Chapter 3

Role of Melatonin and the Biological Clock in Regulating Lactation in Seasonal Sheep

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Additional information is available at the end of the chapter

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Abstract

Impact of light on animal behavior has been known for a long time—from 1925, Rowan [30] showed that lighting conditions influence gonad activity in birds and the related processes are controlled not only by means of intraorganic signals. Studies carried out in subsequent years have established that, also in mammals, the gland reacting to changes in light conditions is the pineal gland, producing a substance called melatonin. Biosynthesis of melatonin in most animals studied to date occurs at a rhythm dependent on the photocycle. The highest concentrations of this hormone—often called "the hormone of darkness"—are recorded at night. Seasonal changes in melatonin secretion conditioned by activity of the biological clock, known also as "biochemical calendar", are the key signals in the annual reproductive cycles of animals exhibiting seasonality of reproduction. Seasonality in sheep refers not only to the reproduction itself but also to lactation. One of the main hormones conditioning initiation and maintenance of lactation, synthesis of milk proteins, fat and immunoglobulins is prolactin (PRL), secreted primarily by lactotrophic cells in the adenohypophysis. Prolactin is also produced locally by the mammary gland—the hormone of this origin is identical to prolactin secreted by the pituitary gland. Until now, it was considered that the level of milk production in mammals is determined by both genetic and environmental factors. However, in recent years, many studies focused on the role of light as a modulator of prolactin levels. In livestock, changes in light-period length play a very important role as this determines their productivity and milk yield. Photoperiod is particularly important in short-day breeder animals (sheep), for which the length of light period is associated with changes in melatonin level. The modulating effect of melatonin on secretion of prolactin may take place via two different mechanisms. One is associated with the circadian rhythm, wherein—directly or through the medium of a factor popularly termed "tuberalin"—melatonin stimulates the release of prolactin. However, this effect is short-lived and is most likely applicable only to prolactin stored in lactotrophic cells of the pituitary. The second mechanism regulating the secretion of melatonin and prolactin is associated with the annual rhythms of secretion—melatonin, due to its lipophilic characteristics, has a direct effect on the secretion of prolactin. Under

natural conditions, the maximum concentration of prolactin in the blood of sheep is observed over the long-day period, during which the melatonin level decreases. The lowest prolactin concentration is observed over the short-day period, where melatonin levels are at their highest. Changes in secretion of prolactin during lactation in sheep undoubtedly affect the amount of milk produced.

Keywords: seasonality sheep, melatonin, length days, biological clock lactotropic and metabolic hormone

1. Introduction

1.1. Genetic predispositions and impact of environmental factors on the lactation process in sheep

1.1.1. Role of melatonin and the biological clock

Sheep are short-day breeders, in which the signal for onset of estrus occurs after the summer solstice and is maintained until winter. Such a model of the reproductive cycle in which the young ones are born in the spring provides favorable conditions for rearing lambs as this period coincides with the time of abundance of food, thereby the young have time to put aside fat for the winter. Seasonality of reproductive cycle in sheep is associated with the season and the day length as a recurring reproductive cycle is an endogenous rhythm, encoded genetically. Information on changes in photoperiod reaches the animal's organism through a multineural tract. Much more studies confirm the presence of a molecular mechanism—located in the SCN (suprachiasmatic nucleus) as well as in pars tuberalis (PT)—involved in decoding the melatonin signal.

Both the SCN and PT host over a dozen genes of the circadian clock, such as *Bmal1*, *Clock*, *Per1*, *Per2*, *Cry1*, *Cry2A*, *Rev-erbα* and *CK1ε*, which are mutually coupled [1, 2]. Most likely, the summer and winter rhythm observable in sheep is conditioned by the biological clock genes. Over 24 hours, changes in the melatonin profile affect the rhythmic changes in the expression of these genes as evidenced by varying levels of clock genes mRNA in the PT and SCN. The peak gene expression of *Cry1* (Cryptochrome) gene occurs at twilight, together with the increase in melatonin level, while expression of the gene *Per1* (Period) is stimulated by approaching dawn [3, 4]. In contrast to *Cry1*, *Cry2* gene is not melatonin-induced [5]. On the other hand, expression of the *Per1* gene is melatonin-dependent as pinealectomy (surgical removal of the pineal gland) blocks the rhythm of the *Per1* gene in the PT, but does not affect the expression of this gene in the SCN.

Studies have reported that repeated multiple injections of melatonin in animals previously subjected to pinealectomy restore the cyclical transcription of *Per1* in PT [6]. The results of studies on biological clock genes in mammalian SCN showed that the BMAL1/CLOCK protein

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complex encoded by the genes of *Bmal1*, *Clock* induces activation of the *Per1*, *Per2*, *Cry1*, *Cry2*, *Rev-erbα* genes [2]. Studies in sheep artificially subjected to a sudden light stimulus characteristic of a long-day period have shown that the gene expression profile of *Cry1* and *Per1* mirrored that occurring in natural conditions, i.e., expression of *Cry1* increased at night, while that of *Per1* was rising during the day, indicating the presence of a rapid mechanism for regulation of *Cry1* and *Per1* gene expression in response to a melatonin impulse [1].

The neurosensory receptor of circadian rhythm in mammals is the retina of the eye, through which light stimuli are transmitted to the suprachiasmatic nucleus of the hypothalamus (SCN). The pathway the light stimulus travels from the retina to the SCN is known as the retinohypothalamic tract. Secretion of melatonin is a biochemical signal informing the body about changes occurring in the external environment. Melatonin has lipophilic properties and is secreted from the pineal gland by simple diffusion [7]. Because of a well-developed network of blood vessels in the pineal gland, this hormone is released directly into the blood and distributed throughout the entire organism. In animals sensitive to changes in day length, the melatonin profile is a biochemical signal regulating the processes of reproduction and lactation [8]. In sheep, which are short day breeders, seasonal changes in melatonin levels inform the fetus of the environmental conditions.

