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Toolbox

Non-invasive assessment of hormonal fluctuations during pregnancy in guanacos (*Lama guanicoe*) and its application in a wild population

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Obtaining endocrinological profiles using non-invasive methodologies by the measurement of hormone fecal metabolites is a widely used method to monitor ovarian activity and pregnancy in wild species. These tools allow the obtention of physiological information without causing capture-related stress on the individuals. In this research, we aimed to 1) biologically validate a non-invasive method to assess fecal progestagens and estrogens fluctuations during gestation in guanacos (*Lama guanicoe*) and 2) apply this technique to assess pregnancy in a wild free-ranging population. Fecal samples were collected through the gestation period (∼12 months) of female guanacos in a 6.5-ha paddock. An increase in fecal metabolites of both hormones was detected. Progestagens increased gradually, in contrast to estrogens, which remained at basal values for most of the gestation period and peaked only a few days before calving. To assess pregnancy in wild free-ranging animals, fecal samples were collected from a population of La Payunia provincial reserve (Mendoza, Argentina) during the beginning of gestation and at the end of gestation. Through the first months of possible gestation, pregnant females represented between 40 and 80% of the population; at the end of gestation, only 20–40% of the females had confirmed pregnancies. Our results demonstrated that the polyclonal antisera and sexual hormone metabolite assays used here detect variations in the metabolites excreted through feces in guanacos and provide the possibility of non-invasive hormone monitoring of female reproductive status. Also, the findings in wild conditions suggest that natural abortions could have occurred during the first months of gestation. Although some abortions may be natural, the harsh environmental conditions that challenge the support of such a long gestational process may be another relevant factor to consider. The results obtained here enhance our understanding of the reproductive physiology of one of the most emblematic ungulates in South America.

Key words: Estrone conjugates, gestation, pregnancy rate, pregnanediol glucuronides

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Introduction

Steroid hormones play a fundamental role in the reproductive physiology of females. These hormones participate in gamete production and the maintenance of pregnancy (Christensen *et al*., 2012). As a consequence, the proportion of pregnant females can be considered a starting point for assessing vital parameters like birth or recruitment rate (the process of adding new individuals to a population through reproduction or immigration; Gaillard *et al*., 2008) because individuals must reproduce to sustain population dynamics (Cain *et al*., 2012; Decesare *et al*., 2012; Kersey and Dehnhard, 2014). Gestation success can be considered an indicator of population health (Lasley and Kirkpatrick, 1991; Kirkpatrick *et al*., 1993), given that if a population is in a challenging environment, resource allocation to reproduction may not be an obvious choice, and most individuals might favor survival over reproduction (Piasecke *et al*., 2009; Nystrand and Dowling, 2020). In this sense, monitoring ovarian activity is one of the first actions we should consider to infer pregnancy rates and gestation success in wild populations (Hodges *et al*., 2010).

Obtaining endocrinological profiles using a non-invasive methodology is one of the most widely used tools to monitor ovarian activity and pregnancy in wild species (Sontakke, 2018). These techniques allow stress-free sampling with no need to capture and extract blood (Kirkpatrick *et al*., 1993; Schwarzenberger *et al*., 1995; Schwarzenberger, 2007; Mastromonaco *et al*., 2015; Flacke *et al*., 2017; Valenzuela–Molina *et al*., 2018; Miller *et al*., 2021; Watson *et al*., 2023). They rely on the fact that blood-circulating hormones are metabolized in the liver and excreted in the feces. As a result, the variation in hormone levels can be estimated using fecal metabolite dosages (Palme *et al*., 2005; Schwarzenberger and Brown, 2013). In general, the excretion rate is proportional to the amount of circulating hormone; therefore, the values obtained reflect individual endocrinological variations (Kersey and Dehnhard, 2014). Nevertheless, because secretory profiles differ across species, it is critical to demonstrate that hormonal fluctuations in the ovary are reflected in fecal metabolite concentrations via biological validation of the method in the study model (Palme *et al*., 2005).

