing ewes; 100% of those with irregular progesterone profile) remains non-pregnant following mating [Exp. 1].
2. Contrary to the autumn-lambing ewes resumption of ovarian cyclicity happens later (only 29% before day 35 PP) in spring-lambing ewes, and irregular cycles are rare [Exp. 1].
3. In autumn-lambing dams additional artificial light (up to 16 hours/day) delays the time of first postpartum ovulation, and thus may be a suitable tool to avoid the unwanted premature ovulation. At the same time long-day photoperiodic treatment also prevents early drying-off of autumn-lambing animals [Exp. 2].
4. Melatonin receptor 1a gene (MT1) is polymorphic at both the RsaI and MnlI restriction sites, with high incidence of R (0.83) and M (0.55) alleles in the examined Awassi flock [Exp. 3].
5. The preferable allele (R at a significant level and M tendentiously) can enhance out-of-season cyclicity only in dams with adequate fat stores and high milk yield (suboptimal metabolic condition related to low plasma IGF-I and high plasma leptin level). Nevertheless, these polymorphisms are not suitable for marker assisted selection because only the RsaI RFLP site showed significant effect and it was limited to a subgroup of the flock [Exp. 3].
6. When applying melatonin treatment in lactating ewes, due to the depression of IGF-I level, there is a risk of decline in milk yield and shortening of lactation [Exp. 4]. Plasma IGF-I level is not influenced by melatonin treatment in rams [Exp. 5].

7. Implementation of new scientific results in dairy sheep breeding

Proposal for developing reproduction technology to provide continous milk production in intensive dairy Awassi flocks („know-how")

Relying on the previously described series of experiment put side by side with literature data we suggest the following reproductive management system for intensive dairy Awassi flocks (suggestions relying on the new findings of the present thesis are highlighted as ***italic bold***):

1) Rams and ewes should be kept separately, far enough from each-other to exclude pheromone effect.

2) Feeding of lactating dams must strictly be adjusted to the energy and protein demand of their actual milk production. Rumen degradable protein overfeeding (specially overfeeding of fresh alfalfa) has to be avoided.

2) ***To provide continuos milk production the breeding technology must rely on year-round handling of two set of dams kept separately. (a) half of the flock would conceive in the tradicional breeding season (September-October) from natural cycle, and lamb at the end of winter – beginning of spring; (b) other half of the flock would conceive in April following cycle induction and lamb in September.***

3) Animals conceiving during the natural breeding season (in autumn) and lambing at the end of winter – beginning of spring (mating between 1st of September – 25th October → pregnancy check between 5th to 20th of December):
   - This set of animal can be easely formed of (i) dams lambed in spring of the given year, and (ii) of ewes born during the autumn of the previous year or the spring of the given year (≥7 months old ewes) and having adequate body condition
   - ***Ewes became spontaneously cycling at the end of August (first parity animals mainly at early September)***. The onset of cyclicity may be enhanced by the following feeding and zootechnological tools:
     - Extra energy provided from 15th August for a maximum 3 week-long period – flushing (providing adequate protein supply and approximately 15% of extra energy relative to the energy demand of the actual milk production).

       Cautions!
       - Overfeeding of rumen degradable protein (ex. fresh alfalfa) is strictly prohibited from mid-August.

- Extra energy provided for more than 20-21 days may increase the risk of early embryo mortality, and thus decrease lambing rate.

- Vasectomised rams should be introduced around day 10-14 of flushing (approximately one week before the planned mating) to provide *pheromone* effect; vasectomized or breeding rams will remain among ewes until the end of the breeding season.

Altough ovarian cyclicity can be induced effectively with hormonal treatments (gestagen + eCG from mid August, ***or slow release melatonin implant from the summer solicite***), the use of these treatments is not needed at a flock level, and is furthermore costly.

