Efecto del fotoperiodo sobre la estacionalidad reproductiva en guanacos (*Lama guanicoe*) hembras

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" Begin by doing what is necessary; Then do, the possible and suddenly you will be doing the impossible."

San Francisco de Asís

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Chapter 1

General Introduction

Guanaco belongs to the Camelidae family. It is the most abundant native of the ungulates in South America (Gonzalez *et al*., 2006). It is also a geographically widespread species within the Southern part of the American continent, and it can be found in areas from sea level up to 4,500 m.a.s.l (Ortega and Franklin, 1995).

Given its considerable flexibility, both ecological and behavioral, it is a remarkably resistant ungulate to extreme conditions (Franklin and Fritz, 1991; Puig *et al*., 1997). Nevertheless, the guanaco has been categorized as a vulnerable species in Chile. Currently, the main threats to this species are dog predation, illegal hunting, intensive agriculture destroying its natural habitat (Baldi *et al*., 2016), and fierce competition for forage between guanacos, livestock and introduced mammals (González *et al*., 2006).

Since the early 1960s, this species has been included in Appendix II of the list of species threatened by Cites (the Convention on International Trade in Endangered Species of Wild Fauna and Flora and thus, guanaco-hunting and trade in these camelids are prohibited. As a consequence, guanacos have become the most abundant species in Patagonia for over a decade, competing for the few pastures with domestic cattle, which has been generating a social and territorial conflict in the area (Baldi *et al*., 2016).

Guanaco is characterized by specific anatomical, physiological, and reproductive adaptations to thrive on and survive in extreme environments (González *et al*., 2006). This species exhibits a seasonal reproduction strategy (Riveros *et al*., 2017), having the mating season during long photoperiod (Schmidt, 1973; Garay *et al*., 1995). Female guanaco is an induced ovulatory, which means that ovulates in response to mating, whereas in the absence of mating or ovulation-inducing, mature follicles start to regress (Riveros *et al*., 2010).

Previous studies have demonstrated that one of the physiological responses of guanacos to daily light variations is fluctuations in plasma melatonin concentration (Riveros *et al*., 2017). Melatonin regulates the hypothalamus-hypophysis-gonadal axis (HPG) and also plays a key role in reproduction control in seasonal breeders (Legan and Karsch, 1980).

The action of melatonin in the HPG axis is predominantly inhibitory (Dubocovich and Markowska, 2005). The downregulation of kisspeptin signaling by melatonin in short days may be the cause of the HPG inhibition (Revel *et al*., 2006). KiSS-1 expression in the arcuate nucleus of ovariectomized ewes has been reported to be higher between the end of the anoestrus season and the onset of the breeding season, compared to the anoestrus season (Smith *et al*., 2007).

Melatonin regulates the pulsatile secretion of GnRH from the hypothalamus, thereby influencing gonadotropin secretion from the adenohypophysis (Vanecek and Vollrath, 1990). The increase in melatonin concentration results in a decrease in serum gonadotropins and prolactin, accompanied by a massive reduction of gonadal hormones biosynthesis (Pevet, 1988; Dubocovich, 2007).

The effect of the natural photoperiod on reproductive hormone concentrations in female guanacos is still not fully known. Therefore, a better understanding of the reproductive physiology is needed in order to design a sustainable management strategy for this species. Furthermore, plans aimed at the management of biological control in areas with high population density can be subsequently developed.

Therefore, our hypothesis in this thesis is that the photoperiod regulates reproductive seasonality in female guanacos, by activating the hypothalamic-hypophyseal-gonadal axis in the phase of long days and deactivating it in the phase of short days. Consequently, this thesis aims to evaluate the photoperiod influence on reproductive hormones in the hypothalamus-hypophysis-gonadal axis in female guanacos. In general, this thesis investigates the effect of photoperiod on follicular development and blood concentrations of reproductive hormones, in addition to the effect of exogenous melatonin on reproductive hormone concentrations in female guanacos in captivity.

The work displayed in this thesis includes three chapters: the first chapter makes a morphological and histological description in the guanaco ovary in short and long luminosity days, as well as the presence of estrogen receptor alpha (ERα) and beta (ERβ) in the different cell types of the ovary.

The second chapter compares the response of the hypothalamic-hypophyseal-gonadal axis of female guanacos during short and long luminosity days, analyzing reproductive hormone secretion patterns and the expression of the melatonin receptor and kisspeptin in the hypothalamus-hypophyseal glands. The last chapter shows the effects of different photoperiods and melatonin-treated on diurnal plasma prolactin secretion.

Hypothesis

The photoperiod regulates reproductive seasonality in female guanacos *(Lama guanicoe*), activating the hypothalamic-hypophyseal-gonadal axis in the phase of luminosity long days and deactivating it in the phase of short luminosity days.

General Aim

To evaluate the photoperiod influence on reproductive hormones and follicular development in female guanacos (*Lama guanicoe*).

Specific Aims

1. To determine whether follicular development has a variation between the short days phase and the long days phase in female guanacos kept in captivity.

2. To determine whether reproductive hormone concentrations fluctuate between short days and long days in female guanacos kept in captivity.

3. To determine whether exogenous melatonin- treatment concomitantly affects reproductive hormone concentrations in female guanacos kept in captivity.

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Chapter 2

Histomorphological changes of ovary between short and long days in guanacos (*Lama guanicoe*)

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Histomorphological changes of ovary between short and long days in guanacos (*Lama guanicoe)*

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Simple Summary: A better understanding of the follicular development of guanacos ovary, in different seasons, will help to understand the reproductive physiology and to develop sustainable management strategies for this species. Guanaco is the most abundant native ungulates in South America and is characterized by seasonal breeding strategy. This is the first comparative histologic study of guanacos ovaries in different seasons. We reported differences in follicular development between periods of short and long days, probably with higher ovarian activity during periods of long days..

Abstract: Morphological and histological description in the guanaco ovary in short and long days, as well as the presence of estrogen receptor alpha (ERα) and beta (ERβ) in the different cell types of the ovary, were studied. Tissue samples were collected from the right and left ovaries of two non-pregnant adult female guanacos, during the short and long days. Samples were fixed and processed for the histological sections, stained with hematoxylin and eosin; and the ERα and ERβ were detected using an indirect immunohistochemistry method. Ovarian follicles were located in the cortical area, at different stages of development. Preantral and antral follicles, as well as follicles with varying degrees of atretia were identified, but no corpus luteum was detected in neither ovaries, due to the lack of contact with a male guanaco. The number and size of ovarian antral follicles and large atretic follicles were detected in long days. Showing a higher ovarian activity in this season. Significant differences were observed on the frequency and size of the atretic follicles during long days between right and left ovaries. Differential expression of ERα and ERβ was observed in follicles at different stages of development, in short days and long days.

Keywords: Guanaco, Photoperiod, Seasonal breeders, Ovary, Histology, Estrogen receptors.

1. Introduction

Guanaco belongs to the family Camelidae, and it is one of the most abundant native ungulates in South America [1]. Since the early 1960s the species has been included in Appendix II of the list of species threatened by Cites (the Convention on International Trade in Endangered Species of Wild Fauna and Flora), prohibiting hunting and commercialisation of this Camelid. As a result, more than a decade ago guanacos have become the most abundant species in Patagonia, competing for the few pastures with domestic cattle, generating a social and territorial conflict in the area [2]. Knowledge of its reproductive physiology is necessary for the sustainable care and/or management of the species, and thus generate plans for the management of biological control in areas with higher population density.

Guanaco is characterized by specific anatomical, physiological and reproductive adaptations to thrive and survive in extreme environments [3]. The main reproductive characteristics of female guanaco include seasonal breeding strategy [4] and narrow reproductive season in the months of long days [5,6]. Also, follicular activity occurs in repeated cycles of growth and regression in the absence of ovulatory stimulus or mating [7]. The development of dominant follicles is found equally distributed between the left and right ovary, although all gestations are developed in the left uterus horn [4]. Seasonal effects on the morphology and ovarian follicular growth of seasonal breeders have been reported [8]. The development of large follicles waves have been described in camels (*Camelus dromedarius*). Females with active ovaries shows a significant seasonal effect [9].

Estradiol is an important local modulator of ovarian function and influences the growth, differentiation, and functions of female reproductive tissues [10]. In guanacos, the plasma concentration of 17β-estradiol increased gradually and concomitantly with an increase in follicular diameter [7]. Estradiol act through the estrogen receptors (ER), and two estrogen receptors structurally related subtypes, ERα and ERβ, have been characterized by several mammals such as ewes [11] and rats [12]. The distribution of both receptors is differential, demonstrating the selective action of estrogen in the ovary [13]. Previous studies on rodents have shown photoperiod-related differences in the distribution of steroid receptors [14]. However, there are no studies in camelids that shows the effect of photoperiod in steroid receptos distribution.

Therefore, a better understanding of the morphology, histology, and follicular development of guanacos ovary, in different seasons, will help to understand the reproductive physiology of females. This work describes a morphological and histological description in the guanaco ovary in periods with short and long days, as well as the presence of estrogen receptor alpha (ERα) and beta (ERβ) in the different cell types of the ovary in guanacos. Significant differences were observed in ovarian histology in short days and long days, with increased reproductive activity on long days. Differential expression of ERα and ERβ was observed in follicles at different stages of development, in short days and long days.