The guanaco (*Lama guanicoe*) is the most important native herbivore in the Patagonian steppe (Carmanchahi *et al*., 2022); however, several facts about its reproductive physiology remain unclear. This species is known to be an induced ovulator (Fowler and Bravo, 2010; Riveros *et al*., 2010). Estrogens vary their concentrations according to follicular recruitment, but in the absence of mating, there is no ovulation or luteal phase. Progestagens only increase if females become pregnant; otherwise, they remain basal (Bravo *et al*., 1990; Miragaya *et al*., 2004; Riveros *et al*., 2009, 2010). In wild conditions, reproduction only occurs in austral, late spring and early summer, i.e., December, possibly due to environmental factors such as nutrient availability (Sumar, 1994; Urivola García and Riveros, 2017), photoperiod (Urivola García and Riveros, 2017; Correa *et al*., 2020), climate conditions and migratory movements (Candino *et al*., 2022).

Gestation in the guanaco lasts almost a year, between 335 and 360 days (Riveros *et al*., 2010). Studies performed on serum samples obtained every 15 days indicated that progesterone levels reach their maximum values between 260 and 290 days of gestation. After that, progestagens decrease, returning to baseline levels after calving. On the other hand, estradiol increases from day 290, reaching its maximum levels in the postpartum period (Vaughan and Tibary, 2006; Riveros *et al*., 2009). Competent dominant follicles are rapidly developed after calving to be ovulated during the early post-calving period (Riveros *et al*., 2015).

Although understanding gestation success in wild populations can provide insight into population health and dynamics, a comprehensive study of the ovarian activity using non-invasive methods has never been conducted in guanacos. To successfully study wild populations, it is necessary to develop protocols that allow the sampling of wild individuals while avoiding or minimizing human contact. In this study, we aimed to 1) biologically validate a non-invasive method to assess sexual steroid hormonal changes during gestation in guanacos kept in captivity through fecal progestogens and estrogens metabolites quantification and 2) apply this non-invasive method to diagnose early and late pregnancy stages in a wild guanaco population.

Materials and Methods

Ethical Statement

The experimental methodology described here was evaluated and approved by the CICUAL (Institutional Committee for

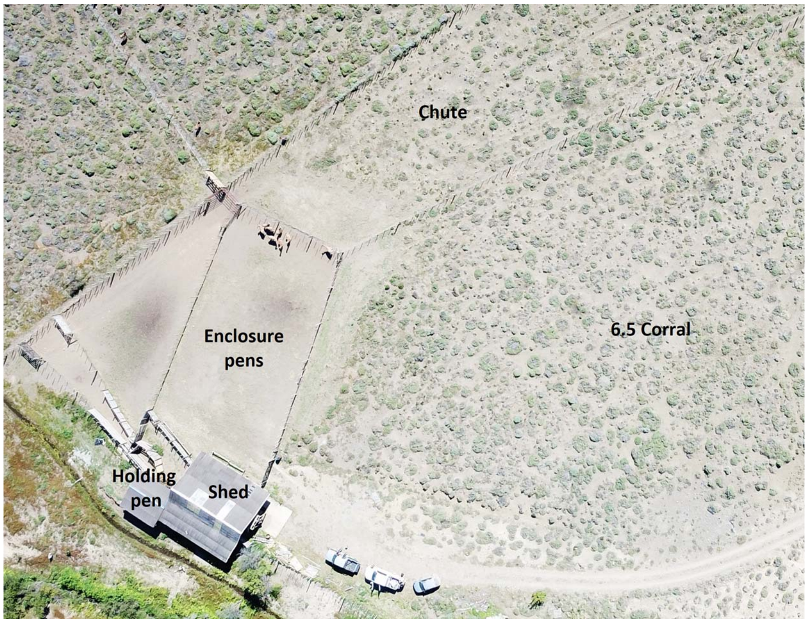

Fig. 1: Structure used for animal handling. 6.5-ha paddock with natural pasture, free water and shelter with a chute that leads to pens for enclosing the animals. These enclosure pens open up to a holding pen that connects to a shed for handling the guanacos.

Table 1: Females' ID, birth date, age and body condition score at the beginning of the experiment

ID	Females' birth date (mm/dd/yyy)	Age in months	Body condition score
I-432	01/26/2009	107	2.5
I-436	01/28/2009	107	3
K-504	12/22/2010	84	3
K-514	12/17/2010	84	3
K-550	01/12/2011	83	3.5
K-568	01/15/2011	83	3.5
K-592	01/26/2011	83	3

the Care and Use of Laboratory or Experimental Animals) of INIBIOMA-CONICET-UNCo, Argentina, under protocol N° 2020–021. The research was also approved by the Secretary of Territorial Development and Environment (Disp. 002/20) of Neuquén Province (Argentina).

