Seasonal changes in the interactions among leptin, ghrelin, and orexin in sheep

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ABSTRACT: The adaptation of the physiology of an animal to changing conditions of light and food availability is evident at the behavioral and hormonal levels. Melatonin, leptin, ghrelin, and orexin, which exhibit rhythmic secretion profiles under ad libitum feeding conditions, are sensitive to changes in daylength, forming a tight web of interrelationships in the regulation of energy balance. The aim of this study was to determine the effects of central injections of leptin, ghrelin, and orexin on the reciprocal interactions among these hormones and the influence of photoperiod on these responses. Twenty-four ovariectomized and estradiol-implanted ewes were used in a replicated switchback design. The ewes were assigned randomly to 1 of 6 treatment groups, and the treatments were infused into their third ventricles 3 times at 0, 1, and 2 h, with 0 h being at dusk. The treatments were as follows: 1) control, Ringer-Locke buffer; 2) leptin, 0.5 μg/kg BW; 3) ghrelin, 2.5 μg/kg BW; 4) orexin B, 0.3 μg/kg BW; 5) leptin antagonist, 50 μg/kg BW, then ghrelin, 2.5 μg/kg BW; and 6) leptin antagonist, 50 μg/kg BW, then orexin B, 0.3 μg/kg BW. Blood samples (5 mL) were collected at 15-min intervals for 6 h. The administration of leptin increased (P < 0.05) plasma concentrations of melatonin during short-day (ShD) photoperiods and decreased (P < 0.05) them during long-day (LD) photoperiods, whereas ghrelin decreased (P < 0.05) melatonin concentrations during ShD photoperiod, and orexin had no effect (P > 0.1). Leptin attenuated (P < 0.05) ghrelin concentrations relative to the concentration in controls during ShD. The plasma concentrations of orexin were reduced (P < 0.05) after leptin infusions during LD and ShD photoperiods; however, ghrelin had the opposite effect (P < 0.05) on orexin concentration. Orexin increased (P < 0.05) ghrelin concentrations during LD. Ghrelin and orexin concentrations were increased (P < 0.05) after leptin antagonist infusions. Our data provide evidence that the secretion of leptin, ghrelin, and orexin are seasonally dependent, with relationships that are subject to photoperiodic regulation, and that leptin is an important factor that regulates ghrelin and orexin releases in sheep.

Key words: ghrelin, leptin, leptin antagonist, orexin, sheep

INTRODUCTION

Hormones involved in the maintenance of energy homeostasis, such as leptin, ghrelin, and orexin, are characterized by daily and annual concentration changes in the bloodstream. The seasonal pattern of circulating leptin concentrations reflects adiposity and matches the seasonal pattern of appetite drive (Marie et al., 2001). Harrison et al. (2008) demonstrated that photoperiod influences the effects of ghrelin on appetite and reproductive neuroendocrine axes. Interestingly, there is evidence indicating that some of the in vitro effects of ghrelin are affected by photoperiod (Zieba et al., 2011). Based on these data, it has been hypothesized that leptin and ghrelin act jointly to regulate energy homeostasis in response to daylength. Furthermore, orexins have attracted considerable attention in recent years. In sheep, orexin gene expression varies depending on the length...
of day: gene expression is greater during short-day (ShD) photoperiods than during long-day (LD) photoperiods (Archer et al., 2002). Moreover, prepro-orexin mRNA and orexin immunoreactivity display diurnal variations in the hypothalamic area, which led to the hypothesis that this hypothalamic peptide is involved in the daily rhythm of melatonin synthesis (Archer et al., 2002). Considering the seasonal changes in the concentrations of leptin, ghrelin, and orexins, the present experiment tested the hypothesis that the interactions among leptin, ghrelin, and orexin B are intimately related to the time of year. To test this hypothesis, we used a specific leptin antagonist (L39A/D40A/F41A) to investigate the effects of leptin on the secretion of ghrelin and orexin B proteins involved in the regulation of metabolism, reproduction, and feed intake. Furthermore, we used sheep as a model to determine whether the aforementioned relationships are seasonally influenced.

**MATERIALS AND METHODS**

All animal-related procedures were approved by the Local Agricultural Animal Care and Use Committee of Krakow.

