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The Effect of Physiological and Environmental Factors on the Prolactin Profile in Seasonally Breeding Animals

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1. Introduction

Light, being an environmental factor, has a significant effect on reproductive functions of animals exhibiting sensitivity to changes of the day length [1]. Among mammals there are many species displaying seasonality of reproduction, and given that, two models of seasonal sensitivity were distinguished. The first one refers to long-day animals (horses), in which reproductive processes are induced by lengthening days, i.e. in the spring. The other model concerns short-day animals, which include sheep, goats and deer; in these animals the reproduction system is stimulated and estrus takes place in the autumn and winter period [2]. In sheep the phenomenon of seasonality relates not only to reproduction but also to lactation. Following the process of mammogenesis in mammals, a mammary gland is developed, which is a complex cutaneous acinotubular gland [3]. The endocrine mechanism of entering and maintenance of lactation in sheep involves a number of hormones, which proves that the process relies basically on the activity of hypothalamus and pituitary gland [4,5,6]. One of the principal hormones conditioning both triggering and maintenance of lactation, synthesis of milk proteins, fat and immunoglobulins is prolactin (PRL), which is secreted chiefly by lactotroph cells of the anterior pituitary gland [7]. Prolactin is also produced locally by the mammary gland of mammals and does not differ immunologically from prolactin produced by the pituitary gland [8]. An important role in the process of mammogenesis and lactogenesis is assigned also to glucocorticoids, insulin and growth hormone and estrogens [9].

The fundamental feature of all living organisms is the ability to receive and process information about changes in the environment. Succession of physiological changes is synchronized with changes of environmental conditions and conditioned by the activity of

the biological clock [10,11]. It is confirmed via seasonal changes of the activity of the hypothalamic-pituitary axis in animals kept under permanent light conditions. Thanks to constant and cyclical factors physiological processes can be synchronized with a relevant season of the year. The synthesis of melatonin is a biochemical signal informing the organism about changing light conditions [12,13,14]. Numerous studies go to show the existence of a molecular mechanism involved in deciphering of the melatonin signal which is found in the SCN (Suprachiasmatic Nucleus) and in the PT (Pars Tuberalis). Both in the SCN and PT there are several dozens of genes of the biological clock such as *Per1*, *Per2* or *Cry 1*, *Cry2*, which are associated with each other [15,16]. The melatonin profile changing in a 24-hour cycle affects the rhythmical changes in the expression of the clock genes, which is reflected via their different amounts of mRNA in the PT and SCN. The maximum expression of the *Cry1* gene occurs during the dusk period parallel to the growth of the melatonin concentration, whereas the expression of the *Per1* gene is induced at dawn [17,18].

2. The effect of diverse photoperiod and exogenous melatonin on the secretion of prolactin under *in vivo* conditions

According to Misztal et al. (1999) [19] the modulating effect of melatonin on the secretion of prolactin can be exerted via two various mechanisms. The first one refers to the circadian rhythm and applies probably only to the prolactin stored in lactotroph cells of the pituitary gland. Tuberalin - a factor produced in the infundibular part of the gland probably triggers the expression of the PRL gene in lactotroph cells [20]. The daily secretion of prolactin is also controlled by the dopaminergic system because even the short-lasting growth of prolactin under the influence of melatonin is observed only in a situation when the activity of the dopaminergic system is weakened [21]. It must be stressed that the daily rhythm of prolactin displays a high seasonal variability; in the spring a higher concentration of the hormone is observed in morning and evening hours, and in the summer the daily secretion peak falls at night. In the autumn the rhythm has a two-phase character, like in the spring, whereas the concentration rises in morning and evening hours. In the winter, though, no specific release of prolactin is observed at all.

The other mechanism for regulation of the prolactin secretion is related to its circannual secretion rhythm, when melatonin, owing to its lipophilic, exerts a direct effect on lactotroph cells of the pituitary gland and, accordingly, on the secretion of prolactin [19,22,23]. Under natural conditions the maximum PRL concentration in the sheep's bloodstream is recorded in the long-day period; whereas at this time the melatonin level drops. The lowest level of prolactin is observed during short days, when the melatonin level is the highest [24,25]. Shortening of the day length or prolonged administration of melatonin in the period of physiologically increased concentration of prolactin leads to a reduced secretion of this hormone [1,13]. Seasonal changes in the prolactin secretion in the lactation period in sheep undoubtedly affect milk yields. Rhythmical changes of the level of prolactin and melatonin throughout the year were observed mainly in barren sheep and rams; however, studies carried out in the group of sheep used for dairy production confirmed the presence of the

seasonal rhythm of these two hormones. The experiments demonstrated a definite influence of the day length on the parameters of ewes' milk yields. Mothers entering lactation in the period of shortening days yielded 50% less milk as compared to ewes milked in the long-day period. The drop in milk yields in the shortening photoperiod resulted from the change in the prolactin secretion. The highest PRL concentration in sheep milked in the long-day period was identified in May 312.6 ± 45.2 ng/ml, at this time the concentration of melatonin amounted to 33.5 ± 11.2 ng/ml. As lactation progressed and days became shorter, the concentration of prolactin declined, and that of melatonin increased (table 1).