Study under captive conditions

This study was performed at 'Los Peucos' ranch (39°43´40.12" S; 71°03′37.58" W; Neuquén Province, Argentina). The site holds a herd of 400 guanacos in extensive farming, maintained for fiber production. To fulfill the first objective, during late spring (November 2018), seven female guanacos and one male were placed in a 6.5-ha paddock with access to natural pastures, water and shelter (Fig. 1). This time of year matches the beginning of the reproductive season in wild conditions (Franklin, 1983; Young and Franklin, 2004). Each female was identified with a different colored collar to facilitate recognition from a distance. In addition, the ranch keeps individuals marked with a tag containing a combination of numbers and letters, allowing us to know their age. We selected middle-aged females between 7 and 9 years old (Table 1). We assessed body condition by palpating the degree of sharpness of spinous processes, muscle mass and fat cover adjacent to the lumbar vertebrae (Audige *et al*., 1998; Taraborelli *et al*., 2017). Scores range from 1 (thin) to 5 (obese) (Table 1). The females remained in the paddock with the male until late March 2019, after which it was removed. Abdominal ultrasound scans were performed on the females using a scanner (SonoScape A5; SonoScape Medical Corp.) with a multifrequency probe (3–7 MHz) to confirm pregnancy in March. Additionally, we did a monitoring

Table 2: Estimated conception date of each female in the study

ID	Calving date	Estimated conception date
I-432	12/09/2019	27/12/2018
I-436	12/18/2019	5/1/2018
K-504	12/13/2019	31/12/2018
K-514	Abortion	Undetermined
K-550	Abortion	Undetermined
K-568	2/1/2020	19/2/2019
K-592	1/1/2020	19/1/2019

Table 3: Summary of the analysed periods according to gestation time; calving day is considered as day zero; gestation days are counted in negative numbers and postpartum days, with positive numbers. The beginning of gestation is considered in the interval between (−320, −240) days and the end of gestation corresponds to the interval between (−60, 0) days

Stage of gestation	Days until/after calving
Beginning	(−347, −240)
Early	(−220, −160)
Middle	(−140, −80)
Advanced	(−60, 0)
Postpartum	(1, 6)

ultrasound in November 2019. Fecal samples were collected from all pregnant females once every 20 days from December 2018 until the last calving in February 2020.

For sample collection, each female was followed around the paddock at a distance of ~100 m and monitored with binoculars and telescopes to collect the samples immediately after defecation. Since the beginning of the birth season, i.e. December, we monitored the individuals every day between 8:00 and 18:00 Hs. to ensure that the collection of postpartum fecal samples began immediately after calving. Postpartum samples were collected every day for 6 days. All samples were placed in individual plastic bags, stored in a cooler with refrigerant gels during the day and then in a freezer at −20°C until analysis.

To estimate the day of conception, a mean gestation time of 347 days was assumed (Fowler and Bravo, 2010). We recorded the calving date and, considering it zero counted 347 backwards as the date of conception (Table 2). Of the seven pregnant females, confirmed by ultrasound at the beginning of the experiment, five gave birth to a living calf, while two had an abortion.

For analysis of hormones fecal metabolites data during gestation, a mixed effect model was used with time as a fixed effect and females as a random effect with the MCMCglmm package of R (Hadfield, 2021). A normal prior distribution

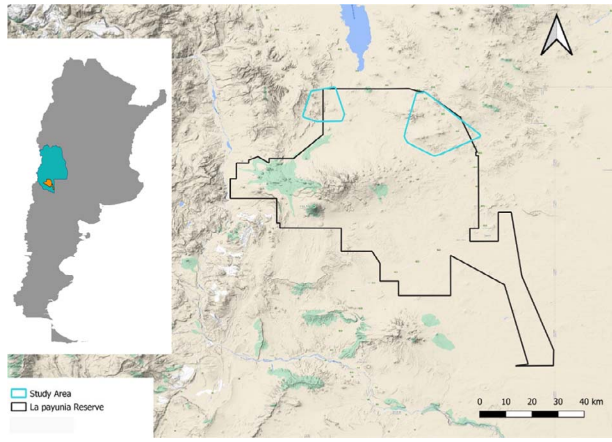

Fig. 2: imits of La Payunia Reserve (Mendoza, Argentina). The small polygons in the north represent the areas under study.