A large number of MT_1 melatonin receptors are present in pars tuberalis (PT) of sheep, while no similar high concentration is noted in the tuberal region of the hypothalamus and in the SCN. This suggests that melatonin may modulate the secretion of hormones secreted only in the pars tuberalis [9, 10]. Increase in melatonin secretion in sheep is stimulated already within 1 or 2 hours after sunset and lasts until the onset of dawn. According to Misztal et al. (1999) [11], the modulating effect of melatonin on prolactin (PRL) secretion could be explained by two different mechanisms. One is linked to the circadian rhythm, which may either have direct impact or act through the factor conventionally known as tuberalin. However, this effect is short-lived and most likely is only applicable to prolactin stored in the lactotropic cells of the pituitary. It is possible that tuberalin activates the expression of the prolactin gene in lactotropic cells [9]. The second mechanism modulating melatonin secretion is related to the annual rhythm of secretion—this means that melatonin, due to its lipophilic properties, has a direct effect on the lactotrophic cells of the pituitary and thus also impacts the secretion of prolactin [11, 12]

1.1.2. Role of prolactin and the growth hormone (GH)

Changes in melatonin and prolactin profiles in sheep are closely interlinked. Regulation of PRL secretion by melatonin may occur via two different mechanisms. In the case of the circadian rhythm mechanism, melatonin may directly affect PRL secretion—this option applies to the hormone stored in the lactotropic cells; the process may also be mediated by the aforementioned peptide—tuberalin, which activates PRL gene expression in lactotropic cells of the anterior pituitary [9]. In contrast, the process of annual PRL secretion rhythm is directly induced by melatonin that—due to its lipophilic nature—affects the lactotropes [13]. Synthesis of prolactin occurs in the anterior pituitary in the lactotropic cells. The main role of PRL is to initiate and control processes such as mammogenesis, lactogenesis, galactopoesis and involu-

tion. Moreover, this hormone plays an important role in biosynthesis of milk proteins (β-casein, α-lactalbumin, lactose). It has been shown that in sheep, the daily and seasonal rhythms of PRL and melatonin are characterized by volatility. Changes in prolactin levels throughout the day are strongly associated with the season. Increases in PRL concentration in the spring are observed at dawn and before dusk. In the summer, the peak level is recorded halfway through the dark cycle, while the autumn PRL profile is characterized by a spike in the first half of the photoperiod and near its end. In short-day breeders, such as sheep, the seasonal lengthening of day-light hours (spring, summer) resulting in a short melatonin signal (4–8 hours a day) does not inhibit the secretion of PRL, while in autumn and winter, a long-lasting melatonin impulse (>10 hours per day) causes a decrease in prolactin concentration [6, 14]. Melatonin modulates PRL secretion also through the intermediary of dopamine. The neurotransmitter stimulates PRL secretion acting through dopamine D1 receptors and inhibits the secretion of this hormone via its effect on dopamine D2 receptors. The presence of seasonal changes in melatonin and prolactin profiles was also confirmed by tests carried out on sheep kept for dairy purposes. It has been shown that key factors affecting milk yield in ewes are changes in the photoperiod. It has been found that milk yield in females entering lactation during the day-light lengthening season is by far (50%) higher than that in animals starting milk synthesis in short-day conditions [15]. The reaction to the shortening of the photoperiod was an increase in melatonin levels, decrease in PRL concentration and lower milk yield. Subjecting sheep to conditions of artificially prolonged day-light cycle (16L:8D) resulted in light-induced inhibition of melatonin synthesis in the pineal gland. Decrease in PRL concentration and lower milk yield were observed simultaneously. Thus, the artificial prolongation of the photoperiod during short-day season is not enough to maintain lactation in seasonal sheep [10, 14, 16, 17]. Previous observations indicate that such processes as mammogenesis, lactogenesis, galactopoesis and involution in sheep require the presence of multiple factors, strongly interdependent. Milk production is based on the impact of a number of factors and day length, as well as changes in PRL profile are only some of many [18]. An important role is also played by the somatotropic system (GH, IGF-1). The growth hormone, similar to PRL, is produced in the anterior pituitary and is involved in synthesis of proteins and fatty acids; it also lowers the concentration of glucose in the blood and is partly responsible for synthesis and secretion of prolactin [19]. Increase in concentration of the GH is stimulated by the "suckling factor"—higher growth hormone levels are observed at the beginning of lactation. Studies carried out on sheep have shown that changes in concentration of both the GH and PRL are dependent on the length of day and are linked to changes in the profile of pineal melatonin. Periodic changes in melatonin levels result in rhythmic inhibition of PRL secretion [20]. It is known that during lactation, under the impact of suckling, the GH and PRL levels in the blood are boosted [19]. Increase in GH secretion during lactation is controlled by GHRH and endogenous opioids. Recently, attention has been drawn to a compound, derivative of dopamine, known as salsolinol—it has been shown that concentration of this substance increases in the case of various dysfunctions in the dopaminergic system. Salsolinol stimulates the release of PRL in rodents and ruminants. In lactating sheep, the presence of salsolinol was confirmed in MBH and increase in its concentration in response to suckling was recorded [21]. Salsolinol administered to the third ventricle of the brain during lactation increases prolactin concentration.

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Salsolinol antagonist is a compound called 1-MeDIG—this substance inhibits the release of PRL and cancels the stimulatory impact of the "suckling factor" on PRL secretion in rats. A similar effect was observed in sheep in which 1-MeDIQ acts directly on the central nervous system [22]. The compound inhibits the increase in the level of noradrenaline (NA), which acts as a mediator between salsolinol and GH. Interestingly, 1-MeDIQ does not affect the changes in GH concentration induced by stimulation of the mammary gland during suckling. In sheep, both over the period of lamb rearing and beyond, PRL levels decreased after 1-MeDIG was administered. It was proven experimentally that salsolinol has no direct influence on GH profile during lamb rearing. However, in rats subjected to simultaneous administration of both salsolinol and 1-MeDIQ, no statistically significant changes in pituitary hormone levels were observed with the exception of prolactin.

1.1.3. Role of metabolic hormones

The role of metabolic hormones in the process of lactation has garnered a lot of attention. An important role in initiating and maintaining lactation in small ruminants is played by the thyroid hormones, ghrelin and orexin. The production capacity of animals (milk yield, growth and development, coat growth) is largely dependent on proper functioning of the thyroid hormones. These hormones influence also the processes of reproduction in many species of animals, including sheep and goats [23]. In seasonal species, T4 and T3 are obligatory for the annually recurring termination of reproductive activity [24, 25]. Thyroxine in sheep with normally functioning thyroid will shorten the reproductive period and quicken the transition into anoestrus. Thyroxine level peaks in ewes in early pregnancy and decreases just before lambing and after the offspring is born. The level of thyroid hormones in sheep varies throughout lactation. At the start of the process, concentration of these substances is low [26]; however, over time, the thyroxine concentration increases. It has been shown that thyroid hormones, especially tri-iodothyronine, have a suppressive effect on expression of the prolactin gene, which can translate into milk yield as well. An important role in lactation belongs to calcitonin and the parathyroid hormone, responsible for modulation of phosphorus (P) and calcium (Ca) levels. Concentration of these elements in milk has major impact on the chemical composition of the product. The presence of the parathyroid hormone is essential for calcium absorption from the gastrointestinal tract; it also enhances synthesis of active D3 (1,25-di-hydroxycalciferol) that stimulates the process of calcium binding by proteins. It has been experimentally demonstrated that thyroid hormone secretion is correlated with day length in sheep. In vitro studies in thyroid gland explants showed higher levels of thyroxine under short-day conditions and lower in the season of elongating photoperiod (spring), while T3 reached higher levels in the summer and lower when the photoperiod was shortening. In addition, there was an increase in T3 concentration induced by exogenous melatonin [25]. Productivity of the animals depends not only on the level of nutrition, environmental factors and their genetic potential. Thyroid hormones are an important link in the key stages of life (reproduction and lactation) of all living organisms [27]. Orexin A is of particular importance in the reproductive process of animals sensitive to changes in day length. The process of initiating and maintaining lactation in sheep requires the presence of many hormones. Defining the role

of orexin in regulating their secretion, especially that of prolactin, may allow to better understand the process of maintaining lactation in sheep, in particular over short-day period.