Months	May		June		July		August		September	
	\bar{x}	SE	\bar{x}	SE	\bar{x}	SE	\bar{x}	SE	\bar{x}	SE
Melatonin pg/ml	133.5	11.3	77.8	18.9	73.3	15.1	124.7	21.6	91.3	22.2
Prolactin ng/ml	312.6	45.2	185.7	34.7	247.0	48.9	151.6	33.9	43.9	10.1

Table 1. Changes in the concentration of melatonin and prolactin in sheep milked in the long-day period

As regards sheep lambled and milked in the short-day period and kept under the natural photoperiod conditions the highest level of prolactin was observed in August, i.e. 124.0 ± 48.8 ng/ml. In the first month of milking the concentration of prolactin in sheep corresponded to its seasonal rhythm and declined in subsequent months (table 2).

Months	August		September		October		November	
	\bar{x}	SE	\bar{x}	SE	\bar{x}	SE	\bar{x}	SE
Melatonin pg/ml	87.8	15.5	82.3	15.0	77.5	16.1	93.2	17.4
Prolactin ng/ml	124.6	48.8	60.5	11.1	30.8	9.7	16.8	4.1

Table 2. Changes in the concentration of melatonin and prolactin in sheep milked in the short-day period

As the light day shortened, the secretion of PRL declined as the level of the hormone in September was lower by 15% as compared to the concentration observed in August. A distinct drop in the prolactin level was observed in the period of the last two months of lactation, i.e. in October and November, and amounted respectively to 30.8 ± 9.7 ng/ml and 16.8 ± 4.1 ng/ml. The low concentration of prolactin already in the first month of milking and systematic growth of the melatonin secretion in the period of shortening days exerted an impact on the parameters of sheep milk yields, causing a drop of ewes' milk yields. In the

group of sheep kept under artificially simulated long-day conditions, 16 hours of light and 8 hours of darkness (16L:8D), in the period from August to November, the concentration of PRL already in the second month of milking, i.e. from September to November, decreased. In October and November a sharp drop in the concentration of prolactin was observed. The parameters of milk yields of sheep kept under conditions of artificially extended light day decreased proportionately to the drop in the concentration of prolactin (table 3).

Months	August		September		October		November	
	\bar{x}	SE	\bar{x}	SE	\bar{x}	SE	\bar{x}	SE
Melatonin pg/ml	60.4	19.8	17.6	7.6	4.4	1.1	17.0	5.5
Prolactin ng/ml	132.7	37.4	147.9	22.4	84.3	12.5	38.3	15.2

Table 3. Changes in the concentration of melatonin and prolactin in sheep kept under simulated long-day conditions -16L:8D

In the short-day period even in lactating sheep it is highly difficult to maintain an appropriately high concentration of prolactin. Studies carried out by Molik et al. (2007) [26] demonstrated that in sheep under artificially extended long-day conditions (16L:8D) it is impossible to maintain a high concentration of prolactin as well as to maintain lactation during shortening days.

Subsequent studies conducted on sheep milked in the long-day period showed that introduction of exogenous melatonin and initiation of artificial short-day conditions (16D:8L - 16 hours of darkness 8 hours of light) in the long-day period gave rise to reduction of the prolactin concentration (table 4).

Months	Concentration of prolactin ng/ml									
	May		June		July		August		September	
	\bar{x}	SE	\bar{x}	SE	\bar{x}	SE	\bar{x}	SE	\bar{x}	SE
Control group milked in the long day period	220.5 2	8.1	199.1	5.6	137.8	9.4	125.7	9.2	84.3	4.4
Sheep with melatonin implants	160.8	5.6	155.3	5.4	117.1	9.3	84.6	11.3	60.3	4.7
Sheep group under artificial short-day conditions 16D:8L	192.8	9.3	82.8	4.2	163	6.2	78.6	8.6	52.8	2.7

Table 4. Changes in the concentration of prolactin in sheep exposed to the effects of exogenous melatonin and simulated short-day conditions 16D:8L