2. Materials and Methods

All protocols adhered strictly to the requirements of the Animal Ethics Committee of the Pontificia Universidad Católica de Chile and had its formal approval.

2.1 Collection of sample:

Tissue samples were collected from two adult female guanacos (8-10 years old approximately), not gestating or lactating and isolated from males, that died. One during the short days period (April; bodyweight: 74kg) and the other during the long days period (October; bodyweight: 85kg). Both animals were raised in captivity in the Mediterranean ecosystem of Chile (33°38′28″S, 70°34′27″W). Ovaries were removed and fixed in PFA solution then embedded in paraffin blocks. Whole ovaries were cut into 12 μm sections using a microtome Leica. One out of sections was mounted on microscope slides and stained with Hematoxylin Eosin, or immunostaining was performed.

The length and width of ovaries were recorded with Vernier Digital. The slides were analyzed by Nikon Eclipse E600 microscope at magnification X400 and X600. Using the ImageJ software (NIH, USA), the number and diameter of pre-antral [15], antral and atretic follicles were counted for each section of the ovary [16].

2.2 *Estrogen Receptor Immunohistochemistry:*

Estrogen receptor alpha (ERα) and beta (ERβ) proteins were assayed in paraffin-embedded cross-sections of guanacos ovaries fixed in PFA solution. The protocol used for immunostaining is described by Urriola-Muñoz et al., 2014. The primary antibodies used were anti-Erα (200 μg/ml; Santa Cruz Biotechnology, USA) and anti-Erβ (1.0 mg/ml; SEROTEC). Immunohistochemistry detection was performed with the Ultra vision kit: HRP polymer/DAB plus chromogen (Thermo Science). As a negative control, the primary antibody was omitted. Lack of nonspecific immunostain in samples incubated in medium lacking the primary antibody shows the specificity of negative controls in the assay.

2.3 Data analysis:

The number of follicles at all stages, preantral, antral, and atretic follicles, in different seasons were calculated and analyzed by ANOVA. Values of $P \leq 0.05$ were considered significant. A P value of ≤ 0.05 was considered statistically significant. The diameters of the preantral, antral, and atretic follicles were expressed as mean ± S.D. Statistical analyses were conducted using the statistical software SPSS Version 18.

3. Results and discussion

The results of the present study provide information about the structure of guanaco ovaries during short and long photoperiods.

3.1. Histology of the guanaco ovary

The histology of the guanaco ovary is shown in Figure 1. The limit between cortical and medullary areas was clearly defined and is a highlight in the figure. In the cortical area, the germinal epithelium was detected (Figure 1A.), where ovarian follicles are located in their different stages of development. On the contrary blood vessels are located in the medullary area.

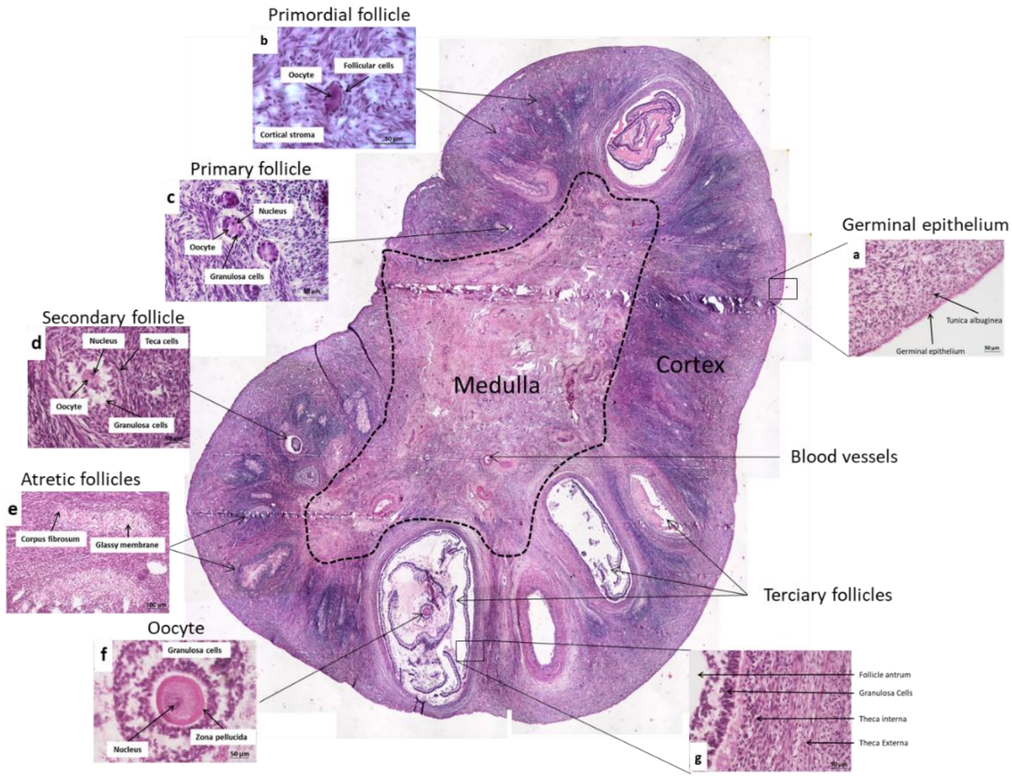

Figure 1. A cross-section of guanaco ovary. (**a**) Ovarian germinal epithelium. (**b** to **g**) Follicles in different stages of development with their follicular cells: (**b**) Primordial follicle, (**c**) Unilaminar primary follicle, (**d**) Secondary follicle, (**e**) Atretic follicle, (**f**) Oocyte of tertiary follicles, (**g**) Granulosa and theca cells of tertiary follicle. a-g scale bar: 50µm. Stain: Hematoxylin and Eosin.

The classification of the different stages of follicle was performed according to the criteria previously defined in other species (Table 1.) [16, 17]. The primordial follicles, with the oocyte surrounded by flat follicular cells, are grouped in the region adjacent to the tunica albuginea (Fig 1B.). A similar distribution of primordial follicles in the cortical area has been previously described in llamas [18]. The primary (Fig 1C.) and secondary follicles (Fig 1D.) were identified as having an oocyte with an obvious nucleus surrounded by a simple or a multilaminar cubic epithelium, respectively. The tertiary follicles were characterized by their large size, the presence of an antral cavity, and internal and external theca cells. Antral follicles had an oocyte of approximately 120 µm, surrounded by the zona pellucida and then a compact layer of granulosa cells (Fig 1F.). The boundary between internal theca cells and mural granulosa cells was clearly observed in these follicles (Fig 1G.).

Table 1. Description of the histological characteristics of follicular development in guanacos (Modificate by [17]).

	Developmental stage	Description
Preantral	Primordial	Single-layer of follicular cells surrounding the oocyte.
	Primary	A larger oocyte with a visible nucleus surrounded by cuboidal granulosa cells.
	Secondary	Enlarged oocyte surrounded by 3 to 4 layers of cuboidal granulosa cells and theca cells around ovarian stroma.

Antral	Tertiary	Presence of liquid-filled cavity, antral follicle. The oocyte lies at the edge in a mound made of granulosa cells, the cumulus oophorus. The connective tissue around the follicle differs in internal and external thetic cells.
Atretic		Thick underlying basement membrane, glassy membrane. Fibrous tissue infiltration of the follicular space, corpus fibrosum.
Corpus luteum		A large rounded structure in the periphery of the ovary with theca lutein cells in the periphery and granulosa lutein cells forming most of the corpus luteum.

Atretic follicles were identified as having a thick and wave-shaped membrane with infiltration of fibrous tissue from the follicular space (Fig 1E.). No corpus luteum was identified in the samples. Previous studies indicate that guanaco as South American camelid (SAC) is an induced ovulator and therefore only ovulates in response to mating, and in the absence of mating or ovulation-inducing, mature follicles naturally regress [5]. The absence of males in this group of females suggests that these structures correspond to follicles in regression and not to corpus albicans, the regressed forms a corpus luteum.

3.2. Morphology and histology of the guanaco ovary in short and long days

Previous studies show that guanacos concentrate their reproductive season in the months of long days [5, 19]. However, changes in morphology and histology of the guanaco ovary between breeding and non-breeding seasons have not been described. The weights of the ovaries in this study (Table 2.) are lower than those observed in domestic SAC [20]. The size was less than alpacas (16 X 11 mm) and llamas (13-25 X 14-2.5 mm) but similar to vicuñas (13 x 0.7 mm) [21]. Ovarian size varies within species depending on the structures present in each ovary, especially by the size of the follicles or the presence of corpus luteum [18]. However, this statement does not match what is histologically observed in guanaco ovaries on short and long days (Fig. 2.). There was no relationship between ovaries weight and antral follicles sizes (Table 2.).

Table 2. Morphometric measurements of the right and left ovaries and follicles at different stages of the short and long photoperiod development of guanaco.