2. Influence of day length and melatonin on milking yield

Changes in day length and the related secretion of melatonin and prolactin are of particular significance in sheep as they determine reproductive processes, the last stage of which is lactation. The possibility of artificial extension of the milking period in late-lambing ewes by application of prolonged day length, 16 hours of light—8 hours of darkness (16L:8D), was introduced additionally (Group III). Measurements of plasma levels of prolactin and melatonin were used as parameters of season-dependent hormonal regulation of milk production in this seasonally breeding species [28]. Lambs remained with their mothers up to 56th day of life. Then lambs were separated from their mothers, which were allocated to the milking. During milking period, ewes were milked twice a day using Alfa-Laval machine. Individual milk yield checks were carried out every 10 days. From the 20th day of lactation to the end of this process, the blood samples were carried out from each sheep every 30 days to determine concentration of melatonin and prolactin. Blood sampling started after sunset and continued for 6 consecutive hours with a frequency of every 60 minutes. Blood after collection were centrifuged and the resulting plasma was stored at temperature −20°C until analysis. Hormones have been determined by radioimmunologically (RIA) method. During lambs' rearing period, sheep produced similar quantities of milk, since ewe Group 1 produced 48.2 ± 12.9 liters whereas Group II produced 42.4 ± 16.4 liters. Higher productivity was observed in Group III 60.5 ± 16.6 liters, which was kept in artificial light conditions. The observed differences were statistically insignificant. Distinct differences in milk yield were

Groups of sheep	Milk yield of the first 28 days of lactation (l)		Total length of lactation (days)		Days of milking (days)		Milk production during milking (l)	
	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM
Group I Sheep lambed in January	48.2	2.3	177	8.6	102	4.8	33.0	3.6
Group II Sheep lambed in June	42.4	3.1	147	3.5	77	4.0	16.8	1.4
Group III Sheep lambed in June (16L:8D)	60.5	3.2	160	4.1	90	3.9	21.2	1.7

Table 1. Parameters characterizing lactation duration and efficiency of Polish Longwool sheep lambing in January (Group I), in June, kept under natural lighting conditions (Group II) and in June, kept under the long-artificial photoperiod (16L:8D, Group III). See text for statistical comparisons.

observed between the groups of sheep milk in the period of use. The highest milk yield of 33.0 ± 11.2 liters was found in Group I, while Group II produced only 16.8 ± 4.4 liters, the obtained differences were statistically significant ($P \leq 0.01$). Mothers who remained in artificial light conditions (Group 3) produced 21.2 ± 5.5 liters of milk (**Table 1**). The results of the total lactation length and days of milking show conclusively that the lactation period in Group I was significantly longer than that in Groups II and III ($P < 0.05$, **Table 1**). Analysis of the course of lactation with regard to the mean amount of milk obtained in particular months of milk use revealed that the milk yield of Group I in the first month of milking (0.43 ± 0.09 liters/day) was similar to the milk yield of Group III (0.42 ± 0.07 liters/day), with only 0.18 ± 0.08 liters/day in Group II ($P < 0.01$, **Figure 1**, **Tables 2–4**).

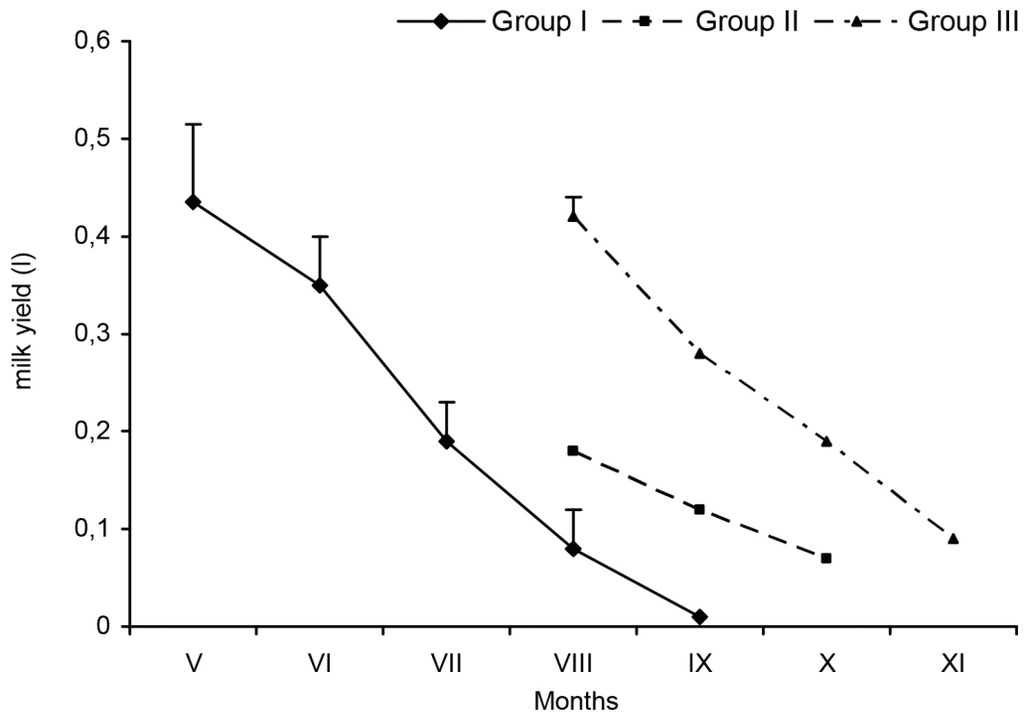

Figure 1. Mean monthly milk yield of Polish Longwool sheep lambed in January (Group I), in June, kept under natural lighting conditions (Group II) and in June, kept under the long-artificial photoperiod (16L:8D, Group III), during the milking period. See text for statistical comparisons.