In the first month of milking, in May, the highest concentration of prolactin was identified in sheep kept under natural day length conditions, with its level amounting to 220.52 ± 8.1 ng/ml. As lactation progressed and days shortened, the secretion of prolactin dropped and in the last two months it amounted respectively to 125.7 ± 9.21 ng/ml and 84.3 ± 4.4 ng/ml. Use of subcutaneous melatonin implants in ewes caused a drop in the concentration of prolactin. Already in the first month of milking, in May, the concentration of prolactin amounted to 160.8 ± 5.6 ng/ml. In subsequent 60 days of lactation (June) a further bigger decrease of the concentration of prolactin was recorded, reaching the level of 82.8 ± 4.2 ng/ml. As lactation progressed and melatonin implants started to exert their effects, a further drop in the secretion of prolactin was observed. In the group of sheep exposed to the effects of artificially simulated short-day conditions (16D:8L), the highest concentration of prolactin was identified in May, equaling 192.8 ± 9.3 ng/ml. In subsequent months of lactation keeping sheep under 16D:8L conditions caused a drop in the secretion of prolactin. In the fourth and fifth month of milking the secretion of prolactin decreased again, reaching in August the level of 78.97 ± 8.63 ng/ml, and in September 52.83 ± 2.73 ng/ml. The studies revealed that simulation of the long signal of melatonin in the spring and summer period contributes to disturbance of the endogenous rhythm of prolactin.

In studies carried out to date no differences have been identified as to the amount of milk obtained in the period of lambs rearing. In the early lactation period sucking is an important factor stimulating the PRL secretion in the mother's organism [27]. The sucking impulse induces release of serotonin [28] and oxytocin in the central nervous system (CNS) which give rise to the release of prolactin from the pituitary gland into the peripheral blood [29]. Experiments conducted on lactating rat females demonstrated that intravenous administration of specific oxytocin antagonist (desGly-NH₂-d(CH₂)₅[D-Tyr₂,Thr₄]OVT) completely inhibits the release of prolactin triggered by the sucking impulse [30]. Another important compound stimulating the release of prolactin induced by sucking is represented by salsolinol produced by the dopaminergic system in the lactation period. Infusion of exogenous salsolinol into the CNS in the group of lactating sheep gave rise to the release of prolactin into blood [31].

With that in mind, further experiments were carried out, which aimed at determining changes in the secretion of prolactin in sheep feeding lambs in the period of lengthening and shortening days, and verifying the hypothesis that melatonin can modify the secretion of prolactin despite strong stimulation of the mammary gland by sucking in the early lactation period [32]. Results of these studies demonstrated that administration of exogenous melatonin to ewes feeding lambs in the long-day period caused a significant drop in the concentration of prolactin. The identified changes in the PRL concentration in sheep entering lactation in the long-day period are comparable with the studies by Rhind et al. (1991) [33] which proved that in mothers rearing lambs in the period of lengthening days the concentration of prolactin rises. The studies conducted showed that despite intensive sucking the secretion of prolactin in sheep with melatonin implants dropped significantly. It must be stressed that the melatonin signal, acting as a marker of the biochemical biological clock, is evolutionarily so strong that the secretion of prolactin is reduced despite the stimulating sucking impulse.

Introduction of melatonin implants for sheep rearing lambs in the short-day period did not cause significant changes in the profile of the prolactin secretion. By analyzing the profile of the PRL secretion in both sheep groups, a conclusion can be drawn that in sheep lambled in November the concentration of prolactin in the first control sample drawn was lower by 50% as compared to the control sample drawn in March. In the long-day period the concentration of prolactin in the control group increased, and the secretion of melatonin decreased. In the autumn and winter period, though, as natural conditions set in, the concentration of melatonin increased and the level of prolactin dropped. The extending signal of melatonin observed in the autumn and winter period causes under natural conditions a decrease of the concentration of prolactin in sheep [34,35].

3. The effect of the day length and exogenous melatonin on the secretion of prolactin under *in vitro* conditions

Studies carried out on a group of lactating sheep under *in vivo* conditions showed modulating effects of melatonin with respect to the secretion of prolactin. The above mentioned relations demonstrate that melatonin can modulate the seasonal rhythm of the prolactin secretion affecting directly the pituitary gland. The highest concentration of melatonin receptors in the sheep's pituitary gland was identified in the pars tuberalis [36]. This structure is located precisely between the eminentia medialis of the hypothalamus and the distal part of the gland, which enables its mediation in the communication of the brain with the pituitary gland. In the sheep's PT only a MT1 melatonin receptor is found, and surgical separation of the pituitary gland from the hypothalamus does not have any influence on the concentration and location of this receptor and its sensitivity to binding melatonin [37]. Despite the fact that it was demonstrated that the activity of PT secretory cells is seasonal and melatonin-dependent, it was not confirmed that it is involved in the control of seasonal changes in the sexual activity.