**Animal Model**

Studies were performed at the Experiment Station in the Department of Swine and Small Ruminant Breeding, Agricultural University of Krakow (longitude: 19° 57´ E, latitude: 50° 04´ N). This study included 24 female Polish Longwool sheep, which is a breed that exhibits strong reproductive seasonality. The animals were 2 to 3 yr of age, weighed 60 ± 5 kg, and were housed in individual pens under natural photoperiodic and thermoperiodic conditions. The sheep were in moderate body condition (BCS = 3 on a scale of 1 to 5, following Russel et al., 1969) and were fed a diet formulated to provide 100% of the National Research Institute of Animal Production recommendations for maintenance. Sheep were fed twice daily at 0700 and 1400 h (Norms, 1993). Water was available ad libitum.

In the current experiment, estradiol-implanted and ovariectomized ewes were used, as in our earlier studies on the effects of leptin (Zieba et al., 2003, 2004). This sheep model is a widely accepted neuroendocrine model commonly used to avoid the confounding effects of the ovarian cycle on hormonal interactions. It is used to provide a model with constant physiological concentrations of gonadal steroid feedback.

**Procedures and Treatments**

The mature ovariectomized female sheep, each harboring an estradiol implant to maintain circulating concentrations of estradiol at 2 to 4 pg/mL, were fasted for 24 h and fitted surgically with intracerebroventricular (ICV) cannulae according to the methodology of Traczyk and Przekop (1963). The location and function of the cannulae were verified by the continuous flow of cerebrospinal fluid. A period of at least 3 wk was allowed for the sheep to recover from the neurosurgery.

Before the study began, the ewes were frequently placed into individual sampling carts to familiarize them with the experimental conditions. Carts were constructed of wood with solid floors, and allowed animals to stand or lie down freely during sampling procedures. All of the experiments began at dusk. In Poland, during LD photoperiod (May), the length of the night lasts approximately 9 h and during ShD photoperiod (November), the length of the night lasts approximately 14 h.

In the morning on the day of each experiment, 4 randomly chosen sheep were fitted with jugular catheters for intensive blood sampling. Polyethylene tubing (70 mm; 0.58-mm i.d., 0.96-mm o.d.; Intramedic Clay Adams Brand, Becton Dickinson, Sparks, MD) was inserted using an aseptic technique through each ICV-guided cannula so that the distal end projected 3 to 5 mm past the end of the cannula and into the ventricle. The proximal end of the tubing extended approximately 5 to 10 mm beyond the tip of the guide cannula. The tubing was adjusted until the cerebrospinal fluid flowed easily using a blunt 22-gauge needle and a tuberculin syringe. The tubing was plugged until later use.

In the afternoon on the day of each experiment, ewes were assigned to 1 of 6 groups (n = 4/group). The animals were placed into the carts, and the flow of cerebrospinal fluid was confirmed. Recombinant ovine leptin (roleptin) and ovine pegylated leptin antagonist (mutant D23L/L39A/D40A/F41A) were purchased from PLR Laboratory (Rehovot, Israel). Ovine ghrelin and rat orexin B were purchased from PolyPeptide Laboratories (Strasbourg, France). The treatment groups consisted of 1) Control: Ringer-Locke buffer, pH 7.4; 2) Lept: roleptin (0.5 μg/kg BW); 3) GHRL: ghrelin (2.5 μg/kg BW); 4) ORX: orexin B (0.3 μg/kg BW); 5) LAG: leptin antagonist (50 μg/kg BW) and GHRL at the same dose as above; and 6) LAO: leptin antagonist (50 μg/kg BW) and ORX at the same dose as above. The Ringer-Locke buffer, roleptin, ghrelin, and orexin were infused centrally into the third ventricle at 0, 1, and 2 h of the 6-h experiment (3 single infusions, Figure 1A). In groups 5 and 6, the leptin antagonist was centrally infused twice at 0 and 1 h of the experiment, and the ghrelin or orexin was infused at 15 and 60 min after the leptin antagonist (Figure 1B). The treatments for each group were replicated twice in 4 randomly chosen ewes approximately 1 wk apart (n = 8/treatment). The appropriate dose of leptin for the ICV study was...
determined in our previous experiments (Zieba et al., 2008), and the doses of ghrelin or orexin and the leptin antagonist were based on both theoretical calculations and published studies of ICV injections in sheep (Sartin et al., 2001; Harrison et al., 2008, Shpilman et al., 2011). Blood samples (5 mL) were collected at 15-min intervals for 6 h beginning immediately before the first infusion at dusk and were continued under red light.