- Suggested breeding technology at flock level is natural mating in harems; insemination with fresh or frozen semen should be restricted to high genetic value dams.
- For management reasons mating should be restricted to the time interval between 1st of September and 25th of October.
- Synchronization of cycle is needed only in cases when dams are inseminated artificially. ***The recommended method of synchronization is the long-term gestagen sponge combined with eCG injection at sponge removal. If the consumer requirements desires and economical background allows, cycle synchronization before insemination is also possible by GnRH + PGF2α + GnRH combination***, however this method is more labor intensive and more costly compared to the gestagen + eCG protocol.
- To increase conception rate, drying off of lactating dams at early September can be considered.
- *Pregnancy check*: transabdominal ultrasonography should be performed between the 5th and 20th of December (2.5 - 3.5 MHz, B-mode, sector probe). The method allows the identification of pregnant and non-pregnant dams (from day 30 of pregnancy) and the recognition of twin pregnancy (from day 45-50).

  ↳ *pregnant dams*: remain with the spring lambing set of animals (from day 130-135 of gestation twin pregnants should be sparated from the rest of the flock and feeding has to be adjusted to the increased energy demand of multiple pregnancy).

↳ *non-pregnant dams*: healthy animals with adequate milk yield can be moved to the autumn-lambing set of animals. If they do not meat the above requirements, it is reasonable to cull them.

Cautions: ***To securely use the above descriebed management system, the mating period in autumn has to be finished approximately 45-50 days before pregnancy check, thus the breeding season should end by 25th October.***

- Lambing date of conceived ewes is expected between 1st February and 31th of March. The following breeding of dams will take place similarly, between 1th September and 25th October.

4) Animals conceiving from induced cycle (in April) and lambing in September (mating between 1st and 30th of April → pregnancy check between 15th May and 50th June):
   - This set of animal can be formed of (i) dams lambed in the previous autumn, (ii) of ewes born during the end of winter – beginning of spring of the previous year (≥12 months old ewes) having adequate body condition, and (iii) those healthy dams which were found to be non-pregnant at the pregnancy check in december.

     Cautions: technology ***will fail if the formation of the autumn-lambing flock is relying*** mostly on dams which did not conceive in the normal breeding season, ***on young ewes (<10-11 months-old), or on animals wich do not reach adequate body condition.***
   - ***Most of the dams and first-parity ewes are acyclic in April. Thus cycle induction with hormonal treatment is necessary*** (12-14 day-long gestagen treatment[1] started in the last days of March, beginning of April, and 500-600 IU eCG injection at removal). First ovulation is expected between 48-65 hours following gestagen removal***. If the animal does not conceive from the first ovulation, one or two additional cycles can be expected, but ovarian activity will become acyclic again thereafter.***
   - The succes of cycle induction treatments may be enhanced by the following feeding and zootechnological tools:
     - Extra energy provided from the insertion of gestagen sponge for a maximum 3 weeks-long period – flushing (providing adequate protein supply and approximately 15% of extra energy relative to the energy demand of the actual milk production).

[1] We recommend to use products with reduced amount of active ingredient (20 mg cronolon, syn.: fluorogeston).

Cautions: Similarly to the normal breeding season, extra energy provided for more than 20-21 days may increase the risk of early embryo mortality, and thus decrease lambing rate.

- Vasectomised rams should be introduced from day 5-7 of the gestagen treatment, (approximately one week before the planned mating) to provide pheromone effect.