The presence of melatonin receptor limited to the PT of the pituitary gland, and lack of direct effects of melatonin both on the prolactin gene expression and release of prolactin in cultures of lactotroph cells under *in vitro* conditions [38] suggests that melatonin regulates the seasonal secretion of prolactin by way of a specific compound synthesized in the PT. The presence of such a compound in the PT, with a peptide structure and stimulating secretion of prolactin from lactotroph cells of the pituitary gland, was confirmed in experiments, and the peptide itself was called tuberalin [36,39]. Own studies carried out under *in vitro* conditions demonstrated that both the day length and administration of exogenous melatonin affect the profile of the prolactin secretion. *In vitro* incubations of pituitary explants taken from sheep on 30th day of lactation were held in three periods: in the period of lengthening days (March), in the period of shortening days (August) and in the short-day period (November). The pituitaries taken were divided along the sulcus into two halves so that each one contained the adenohypophysis and the neurohypophysis. *In vitro* incubation was held during 3 hours in the Parker medium at a temperature of 37°C. The control group (G1) was incubated in a medium without any hormonal additives,

whereas the experimental group (G2) was incubated in a medium with exogenous melatonin. The experiments performed demonstrated that administration of exogenous melatonin in the long-day period caused a decrease of the secretion of prolactin in the pars tuberalis (table 5)

Group	Secretion of prolactin $\mu\text{g/ml}$					
	March		August		November	
	\bar{x}	SE	\bar{x}	SE	\bar{x}	SE
Parst tuberalis – control group (G1)	145.4	21.2	65.4	15.7	13.1	2.8
Parst tuberalis – with melatonin (G2)	105.0	11.4	45.4	9.2	13.7	2.2

Table 5. Effects of the day length and exogenous melatonin on the secretion of prolactin under in vitro conditions.

The concentration of prolactin in the period of lengthening days (March) in the control group was at the level of $145.45 \pm 21.2 \mu\text{g/ml}$, whereas in the group incubated with melatonin it was lower and amounted to $105.06 \pm 11.4 \mu\text{g/ml}$. In the period of shortening days (August), the concentration of prolactin in the medium with exogenous melatonin was lower, equaling $45.4 \pm 9.2 \mu\text{g/ml}$, as compared to the control group, being at the level of $65.4 \pm 15.7 \mu\text{g/ml}$. The lowest concentration of prolactin both in the control group and experimental one was recorded in the short-day period. The experiments conducted confirmed the seasonal rhythm of prolactin, because the highest concentration of this hormone was observed in the period of lengthening days ($145.45 \pm 21.2 \mu\text{g/ml}$). At the same time the lowest one was recorded in the short-day period ($13.1 \pm 2.8 \mu\text{g/ml}$). The experiments conducted under in vitro conditions showed that in the long-day period melatonin exerted a strong inhibitory effect on the secretion of prolactin. The studies confirmed also a direct influence of melatonin on cells of the sheep's pars tuberalis. It was demonstrated that the secretory activity of lactotroph cells of the pituitary gland under in vitro conditions is closely linked to the day length, and at the same time to the secretion of melatonin. It must be stressed at this point that the obtained seasonal distribution of prolactin concentrations in lactating sheep resembles a seasonal rhythm of the prolactin secretion in barren sheep [19]. However, it should be noted that in the group of lactating sheep the secretion of prolactin is much more intensive. Melatonin administered on the 30th day of lactation contributed to the reduction of the prolactin secretion [40].

4. The role of orexins and leptin in the regulation of the secretion of prolactin in sheep

The process of entering and maintenance of lactation in sheep involves a great number of hormones, with prolactin playing a key role. In recent years, though, a focus has been placed on orexins and their role in the secretion of prolactin in sheep. In 1998 a new group of

neuropeptides was identified. It was demonstrated that they exert a significant influence on food intake, and in this manner they were dubbed orexins (Greek *orexis* – appetite) [41].

Orexins derive from the same precursor – prepro-orexin, but they are a product of different posttranslational modifications. It is a protein built of 130 amino acid residues (mouse, rat) or 131 amino acid residues (human). In terms of the amino acid sequence a rat's prepro-orexin displays 83% homology with a human protein and 95% with a mouse's protein [41]. Two forms of the newly discovered compounds are distinguished: orexin A (OxA) and orexin B (OxB), which are molecules whose amino acid composition is similar to the gut hormone - secretin [42]. Orexin A and orexin B bind specifically and activate two GPCR receptors (G-protein coupled receptors), which – before discovery of the ligands - were called orphan receptors, and now they are known as orexin receptors – 1 and – 2 (Ox1R and Ox2R). Studies carried out in 2000 by Date et al. [43] confirmed that matrix RNA of Ox1R and Ox2R exhibits strong expression in the middle, anterior (adenohypophysis) and posterior (neurohypophysis) lobe of the rats' pituitary. At the same time it was noticed that in the anterior part of the pituitary gland Ox1R is subject to stronger expression than Ox2R. The observations go to show that the rat's pituitary is a gland capable of receiving the orexin signal.

Studies related to the synthesis of orexins and distribution of their receptors reveal that they are found in numerous brain sections and confirm the theory, according to which these compounds are regulatory proteins active in the central nervous system [41,44].