		Short days		**Long days**	
		Left	**Right**	**Left**	**Right**
Weight (gr)		0.51	0.41	0.49	0.58
Size (mm)		12.25 x 8.81	10.76 x 8.29	12.38 x 8.91	13.38 x 9.41
Preantral follicles	No.	126	123	55	80
	diameter (μm)	43+14	28+14	17+5	70+32
Antral follicles	No.	7	5	6	5
	diameter (μm)	1448+895	620+314	3042+466	2894+2081
Atretic follicles	No.	18	13	4	7
	Length (μm)	390+228	358+180	249+40	934+472

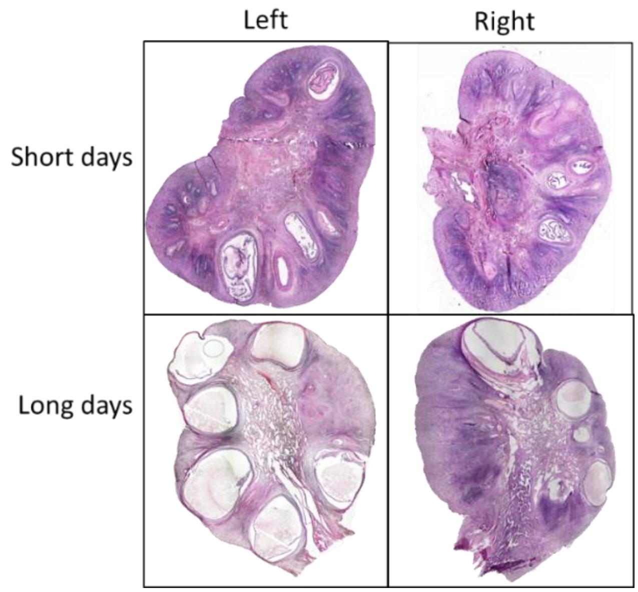

Figure 2. The left and the right ovarian section in the short and long photoperiod of female guanaco. Stain: Hematoxylin and Eosin.

The ovaries of females exposed to short days and long days showed follicles at various stages of development, including preantral and antral follicles, as well as follicles with varying degrees of atresia, but no corpus luteum was detected in neither of them (Fig 2.). A similar proportion of preantral follicles were observed in short (85%) and long (86%) days ovaries. These results are consistent with those reported in old-world camelids, where a proportion of females presenting medium-sized follicles is similar between the breeding and non- breeding season [9]. The range of diameters for preantral follicles in the ovaries observed in this study was 15-150 μm (Table 2), similar to that reported in alpacas [15].

The size of the antral follicles was 215- 2800 μm in short days and 560- 5820 μm in long days (Table 2). A greater proportion of antral follicles was observed on long days (7%) as compared to short days (4%). Also, a significant ($P < 0.05$) increase in the diameter of the long-day antral follicles was observed. However, only one follicle > 5 mm, the characteristic size of follicles intended to be dominant [22], observed in a long day ovary (Table 2.). These results are consistent with those observed by Admas et al., 1989 [18], where llamas without a detectable corpus luteum had follicles of < 5mm. Ovaries in short days had a higher amount of atretic follicles (11%) compared to long days (7%), but with smaller sizes ($P < 0.05$) (Table 2.). The length of the atretic follicles was 215- 2800 μm in short days and 560- 5820 μm in long days (Table 2). The presence of large atretic follicles observed in this study mainly on long days, in addition to the increase in the number and size of antral follicles, is indicative of higher ovarian activity in this season. In female Siberian hamsters, long-day seasonal breeders, exposure to short photoperiod induces an increased number of atretic follicles, decreased folliculogenesis and consequently, the cessation of ovulation [23].

Previous studies in the follicular dynamics of guanacos have shown that the dominant follicles are equally distributed between the left and right ovary. A dominant follicle appeared in the left ovary on 12 occasions (48%) and the right ovary 13 times (52%) [7]. In the present histology study, there are no significant differences ($P > 0.05$) in the frequency and size of the antral follicles in the left and right ovaries. But, significant differences were observed on the frequency and size of the atretic follicles during long days. A 2-fold increase in the frequency and 3-fold in the sizes ($P < 0.05$) of right ovary were noted compared to the left ovary. During short days, no significant differences were observed in follicular development.

3.3. Distribution of estradiol receptors in the guanaco ovary in short and long days

Finally, we study the distribution of estradiol receptors in the guanaco ovary in short and long days. Differential expression of ERα and ERβ in preantral and antral follicles, and their different cell populations (oocytes of preantral follicles, granulosa and theca cells, atretic follicles, and surrounding stromal cells) in different seasons was observed (Fig. 3 and Table 3). In general, ERα was detected in granulosa cells and oocyte and of preantral and antral follicles, and in surrounding stromal cells of primordial and preantral follicles. A low immunoreactivity was observed in theca cells. ERβ protein was detected in the oocyte and their nucleus, surrounding stromal cells and corpus fibrosum. A weak ERβ signal was observed in granulosa cells. Unlike those observed in sheep [24], the expression of ERβ on granulosa cells did not decrease by increasing the size of the follicle. The differential expression of ERα and ERβ in ovarian cells shows an important role in the regulation of follicular development and maturation in each of the follicle states. In our immunocytochemical study, the distribution of ERα and ERβ proteins showed a similar distribution pattern like in dromedary camels [25].

Table 3. Estrogen receptor alpha (ERα) and beta (ERβ) expression in follicular cells of primordial, preantral, antral and atretic follicles in short and long days in guanaco ovaries.
Grading by intensity; -, not detectable; +, weak but definitely detectable; + +, strong staining; + + +, very intense staining. *Higher intensity at the nucleus. / The structure is not found or could not be observed in that follicular state.

	Short days										**Long days**									
	Oocyte		Granulosa cells		Theca cells		Surrounding stromal cells		Corpus fibrosum		Oocyte		Granulosa cells		Theca cells		Surrounding stromal cells		Corpus fibrosum	
	ERα	ERβ	ERα	Erβ	ERα	ERβ	ERα	ERβ	ERα	ERβ	ERα	ERβ	ERα	ERβ	ERα	ERβ	ERα	ERβ	ERα	ERβ
Primordial	+++	+++	-	-	-	-	++	+++	/	/	++	++*	-	-	-	-	++	++	/	/
Preantral	++	++	+	+	-	-	++	+++	/	/	++	++*	+++	+++	-	-	++	+++	/	/
Antral	/	/	++	-	+	+	+	+++	/	/	/	/	++	+++	+	+++	+	+++	/	/
Atretic	/	/	/	/	/	/	/	/	++	+++	/	/	/	/	/	/	/	/	+	+

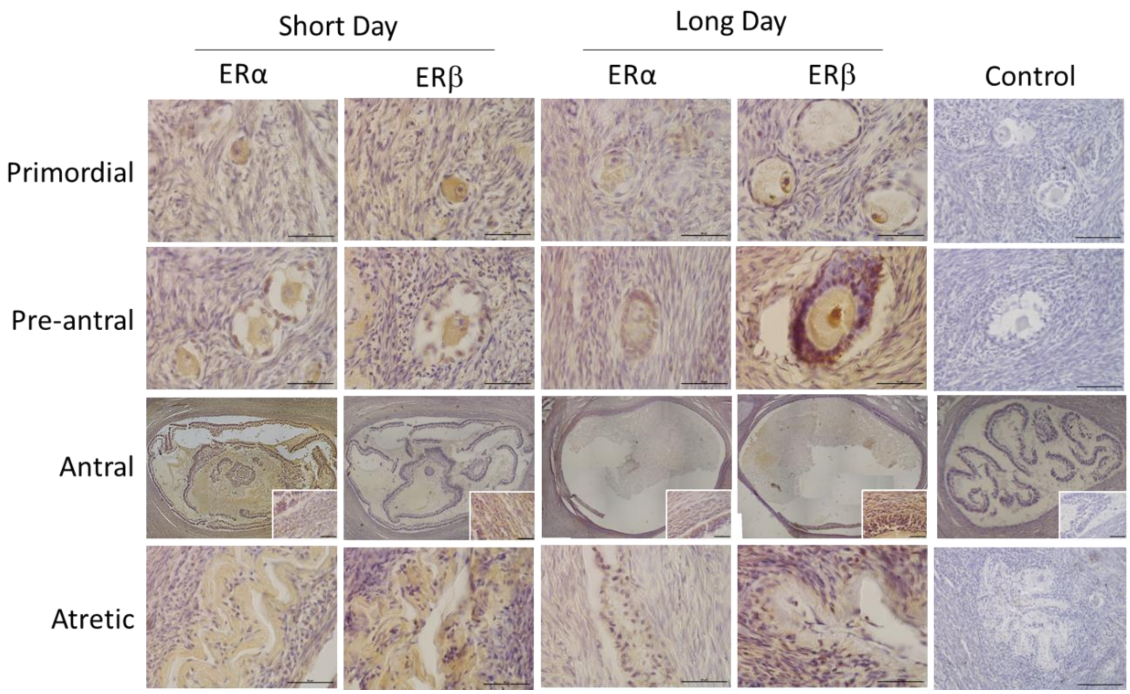

Figure 3. Immunolocalization of estrogen receptor alpha (ERα) and beta (ERβ) of guanaco ovaries in short and long days. Intense immunostaining was detected in follicular cells of primordial, pre-antral, antral and atretic follicles at different stages of development. The inserts in antral follicles show a digital zoom of granulosa and theca cells. The last column shows the negative control of the immunohistochemistry. Counterstained with hematoxylin.