- Suggested breeding technology at flock level is artificial insemination with diluted fresh semen (produced locally). Semen deposition is cervical or cervico-vaginal. Timing of AI should be 48-50 and 58-60 hours after sponge removal. 14 days later rams can be introduced to ewes and natural mating in harems will las for 24-28 days (max 20-25 ewes/ram)
- ***Rams has to be removed from the flock 26-30 days following sponge removal.***
- *Pregnancy check*: 45-50 days after the end of the mating period (method: same as in December).
  - *pregnant dams*: remain with the autumn-lambing set of animals (from day 130-135 of gestation twin pregnants should be separated from the rest of the flock and feeding has to be adjusted to the increased energy demand of multiple pregnancy).
  - *non-pregnant dams*: healthy animals with adequate milk yield can be moved to the spring-lambing set of animals. If they do not meat the above requirements, it is reasonable to cull them.
- Lambing date of conceived ewes is expected in September-October.
- ***In autumn-lambing dams the first postpartum ovulation, in part because of the early weaning, and in part because of the stimulatory photoperiodic signal, will take place soon, in many cases even before the completition of uterine involution. This presumably increases the risk of subclinical alterations of uterine involution. At the same time lactation length is shorter and re-conceiving dams dry off soon after getting pregnant. To prevent these negative effects (thus to increase lactation length and to delay the first postpartum ovulation), the following management interventions are suggested:***
  - ***Dams has to be kept separately from rams, far enough from each-other to exclude pheromone effect.***
  - ***Additional artificial lightening should be applied from lambing (beginning of September). Proportion of light and dark hours for long-day photoperiodic signal***

has to be 16:8, or additional lightening has to be applied during the photo-sensitive phase (between 16-18 hours after sunrise).

- The following breeding of dams will take place similarly, throughout April of the next year. To increase the success rate, drying off of lactating dams before starting the cycle induction treatment can be considered.

5) ***Determination of RsaI polymorphism of MT1 gene in all animals, and following-up of reproductive performance can provide additional information on photoperiodic sensitivity. Although in our study beneficial effect of the presence of R allele was only significant in a subset of animals with suboptimal metabolic status, further research with a higher number of sheep may justify the utility of the above mentioned mutation as a genetic marker.*** Decreased seasonality would augment the number of animals cycling throughout the year, and allow for spring mating without hormonal treatments, profiting only from the use of ram-effect and flushing.

8. References

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9. Publications

8.1. Publications related to the topics of the present thesis

Full text papers published in peer-reviewed journals in English:

1. **Faigl V.,** Keresztes M., Kulcsár M., Nagy S., Keresztes Zs., Amiridis, S., Solti L., Huszenicza Gy., Cseh S.: Testicular function and semen characteristics of Awassi rams treated with melatonin out of the breeding season. Acta Vet. Hung., 2009. 57: 531-540. (**IF:** 0.642)

2. **Faigl V.,** Keresztes M., Márton A., Fébel H., Kulcsár M., Nagy S., Cseh S., Solti L., Huszenicza Gy.: Effect of season and photoperiod on the time of first postpartum ovulation in Awassi ewes. Acta Vet Hung., 2011. 59(4): 497-510. (last known **IF:** 1.264)

3. **Faigl V.,** Keresztes M., Reiczigel J., Kulcsár M., Dankó Novotni G., Chilliard Y., Jávor A., Cseh S., Huszenicza Gy., Árnyasi M.: Relationship between seasonality of reproduction, metabolic status and MT1 receptor gene polymorphism in Awassi sheep. [Submitted for publication to Theriogenology]

4. Cseh S., **Faigl V.,** Amiridis, G.: Semen processing and artificial insemination as part of health management of small ruminants. A review. Anim Rep Sci., 2012. 130(3-4): 187-92. (last known **IF:** 1.721)

5. Radoslava, V., Kostecká, Z., **Faigl, V**., Marton, A., Keresztes M., Árnyasi M., Kulcsár, M, Dankó G., Svantner R., Nagy, S., Csatári G., Cseh S., Solti, L., Huszenicza Gy., Maracek, I.: Recent progress in endocrine, nutritional and genetic aspects of ovine reproduction. Folia Veterinara (Kosice), 2006. 50: 157-166. (IF: 0.000)

6. Radoslava, V., Kostecka, Z., **Faigl, V.,** Marton, A., Keresztes, M., Novotni-Danko, G., Csatari, G., Nagy, S., Arnyasi, M., Kulcsar, M., Cseh, S., Huszenicza, Gy., Svantner, R., Maracek, I., Recent progress in the physiology and biotechnical control of reproduction in dairy ewes. Slovensky Veterinarsky Casopis (Kosice) 2006. 31:(5) 309-314. (IF: 0.000)