Experiments conducted on rats demonstrated that administration of exogenous orexin causes increased intake of food by animals [41]. OxA stimulates appetite in a dose-dependent manner and this effect prevails even for up to 4 hours after injection. The effect of OxB injection does not prevail as long as that of OxA; after two hours stimulation of food intake is low. The longer effects of OxA probably are attributable to the molecular structure, thanks to which it is more resistant than OxB to an attack of inactivating peptidases [41]. The experiments also displayed functions of orexins – other than the metabolic one. It was confirmed that orexins play a modulating role in the regulation of the hypothalamic-pituitary-thyroid axis [45], - adrenal axis [46] and – gonadal axis [47].

Results of *in vivo* studies conducted in 2000 by Russell et al. [48] showed for the first time that injection of orexin A reduced the level of prolactin in rats' blood. Moreover, it was concluded that the mechanism is not connected with the activity of TIDA neurons synthesizing dopamine (PRL inhibitor). In *in vitro* experiments it was proven that OxA exerts a direct influence on the secretory activity of explants of the hypothalamus and pituitary gland. Orexin A is also described as an inhibitor of the PRL secretion by lactotroph cells of the pituitary anterior lobe by Dusza and Ciereszko (2007) [49]. However, results of studies carried out on immature female rats under primary culture conditions are completely different as they reveal a statistically significant growth of the prolactin secretion depending on a dose and time of incubation of pituitary glands [50].

The orexin gene expression in ruminants was defined for the first time only in 2002 and it was shown that the location of the mRNA of prepro-orexin in the hypothalamus in sheep

corresponds with the published pattern for rodents. Results of the studies confirmed also that the orexin gene expression is sensitive to changes in the day length (higher during shorter days). However, no starving-induced changes were identified, which questions the key role of orexins in the regulation of appetite in sheep [51,52].

In the light of the presented results of the studies orexins can be perceived as a link integrating the processes of maintaining energy, hormonal and reproductive balance. Orexin A can be of special importance to animals exhibiting sensitivity to changes in the day length, which include sheep. The process of entering and maintenance of lactation in the animals involves a number of factors, whereas the key role is attributed to prolactin and growth hormone. Unfortunately, mechanisms regulating secretion of these compounds in sheep in the lactation period still remain unclear. It is certain, though, that defining functions of orexins - above all - in the regulation of the prolactin secretion will make it possible to understand the process of maintaining lactation in sheep, in particular in the short-day period. Thus, the purpose of the initiated studies was to determine the role of orexin A in the regulation of the prolactin secretion in sheep under different day-length conditions in *in vitro* experiments.

The experiments were performed on 15 sheep. Pituitary glands were taken from sheep on the 30th day of lactation in the long-day period (May n=5), in the period of shortening days (August n=5) and in the short-day period (December n=5). The pituitaries taken were divided along the sulcus into two halves so that each one contained the adenohypophysis and the neurohypophysis. *In vitro* incubation was held during 3 hours in the Parker medium at a temperature of 37°C. The control group (G1) was incubated in a pure medium, whereas the experimental group (G2) was incubated in a medium with exogenous orexin A. Injection of exogenous orexin caused an increase of prolactin secretion already in the first hour of incubation (451.6 ± 9.4 µg/ml) (table 6).

Group	Secretion of prolactin µg/ml					
	Hours of incubation					
	First hour		Second hour		Third hour	
	\bar{x}	SE	\bar{x}	SE	\bar{x}	SE
Parst tuberalis – control group (G1)	401.8	6.0	344.6	8.2	155.8	3.6
Parst tuberalis – with orexin A (G2)	451.6	9.4	346.5	7.0	196.3	4.7

Table 6. Effects of orexin on the secretion of prolactin in the long-day period

In the control group in the second hour of incubation the concentration of prolactin was lower (344.6 ± 8.2 µg/ml) than in the group with orexin (346.5 ± 7.0 µg/ml). But in the third hour of incubation the concentration of prolactin in the experimental group was higher by 40.5 µg/ml than in the control group. In general, the PRL concentration in the long-day period during three-hour incubation in the group with orexin A equaled (331 ± 15.2 ng/ml) and was higher as compared to the control group (300.0 ± 16.6 ng/ml). The experiments

demonstrated that irrespective of how the secretory capabilities of cells deteriorated in the course of incubation, exogenous orexin A stimulates the PRL secretion in the long-day period in lactating ewes (53).

Studies conducted in the period of shortening days (August) showed that administration of orexin A can modulate prolactin secretion (table 7).