The blood samples were dispensed into tubes containing 150 μL of a solution of heparin (10,000 IU/mL) and 5% (wt/vol) EDTA and were immediately placed on ice. The plasma was separated by centrifugation at 3000 × g at 4°C for 10 min and stored at −20°C until the estradiol, melatonin, ghrelin, and orexin analyses.

**Hormone Assays**

Melatonin was assayed in unextracted plasma according to the method of Fraser et al. (1983) and modified by Misztal et al. (1993), as described previously (Zieba et al., 2007, 2011). The sensitivity of the assay was 16.8 ± 8.0 pg/mL, and the intra- and inter-assay CV were 9.6% and 11.1%, respectively.

The concentrations of estradiol were determined by enzyme immunoassay using commercially available kits (DRG Instruments GmbH, Marburg, Germany) according to the manufacturer’s instructions. All of the samples were assayed in duplicate in the same assay. An intra-assay CV was 6.7%. The sensitivity of the assay was 1.71 pg/mL.

The circulating concentrations of ghrelin in the extracted samples (using the C18 Sep-Column extraction method) were determined using an enzyme immunoassay kit (human, EIA-3706, Phoenix Pharmaceuticals, Inc., Ghrelin Phoenix Europe GmbH, Karlsruhe, Germany). This kit detects acyl and des-acyl ghrelin, and the manufacturers claim 100% cross-reactivity for human, rat, and mouse ghrelin. The intra-assay and inter-assay CV were 8.9% and 4.3%, respectively, and the assay sensitivity was 0.09 ng/mL.

The circulating concentrations of orexin B in the extracted samples (using the C18 Sep-Column extraction method) were determined using an enzyme immunoassay kit for orexin B (human, EIA S-1147, Peninsula Laboratories, LLC, San Carlos, CA). The manufacturers claim 100% cross-reactivity for human, rat, and mouse orexin B. The intra- and inter-assay CV were 7.1% and 5.3%, respectively, and the sensitivity of the orexin assay was 0.2 ng/mL.

**Statistical Analysis**

The hormone data were analyzed using the general linear models procedure (**PROC GLM**; SAS Inst. Inc., Cary, NC). For the hormone comparisons, the overall ANOVA model included treatment, season, and replicates within seasons, time within seasons, and all 2- and 3-way interactions for repeated measures in a switchback design. Significant treatment × season interactions resulted in a within-season model that included treatment, time, and treatment × time. After a significant F-test result, the SAS Pdiff procedure was used for means comparison. After the determination of a significant F-value, the means were compared using a Duncan’s multiple-range test. Differences with \( P < 0.05 \) were considered statistically significant. Data are expressed as means ± SEM (means represent concentrations of each hormone from samples harvested every 15 min during a 6-h experiment from a particular experimental group analyzed separately for ShD and LD photoperiods).

**RESULTS**

**Plasma Concentrations of Estradiol and Melatonin**

The mean circulating concentration of estradiol in sheep plasma was 4.3 ± 0.2 pg/mL. In the absence of hormonal treatments (Control group), the mean circulating concentration of melatonin was greater (\( P < 0.001 \)) during ShD than LD photoperiods (87.3 ± 1.2 pg/mL vs. 59.7 ± 3.1 pg/mL, respectively). The ICV treatment with roleptin increased (\( P < 0.05 \)) the mean concentration of circulating melatonin relative to the concentration in the controls during ShD to 105.6 ± 7.1 pg/mL (Figure 2). The concentration of melatonin after roleptin treatment was greater (\( P < 0.01 \)) during ShD compared with LD photoperiod. Exogenous ovine ghrelin reduced (\( P < 0.05 \)) the mean melatonin concentra-
tion relative to the concentration in the controls during ShD photoperiod. Orexin had no effect ($P < 0.07$) on the melatonin concentration during ShD compared with Control group and increased ($P < 0.05$) its concentration during LD relative to the concentration in the controls (Figure 2). No difference ($P < 0.07$) was observed between melatonin concentrations after orexin treatment between LD and ShD (Figure 2).