Full text papers published in peer-reviewed journals in Hungarian:

7. **Faigl V.,** Marton A., Keresztes M., Novotniné Dankó G., Csatári G., Antal J., Nagy S., Árnyasi M., Kulcsár M., Cseh S., Huszenicza Gy.: Az anyajuhok szaporodási teljesítményének növelésével összefüggő egyes újabb élettani kérdések és ezek technológiai vonatkozásai. Irodalmi áttekintés. Magy. Áo. Lapja, 2005. 127: 586-593. (**IF**: 0.114)

8. **Faigl V.,** Keresztes M., Márton A., Schneider Z., Korvin L., Nagy S., Novotniné D. G., Árnyasi M., Jávor A., Cseh S., Huszenicza Gy.: Melatonin-, ill. fénykiegészítés alapú ciklusindukciós technikák kiskérődzőkben. Élettani vonatkozások és gyakorlati alkalmazás. Irodalmi áttekintés. Magy. Áo. Lapja, 2007. 129. 219-230. (**IF:** 0.104)

9. **Faigl V.,** Keresztes M., Árnyasi M., Kulcsár M., Balogh O., Nagy S., Szenci O., Cseh S., Huszenicza Gy.: Melatonin alapú ciklusindukciós technikák tejelő Awassi juhokban. Magy. Áo. Lapja, 2012. 134: pp. 24-29. (last known **IF:** 0.3)

Poster or oral presentation on international conferences:

10. **Faigl V.,** Keresztes M., Kulcsár M., Márton A., Nagy S., Cseh S., Huszenicza Gy.: Effect of melatonin treatment on plasma IGF-I and thyroxin level, and endocrin function of testicles in Awassi sheep. Magyar Buiatrikus Társaság XVIII. Nemzetközi Kongresszusa, Siófok, 2007. okt. 10-13, oral presentation

11. Cseh S., **Faigl V**., Keresztes M., Kulcsár M., Nagy S., Amiridis G., Solti L., Huszenicza Gy.: Testicular function and semen characteristics of rams treated with melatonin out of the breeding season. 11th Annual conference of the European Society of Domestic Animal Reproduction (ESDAR), Celle, Germany. Abstract. Reprod. Dom. Anim., 2007. 42. 71. oral presentation

12. **Faigl V.,** Keresztes M., Kulcsár M., Márton A., Nagy S., Dankó G., Magyar K., Solti L., Cseh S., Huszenicza Gy.: Postpartum resumption of cyclic ovarian function on non-suckling dairy (Awassi) ewes lambed in different seasons. 11th Annual conference of the European Society of Domestic Animal Reproduction (ESDAR), Celle, Germany. Abstract. Reprod. Dom. Anim., 2007. 42. 140. poster presentation

13. Árnyasi M., **Faigl V.,** Keresztes M., Kulcsár M., Reicziegel J., Jávor A., Cseh S., Huszenicza Gy.: Effect of MT1 gene polymorphism and some metabolic factors on out-of-season ovarian cyclicity in dairy Awassi sheep. 12th Annual conference of the European Society of Domestic Animal Reproduction (ESDAR), Utrecht, Netherland. Abstract. Reprod. Dom. Anim., 2008. Vol: 43(5): 87. poster presentation

14. **Faigl V.,** Keresztes M., Árnyasi M., Kulcsár M., Nagy S., Cseh S., Huszenicza Gy. Melatonin-based induction of cyclicity in intensive dairy (Awassi) flocks. International Congress on Animal Reproduction, Budapest (Hungary) 13-17 July 2008. Reprod. Dom. Anim., Vol. 43. Suppl 3. page 74. Poster presentation