Table 4: Fieldwork summary conducted at La Payunia and the number of samples taken in each survey

Year	Month of collection	Number of samples (*n*)
2007	February–March	9
2007	September–October–November	32
2008	April	16
2008	October	18
2016	September–October	63
2017	February	44
2017	September	31
2018	February	31

was established for the random variable. The hormonal data was re-grouped into four periods: beginning of gestation (the first 107 days), early gestation (next 60 days), mid-gestation (next 60 days) and late gestation (the last 60 days; Table 3).

Data on pre-calving and post-calving hormonal variations were separately analysed due to differences in sampling frequency (once every 20 days approximately for pre-calving samples and once a day for post-calving samples). In the case of pre-calving, we considered advanced gestation (between 0 and 60 days) as the reference level. In the case of postpartum samples, day zero (calving day) was taken as the reference level (Hadfield, 2021). We considered significant differences among stages of gestation if credible intervals did not overlap zero. Also, the effective number of Markov chains (n_{eff}) was assessed.

Study in wild conditions

To evaluate the gestation rate success in the wild, we worked with a guanaco population at La Payunia Provincial Reserve (Mendoza, Argentina; 36°25′S; 69°12′W) with an area of 6.641 km^2. This protected area presents a transitional

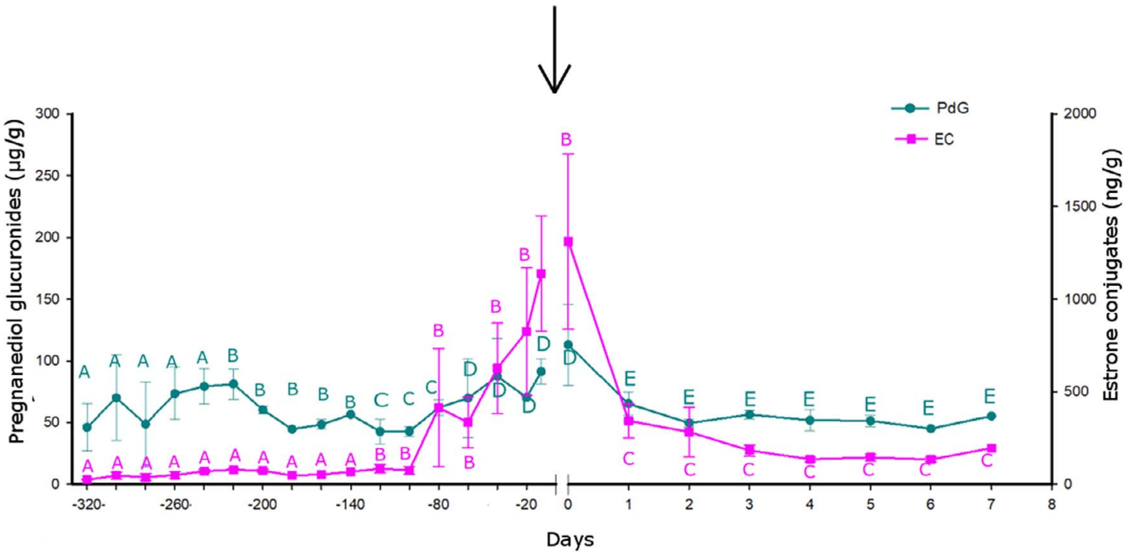

Fig. 3: Average PdG and EC concentrations in pregnant females from Los Peucos ranch. The arrow indicates the time of birth. Different letters indicate that credible intervals do not overlap zero; consequently, there are meaningful differences in fecal metabolite concentrations. The same letter indicates no meaningful differences among concentrations.

environment between the Patagonian steppe and the Monte (Martínez Carretero, 2004) and has one of the most important wild partially migratory guanaco populations in South America, estimated at 26 000 individuals (Schroeder *et al.*, 2013). The north of the reserve (Fig. 2) is the preferred area of the population during the breeding season from September to March (Bolgeri, 2016). The sampling designed for this research consisted of traveling along the available roads of the north of the reserve to collect fecal samples of female guanacos randomly.