**Plasma Concentration of Ghrelin**

Exogenous ghrelin treatment increased ($P < 0.05$) the circulating ghrelin concentration relative to Controls during both ShD (3.0 ± 0.1 vs. 4.4 ± 0.3 ng/mL in the control) and LD photoperiods (2.5 ± 0.2 ng/mL vs. 4.0 ± 0.1 ng/mL; Figure 3). Leptin decreased ($P < 0.05$) ghrelin concentration during ShD, whereas ghrelin concentration after the orexin treatment was greater ($P < 0.01$) than in the Controls during LD (3.05 ± 0.04 ng/mL vs. 2.49 ± 0.09 ng/mL). Orexin had no effect ($P > 0.1$) on ghrelin during ShD (Figure 3). The leptin antagonist increased ($P < 0.05$) ghrelin concentration during both ShD (3.52 ± 0.01 ng/mL) and LD (3.4 ± 0.03 ng/mL) photoperiods (Figure 3, 4A, and 4B).

**Plasma Concentration of Orexin**

Mean orexin concentration was greater ($P < 0.01$) during ShD (0.59 ± 0.05 ng/mL) than during LD (0.39 ± 0.01 ng/mL) in the Control group. Exogenous orexin treatment increased ($P < 0.05$) plasma orexin concentrations (0.62 ± 0.01 ng/mL and 0.71 ± 0.03 ng/mL during LD and ShD, respectively; Figure 5), whereas treatment with leptin decreased ($P < 0.05$) circulating orexin concentration during the LD and ShD photoperiods (Figure 5). Orexin concentrations were greater ($P < 0.05$) in Lept, GHR, and LAG groups during ShD compared with LD photoperiods. Ghrelin increased ($P < 0.01$) orexin concentrations during LD (0.49 ± 0.01 ng/mL) relative to concentrations in Controls (Figure 5). This same effect was noted for leptin antagonist, which increased ($P < 0.05$) the orexin concentrations to 0.68 ± 0.01 ng/mL and 0.79 ± 0.02 ng/mL for the LD (Figure 6A) and ShD (Figure 6B) photoperiods, respectively compared with Controls.

**DISCUSSION**

Studies in explants of ovine pineal glands performed by our group (Zieba et al., 2011) have demonstrated a dose-dependent orexin B stimulatory effect on melatonin release, mainly during a LD photoperiod, with a lesser dose having a stronger effect. Therefore, it is possible that orexins modulate feed intake and energy metabolism via an interaction with melatonin or leptin. There are data indicating that circadian rhythms and the expression of clock genes are modified by diet (Mendoza, 2007). Given this evidence, Cassoni et al. (2004) concluded that the secretion of appetite-regulating peptides was closely connected to the circadian rhythm and metabolic processes. The current study demonstrates that photoperiod is able to influence the secretion of leptin, ghrelin, and orexin in seasonally breeding sheep. Furthermore, the experiments using a leptin antagonist revealed an important role of leptin in these relationships.

Interesting links also have been found between ghrelin and orexins. In rats, the ICV injection of ghrelin indicated anatomical and functional synaptic connections between neurons that secrete orexins and neurons that secrete ghrelin in the region of the lateral hypothalamus (Toshinai et al., 2003). Furthermore, ghrelin injections can induce the immediate expression
of c-Fos protein, which is a marker of neuronal activity in neurons that synthesize orexin (Pirnik et al., 2008). In sheep, it was demonstrated by Qi et al. (2010) that leptin treatment reduced the percentage of orexin Fos-responsive neurons. Our previous study indicated that photoperiod affected the expression and concentrations of leptin in sheep (Zieba et al., 2007, 2008). In this ruminant species, the hypothalamus is resistant to leptin during certain periods, and this phenomenon is related to the adaptation of sheep to the annual changes in energy supply and demand (Marie et al., 2001).

During LD photoperiod, when there is an abundance of feed that is readily accessible, sheep exhibit an increased appetite and appear to be insensitive to high concentrations of leptin, which results from an increase in adiposity. Seasonal leptin resistance allows these animals to live in a changing climate and to store energy for use during periods of reduced feed availability. In the autumn and winter, sheep exhibit sensitivity to leptin at physiological concentrations, and their appetite adjusts to approximate their nutritional status. This paradox can be explained by the state of leptin resistance or leptin insensitivity that occurs during the LD season, and a greater suppressor of cytokine signal-3 (SOCS-3) protein expression in the hypothalamic nuclei seems to be partially responsible (Adam et al., 2008; Zieba et al., 2008). The same phenomenon of leptin resistance is observed in the state of human obesity. Myers et al. (2008) demonstrated that pathologic leptin resistance may be mediated by changes in second messengers including the long form of leptin receptor, downstream signal transducer and activator of transcription-3 (STAT3), or the feedback of SOCS-3.