Group	Secretion of prolactin $\mu\text{g/ml}$					
	Hours of incubation					
	First hour		Second hour		Third hour	
	\bar{x}	SE	\bar{x}	SE	\bar{x}	SE
Parst tuberalis – control group	38.05	12.1	32.1	9.6	19.2	7.3
Parst tuberalis – with orexin A	40.6	13.5	38.7	10.1	28.6	9.7

Table 7. Effects of orexin on the secretion of prolactin in the period of shortening days (August)

While analyzing changes in the prolactin secretion in sheep during 3-hour incubation, the highest secretory activity of cells was observed in the first two hours of the experiment. In the first hour of incubation the concentration of prolactin in the group incubated with orexin A equaled ($40.6 \pm 13.5 \mu\text{g/ml}$) and was higher as opposed to the control group ($38.05 \pm 12.1 \mu\text{g/ml}$). The concentration of prolactin in the second hour of the experiment was still higher than that in the control group and equaled respectively (38.7 ± 10.1 , $32.1 \pm 9.6 \mu\text{g/ml}$). In the third hour of the experiment it was demonstrated that in the experimental group the concentration of prolactin was higher ($28.6 \pm 9.7 \mu\text{g/ml}$) as compared to the control group ($19.2 \pm 7.3 \mu\text{g/ml}$).

In the period of shortening days, when the concentration of prolactin under natural conditions is reduced, administration of orexin A caused a growth of the prolactin secretion; similarly, injection of exogenous orexin A in the short-day period caused an increase of the prolactin secretion (table 8)

Group	Secretion of prolactin $\mu\text{g/ml}$					
	Hours of incubation					
	First hour		Second hour		Third hour	
	\bar{x}	SE	\bar{x}	SE	\bar{x}	SE
Parst tuberalis – control group	135.1	8.4	107.8	7.5	65.7	5.9
Parst tuberalis – with orexin A	158.9	9.2	164.8	7.5	79.9	7

Table 8. Effects of orexin on the secretion of prolactin in the short-day period (December)

In the first hour of incubation of the pituitaries in the short-day period it was observed that the prolactin secretion in the experimental group increased ($158.9 \pm 9.2 \text{ pg/ml}$) as contrasted with the control group ($135.1 \pm 8.4 \text{ pg/ml}$). In the second hour of incubation it was recorded that the concentration of prolactin in the experimental group grew ($164.8 \pm 7.5 \text{ pg/ml}$), whereas in the control group the concentration of prolactin amounted to ($107.8 \pm 4.0 \text{ pg/ml}$).

Results obtained in the third hour of incubation revealed that the concentration of prolactin in the control group equaled (65.7 ± 5.9 pg/ml), whereas in the group treated with exogenous orexin it amounted to (79.9 ± 7.0 pg/ml). Despite the fact that secretory capabilities of cells deteriorated in the course of incubation, the PRL concentration in the examined samples grew.

The experiments conducted under *in vitro* conditions confirmed that the annual rhythm of the PRL secretion in sheep is characterized by a higher concentration in the long-day period (summer) and lower concentration in the short-day period (winter).

Orexin exerts its effects on the PRL secretion in sheep, like in case of rodents, thanks to the presence of specific receptors Ox1R and Ox2R in the cytoplasmatic membrane of cells. Their existence in the sheep's adenohypophysis was described in 2002 by Xu et al. [54], who identified a high homology of the structure of a gene encoding sheep's Ox1R and a gene of the receptor in a rat (87%) and human being (89%). The presence of both of these forms of orexin receptors in the sheep's pituitary gland was also confirmed by Zhang et al. in 2004 [55].

Studies carried out to date with a view to explaining the relations between orexin and prolactin have been conducted mainly on rats. Thus, results of experiments on sheep in the period of lactation offer new information about the role of orexins in seasonally breeding animals. The conducted experiments proved that orexin A exerts a greater stimulating effect on the level of the PRL secretion in the summer season than in the winter season. The weaker response of sheep's pituitaries to orexin during short days as compared to the response during long days is a phenomenon of resistance of lactotroph cells to the orexin signal. Such a reaction of endocrine cells can be explained by the seasonal rhythm of biosynthesis and secretion of orexin in sheep, regulated by the photoperiod. It was proven that under conditions of shortening light days the expression of the orexin precursor gene in these ruminants is on a higher level than during long days [51]. As a result, an increased concentration of the mRNA of prepro-orexin in the winter season is observed, followed by an increased concentration of orexin in the organism. It is probable that at this time the saturation degree of orexin receptors of lactotroph cells with endogenous ligand is so high that adding exogenous orexin A does not cause such a distinct response as that occurring during long days. While attempting to identify interactions between the secretion of prolactin and growth hormone and the level of melatonin in lactating sheep an analogous phenomenon was noticed. Despite the fact that ewes lambed in June were kept under artificial long-day conditions, as the day became shorter, a drop in the PRL level was observed. In consequence, it was concluded that the secretion of the hormones retained its endogenous seasonal rhythm. The effect of the loss of the pituitary cells' sensitivity to the summer signal of the hormone of darkness repeating for too long does not allow for extension of sheep's lactation to the autumn and winter period [57].