High levels of melatonin have been detected in follicular fluid and the presence of melatonin receptors in granulose cells in cattle [26] and goats [27], suggesting a direct effect of melatonin on ovarian functions. Studies in llamas show that uterine ERα varies depending on the phase of ovarian activity and cell type, with a greater expression during the follicular phase than during the luteal phase [28].

The different expression of ERα and ERβ in guanaco ovaries of short and long days was mainly evident in the nucleus of the oocyte of preantral follicles, granulosa cells, and atretic follicles (Fig. 3). In long days, the strongest ERβ immunolabeling was observed in the nuclei of the oocyte of primordial and preantral follicles, and theca cells of antral follicles. ERα and ERβ immunoreaction of preantral and antral granulosa cells were stronger in long day than in short days. Strong immunoreactivity of both estrogen receptors on short days was observed in the atretic follicles compared to long days. Similar to that observed in equines [29], in guanaco ovaries the expression of ERα increased along with an increase in follicle diameter in both seasons. The differential expression of these two receptors may partly explain the selective action of estrogen in different types of cells in different physiological states. These results provide us with important information about ovarian physiology in the reproductive seasonality of guanacos.

4. Conclusions

This study presents the first histological characterization of adult guanaco ovary. Ovarian follicles are located in their different stages of development in the cortical area. Preantral and antral follicles, as well as follicles with varying degrees of atresia were identified, but no corpus luteum was detected in neither ovaries, probably due to the absence of a male. The distribution of ERα and ERβ in guanaco ovaries in their different cell types was shown. Also, significant differences were observed in ovarian histology in short days and long days, with increased reproductive activity on long days. Differential expression of ERα and ERβ was observed in follicles at different stages of development, in short days and long days. More studies are necessary to understand the implications of these results the reproduction of seasonal breeders.

6. Patents

Author Contributions: L.C was responsible for the experimental design, conceived and carrying out the experiments, analysing and interpreting the data, and drafting the manuscript for publication. R.M. involved for the experimental desing, anlysing and interpreting the data and involved in drafting the manuscript. and funding acquisition J.R involved for the experimental design, conceived the experiments, analysing and interpreting the data and involved in drafting the manuscript.

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Chapter 3

Hypothalamic-pituitary-gonadal axis response to photoperiod changes in female guanacos (*Lama guanicoe*)

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Hypothalamic-pituitary-gonadal axis response to photoperiod changes in female guanacos (*Lama guanicoe*)

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Highlights

- The pineal gland of adult guanacos is sensitive to seasonal changes in day length.
- E2, FSH and LH concentrations are higher during long days than short days.
- Melatonin receptor 1A (Mel1A) and kisspeptin (KISS1) were detected in guanacos.

Abstract

The guanaco, a wild South American camelid, is considered one of the ungulates most resistant to extreme environmental conditions. Guanacos breed seasonally during the season of the year when the photoperiod is longer, but little is known about how females modulates the concentration of reproductive hormones. This study examined the response of the hypothalamic-hypophyseal-gonadal axis in female guanacos during short days compared to long days. Blood samples were collected from 14 adult female guanacos on short days (10L:14D; July) and long days (16L:8D; December) in the Mediterranean ecosystem (33°38′28″S, 70°34′27″W). Melatonin, 17β-estradiol, FSH and LH concentrations were measured. Results showed that melatonin concentration was significantly higher ($P<0.05$) during long days than during short days. Similarly, 17β-estradiol, FSH and LH concentrations were significantly higher ($P<0.05$) during long days than during short days. Expression of the melatonin receptor 1A and kisspeptin were detected in the hypothalamus and hypophysis. These results suggest that the pineal gland of female guanacos is sensitive to seasonal changes in the length of the day. They also indicate a seasonal variation in the concentration of reproductive hormones, which may be associated with the differential modulation of the hypothalamic-hypophyseal-gonadal axis of female guanacos on short and long days.

Keywords

Guanaco; photoperiod; melatonin; gonadotrophins, kisspeptin.

1. Introduction

The guanaco is a wild ungulate belonging to the *Camelidae* family and has a wide distribution in South America, from sea level up to 4,500 m.a.s.l (Franklin, 1983; Torres, 1986). Given its great ecological and behavioral flexibility, it is considered one of the ungulates most resistant to extreme conditions (Franklin and Fritz, 1991; Puig et al., 1997).

Previous studies have shown that, regardless of latitude and of whether guanacos live in the wild or in captivity, they breed and give birth in the spring-summer months or, in other words, when days are longer. In northern hemisphere zoos (~50°N), 74% of guanacos breed between May and July (Schmidt, 1973) while, in Patagonia in the southern hemisphere (~50°S), they do so between November and January (Garay et al., 1995; Sarno et al., 2003). It was previously thought that the reproductive seasonality of wild camelids was related mainly to food availability (Koford, 1957; Franklin, 1983). However, as in the case of other ungulates that reproduce seasonally, photoperiod could be the main environmental factor involved (Malpaux et al., 1999). Previous studies have shown that guanacos respond to daily environmental variations in light with changes in plasma melatonin concentration (Riveros et al., 2017); like other ungulates such as caprines (Chemineau et al., 1992), cervids (García et al., 2003), camels (El Allali et al., 2005; Ainani et al., 2018) and ovines (Malpaux et al., 1996)

In South American camelids, the effect of melatonin on the hypothalamic-pituitary-gonadal (HPG) axis is relatively rare. In mammals that reproduce seasonally, ranging from the Syrian hamster to sheep, melatonin acts on the hypothalamic-pituitary axis (HPA) in a predominantly inhibitory manner, decreasing kisspeptin expression (Revel et al., 2006), secretion of the gonadotropin-releasing hormone (GnRH) in the hypothalamus (Dubocovich and Markowska, 2005) and the release of gonadotropins (FSH and LH) from the hypophysis (Vanecek and Vollrath, 1990). Melatonin action is mediated by the transmembrane receptors MT1 (or Mel1A or MTNR1A) and MT2 (or Mel1B or MTNR1B) (Masana and Dubocovich, 2001), located in the central nervous system (CNS) (Mazzucchelli et al., 1996) and peripheral tissues, such as the *pars tuberalis* of the hypophysis gland (de Reviers et al., 1989). The density of melatonin receptors varies by species, location and developmental or endocrine status as well as being affected by environmental factors such as light intensity or day length (Vanecek et al., 1990; Gauer et al., 1994; Vanecek and Kosar, 1994; Dubocovich, 2007).

The effect of natural photoperiod on reproductive hormone concentrations in female guanacos is still not known. In this study, we compare the response of the hypothalamic-pituitary-gonadal axis of female guanacos during short and long days, analyzing reproductive hormone secretion patterns and the expression of the melatonin receptor and kisspeptin in the hypothalamus-pituitary glands.

2. Materials and methods

All protocols adhered strictly to the requirements of the Animal Ethics Committee of the Pontificia Universidad Católica de Chile and had its formal approval. The study was conducted in captive herd maintained in the Mediterranean ecosystem of Chile (33°38′28″S, 70°34′27″W).

2.1. Animals and experimental design

The study group comprised 14 adult female guanacos (8-10 years old, bodyweight 74±17 kg) that were not gestating or lactating, were isolated from males and had been raised in captivity. The animals grazed natural grassland and were given supplementary alfalfa and water *ad libitum*. The study was carried out in July (short days, 10L:14D) and December (long days, 16L:8D). Light intensity was recorded during samplings using an Extech SDL400.

2.2. Blood Sampling and hormonal assays

Each Monday for three weeks during the light phase (9:00-11:00 am), blood samples were collected from the jugular vein in two 5-ml vacutainer tubes, one containing EDTA and the other without additive. The samples were then centrifuged at room temperature for 15 minutes at 300 g and plasma and serum were harvested and stored at −20°C for subsequent hormonal assays. Plasma melatonin concentration was measured using competitive ELISA (Riveros et al., 2017). Plasma 17β-estradiol (E2) concentration was determined using ELISA (E2-EASIA, KAP0621, DiaSource®), with a minimum analytic sensitivity of 5±2 pg/mL. Serum FSH concentration was measured using competitive ELISA with a commercial kit (FSH, KAPD1288, DiaSource®), with a minimum analytic sensitivity of 0.8 mIU/mL and a measurement range of 0.8-100 mIU/mL. Serum LH concentration was measured using ELISA (LH, KAPD1289, DiaSource®), with a measurement range of 1.2-200 mIU/mL. Calibration curves were obtained using 4-parameter logistic regression.

2.3. Tissue collection

The study had access to two adult guanacos that had died during the long photoperiod season due to causes not related to experimental manipulation. The heads were removed and kept under cold chain at -4°C until processing. The brain was removed and a tissue block containing the hypothalamus and hypophysis was dissected (Correa et al., 2017). The tissue was weighed, measured and frozen at -80°C, pending laboratory analysis.