Fecal samples were collected from female guanacos at the beginning of pregnancy (February, March and April, Table 4) and in their final stages (September, October and November, Table 4). The observers moved through the roads inside the reserve at low speed in a pickup truck; when a group of guanacos was spotted, it was observed using binoculars and a telescope. When an individual defecated, we assigned sex and collected the samples from females, stored them in a plastic bag and kept them in liquid nitrogen (−196°C) before arrival at the laboratory, where samples were stored in a freezer at −20°C until analysis. We used samples collected in 2007, 2008, 2016, 2017 and 2018 (Table 4). We estimated the percentage of pregnant and non-pregnant females in the periods studied. To assess differences in the proportion of these two groups among years, we did a proportion test using R (R Core Team, 2020).

Laboratory analysis

The endocrine patterns of dams under captive and wild conditions were estimated through the analysis of fecal estrone conjugates (EC) and pregnanediol glucuronides (PdG) concentrations determined with an in-house enzyme immunoassay (EIA) using polyclonal antibodies. Standards and their corresponding horseradish peroxidase conjugates were used (anti-EC R522–2 and anti-PdG R13904; CJ Munro, UC Davis, CA, USA) as previously described by Marozzi *et al.* (2020). Before the assay, and according to parallelism results, fecal extracts were diluted in EIA buffer (0.1 mM 165 sodium phosphate buffer, pH 7.0, containing 9 g of NaCl and 1 g of BSA per litre; final dilution: EC 1:100, PdG 1:20) and assayed in duplicate.

Cross-reactivity reported for EC is as follows: estrone 3-glucuronide 100%; estrone 3-sulfate 66.6%; estrone 23.8%; estradiol 17b 7.8%; estradiol 3-glucuronide 3.8%; estradiol 3-sulfate 3.3%; estradiol 17-sulfate 0.1%; estradiol 3-disulfate 0.1%; and <0.1 with all other steroids tested. Cross-reactivity reported for PdG is as follows: pregnanediol 3-glucuronide 100%; 20a-Hydroxy progesterone 44.8%; 20b-Hydroxy-progesterone 3.1%; progesterone 0.7%; estradiol 17b 0.04%; testosterone 0.2% and cortisol 0.06%. The assay sensitivities for EC and PdG were 0.0078 and 1.954 ng/ml, respectively. The intra-assay coefficient of variation was <12% for both hormones; the inter-assays were 6.5% for EC and 6.8% for PdG.

Results

Study under captive conditions

The calving season started in December (date of first calving, December 9, 2019) and ended in February (date of last calving, February 1, 2020, see Table 2). During gestation, we detected an increase in fecal metabolites in both

Table 5: Differences between conjugated estrone concentrations in prepartum samples at different stages of gestation. Groups that differ from the reference group (late gestation) are indicated in bold (the credible interval does not overlap zero). Intercept: advanced gestation (between −60 days and parturition); beginning of gestation (days −347 and −240), early gestation (days −220 and −160), middle gestation (days −140 and −80), n_{eff} = effective number of Markovian chains

Stage of gestation	Mean	Credible intervals		n_{eff}
		25%	95%	
Intercept	**983.7**	**769.6**	**1187.7**	**4000**
Beginning of gestation	**−935.7**	**−1253.6**	**−620.6**	**4000**
Early gestation	**−915.3**	**−1258.7**	**−598.8**	**4000**
Middle gestation	**−748.7**	**−1090.0**	**−438.6**	**4000**

Table 6: Differences in estrone conjugates concentrations between calving day and postpartum days. Groups that differ from the reference day (calving day) are indicated in bold (the credible interval does not overlap zero). Intercept: birth date, n_{eff} = effective number of Markovian chains

Postpartum days	Mean	Credible intervals		n_{eff}
		25%	95%	
Intercept	**1719**	**1408**	**2014**	**4000**
One	**−1388**	**−1764**	**−1016**	**4000**
Two	**−1472**	**−1841**	**−1101**	**4000**
Three	**−1558**	**−1998**	**−1164**	**4000**
Four	**−1574**	**−1991**	**−1136**	**4000**
Five	**−1551**	**−2043**	**−1091**	**3795**
Six	**−1580**	**−1962**	**−1208**	**3821**

hormones. Progestagen metabolites showed slight fluctuations through pregnancy, increased gradually from the beginning of gestation (days −347, −240) and immediately decreased to baseline levels after calving (Fig. 3). On the contrary, estrogens persisted at basal values throughout pregnancy and increased abruptly in the last 60 days before calving (Fig. 3; Table 5). Already on postpartum day 1, a sharp decrease in estrogen metabolite concentrations was detected (Fig. 3; Table 6). When concentrations were considered in periods (Table 3), prepartum PdG concentrations were significantly lower between the early and middle gestation compared to the end of gestation (Table 7). Similar to EC, PdG concentrations decreased on postpartum day 1 and maintained baseline levels until the end of our sampling period. (Table 8).