In experiments conducted in 2006 it was proven for the first time that leptin exerts an effect on the melatonin secretion both under *in vitro* [57] and *in vivo* [58] conditions. It was observed that the day length (photoperiod) is one of the factors which modulate the effects

of leptin directly in the sheep's pineal gland, with an inhibitory effect of leptin on melatonin secreted by explants of the pineal gland in the period of lengthening days. However, during shortening days leptin stimulated the melatonin secretion by the gland explants [57]. Results of the *in vitro* experiments were confirmed in *in vivo* experiments, in which leptin was injected into the 3rd brain chamber. They demonstrated that exogenous leptin has an inhibitory effect on the concentration of melatonin during long days and a stimulating dose-dependent effect – during short days [58].

Seasonal insensitivity to leptin is observed in sheep in the spring and summer period, making it possible for the ruminants to make energy reserves, and despite increasing fatness intake of food in sheep is not reduced. Increased intake of food and a growth of the body weight during long days – LD (*Long Days*) spring - summer – is characterized by a high concentration of leptin in blood plasma, which “loses” then its anorectic features. In the short-day period – SD (*Short Days*) autumn - winter, when the availability of food dwindles, the sensitivity of centres regulating food intake in the hypothalamus to the concentration of leptin returns to normal. This paradox is explained by leptin resistance occurring during LD, whereas the neuroendocrinological basis of leptin resistance has not been fully understood yet. One of the phenomena underlying leptin resistance is auto-suppression of the transfer of signal from the receptor to the cell centre resulting from the leptin-induced expression of SOCS-3 factors, being inhibitors of cytokine sygnalisation [58]. The phenomenon is observed in particular in the area of ventromedial hyopothalamus [58], where long-form receptors of leptin Ob-Rb are found in the highest concentration.

Orexigenic and anorexigenic systems are linked in terms of morphology and functionality. It confirms the hypothesis about the existence of a nervous network regulating hunger, located in nervous centres of the hypothalamus. The area of receptors and nerve endings for orexigenic factors overlaps with the area of receptors for anorexigenic factors, which also reveals mutual interactions of the two systems. In animals with strong seasonal breeding characteristics the network of the above described relations overlaps additionally with effects of the photoperiod, which by way of a biological signal in the form of melatonin interferes with the developed relations. The suprachiasmatic nucleus (SCN), which is a part of the biological clock, generates signals adjusting food intake to the circadian rhythm. This element is considerably weakened in the primates, and in particular in humans, but it is very efficient in ruminants. The objective of the conducted experiments was to investigate interactions between the day length and leptin and orexin B in sheep under *in vivo* conditions.

The experiments were carried out on 24 sheep of the Polish sheep breed. The first stage of experiments (n=12) was conducted in the period of lengthening days (spring – summer). The second stage (n=12) was held under conditions of shortening days (autumn – winter). Three weeks prior to the initiation of the planned experiments metal stainless cannulas were inserted surgically into the third brain chamber of ewes (using the stereotactic method by Traczyk and Przekop [1963] [59]). The tests were started at the sunset, and were continued during subsequent 6 hours. The experiments were performed so that each ewe received in a

2-week interval intraventricular infusion of Ringer-Locke's fluid – control, recombinant sheep leptin (roleptin) – in a dose of 0.5 µg/kg body weight (Leptin 1), and roleptin in a dose of 1.0 µg/kg body weight (Leptin 2). On the day of the experiment animals were put in individual cattle crushes, the Ringer-Locke's fluid (control) and roleptin were injected intraventricularly for the first time directly after drawing time-zero blood samples and in the 60th and 120th minute of the 6-hour experiment. During the experiment blood samples were drawn every 15 minutes. Blood was poured into test tubes containing 100 µl of heparin solution (1000 IU/ml).

During lengthening days the total PRL concentration in the plasma of control sheep was significantly higher ($P < 0.001$) than during shortening days (132.28 ± 19.87 vs. 44.41 ± 8.27 ng/ml). Intraventricular infusions of two doses of exogenous leptin reduced the concentration of prolactin during SD ($P < 0.001$) as compared to the values observed in control ewes. It was concluded that the day length is one of the factors which modulate the effects of leptin on the prolactin secretion in sheep. It was observed that central infusions of leptin into the 3rd brain chamber significantly decreased the concentration of prolactin in sheep during short days in a dose-dependent manner; whereas the effect was opposite during a long photoperiod.