2.4. Immunoblot analysis

Expression of the melatonin receptor 1A (Mel1A) and kisspeptin (KISS1) proteins was analyzed using western blot, applying a modified version of the protocol of Gonzalez et al., 2018. Extracts from the hypothalamus and hypophysis were obtained by homogenization at 4°C in a RIPA lysis buffer (Cell Signaling, Mass., USA) containing a protease inhibitor cocktail (Sigma, Mo., USA) and 1 mM phenylmethane sulfonyl fluoride. Proteins were separated with a 4%-20% SDS-PAGE gradient and were then transferred to a PVDF membrane in a semi-dry blotting chamber according to the manufacturer's protocol (Bio-Rad). Blots were blocked with 5% milk in a Tris-buffered saline solution (pH 7.6) containing 0.05% Tween 20 and proofed with mouse primary antibody against MEL1AR (200 µg/mL; Santa Cruz Biotechnology, Inc) and rabbit primary antibodies against KISS1 (0.5 mg/mL; Abcam). Bound antibodies were detected using horseradish-peroxidase-conjugated secondary antibodies and incubated for one hour at room temperature. All immunoreactions were visualized with enhanced chemiluminescence (Pierce, Ill., USA) using a ChemiDoc-It HR 410 imaging system (Upland, Calif., USA).

2.5. Data analysis

Data homogeneity was tested using the Kolmogorov-Smirnov test. A repeated-measures ANOVA test was performed to detect differences in hormone concentrations between photoperiod seasons. A Benjamini-Hochberg test was used to determine significant differences between means. Pearson's correlation analysis was performed to test the relationship between light intensity and hormone concentrations. An error probability of $P \leq 0.05$ was considered significant. Results are expressed as mean ± S.D. Data were analyzed using SPSS Version 18.

3. Results and discussion

Reproductive seasonality is an adaptation mechanism developed by some wild mammals, especially those living at high latitudes, as a means of concentrating reproductive activity when environmental conditions favor the survival of offspring (Lincoln, 1992; Nowak et al., 2000). The guanaco has a gestation period of approximately 11.5 months (Riveros et al., 2009) which, combined with breeding mainly in months with long days, means that the young are born in spring-summer when food is more readily available and the weather is warmer (Garay et al., 1995). The photoperiod could be the main environmental factor modulating seasonal reproduction because, unlike climatic variables such as temperature and rainfall, the length of the day in a particular month does not vary from year to year (Prendergast et al., 2009). As indicated above, guanacos react to the light signal by secreting melatonin during the dark phase in an inverse correlation with changes in light intensity (Riveros et al., 2017). Through patterns of secretion of reproductive hormones and the expression of the melatonin receptor and kisspeptin in the hypothalamus-hypophysis glands, our results for the first time provide a comparison of how the response of the hypothalamic-hypophyseal-gonadal axis of female guanacos kept in captivity varies between short and long days.

3.1. Plasma melatonin concentration

We first tested whether photoperiod length is correlated with plasma melatonin concentration in female guanacos. The intra and inter-assay variation coefficients were 3-9.7% and 6.4-19.3%, respectively. The results showed that mean melatonin concentration on long days (0.3-9.4 pg/mL) was significantly lower ($P<0.001$) than on short days (5.3- 40.8 pg/mL) (Fig.1). This suggests that, in adult female guanacos, as in other seasonally reproducing ungulates such as goats (Carcangiu et al., 2015), mares (Diekman et al., 2002), red deer (Loudon et al., 1992; García et al., 2003) and sheep (Lincoln, 1992), the pineal gland is sensitive to seasonal changes in day length as reflected in different patterns of melatonin secretion.

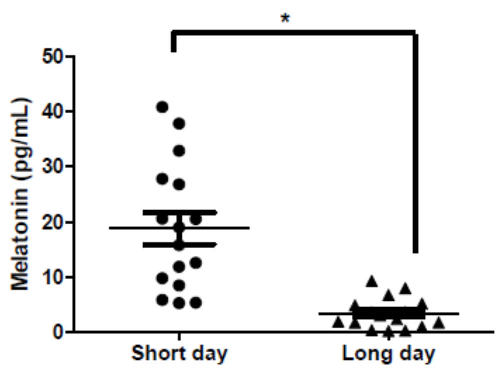

Fig. 1. Comparison of plasma melatonin concentrations on short and long days in female guanacos in captivity. Short days (10L:14D; July) and long days (16L:8D; December). *Significant differences ($P < 0.001$) between groups.

The interpretation of the photoperiodic message transmitted by melatonin in an organism will depend on its pattern of secretion (duration and amplitude) and varies between species (Simonneaux and Ribelayga, 2003; Karsch et al., 2013; Weems et al., 2015). The duration of the nocturnal melatonin peak is the main signal providing photoperiod information (Malpaux et al., 2001). The results show that, in guanacos, baseline daytime melatonin concentrations vary according to the length of the photoperiod, suggesting that, as in mares (Diekman et al., 2002), seasonal variations in the amplitude of melatonin secretion, as well as differences in its duration, could be important for changes in the sensitivity of the hypothalamus-hypophysial gland.

The length of daylight on the short and long days studied was 10.4-10.8 hours and 13.4-14 hours, respectively. A negative correlation was found between daylight hours and melatonin concentration ($r=-0.55$; $P<0.05$). These results are consistent with those obtained in previous work (Riveros et al., 2017) and are similar to those observed for other camelids (El Allali et al., 2005). They are also in line with descriptions in the literature of an inverse relationship between secretion of melatonin and the light phase.

3.2. 17β-estradiol plasma concentration

Table 1 shows 17β-estradiol concentrations during the short and long-photoperiod days. The intra and inter-assay CV were 1.8-9.8% and 6.1-33.6%, respectively. The concentration on short days was lower than on long days ($P<0.001$). The results show a seasonal influence on gonadal steroidogenic activity in female guanacos in response to changes in the photoperiod. The mean plasma 17β-estradiol concentration on short days was similar to that found by other studies of guanacos (25±2 pg/mL) (Riveros et al., 2010) and to values reported for vicuñas (11.7±1 pg/mL) (Miragaya et al., 2004) and llamas (12.8±0.9 pg/mL) (Chaves et al., 2002).

Table 1

Reproductive hormone secretion patterns during short (10 h light, 14 h dark) and long days (16 h light, 8 h dark) in female guanacos in captivity.

	17β-estradiol (pg/mL)	**FSH (mLU/mL)**	**LH (mLU/mL)**
Short days (10L:14D)	21.3[a] (12.5)	0.3[a] (0.1)	1.0[a] (0.7)

Long days (16L:8D)	57.0^{b} (18.0)	0.5^{b} (0.3)	2.2^{b} (0.9)

Group means and standard deviation (in parentheses). Results in the same column with different superscript letters (a and b) are significantly different ($P < 0.05$).

In the dromedary camel (*Camelus dromedarius*), estradiol concentration coincides with ovarian activity, with higher levels in the breeding season (74.7±6.61 pg/mL) and lower concentrations in the non-breeding season (6.8±0.88 pg/mL) (Elias et al., 1984). In addition, ovarian activity during the breeding season is related to the development of follicular waves whereas, in the non-breeding period, it decreases and a little number of small follicles is observed (Sghiri and Driancourt, 1999). Follicular activity in guanacos (Riveros et al., 2010), alpacas (Vaughan et al., 2003), llamas (Chaves et al., 2002) and vicuñas (Miragaya et al., 2004) occurs in waves characterized by the emergence and continuous regression of follicles in the absence of an ovulatory stimulus. It has also been observed that the plasma 17β-estradiol concentration gradually increases with follicular diameter (Riveros et al., 2010) and then decreases during atresia if ovulation is not induced (Aba et al., 1995). The results for 17β-estradiol concentration in female guanacos in this study suggest modulation of ovarian activity by the photoperiod, with increased activity during long days as compared to short days.

3.3. FSH and LH serum concentration

The intra and inter-assay CV in both assays were <10%. FSH and LH concentrations were significantly lower during the month of short days as compared to long days (Table 1, P<0.001). The mean FSH and LH concentrations are similar to those seen in camels, with high concentrations in the breeding season and low concentrations in the non-breeding season (Hussein et al., 2008; Qarawi and ElMougy, 2008).

The different secretion patterns of gonadotropins (FSH and LH) observed in this study suggests a response of the hypothalamus-hypophyseal neuroendocrine system to changes in the photoperiod. Gonadotropin-releasing hormones (GnRH), in response to kisspeptin, act on the adenohypophysis by controlling the secretion and release of gonadotropins (Matsuyama et al., 2011). The activation of melatonin receptors in hypothalamic neurons modulates the pulsatile release of GnRH, which stimulates the secretion of FSH and LH from the hypophysis (Dubocovich and Markowska, 2005). Release of both FSH and LH from the pituitary is under feedback control by estradiol (Irvine et al., 1986). In mares, the reproductive function is regulated mainly by the photoperiod, with steroid feedback mechanisms modulating the cyclic reproductive process during the breeding season. For example, seasonal changes in gonadotropin concentrations in ovariectomized mares have been observed, with high concentrations during long days (Garcia and Ginther, 1976).

In mammals, periodic secretion and pulsatile release of gonadotrophins are responsible for follicle waves and control growth and reproductive activities (Smith and Clarke, 2010). In domestic camelids, the relationship between gonadotropins and follicular development has not been demonstrated (Adams, 1999). No temporal relationship between gonadotrophin secretion and follicular development has been found in llamas, although FSH levels fluctuate throughout follicle growth cycles (Bravo et al., 1990). However, in female camels, serum concentrations of FSH increase with the diameter of the follicles up to 9-19 mm and then gradually decrease. LH concentrations also increase steadily as the growing follicle increases in diameter (Hussein et al., 2008).