Months		**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**
MLT (pg/ml)	$\bar{x}$	168.5	85.5	132.8	133.5	77.	73.3[c]	124.7	91.3
	SD	137.3	45.3	112.6	113.0	38.9	50.1	100.6	42.2
PRL (ng/ml)	$\bar{x}$	128.6	102.8	156.5	312.6[r]	185.7	247.0[j]	151.6	43.9
	SD	46.3	33.8	18.1	45.2	54.7	60.9	43.9	33.1
Milk (l)	$\bar{x}$	–	–	–	0.43	0.35	0.19	0.08	0.01
	SD				0.08	0.05	0.04	0.04	–

Table 2. Mean (±SEM) and SD of plasma melatonin and prolactin and milk concentrations in sheep lambed in January (Group I). See text for statistical comparisons.

Months		VII	VIII	IX	X	XI
MLT (pg/ml)	$\bar{x}$	61.1	87.8	82.3	77.5	93.2[a]
	SD	28.5	45.5	45.0	46.1	57.4
PRL (ng/ml)	$\bar{x}$	234.0	124.6	60.5	30.8	16.8
	SD	39.5	48.8	31.1	17.7	10.4
Milk (l)	$\bar{x}$	–	0.18	0.12	0.07	0.04
	SD		0.05	0.03	0.01	0.01

Table 3. Mean (±SEM) and SD of plasma melatonin and prolactin and milk concentrations in sheep lambed in June kept under natural lighting conditions (Group II). See text for statistical comparisons.

Months		VII	VIII	IX	X	XI
MLT (pg/ml)	$\bar{x}$	41.0[a]	60.4	17.6	4.4	17.0
	SD	19.7	39.8	27.6	4.1	15.5
PRL (ng/ml)	$\bar{x}$	278.8	132.7	147.9	84.3	38.3
	SD	55.3	57.4	82.4	42.5	25.2
Milk (l)	$\bar{x}$	–	0.42	0.28	0.19	0.09
	SD		0.07	0.05	0.03	0.02

Table 4. Mean (±SEM) and SD of plasma melatonin and prolactin and milk concentrations in sheep lambed in June and kept under the long-artificial photoperiod (Group III, 16L:8D, bottom). See text for statistical comparisons.

2.1. Secretion melatonin and prolactin in day length

The highest melatonin level in Group I determined in February was 168.5 ± 137.3 pg/ml, while prolactin level at this time was 128.6 ± 46.3 ng/ml. The highest prolactin concentration determined in May was 312.6 ± 45.2 ng/ml, further growth of prolactin level in July was 247.0 ± 60.9 ng/ml and the resulting differences were statistically significant ($P \leq 0.05$) compared to the level of the hormone in other administrations. With the lengthening of lactation and changes in day length light from August to September significantly decreased prolactin level from 151.6 ± 43.9 ng/ml to 43.9 ± 33.1 ng/ml and increased levels of melatonin. In August, the concentration of melatonin was the highest and amounted to 124.7 ± 100.6 pg/ml, while sheep milk production has decreased to a level of 0.08 ± 0.02 liters per day. In the last month of lactation, melatonin level was 91.3 ± 42.2 pg/ml and sheep milk production at this time was only 0.01 ± 0.02 liters/day (**Figure 4**, **Table 4**). The increase in melatonin levels from July to September of 33.4 pg/ml was accompanied by a decrease in prolactin level of 107.7 ng/ml. At that time, there was a decrease in the secretion of milk by an average of 0.11 liters/day (**Table 2**).

2.2. Secretion melatonin and prolactin in short days

In the case of Group II, the highest level (234.0 ± 39.5 ng/ml) of prolactin was also determined in July and the melatonin concentration in this period was the lowest (61.1 ± 28.5 ng/ml). With the shortening of the light day, prolactin secretion was decreasing and the level of this hormone

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was lower by 25.8% in September compared to the level observed in July. A clear decrease in prolactin level observed during the last 2 months of lactation, i.e, October and November was 30.8 ± 17.7 ng/ml and 16.8 ± 10.3 ng/ml, respectively. In July, as previously indicated, the lowest concentration of melatonin was 61.1 ± 28.5 pg/ml, differing significantly ($P \leq 0.05$) to the identified levels of this hormone in November (93.2 ± 57.4 ng/ml). Changes in the concentration of melatonin and prolactin during the shorter photoperiod influenced the parameters of sheep milk production, causing a drop in milk yield by 22.2% between August and November (**Table 3**). The results obtained in Group III showed that the highest level of prolactin found in July was 278.8 ± 55.3 ng/ml and much lower in September, 147.9 ± 82.4 ng/ml; however, it was higher than the level of prolactin identified in August, 132.7 ± 57.4 ng/ml. The resulting differences in the levels of prolactin in the month of July, August and September were statistically significant ($P \leq 0.05$). Despite ensuring that this group of animals underwent 16-hour lighting intensity of 200 lux, the concentration of prolactin from September to November reduced (**Table 4**).

The sheep of all groups produced similar amounts of milk during the first 28 days of lactation as estimated based on the weight gains of the lambs. The study results showed that the shift in lambing date—from winter to summer—had a negative effect on milk production parameters in ewes. Sheep that gave birth in January and were used for dairy purposes over the long-day period produced 50% more milk than ewes that gave birth in June and were then milked as the day length was gradually decreasing. Lactation in sheep milked in the summer-time was significantly longer than that in sheep milked when the photoperiod duration was shortening. Day length had no effect on milk yield in the period of rearing lambs (i.e., 28 days). Monitoring of hormone levels (prolactin and melatonin) in sheep during lactation allowed to conclude that secretion of melatonin in the fall months increased, while prolactin secretion was decreased over the same period. The increase in melatonin level during the shortening of the day in the Polish Longwool sheep reduces prolactin secretion and inhibition of the synthesis of milk. Introduction of artificial light conditions during the shortening of the photoperiod is not enough to maintain secretion of prolactin in ewes at a level that allows to maintain lactation in the autumn.

Length of illuminating day, but especially profile of melatonin has a particular meaning in sheep, because decidate of trial procreative with last stage physiology that last stage physiology reproduction is lactation. [18]. As the many physiological processes also the reproductive cycle is genetically encoded in the sheep. The course of this cycle is reflected by the seasonal changes in the secretory activity of the hypothalamopituitary gonadotropic GnRH-LH system. Sustaining of the proper duration of this cycle requires, however, constant and periodically repeated factors which enable the synchronization of the physiological processes with a suitable season of the year. Thus, the day length plays the most important role in this aspect. The information about the day length reaches the organism as the biochemical signal generated by the pineal gland via the nocturnal secretion of melatonin. The seasonal changes in the duration of melatonin secretion are of great importance in the modulation of sexual activity and lactation in the sheep with the inherent traits of seasonality. The dependence of milk yield and the duration of lactation on melatonin and prolactin secretion are also demonstrated in

seasonal and aseasonal breeds of sheep lambed during the different seasons of the year. The putative mechanisms of melatonin action on luteinizing hormone and prolactin secretion are also demonstrated with reference to the melatonin-binding sites in the sheep central nervous system and pituitary gland [29].