15. **Faigl V.,** Árnyasi, M., Keresztes M., Kulcsár M., Reiczigel J., Jávor A., Cseh S., Huszenicza Gy.: Seasonality of reproduction and MT1 receptor gene polymorphism in Awassi sheep. International Congress on Animal Reproduction, Budapest (Hungary) 13-17 July 2008. (Reprod. Dom. Anim., 2008. Vol. 43. Suppl 3, page 11). Oral presentation

16. **Faigl V.,** Keresztes M., Márton A., Fébel H., Kulcsár M., Cseh S., Solti L., Huszenicza Gy.: Effect of season and photoperiod on the time of first postpartum ovulation in awassi ewes. World Buiatric Congress, Budapest (Hungary) 6-11 July 2008, Magy. Áo. Lapja. Vol. 130. Suppl. II. (Oral and poster abstracts), page 134. Poster presentation

17. **Faigl V.,** Keresztes M., Márton A., Kulcsár M., Solti L., Cseh S., Huszenicza Gy.: Intenzív tejhasznosítású Awassi anyajuhok egyes szaporodási jellemzői a Kárpát-medence éghajlati és takarmányozási körülményei között / Reproductive characteristics of Awassi ewes kept in intensive dairy flocks in the Carpathian basin. 15. Szaporodásbiológiai Találkozó, Eger, 2009. április 17-18.

18. **Faigl V.,** Árnyasi M., Kerestes M., Kulcsár M., Reiczigel J., Jávor A., Cseh S., Huszenicza Gy.: seasonality of reproduction and MT1 receptor gene polymorphism in awassi sheep. International Symposium on Sustainable Improvement of Animal Production and Health (organized by the International Atomic Energy Agency; Vienna, Austria; 8-11 June 2009). Poster presention

Academical reports:

19. **Faigl V.,** Árnyasi M., Kulcsár M., Nagy S., Gáspárdy A., Reiczigel J., Dankó G., Keresztes M., Marton A., Csatári G., Magyar K., Jávor A., Solti L., Cseh S., Huszenicza Gy.: A laktáció első tíz hetében ciklikussá vált petefészek-működésű állatok arányát befolyásoló tényezők intenzív tejhasznosítású, tavaszi ellésű awassi anyajuhokban. Akad. besz. 2006.

20. **Faigl V.,** Keresztes M., Kulcsár M., Horváth Á., Nagy S., Márton A., Dankó G., Magyar K., Solti L., Cseh S., Huszenicza Gy.: A petefészek-működés ellés utáni ciklikussá válása bárányaikat nem szoptató tejhasznú (Awassi) juhokban. Akad. besz. 2007.

21. **Faigl V.,** Keresztes M., Kulcsár M., Várnai Zs., Nagy S., Márton A., Solti L., Cseh S., Huszenicza Gy.: A melatonin kezelés hatása a plazma IGF-I és thyroxin (T4) szintjére awassi juhban. Akad. besz. 2007.

22. **Faigl V**., Árnyasi M., Keresztes M., Kulcsár M., Reiczigel J., Jávor A., Cseh S., Huszenicza Gy.: A MT1 gén polimorfizmus és az ivari működés szezonalitásának összefüggése awassi juhon. Akad. besz. 2009.

8.2. Candidate's further peer reviewed full-text publications unrelated to this thesis

- Marton A., Kulcsár M., **Faigl V**., Keresztes M., Cseh S., Nagy S., Pál L., Dankó G., Magyar K., Chilland Y., Husvéth F., Huszenicza Gy.: A tenyésszezon kezdetén alkalmazott takarmányozás petefészek működést befolyásoló intenzív tejhasznú Awassi anyajuhokban. Állattenyésztés és Takarmányozás, 2006. különszám: 137-138. (**IF**: 0.000)