In subsequent experiments a test of the hypothesis about season-dependent effects of leptin and orexin B on the endocrine activity of sheep was performed. Six weeks prior to the start of the experiments ewes were subjected to an ovariectomy procedure, and subcutaneous estradiol implants were inserted into each of them. Three weeks prior to the initiation of the planned experiments metal stainless cannulas were inserted surgically into the third brain chamber of ewes (using the stereotactic method by Traczyk and Przekop [1963] (59). Like in the previous experiment, the tests were started at the sunset, and were continued during subsequent 6 hours. The experiments were performed so that each ewe received in one-week interval intraventricular infusion: 1. Control group (CG) - Ringer-Locke's fluid (pH=7.4); 2. Experimental group 1 (Gr 1) – recombinant sheep leptin (PLR Laboratory, Israel) in a dose of 0.5 µg/kg, the dose was selected based on experiments [58]; 3. Experimental group 3 (Gr 3) – orexins B (PolyPeptides Laboratories, Strasbourg, France) in a dose of 0,3 µg/kg., 4. Experimental group 5 (Gr 5) - superantagonists of leptin (D23L/L39A/D40A/F41A; PLR Laboratory Israel) in a dose of 50 µg/kg, and then orexin B in a dose used previously. In the control group and experimental group 1, 2, and 3 infusions of selected factors were performed three times, every 60 minutes from the start of the experiment, in group 5 the leptin antagonist was infused twice, in the zero and 1st hour of the experiment, and ghrelin/orexin B was infused 15 and 60 minutes after administration of leptin antagonist. During the 6-hour experiment blood samples were drawn every 15 minutes. The average concentration of melatonin in the plasma of ewes from the control group was higher ($P < 0.001$) during LD as compared to the values recorded in animals during short days (87.28 ± 1.2 vs. 59.70 ± 3.1 pg/ml). In the seasons of lengthening days exogenous recombinant sheep leptin significantly reduced the concentration of melatonin ($P < 0.001$) in relation to the values observed in ewes from the control group, whereas during a short day it significantly increased ($P < 0.01$) the concentration of melatonin. Orexin B did not have any impact on the

concentration of melatonin during a short day, but it caused a significant growth ($P < 0.05$) of the concentration of melatonin on a long day.

During a lengthening day the total PRL concentration in the plasma of sheep from the control group was markedly higher ($P < 0.001$) as contrasted with shortening days (132.28 ± 19.87 vs. 44.41 ± 8.27 ng/ml). Intraventricular infusions of exogenous leptin reduced the concentration of prolactin during SD ($P < 0.001$) as compared to the values observed in ewes from the control group. The average concentration of endogenous orexin was higher ($P < 0.01$) during short days (0.59 ± 0.05 ng/ml) as opposed to LD (0.39 ± 0.01 ng/ml) in the control group of sheep. Exogenous orexin caused a growth ($P < 0.05$) of the plasma concentration of endogenous orexin respectively on a long and short day (0.62 ± 0.01 ng/ml and 0.71 ± 0.03 ng/ml), whereas the effects of leptin reduced ($P < 0.05$) the concentration of orexin during LD and SD. Based on the conducted experiments it was concluded that orexin B and anorectic hormone – leptin directly interact closely with each other, regulating not only the processes of metabolism but influencing jointly release of melatonin and prolactin, and the interactions additionally depend on the prevalent photoperiod.

5. Summary

So far it has been believed that milk yields in mammals are determined by genetic and environmental factors. In recent years, though, a special focus has been placed on light, being the modulator of the prolactin level. In farm animals changes of the light day play a very important role as they determine their yields. The length of the light day, and in particular the melatonin profile, is of special importance in sheep as they determine reproductive processes, in which lactation is the last stage of reproductive physiology. Experiments carried out on sheep demonstrated that both the melatonin profile and prolactin profile retain features of a seasonal rhythm depending on the day length. The synthesis of melatonin by the pineal gland is a biochemical signal informing the organism about the break of the day or night. This hormone regulates activities of numerous organs. Until now the activity of melatonin has been associated with the impact on the reproductive system. Experiments carried out in recent years have shown that melatonin can modulate the level of prolactin. Under natural conditions the maximum concentration of prolactin in sheep blood is observed in the long-day period, and at this time the level of melatonin drops. The lowest concentration of prolactin is recorded during short days, when the level of melatonin is the highest. Shortening of the day length or long-term injection of melatonin in the period of physiologically increased concentration of prolactin leads to a reduced secretion of this hormone. Lactation in sheep involves a number of hormones, and for that reason in recent years a special focus has been placed on the role of orexins in the regulation of the prolactin secretion. Experiments conducted demonstrated that orexin A exerts a greater stimulating effect on the level of the PRL secretion in the summer season than in the winter season. The weaker reaction of sheep's pituitaries to orexin during short days as compared to the response during long days is a phenomenon of resistance of lactotroph cells to the orexin signal. Such a reaction of endocrine cells can be explained by the seasonal rhythm of biosynthesis and secretion of orexin in sheep, regulated by the photoperiod.

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