2.3. Influence of day length and melatonin in prolactin secretion and growth hormone in suckling sheep

The effects of melatonin on the secretion of prolactin (PRL) and growth hormone (GH) were studied in ewes' nursing lambs (Polish Longwool, n = 20) under different photoperiods (March and November). The animals were divided into four groups: (a) (LDC—long-day control group, n = 5), (b) melatonin-treated (LDM—long-day group, n = 5), (c) (SDC—short-day control group, n = 5) and (d) (SDM—short-day melatonin, n = 5). Blood samples were collected from ewes 5 days after lambing. Four blood collections were performed at 10-day intervals, over a 40-day time period. Sampling started at sunset and continued for 6 hours at 20-minute intervals. Melatonin implants (exogenous melatonin) were inserted in ewes of the LDM and SDM groups after first blood collection. The plasma concentrations of PRL and GH were assayed using RIA. In ewes from the LDC group, the mean plasma PRL concentration increased gradually, reaching a significantly ($P < 0.001$) higher level, after 3 weeks. In contrast, in the LDM group, PRL concentration decreased significantly ($P < 0.001$) following 10 days, compared to that in ewes from the LDC group. The mean plasma GH concentration was significantly ($P < 0.001$) higher in the LDC group than that in the LDM group, the for the entire experimental period during the experimental period. In the SDC and SDM groups, plasma PRL concentrations did not decrease significantly ($P < 0.001$) 2 weeks after the onset of the experiment and did not differ significantly between these groups. The mean plasma GH concentration increased significantly ($P < 0.001$) in the SDM group compared with the SDC group only after the third week. The mean plasma GH concentration in the SDM group and the SDC group reached a similar level by the end of the trial. It would appear that melatonin may effectively inhibit PRL secretion in nursing ewes during long photoperiod and stimulate GH release during short photoperiod. The inhibition of PRL secretion in nursing ewes during increasing photoperiod (long days) occurs, despite the strong stimulation of suckling. At the onset of the experiment, the mean plasma PRL concentrations in the LDC and LDM groups were similar (193.2 ± 10.9 and 192.3 ± 8.7 ng/ml, respectively) (**Figure 2**). During the subsequent collection (second), the PRL concentration in the LDC group was 166.2 ± 8.0 ng/ml; however, in the LDM group a significant decrease in the plasma PRL concentration was recorded as 56.5 ± 4.2 ng/ml ($P < 0.001$). During this time, the mean concentration of PRL in LDC ewes was significantly higher ($P < 0.001$) than the LDM ewes (**Figure 2**). The mean concentration continued to increase as day length (photoperiod) increased.

During the decreasing day length period, the PRL secretion profile was similar in SDC and SDM groups (**Figure 2**). In both groups, there was a significant ($P < 0.05$) decrease in plasma of PRL concentration in during the third week of lactation (14.7 ± 1.4 and 12.6 ± 1.0 ng/ml) compared with the initial concentration (60.6 ± 7.3 and 53.4 ± 6.0 ng/ml, respectively). No differences in PRL plasma concentrations were recorded between the SDC and SDM groups.

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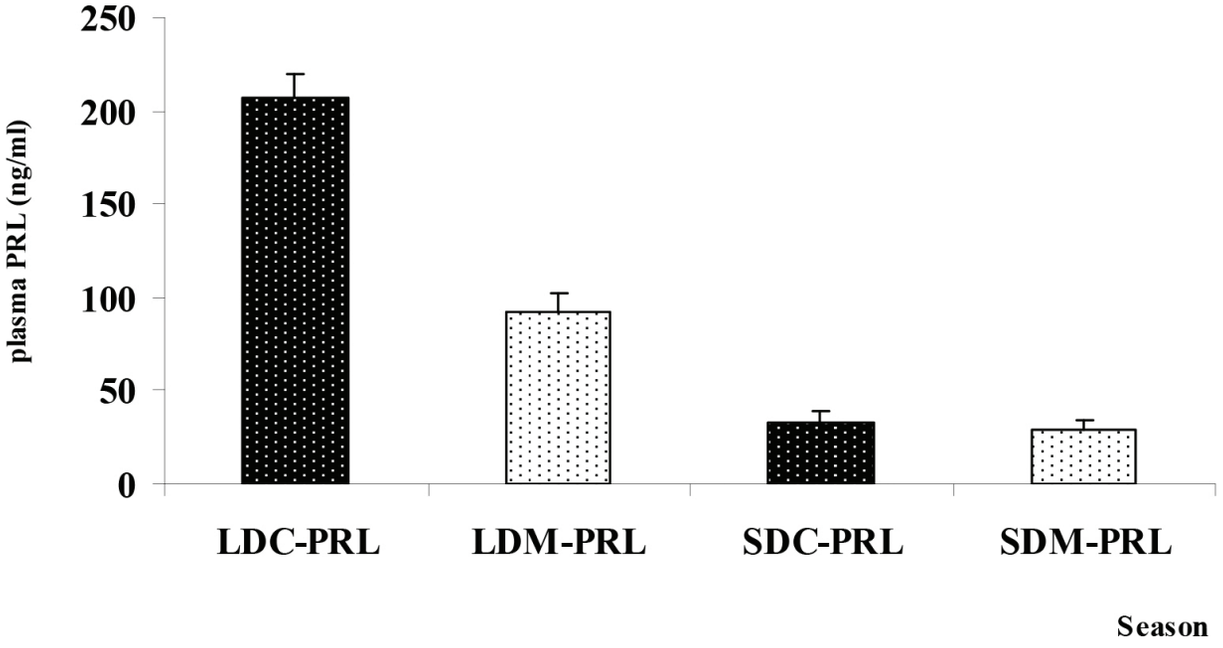

Figure 2. Mean plasma PRL concentrations in nursing-sheep lambing (LDC—a long-day control and LDM—a long-day melatonin-treated group, SDC—a short-day control and SDM—a short-day melatonin-treated group). See text for statistical comparisons.