Little is known about the relationship between gonadotropins and reproductive activities in South American camelids, but our results reveal differences in the concentration of reproductive hormones on short days as compared to long days. This suggests a possible increase in reproductive activity on long days, in line with what has been observed behaviorally in guanacos in the wild and in captivity (Schmidt, 1973; Garay et al., 1995; Sarno et al., 2003).

3.4. Melatonin receptor and kisspeptin in hypothalamus and hypophysis

Since our results suggest that the natural photoperiod influences the concentration of melatonin, estradiol, and gonadotropins, it was important to evaluate the presence of the melatonin receptor and kisspeptin in the hypothalamus and hypophysis of female guanacos. We found that the hypothalamus and hypophysis weighed 12 and 0.5 g, respectively, and the ratios of organ weight to body weight were 0.14 g/kg and $6*10^{-3}$ g/kg, respectively. The weight of the hypophysis is less than that described in camels (1.5$\pm$0.1 g) and sheep (1.75 g) while the ratio to body weight is similar to that found in large ungulates such as the Chinese buffalo ($5.1*10^{-3}$ g/kg) and the Mongolian pony ($6.6*10^{-3}$ g/kg), and less than that for sheep (0.025 g/kg) (Francis and Mulligan, 1949; Das et al., 1971; Ye et al., 2018). These results are in line with suggestions in the literature that the weight of mammals' hypothalamus and hypophysis may be correlated with body weight and length (Francis and Mulligan, 1949).

Western blot, using an antibody against the melatonin receptor (Mel1AR), with a total protein extract from the hypothalamus and hypophysis, showed a unique band of 40 kDa (Fig 2), similar to that described in humans (Tachibana et al., 2014). In addition, using an antibody against the kisspeptin (KISS1), we found a unique band of 16 kDa, similar to that corresponding to the pre-pro-protein KP-145 in humans, rats and mice (Mead et al., 2007). No significant differences were observed in the expression of Mel1AR and KISS11 in the hypothalamus and hypophysis of either of the dead guanacos studied. The expression of the melatonin receptor in the hypothalamus and hypophysis suggests that melatonin may be acting through Mel1AR in these organs. The modulation of the receptor by melatonin concentrations can act as a direct circadian message to regulate the neuroendocrine activity, as has been observed in other ungulate species (Nonno et al., 1995).

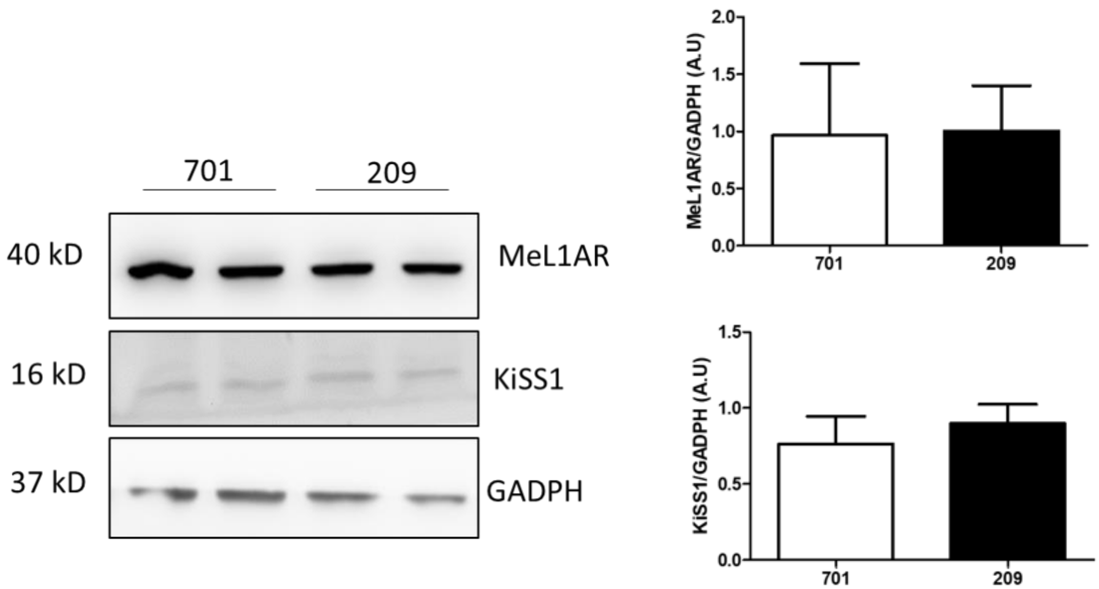

Fig. 2. Expression of melatonin receptor 1A (Mel1AR) and kisspeptin (KISS1) in the hypothalamus and hypophysis of female guanacos in captivity. Western blot analysis of Mel1AR and KISS1 levels in the hypothalamus and hypophysis of the corpses of two adult female guanacos (701, 209). GAPDH was used as an internal control.

Studies of KISS1 in camels (El Allali et al., 2017) and ewes (Chalivoix et al., 2010) found a relationship with seasonal reproductive activity. Changes in the expression of the kisspeptin gene (KISS1) with different photoperiods have been observed in ewes (Smith, 2012). Kisspeptin directly regulates GnRH secretion through their receptor GPR54, as it is expressed in the GnRH neurons (Smith et al., 2008). The presence of kisspeptin in the hypothalamus and hypophysis of female guanacos would indicate possible action on the neuroendocrine reproductive axis.

4. Conclusions

In female guanacos, the hypothalamic-pituitary-gonadal axis responds differently when the photoperiod is short or long. Plasma melatonin concentration in female guanacos is higher during days with a short photoperiod than during days with a long photoperiod, showing a negative correlation with the number of hours of daylight. Concentrations of 17β-estradiol and gonadotropins were significantly higher during the month of long days as compared to that of short days. Expression of the melatonin receptor 1A and kisspeptin were detected in the hypothalamus and hypophysis. Our results suggest an increase in hypothalamic-hypophyseal-gonadal axis activity during long days as compared to short days. This would indicate a possible increase in the reproductive activity of female guanacos when days are long. These results open new avenues of research into reproductive seasonality in wild South American camelids.

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Chapter 4

The effect of different photoperiods and melatonin-treated on plasma prolactin in female guanaco in captivity (*Lama guanicoe*)

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The effect of short and long photoperiods and melatonin treatment on prolactin concentrations in female guanaco (Lama guanicoe)

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Highlights

- The plasma concentration of prolactin in female guanacos is influenced by photoperiod and melatonin concentrations.
- Prolactin secretion pattern varies between seasons, with higher concentrations during short days than long days.
- The melatonin treatment significantly decreases the plasma prolactin secretion.

Abstract

The present study examined the effects of different photoperiods and melatonin treatment on plasma prolactin concentrations in female guanaco (*Lama guanicoe*) kept in captivity. Fourteen adult female guanacos, not gestating or lactating and isolated from males, were studied. The control group was exposed to natural daylight, during short (N=7, 10L:14D) and long days (N=7, 16L:8D). The treatment group received melatonin implants (Melovine®, 18 mg, CEVA Animal Health) every 23 days for six weeks during long days. Samples of plasma were obtained at weekly intervals for three weeks beginning in mid-July (short days) and mid-December (long days), and treatment group 21, 28 and 35 days after receiving the first melatonin implants. Plasma prolactin concentration was measured using competitive ELISA. Plasma concentrations of prolactin in non-lactating female guanacos have seasonal changes, with a higher concentration ($P < 0.001$) in short days (3.50 + 2.24 ng/mL) than long days (1.10 ± 0.91 ng/mL). Low prolactin concentrations in long days coincide with the start of the breeding season. The melatonin treatment significantly decreases ($P < 0.05$) plasma concentrations of prolactin on the 21st day after the treatment, during long days. These findings document the existence of an endogenous circannual rhythm of plasma prolactin concentration and the action of the melatonin on prolactin secretion in female guanacos.

Keywords: Guanaco, Photoperiod, Melatonin, Prolactin.

1. Introduction

The guanaco is a wild ungulate which belongs to the Camelidae family. This species exhibits seasonal reproduction (Riveros et al., 2017). The guanaco has the mating season during the long photoperiod (Schmidt, 1973; Garay et al., 1995), with mating-induced ovulation and a gestation period lasting 11.5 months (Riveros et al., 2009).

Wild guanacos calving season occurs between November and January each year (Garay et al., 1995). Females are sexually receptive to males within fifteen days after calving, showing an early follicular development after a long gestation time (Riveros et al., 2014). The lactation period in guanacos lasts between six and eight months (Riveros et al., 2014), with a maximum suckling frequency during the first four months (summer months) (Garay, et al., 1995).

Previous studies have shown that the guanaco is capable of interpreting variations in light and translating them into changes in plasma melatonin concentration (Riveros et al., 2017). Melatonin mediates changes in the hypothalamus-hypophysis-gonadal axis and plays a key role in controlling reproduction in seasonal breeders (Legan and Karsch, 1980).

Prolactin is a pituitary hormone which influences and participates in reproductive activities, some of which stimulate the development of the mammary gland during pregnancy and regulate postpartum lactation (Kann and Denamur, 1974). Also, prolactin regulates the gonadal function and the development involved in the steroidogenesis (Baird et al., 1981) and the modulation of the gonadotropins effects (McNeilly, 1987). Prolactin has been shown to reduce the secretion of gonadotropins, which tend to be low during the breeding season and high in anestrus (Curlewis, 1992).