Extrapolation of experimental results to the wild population

The proportion of pregnant and non-pregnant females at the beginning and end of gestation was assessed in a wild, free-ranging guanaco population. In the study under captive conditions, all females resulted pregnant; therefore, retrospective hormonal data of non-pregnant females obtained in previous work by our research group was used (Marozzi *et al.*, 2020). As stated before, estrogens remained basal during the first stages of gestation (Fig. 3); hence, only variations in progestogen metabolite concentrations were used to diagnose early pregnancy.

More than 50% of the females were pregnant during the summer season (early gestation, Fig. 4), except in 2007 in which the proportion of pregnant females was lower. Some samples could not be assigned to the pregnant/non-pregnant categories (4 in 2017 and 11 in 2018) because PdG concentrations did not fit into either of the two; thus, they were discarded.

To assign the percentages of pregnant and non-pregnant females in the spring season (advanced gestation), we combined the information obtained from EC and PdG concentrations (Fig. 4). At this stage, the proportion of pregnant females was lower than the proportion of non-pregnant females (<50% each year, Fig. 4). The proportion test indicated that the proportion of pregnant females was significantly different among years compared with non-pregnant females during early pregnancy and late pregnancy (early pregnancy: $\chi^2 = 21.392$, $P = 0.00008727$; late pregnancy: $\chi^2 = 13.746$, $P = 0.003272$), indicating how variable this parameter was among years in a wild population.

Discussion

This is the first study that biologically validates a non-invasive method for monitoring hormonal fluctuations during gestation in guanacos. Our results demonstrate that the hormonal changes that support gestation can be appropriately assessed in guanaco feces. As expected, the concentration of progestagens increased gradually at the late stages of pregnancy and decreased sharply after calving. The secretory profile evaluated in blood samples reported by Riveros *et al.* (2009) indicated a gradual decrease in progesterone levels during the last 4 weeks of gestation. Due to the mechanisms involved in steroid hormone excretion, the results observed in feces have a 24- to 72-h delay compared to what occurs in the blood (Palme, 2005; Kersey and Dehnhard, 2014). As expected, in

Table 7: Differences between PdG concentrations in prepartum samples at the different gestational stages analyzed. Groups that differ from the reference group (advanced gestation) are indicated in bold (the credible interval does not overlap zero). Intercept: advanced gestation (between −60 days and calving); beginning of gestation (days −347 and −240), early gestation (days −220 and −160), middle gestation (days −140 and −80), n_{eff} = effective number of Markovian chains

Stage of gestation	Mean	Credible intervals		n_{eff}
		25%	95%	
Intercept	88.516	73.548	104.182	4900
Beginning of gestation	−22.269	−44.583	2.935	4997
Early gestation	**−25.164**	**−50.897**	**−2.511**	**4900**
Middle gestation	**−37.819**	**−63.682**	**−12.448**	**4900**

Table 8: Differences in pregnanediol concentrations between calving day and postpartum days. Days that differ from the reference day (calving day) are indicated in bold (the credible interval does not overlap zero). Intercept: birth date, n_{eff} = effective number of Markovian chains

Days postpartum	Media	Credible intervals		n_{eff}
		25%	95%	
Intercept	**139.30**	**102.88**	**178.74**	**4000**
One	**−70.35**	**−120.11**	**−26.88**	**4837**
Two	**−92.43**	**−137.23**	**−41.82**	**4000**
Three	**−86.42**	**−139.03**	**−32.47**	**3855**
Four	**−88.18**	**−145.27**	**−38.75**	**4000**
Five	**−86.81**	**−148.30**	**−27.96**	**4000**
Six	**−62.04**	**−109.45**	**−10.59**	**4000**