Orexin and ghrelin, which are orexigenic hormones, affected the in vitro melatonin secretion from pineal gland explants, and those effects were found to be dependent on both the photoperiod and leptin (Zieba et al., 2011). Because orexigenic and anorectic peptides are a component of a large feeding-related central circuitry, it is reasonable to presume that these peptides interact, for example, by affecting release of each other or acting on the same target cells. Therefore, changes in feed intake behavior in sheep can be attributed to a dynamic balance among the peptides within brain networks and the connections to photoperiod-driven changes.

Melatonin itself controls the impact of feed consumption on the activity of peripheral clocks. Anukulkitch et al. (2011) suggested that changes in the expression of a few neuropeptides engaged in energy and appetite control in sheep are not explained by the changes in the expression of the long-form variant of the leptin receptor in the hypothalamus, and are more likely due to the central effects of the photoperiod acting through melatonin to influence the expression of genes such as neuropeptide Y (NPY) and proopiomelanocortin (POMC). Some of the central effects of orexin and leptin are mediated by NPY and POMC (Lopez et al., 2010). Furthermore, Anukulkitch et al. (2010) demonstrated in lean and fat sheep that photoperiod acts as an overriding factor that is able to affect orexin gene expression. The influence of photoperiod on orexin B concentrations was demonstrated in the present study, with the greater orexin concentrations noted in sheep during ShD photoperiod compared with LD photoperiod in all experimental groups. Orexins A and B control wakefulness, feeding, and energy homeostasis (i.e., functions indispensable for survival) via interaction with many types of neurons in the brain and modulate their activities through the activation of orexin-1 receptor or orexin-2 receptor, respectively (De Lecea et al., 1998). In addition, a new functional role of orexin B is emerging in the regulation of insulin and leptin sensitivities responsible for whole-body glucose metabolism (Funato et al., 2009).

Recent evidence indicates that orexin B efficiently protects against the development of peripheral insulin resistance induced by aging or high-fat feeding in mice. In particular, the orexin receptor-2 signaling appears to

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**Figure 4.** Temporal patterns of circulating ghrelin in 15-min samples (5 mL) collected after control or roleptin- (Lept), ghrelin- (GHRL), orexin- (ORX), and leptin antagonist and ghrelin- (LAG) treated ewes (n = 8/treatment) during long-day (LD; panel A) and short-day (ShD; panel B), photoperiods.
confer resistance to diet-induced obesity and insulin insensitivity by improving leptin sensitivity (Funato et al., 2009). Studies in rats and humans indicated that orexin can pass through the blood-brain barrier and is present in the peripheral circulation (Adam et al., 2002; Sun et al., 2006). Therefore, orexin B and its receptor controlling hypothalamic leptin action, together with the information that orexin receptor is expressed in adipose tissue (Digby et al., 2006), provide evidence that a deep cooperation exist between orexin and leptin concerning feed intake and energy metabolism.

The expression of orexin varies depending on daylength as concentrations are greater during ShD than during LD in sheep (Archer et al., 2002). Some seasonal fluctuations in the orexin concentration were also noted in the present study. Changes in the orexin concentration are unlikely to be the result of reproductive activation, which starts in sheep when daylength becomes shorter. Mikkelsen et al. (2001) demonstrated that orexin (mainly B) decreases melatonin release and reduces the activation of N-acetyltransferase, which is a key enzyme involved in the synthesis of pineal hormone through the activation of its receptors in the pineal gland.

In the present experiment, during the LD condition, orexin increased melatonin concentration, which was also noted during our previous in vitro experiments (Zieba et al., 2011). Furthermore, leptin antagonist treatment during LD blocked the effect of leptin, and increased circulating orexin concentrations were observed during this time, with amounts of orexin that were greater than the concentrations observed in the untreated control animals. Funato et al. (2009) indicated that leptin signaling mediates some of the metabolic effects of orexin. The mechanism by which orexin and leptin signals interact remains unclear. Neurons expressing both leptin and orexin-2 receptor may have convergent intracellular second messenger signaling including extracellular factor-regulated kinase (ERK) and the Janus kinase JAK2/STAT3 pathways (Myers et al., 2008). In the hypothalamic arcuate nucleus (ARC), leptin-responsive neurons such as those expressing NPY are directly excited by orexin, whereas POMC neurons are directly inhibited by orexin (Muroya et al., 2004). But, inhibitory GABAergic interneurons in the ARC may also be activated via postsynaptic orexin-2 receptor (Burdakov et al., 2003), predicting complexity in up- or downregulation of these circuits.