Prolactin secretion is regulated by photoperiod through changes in the duration of nightly melatonin secretion by the pineal gland (Azouz et al., 1992; Curlewis, 1992). Studies in ovines (Milne et al., 1990) and camels (El Allali et al., 2018) have shown that continuous treatment with melatonin, or administration of a short day melatonin pattern, inhibits prolactin secretion. The circannual rhythm of plasma prolactin secretion has been described in ewes (Thimonier et al., 1978), which are short-day breeders, and mares (Johnson, 1987), which are long-day breeders, suggesting that this hormone may play a role in the endocrine regulation of seasonal ovarian activity.

Elucidation of mechanisms regulating seasonality in guanacos could lead to population management plans specific to the species. The present study aimed to examine the effects of different photoperiods and melatonin treatment on diurnal plasma prolactin secretion.

2. Materials and methods

Experimental animals and treatments

This study was carried out in the Mediterranean ecosystem of Chile (33°38′28″S, 70°34′27″W) during the short days (July, 10 hours of light, and 14 hours of darkness) and long days (December, 16 hours of light, and 8 hours of darkness). Fourteen healthy adult female guanacos (8-10 years old), weighing from 60 to 95 kg, not gestating or lactating and isolated from males, were used in this study. The animals were fed on natural grassland, supplemented with alfalfa, and given water *ad libitum*. All protocols adhered strictly to the requirements of the Animal Ethics Committee of the Pontificia Universidad Católica de Chile.

Blood samples and hormonal assays

Blood samples were collected from the jugular vein in 5-ml vacutainer tubes with EDTA, then centrifuged at room temperature for 15 minutes at 300 g. Plasma was harvested and stored at −20°C for subsequent hormonal assays. Plasma prolactin concentration was measured using competitive ELISA (PRL-ELISA, KAPD1291, DiaSource®), with a minimum analytic sensitivity of 0,35 ng/mL and a measurement range of 0,35- 200 ng/mL. Calibration curves were obtained using the 4-parameter logistic regression.

Exp. 1: effects of short and long photoperiods on plasma prolactin concentration

Female guanacos were exposed to natural daylight, during short (July, 10 hours of light and 14 hours of darkness) and long days (December, 16 hours of light and 8 hours of darkness). Samples of plasma were obtained at weekly intervals for three weeks beginning in mid-July (short days) and mid-December (long days). Blood samples were taken in short days (n = 7) and long days (n = 7) groups, at intervals of one week during three weeks, in the light phase period (9:00-11:00 am).

Exp. 2: effects of melatonin treatment during the long days on plasma prolactin concentration

Seven female guanacos received two subcutaneous commercial melatonin implants (Melovine®, 18 mg, CEVA Animal Health), every 23 days for six weeks during the long days, at the base of their left ear. Implants have previously been reported to maintain high plasma concentrations of melatonin for at least 25 days in guanacos (Correa et al., 2015). Blood samples were collected early in the morning (9:00-11:00 am), 21, 28 and 35 days after the first implant.

Statistical analyses

The plasma prolactin concentrations of Exp 1 and Exp 2 were compared by student 't' test-Student. An error probability of $P \leq 0.05$ was considered significant. Results are expressed as mean ± S.D. Data were analyzed with SPSS Version 18.

3. Results and discussion

This study is the first to report plasma prolactin concentration in short and long days in female guanacos. Plasma prolactin concentrations for guanacos under the two different photoperiods and melatonin treatment during long days are shown in Figures 1 and 2, respectively. The intra and inter-assay variation coefficients were 3-22% and 8-20%, respectively. Prolactin concentration was significantly higher ($P < 0.001$) in short days (3.50 + 2.24 ng/mL) than long days (1.10 ± 0.91 ng/mL) (Fig. 1). Prolactin plasma concentrations in short days and long days ranged between 1.51-8.28 ng/mL and 0.35-4.34 ng/mL, respectively. The prolactin concentrations obtained in this study in non-lactating female guanacos are similar to those observed in non-lactating ungulated animals such as mares (Johnson, 1987) or female dromedaries (El Allali et al., 2018).

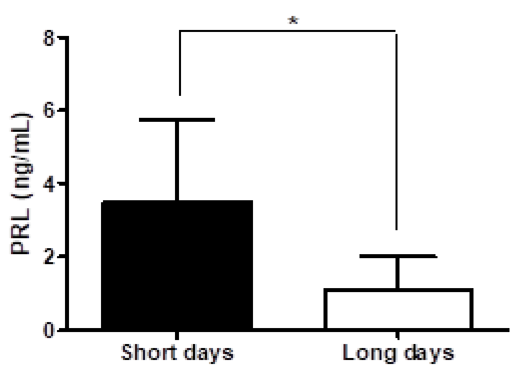

Fig. 1. Experiment 1: Variations of plasma prolactin concentrations (mean ± SD) in female guanacos (33°S) of short days (July, 10L:14D) group (■) and long days (December, 16L:8D) group (□). Prolactin concentration was significantly (*$P < 0.05$) higher in short days group compared to the long days group.

The pineal gland is responsible for translating neural information from the light-darkness cycle into a hormonal signal, through the circadian secretion of melatonin. A negative association between light intensity and melatonin concentration has been shown in guanacos (Riveros et al., 2017). Melatonin appears to have a direct effect on prolactin secretion, mediated by pars tuberalis, which contains the largest component of melatonin receptors (de Reviers et al., 1989). Prolactin is known to exhibit a seasonal rhythm in seasonal breeders, showing a negative relationship with the seasonal rhythm of melatonin in animals such as ewes (Kennaway et al., 1983) and mares (Johnson, 1987). However, some effects of photoperiod on prolactin release occur independently of the pineal melatonin signal, when the prevalent concept that the hypothalamic regulation of prolactin in mammals is predominantly via an inhibitory dopaminergic input (Baldura and Goldman, 1997).

This study shows that guanacos have a prolactin secretion pattern that varies during seasons, with high concentrations during short days compared to long days. These findings suggest a possible photoperiod-independent effect on prolactin secretion in guanacos, where prolactin concentrations in short and long days may be primarily influenced by hypothalamus dopamine (Badura and Goldman, 1994). This could be a mechanism for regulating gonadotropin concentrations in each season when high prolactin concentrations have also been associated with antigonadotrophic effects (Benjaminsen, 1981; Adams et al., 1990). Low prolactin concentrations in long days coincide with the start of the breeding season. A similar observation was reported in yaks (Sarkar and Prakash, 2005), but not in camels (El Allali et al., 2018), ewes (Kennaway et al., 1983), goats (Prandi et al., 1988) and mares (Johnson, 1987), in which prolactin drives the ovarian activity. Further research on the control of prolactin secretion via upstream hypothalamic inhibition of lactotroph activity by dopamine is needed.

Prolactin plasma variations in the melatonin-treated and long-days control animals are reported in Fig. 2. Prolactin plasma concentration was significantly lower ($P < 0.05$) in the melatonin-treated group (0.52 ± 0.15 ng/mL) than in those observed in the long-days control group. In the melatonin-treated group, the prolactin plasma levels ranged between 0.35 and 0.91 ng/mL.

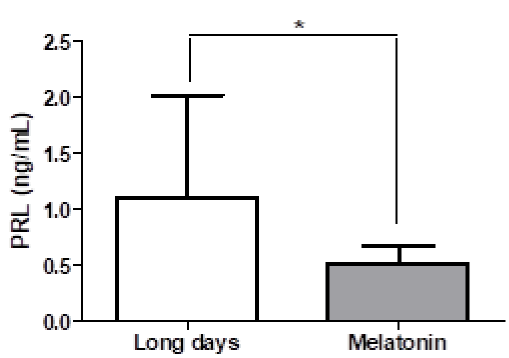

Fig. 2. Experiment 2: Variations of plasma prolactin concentrations (mean ± SD) in female guanacos (33°S) of long days control group (□) and melatonin treatment group (■). Prolactin concentration was significantly (*$P < 0.05$) lower in melatonin treatment compared to the long days control group.

Melatonin treatment is used during anestrus to induce an early onset of reproductive cyclicity with a positive effect on ovarian activity and a suppressive effect on prolactin, in ewes (Malpaux et al., 1988), red deer (Webster et al., 1991) and mares (Guillaume et al., 1995). Previous studies have shown that treatment of exogenous melatonin in guanacos during long days increases plasma concentrations of this hormone (Correa et al., 2015). Our study shows that treatment with exogenous melatonin significantly decreases plasma concentrations of prolactin on the 21st day after treatment (Fig. 2), consistent with those observed in other camelids (El Allali et al., 2018), ewes (Poulton et al., 1986) and mares (Fitzgerald and McManus, 2000). High concentrations of melatonin released from implants could be act on lactotrophs by suppressing prolactin secretion (El Allali et al., 2018). Thus in guanacos, the melatonin could influence prolactin secretion through an independent dopamine mechanism (Lincoln and Clarke, 1995). In this way, the reproductive activity in female guanacos could be modulated by manipulating plasma concentrations of prolactin, through exogenous melatonin treatments.