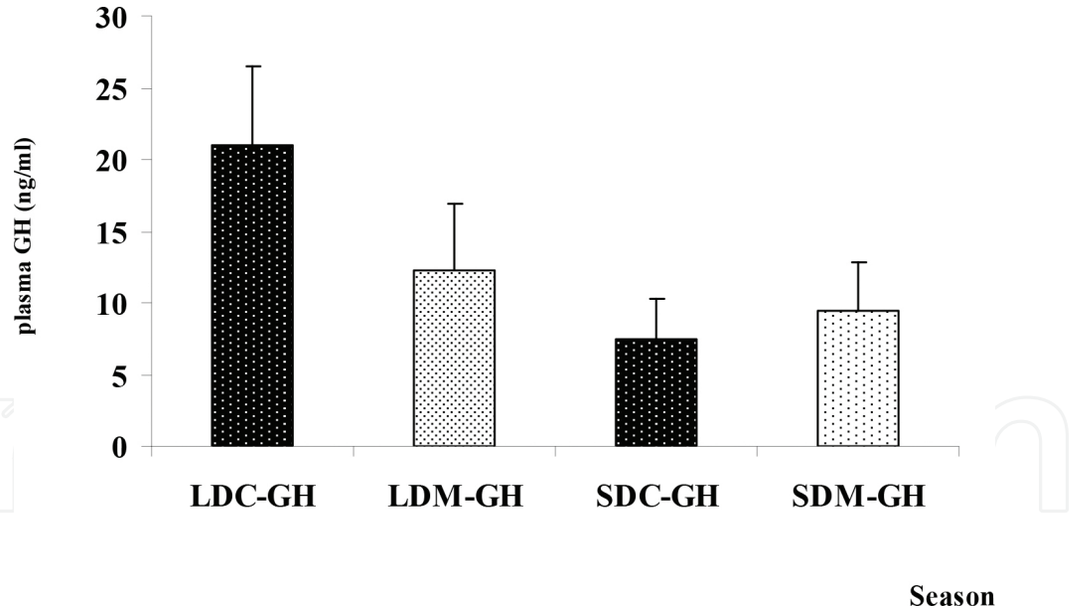

Figure 3. Mean plasma PRL concentrations in nursing-sheep lambing (LDC—a long-day control and LDM—a long-day melatonin-treated group, SDC—a short-day control and SDM—a short-day melatonin-treated group). See text for statistical comparisons.

The mean plasma GH concentration was significantly ($P < 0.001$) higher in the LDC group than the LDM group for the entire trial period. It was also observed that a gradual decrease in GH concentration took place in both groups (**Figure 3**). The mean GH concentrations in

SDC and SDM ewes were at a similar level up to the second time of blood collection day (**Figure 3**). The only significant ($P \leq 0.001$) rise in plasma GH secretion was recorded in the third week of lactation in the SDM group (12.23 ± 5.36 ng/ml) compared with the SDC group (6.58 ± 2.36 ng/ml).

In conclusion, the long-term treatment with exogenous melatonin of early-lactating sheep reduced the PRL secretion during the increasing photoperiod, despite strong stimulation by suckling. Moreover, in nursing ewes, melatonin stimulated GH secretion during the short photoperiod. It can therefore be assumed that melatonin may be indirectly affected by the level of milk production in sheep, especially following the nursing period.

3. Influence of metabolic hormones on prolactin secretion in lactation sheep

3.1. Role of orexin

Studies on the role of orexin A in the control of prolactin (PRL) and growth hormone (GH) secretion in rodents have produced inconsistent results. Orexin A may play a special role in animals' sensitivity such as sheep to the day length changes. The aim of the study was to determine the role of orexin A in the control of prolactin secretion and growth hormone in sheep during different photoperiods. In vitro studies were carried out on 10 Polish Long-wool ewes on 30 days of lactation during long photoperiod (May, LD, n = 5) and short photoperiod (December, SD, n = 5). After rearing lambs to 30 days of age, ewes were decapitated and the pituitaries were dissected and then cut along the longitudinal fissure into two halves, so that each half contained the glandular and nervous parts. Pituitary glands were collected and divided along the longitudinal fissure into two halves. Glands were incubated for 3 hours at 37°C in Parker medium with addition of orexin A—experimental group or in medium alone—control group. During the following 3-hour incubation, medium was exchanged every 15 minutes and a sample of 1 ml was collected and immediately frozen at −80°C until assay. Prolactin concentrations in the medium were determined radioimmunologically (RIA).

In the long-day conditions (May), the pituitary explants of lactating sheep exhibited the strongest secretory activity during the first hour of incubation—significantly higher in orexin-treated group (O1) than the control group (K1), ($P < 0.01$). During the second hour of the incubation, PRL concentration decreased and reached the similar values in both groups. During the third hour, PRL concentration in O1 group was again significantly higher than that noted in K1 group ($P < 0.01$). In the short-day conditions (December), PRL concentration was significantly higher in orexin-treated group O2 during the first hour of incubation than the value observed in the control group—K2 ($P < 0.01$). The inverse relationship in prolactin release was observed during the second hour of incubation ($P < 0.01$), however, during the third hour, PRL concentration was again significantly higher in O2 group than the concentration noted in K2 group ($P < 0.05$). Collective analysis of the data showed that PRL concentrations were higher in experimental groups (O1 and O2) than the concentrations noted in control groups (K1 and K2) under both the long (May) and short (December) photoperiods (**Figure 4**).

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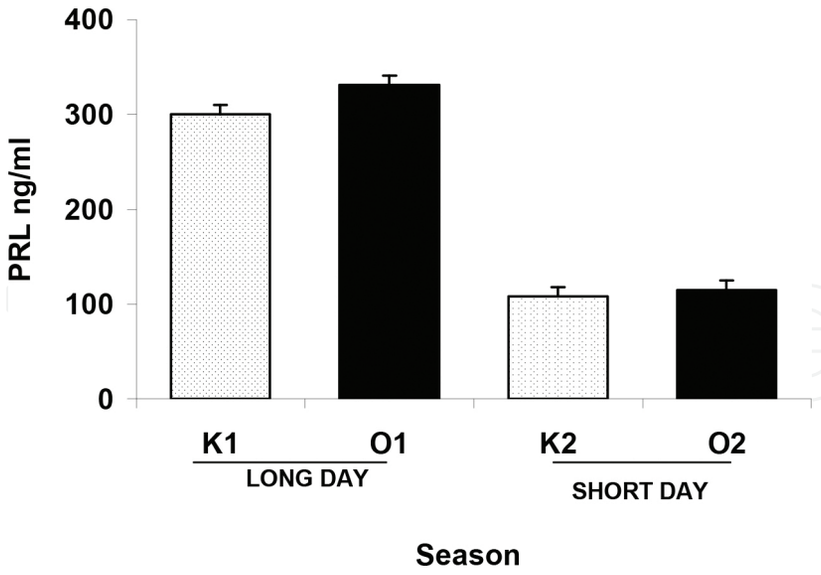

Figure 4. Mean concentrations of prolactin in control and orexin A-treated pituitary explant cultures during long-day (LD) and short-day (SD) photoperiods. See text for statistical comparisons.