In analyzing the interactions between leptin and ghrelin, opposite effects were observed in the present experiment. Specifically, during ShD photoperiod, centrally infused leptin decreased the ghrelin concentration, which was likely due to the anorectic properties of leptin. This effect contrasts with the stimulatory action of ghrelin on food intake (Kirsz and Zieba, 2011). Ghrelin, which is the endogenous ligand for the GH secretagogue receptor (GHS-R), induces its metabolic effects via a multitude of central and peripheral mechanisms. Ghrelin increases feed intake and adiposity through the stimulation of orexigenic peptides. For example, an increased orexin concentration in the plasma was seen in the present study during LD photoperiod, when the feed intake of a sheep is usually increased. Ghrelin acts primarily in the hypothalamic ARC, which contains neurons with receptors for most, if not all, of the hormones associated with energy balance, including leptin and orexin receptors. Furthermore, the presence of ghrelin receptors in the suprachiasmatic
nucleus (SCN) suggests a possible role for this hormone in melatonin secretion.

Exogenous ghrelin administration to the SCN shifts the phases of the circadian rhythm, indicating that ghrelin participates directly in the central regulation of the periodicity of sleep and nutritional processes (Li et al., 2002). Ghrelin also changes the rhythmicity of the peripheral clock, as shown by the increase in the transcription of the PER1 and PER2 genes in the alimentary duct in response to the direct action of ghrelin (Ueberberg et al., 2009). The relationship among ghrelin and clock peptides sheds new light on the mechanism by which the energy economy of the body is regulated in the context of diurnal changes and demonstrates the participation of peripherally synthesized factors, such as ghrelin and leptin, in these processes (Abizaid et al., 2006).

In the present experiment, the central administration of ghrelin reduced the circulating melatonin concentrations during both of the photoperiodic seasons, which confirmed our previous in vitro data (Zieba et al., 2011). Those experiments showed that the addition of ghrelin to a pineal gland explant culture inhibited melatonin secretion during ShD and LD. However, leptin supplementation of ghrelin-treated cultures increased melatonin concentrations relative to the concentrations in the cultures with ghrelin alone during both of the photoperiods (Zieba et al., 2011).

The highly specific ovine leptin antagonist had an interesting effect in the present set of experiments. A significant increase in circulating ghrelin concentrations was noted when the ovine pegylated super active leptin antagonist was infused during the LD and ShD photoperiods, which reflected the inhibitory effects of leptin on ghrelin activity regardless of daylength. The circulating concentration of ghrelin observed in the untreated ewes did not show any photoperiod-dependent changes, a result that confirms our previous in vitro data (Zieba et al., 2011) and results of Harrison et al. (2008). In the study of Harrison et al. (2008), ICV infusions of ghrelin in sheep led to a temporary increase in feed intake during LD, but a similar effect was not observed during ShD photoperiod.

Centrally administered ghrelin also regulates the hypothalamic-pituitary-gonadal axis. Under ShD, exogenous ghrelin inhibits the synthesis of GnRH, which decreases secretion of LH (Bewick et al., 2005). These results show that photoperiod is a factor that modulates the periodicity of reproductive processes, feed intake, and growth in sheep. Nevertheless, the primary role in these processes is played by the seasonal changes in the activity of the hormones, such as ghrelin and leptin, which are responsible for maintaining the energy homeostasis.

The dietary behaviors of animals, which are aimed at satisfying hunger and achieving satiety, result from the activity of a complicated neuroendocrine network that transfers orexigenic or anorexigenic signals from peripheral tissues to the hypothalamus, which is the most important part of the encephalon controlling feed intake in sheep (Smith and Clarke, 2010). Taken together, these findings are indicative of an abundance of functions that are performed by the proteins engaged in the regulation of energy homeostasis. In addition, these proteins form a close web of interactions in which leptin, ghrelin, and orexin play very important roles.

In conclusion, this study is the first to report that photoperiod affects the close interactions among leptin, ghrelin, and orexin in regulating energy homeostasis in sheep. How the hypothalamic nuclei that are involved in energy balance communicate and how melatonin influences that relationship in sheep is a research area of considerable interest.

**LITERATURE CITED**