4. Conclusion

The results of this experiment provide evidence that in female guanacos, the pituitary ability to secrete prolactin is influenced by the season. Plasma concentrations of prolactin in non-lactating female guanacos have seasonal changes, with high concentrations in short days and low concentration in long days. Also, the administration of exogenous melatonin is effective in decreasing prolactin in long days. This research opens new questions to unravel the interrelationship of melatonin and prolactin in the seasonal reproductive activity of camelids and wild ungulates.

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Conflict of interest

The authors declare that is no conflict of interest.

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General Discussion

This thesis shows how the hypothalamic-hypophyseal-gonadal axis of female guanacos responds to changes in the photoperiod, which gives the physiological basis to explain the seasonal reproduction of the species.

Reproductive seasonality is an adaptative mechanism for concentrating reproductive activity when environmental conditions are favorable for the survival of offspring. It is an adaptation developen in some mammals, especially those that live at high latitudes (Lincoln, 1992; Nowak *et al*., 2000).

Variations in annual temperature and nutrition were initially believed to be important in domestic South American Camelids (Sumar, 1985). However, the reproductive season of guanacos takes place during the spring-summer months, regardless of latitude or food availability (Schmidt, 1973; Garay *et al*., 1995; Sarno *et al*., 2003). As daylight hours gradually increase over spring and summer, the photoperiod is indeed a major determinant of the timing of the breeding season.

Moreover, photoperiod is the main environmental factor that regulates reproductive cyclicity in seasonal breeders. The information relating to the annual changing photoperiod transduced by the pineal gland hormone, melatonin, to the HPG axis (Malpaux *et al*.,1999). The main characteristic of the melatonin secretory rhythm is the duration of secretion, positively correlated to the length of the night in all species (Malpaux *et al*., 2001).

The response to annual changes in the hours of daylight requires a variation in melatonin concentration (night vs. day), detection of melatonin duration (long vs. short days) and detection of changes in the duration of melatonin presence relative to previous melatonin exposure (increasing vs. decreasing day length) (Malpaux *et al*., 2001).

The pineal gland of the guanaco is sensitive to seasonal changes of day length. According to previous studies, there is evidence suggesting that guanacos respond to daily environmental variations in daylight and translate them rapidly into plasma melatonin concentration, with high concentration during the dark phase and low during the light phase (Riveros *et al*., 2017).

The diurnal rhythm of melatonin secretion reported in this research has demonstrated that the guanaco, under natural conditions, displays a normal pattern of low daytime levels, with different patterns of secretion in short and long days similar to those observed in camels (El Allali *et al*., 2005). In addition, previous studies have indicated that treatment with exogenous melatonin in guanacos is effective and causes an increase in blood concentration 25 times the baseline concentration, (Correa *et al,* 2015). Exogenous melatonin may be a tool for the study of the effect of melatonin on reproductive parameters in the guanacos.

Melatonin has been described as mediating changes in the hypothalamus-hypophysis-gonadal axis and playing a key role in controlling the reproduction of seasonal breeders (Legan and Karsch, 1980). Studies have provide evidence that the hypothalamus and the hypophysis are the important melatonin target for transducing the effects of this hormone on the reproductive neuroendocrine axis (Malpaux *et al*., 1998).

The activation of melatonin receptors in hypothalamic neurons modulate the pulsatile release of GnRH, which stimulates the secretion of FSH and LH from the hypophysis (Dubocovich and Markowska, 2005). Most of the melatonin effects are mediated through its binding and activation to melatonin receptors (MTNR1A and MTNR1B) (Masana and Dubocovich, 2001).

The expression of the melatonin receptor observed in the hypothalamus and hypophysis of guanacos suggests that in both this species and other ungulates (Malpaux *et al*., 1999), melatonin may be acting through Mel1AR in these glands. However, these studies have failed to define a precise target within the hypothalamus and hypophysis. A further research into this field is needed, aiming to describe the photoperiod-induced changes in melatonin binding sites in the hypothalamus and hypophysis of female guanacos.

Follicular activity in guanacos occurs in waves characterized by the emergence and continuous regression of follicles in the absence of an ovulatory stimulus (Riveros *et al*., 2010). The histological data obtained in this study from ovaries of isolated female guanacos show the presence of follicles in different stages of development and the presence of atretic follicles, but no corpus luteum was detected in any of the ovaries. These results are consistent with the literature, where it is shown that camelids usually require copulatory stimulation to induce ovulation and the subsequent development of a corpus luteum (Adams *et al*., 1991).

Both the melatonin treatment (Dholpuria *et al*., 2012) and the photoperiod (Malinowski et al.,1985) affects gonadotropin levels, probably affecting gonadal function by altering gonadotropin secretion. In seasonal breeders, exposure to inhibitory photoperiods causes a decline in hypophysis and blood concentrations of gonadotropins, while exposure to stimulatory day lengths causes opposite effects (Hasen, 1985).

In the dromedary camel (*Camelus dromedariu*), the ovarian activity during the breeding season is related to the development of follicular waves, whereas in the non-breeding period it decreases, and a few small follicles are observed(Sghiri and Driancourt, 1999). Besides, in mares increasing the daily photoperiod with artificial lighting during the non-breeding period can induce the early resumption of ovarian activity (Malinowski *et al.,*1985).

The current study has demonstrated that follicular development varies between seasons in guanacos. Histological results in ovaries of guanacos in different seasons show an increase in the number and size of antral follicles on long days compared to short days. The presence of large atretic follicles observed in this study, mainly during long days, in addition to the increase in the number and size of antral follicles, is indicative of higher ovarian activity in spring-summer times.

Histological results are consistent with the results in reproductive hormones concentrations where seasonal variations of gonadotropins (FSH and LH) and estradiol concentrations were recorded. High concentrations of reproductive hormones were showed in long days as compared to short days. The increases in serum FSH and LH during the long days coincide with the increases in the number of large follicles suggesting a gonadotropin-follicle association in guanacos.

In mammals, periodic secretion and pulsatile release of gonadotropins are responsible for follicle waves and control growth and reproductive activities (Smith and Clarke, 2010). In female camels, serum concentrations of FSH increase with the diameter of the follicles up to 9-19 mm and then gradually decrease. LH concentrations also increase steadily as the growing follicle increases in diameter (Hussein *et al*., 2008).

Seasonal influence on gonadal steroidogenic activity in female guanacos in response to changes in the photoperiod was displayed. Photoperiodic control of gonadal function is probably largely a result of changes in gonadotropin secretion (Hasen, 1985). In mares, the increase of follicular waves is temporally associated with a surge in circulating gonadotropins and estradiol during the breeding season (Donadeu and Ginther, 2002; Watson *et al*., 2002).

Regarding 17β-estradiol hormone, the effects of different seasons of the year on the 17β-estradiol concentration on female guanacos were significantly higher during long days or summer time than during short days or winter season. These results are in agreement with those in the dromedary camel (*Camelus dromedarius*), where the estradiol concentration coincides with ovarian activity, with higher levels in the breeding season (74.7±6.61 pg/mL) and lower concentrations in the non-breeding season (6.8±0.88 pg/mL) (Elias *et al*., 1984).

Over the past decade, research has focused on the role of kisspeptins in gonadotropin regulation and the role of the KiSS-1/GPR54 system in seasonal breeding. Studies of KISS1 in camels (El Allali *et al*., 2017) and ewes (*Chalivoix et al*., 2010) found a relationship with seasonal reproductive activity. The presence of kisspeptin in the hypothalamus and hypophysis of female guanacos would indicate possible action on the neuroendocrine reproductive axis and this opens up new avenues of research into reproductive seasonality in wild South American camelids.

Another reproductive hormone that is influenced by melatonin is prolactin. Prolactin is a pituitary hormone characterized principally by stimulating the development of the mammary gland during pregnancy, regulating postpartum lactation, and regulating gonadal function and development involved in steroidogenesis (Kann and Denamur, 1974; Baird *et al*., 1981). Prolactin is known to exhibit a seasonal rhythm in seasonal breeders (Kennaway *et al*., 1983; Johnson, 1987).

In dromedary camels, serum prolactin levels are high during the non-breeding season and decrease significantly in the breeding season (Azouz *et al*. 1992). The authors propose that hyperprolactinemia may cause reduced fertility during the nonbreeding season, probably due to prolatin inhibitory action on the synthesis and secretion of gonadotropins (Azouz *et al*. 1992).

This study shows that guanacos have a prolactin secretion pattern that varies between seasons, with high concentrations during short days compared to long days. Low prolactin concentrations in long days coincide with the start of the breeding season, increase in ovarian activity and the concentration of gonadotropins and 17β-estradiol. Increasing of the prolactin concentration during the short days could be associated with antigonadotrophic effects during the non-breeding season (Benjaminsen, 1981; Adams *et al*., 1990). Also, our study shows that treatment with exogenous melatonin significantly decreases plasma concentrations of prolactin at 21st day after treatment, which is also consistent with those observed in other camelids (El Allali *et al*., 2018).

Finally, our results suggest an increase in the HPG axis activity during long days as compared to short days. Our observations would indicate an increase in the reproductive activity of female guanacos during longer days, in line with what has been observed behaviorally in guanacos in the wild and in captivity (Schmidt, 1973; Garay *et al*., 1995; Sarno *et al*., 2003). Therefore, it is possible that photoperiod length and melatonin treatment could be a new factor to be considered to manage guanacos reproduction.

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