GH release from the pituitary explants during the long-day conditions was maintained on significantly higher level in orexin-treated group O1 than control K1 group ($P < 0.05$), throughout the whole period of the incubation. In contrast, during the short-day period, GH release from the explants was significantly less in orexin-treated group O2 than that in the control K2 group ($P < 0.05$). The suppressive effect of orexin was observed during 2 hours. Collective analysis of the data showed that GH concentrations were higher under long-day conditions than under short-day conditions (**Figure 5**).

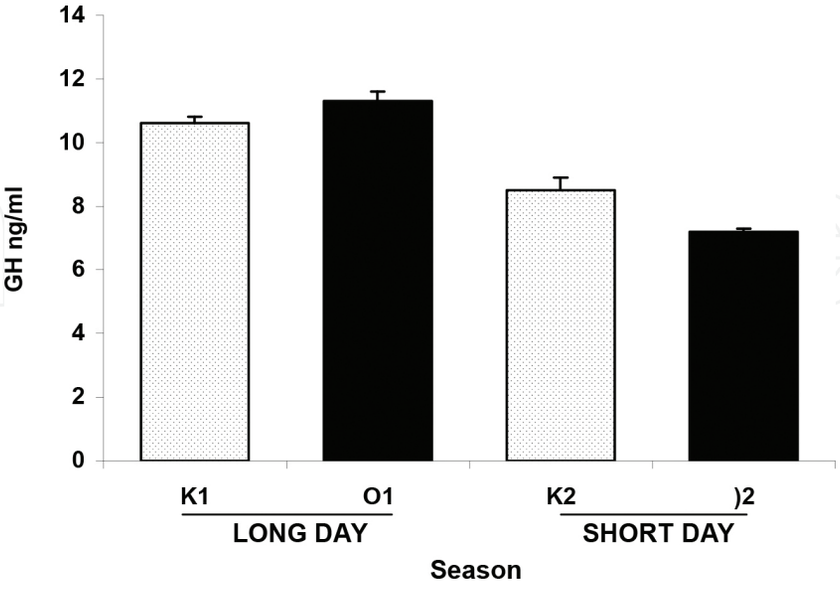

Figure 5. Mean concentrations of growth hormone in control and orexin A-treated pituitary explant cultures during long-day (LD) and short-day (SD) photoperiods. See text for statistical comparisons.

The results of experiments performed on lactating sheep, i.e., animals with strong seasonality characteristics, are difficult to compare with others, however, in ewes as in rodents, orexin A is able to stimulate PRL secretion due to its direct effect on the lactotropic cells. The slight response of ovine pituitary glands to orexin during short days is probably due to the insensitivity of lactotropic cells to the orexin signal. The initiation and maintenance of lactation in sheep require the presence of many hormones, where PRL and GH seem to be the most important. Studies on lactating sheep showed that the ewes starting lactation during the period of increasing day length produced 50% more milk compared with sheep milked during the decreasing day length [15]. When June-lambed ewes were kept under artificial conditions of the long day (16L:8D), PRL level decreased as the natural length of a day became shorter. The fact that pituitary cells become refractory to over-repeated summer signal of the darkness hormone (melatonin) makes it impossible to lengthen lactation in sheep in the autumn-winter period [15]. Determining the role of orexins, especially orexin A, in regulating prolactin secretion may help to clarify the process of lactation maintenance in sheep, especially during the decreasing photoperiod. In conclusion, our results obtained on the pituitary explants demonstrated that the pituitary tissue of lactating sheep was sensitive to photoperiod and orexin A. We conclude that the secretion of PRL and GH from the ovine pituitary gland is negatively responsive to orexin A during SD, whereas orexin may stimulate PRL and GH secretion during LD. Further studies investigating orexin—PRL and GH interactions are needed.

3.2. Role of TRH

Recently, it was observed that TRH has a role to play in the initiation and maintenance of lactation in small ruminants. The aim of the performed study was to determine the impact of the TRH factor on secretion of prolactin in lactating sheep. In vitro studies were carried out on 10 animals. The pituitary gland of each sheep was collected at day 40 of lactation. In vitro incubations were performed on 12 microwell plates in Parker medium for 1 hour at 37°C. One half of the gland was incubated in pure Parker medium (control group), while the second (test group) half was incubated in Parker medium conditioned with exogenous TRH (TRH concentration—36 ug/100 ml medium). The medium was administered every 15 minutes and collected from the wells; in each case, 1 ml of medium was administered. The first 15 minutes served as blank and both halves of the pituitary remained in the same medium; the aim was to stabilize the secretory function of lactotropic cells. Prolactin measurements were made using RIA method. The tests carried out have demonstrated a stimulating impact of the TRH factor on secretion of prolactin. In the first 15 minutes of incubation, PRL concentration in the control group was 81.83 ± 11.4 pg/ml and was significantly ($P \leq 0.05$) lower than the concentration (87.48 ± 11.6 pg/ml) observed in the test group. After 30 minutes of incubation, the control group showed significantly ($P \leq 0.05$) lower prolactin level (74.04 ± 10.03 pg/ml) than the group with TRH-enriched medium (79.9 ± 10.6 pg/ml). After 45 minutes of incubation, the concentration of PRL in the control group was 59.66 ± 9.4 mg/ml and it was significantly ($P \leq 0.01$) lower than that in the experimental group (10.2 ± 65.47 mg/ml) (**Figure 6**).

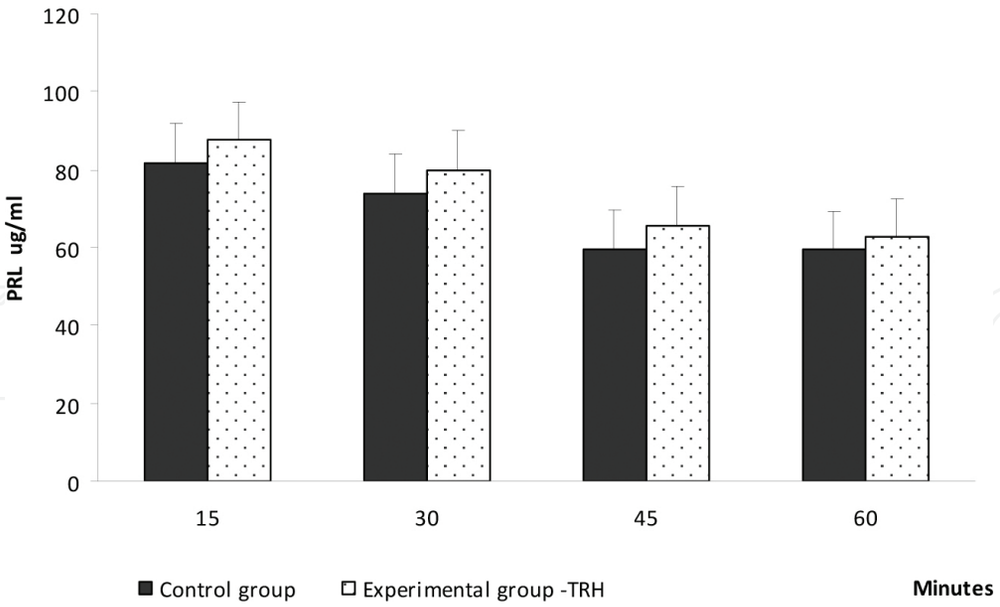

Figure 6. Mean concentrations of prolactin in control and TRH-treated pituitary explant cultures during long-day. See text for statistical comparisons.