- Keresztes M., **Faigl V**., Márton A., Ihnáth Z., Kulcsár M., Mézes M., Husvéth F., Huszenicza Gy. A takarmány védett zsírokkal történő kiegészítésének szaporodásbiológiai vonatkozásai kérődzőkben. Irodalmi áttekintés. 1. rész. Magy. Áo. Lapja, 2007. 129. 525-530. (**IF**: 0.104)

- Keresztes M., **Faigl V.,** Márton A., Ihnáth Z., Kulcsár M., Mézes M., Husvéth F., Huszenicza Gy. A takarmány védett zsírokkal történő kiegészítésének szaporodásbiológiai vonatkozásai kérődzőkben. Irodalmi áttekintés. 2. rész. Magy. Áo. Lapja, 2007. 129. 707-712. (**IF**: 0.104)

- Gyorffy A., Keresztes M., **Faigl V**., Frenyo V.L., Kulcsar M., Gaal T., Huszenicza G., Bartha T.: Effects of peripartum propylene glycol supplementation on energy metabolism: Real-time PCR investigations. Acta Physiol Hung, 2007. 94:(4) 344-345. (**IF**: 0.750)

- Huszenicza Gy., Keresztes M., Balogh O., **Faigl V.,** Kátai L., Földi J., Lemoniati, K., Kulcsár M. Periparturient changes of metabolic hormones and their clinical and reproductive relevance in dairy cows. Proc. XXV Jubilee World Buiatrics Congr., Budapest, July 6-11, Magy. Áo. Lapja, 2008. 130. (1): 45-51. (**IF**: 0.089)

- Győrffy A., Keresztes M., **Faigl V.,** Frenyó V.L., Kulcsár M., Gaál T., Mézes M., Zsarnovszky A., Huszenicza Gy., Bartha. T.: Glycogenic induction of thyroid hormone conversion and leptin system activation in the liver of postpartum dairy cows. Acta Vet. Hung., 2009. 57. 139-146. (**IF:** 0.642)

- Márton A., **Faigl V.,** Kerestes M., Kulcsár M., Nagy S., Fébel H., Novotni Dankó G., Magyar K., Husvéth F., Solti L., Cseh S., Huszenicza Gy.: Milk progesterone profiles, blood metabolites, metabolic hormones and pregnancy rates in Awassi ewes treated by gestagen + eCG at the early breeding season. Vet. Med. Cz., 54, 2009 (11): 507–516 (**IF:** 0.644)

- Kerestes M, **Faigl V.,** Kulcsár M., Balogh O., Földi J., Fébel H., Chilliard Y., Huszenicza G.: Periparturient insulin secretion and whole-body insulin responsiveness in dairy cows showing various forms of ketone pattern with or without puerperal metritis. Dom. Anim. Endocrin., 2009. 37. (4). 250-261. (**IF**: 1.651)

- Tsiligianni T., Valasi I., Cseh S., Vainas E., **Faigl V.,** Samartzi F., Papanikolaou T., Dovolou E., Amiridis G.S.: Effects of melatonin treatment on follicular development and oocyte quality in Chios ewes. Acta Vet Hung, 2009, 57:(2): 331-335. (**IF**: 0.642)

- Novotniné D.G., **Faigl V**.: Kihívások a juh faj szaporodásbiológiai kezelésében a változó fogyasztói igények hatására. Irodalmi Össszefoglaló. Állattenyésztés és Takarmányozás, 2009. 58(6): 539–548. (IF: 0.000)

- Keresztes M., **Faigl V.**, Mézes M., Kulcsár M., Huszenicza Gy.: Glükoneogenetikus takarmány-kiegészítők metabolikus és szaporodásbiológiai hatásai tejhasznú szarvasmarhában. Irodalmi áttekintés. Magy. Áo. Lapja, 2010. 132:(4): 195-203. (**IF**: 0.300)

- **Faigl V.,** Keresztes M., Kollár E., Thuróczy J.: Macskák hyperplasiás fibroadenomatosisa. Magy Áo Lapja, 2010, 132: 601-607. (**IF**: 0.300)

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