3.3. Role of ghrelin

The process of initiation and maintenance of lactation in sheep requires the presence of a number of hormones. The aim of this study was to determine the role of ghrelin in the regulation of prolactin secretion in lactating sheep, based on the culture of in vitro pituitary. The study was conducted in May—long-day period. Pituitary was collected from 10 sheep on day 30 of lactation and divided along the longitudinal grooves so that each contains half of the glandular part and nerves. Incubations were carried out in vitro pituitary in 12 well plates for 1 hour at 37°C. The control group was incubated in a clean Parker medium and experimented in medium supplemented with exogenous ghrelin. The concentration of prolactin in the medium was determined by RIA method. The study showed stimulatory effect of ghrelin on the secretion of prolactin. The tests demonstrated a modulating effect of ghrelin on secretion of prolactin. Significant ($P \leq 0.05$) increase in prolactin secretion after 30 minutes of incubation in the test group (89.6 ± 18.1 mg/ml) compared with the control group (73.6 ± 17.4 mg/ml) was noted. After 45 minutes of incubation, the concentration (69 ± 15.2 mg/ml) of prolactin in the test group was significantly ($P \leq 0.05$) lower than the concentration (77.2 ± 17.6 mg ml) in the control group. After 60 minutes, prolactin level was significantly lower at $P \leq 0.05$ in the test group (46.3 ± 8.4 mg/ml) than that in the control group (51.8 ± 9.6 mg/ml) (**Figure 7**). The results of studies conducted have demonstrated a modulating impact of ghrelin on secretion of prolactin. While increase in prolactin secretion during the incubation period was observed, reduction in prolactin secretion has been recorded in the test group. Administration of exogenous ghrelin during the period of physiologically high prolactin concentration in lactating sheep has not given a clear answer as to whether ghrelin stimulates the secretion of prolactin. The results suggest, therefore, that ghrelin does not directly affect the secretion of prolactin from the pituitary. The hitherto obtained test results showed that the effects of ghrelin

may be dependent on the species of animals. In the case of sheep, seasonal breeders, the mechanism of ghrelin activity is complicated. As revealed by the studies in lactating sheep, administration of exogenous ghrelin modulates the secretion of prolactin.

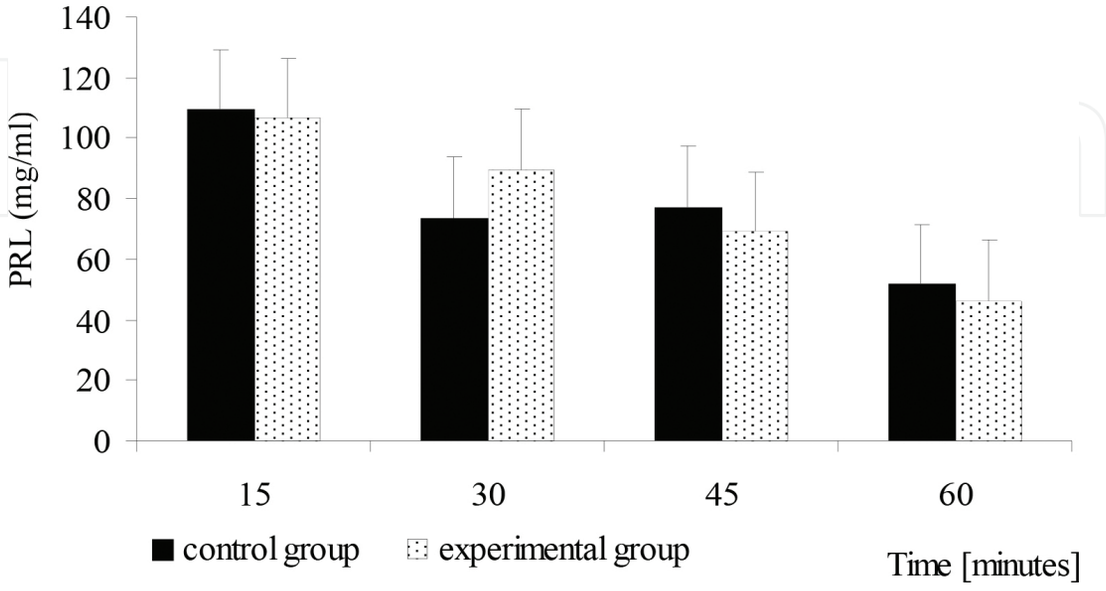

Figure 7. Mean concentrations of prolactin in control and ghrelin-treated pituitary explant cultures during long day. See text for statistical comparisons.

4. Summary

In seasonal animals, the process of triggering and maintaining lactation requires numerous hormones. The interaction of growth factors and other hormones is necessary in processes such as mammogenesis, lactogenesis and galactopoiesis. Due to the proper synchronization of pregnancy and changes in the area of the mammary gland, the gland is ready for the production of milk at the moment the offspring is born. Mammogenesis is a phenomenon that requires the participation of a number of hormones, including prolactin (PRL), growth hormone (GH), estrogens, progesterone, oxytocin, placental lactogen (PL) and insulin-like growth factor (somatomedin, e.g., IGF1). The coparticipation of IGF and GH is necessary in coordinating the differentiation and proliferation of epithelial cells. The manner in which the growth factors stimulate or inhibit the growth of cells or their influence on the cell cycle is not fully understood. The role of IGF in particular stages of functioning of the mammary gland (mammogenesis, lactogenesis, galactopoiesis and desiccation), particularly in the case of ruminants, is highly complicated. Recently, attention has been given to the metabolic hormones, particularly the role of leptin, orexin and ghrelin in mammogenesis, lactogenesis and galactopoiesis, respectively. Due to the recently increased interest in sheep's milk products, an understanding of the endocrine mechanisms facilitating the maintenance of lactation during autumn and winter may contribute to the improved profitability and usefulness of sheep's milk.

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