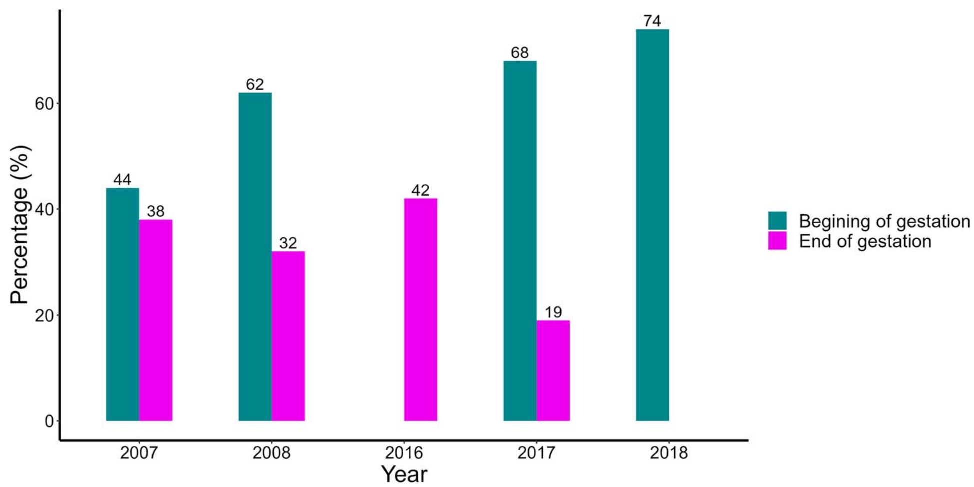

Fig. 4: Percentage of pregnant females during the beginning and end of gestation in the guanaco population of La Payunia. Numbers above each column represent the percentage of pregnant females.

our study, PdG concentrations decreased on the first day post-calving. Estrogens increased in the last days before calving and declined after calving; as with progestagens, a delay was observed compared with the secretory profile in the blood (Fig. 3).

This type of study is of great importance since it sets precedents applicable to other wild populations. Studies investigating progestagens variations in ungulates include multiple species (e.g. *Moschus chrysogaster*; Mithileshwari *et al*., 2016, *Mazama gouazoubira*; Pereira *et al*., 2006, *Cervus elaphus*;

White *et al*., 1995). In particular, Schwarzenberger *et al*. (1995) evaluated hormonal changes during the early stages of gestation in the vicuña (*Vicugna vicugna*), the other wild South American camelid, whose gestation cycle is similar to guanacos' (Fowler and Bravo, 2010). The authors observed an increase in progestagens at the beginning of pregnancy that remained elevated until mid-pregnancy. The maximum concentrations of progestagens were observed in week 10 of gestation (Schwarzenberger *et al*., 1995). However, guanacos showed a marked increase in progestagens only after week 30 of gestation (~80 days before calving) and maintained these levels until the end of gestation. Schwarzenberger *et al's* (1995) study ended several months before calving; therefore, the last stages of gestation cannot be compared, nor can estrogen concentrations, which the authors did not evaluate.

Since progestagens are the best predictors of pregnancy, the study of this hormone variation has generally received more attention from researchers than estrogen fluctuations (e.g. Kirkpatrick *et al*., 1993; Garrott *et al*., 1998; Schoenecker *et al*., 2004; Mithileshwari *et al*., 2016; Flacke *et al*., 2017). Estrogen excretory profile is usually more variable, so they are not considered good predictors of pregnancy (Lasley and Kirkpatrick, 1991; Hundertmark *et al*., 2000; Knott *et al*., 2013; Mastromonaco *et al*., 2015; Nagl *et al*., 2015). However, in guanacos, our results emphasize the fact that the information provided by EC concentrations has the potential to be a calving indicator, using a methodology that avoids animal handling when pregnancy is advanced, and more invasive treatments could put the fetus's life at risk (Solberg *et al*., 2003). Thus, it would be advisable to include the information provided by estrogen fecal metabolites as well, to allow a more accurate pregnancy diagnosis in free-ranging wild animals. Therefore, for late pregnancy diagnosis, between 90 and 30 days prepartum, measuring PdG and EC fecal metabolites is adequate for a proper assessment.

Regarding the results obtained in wild conditions, depending on the stage of pregnancy, i.e. early or late, the timing of sampling in wild conditions is relevant. For early pregnancy diagnosis, it is advisable to sample females at 3–4 months of gestation (March or April), as physiological variability among individuals in the first 2 months hinders the correct assignment of pregnancy status. Previous research on guanacos indicates that hormone concentrations in non-pregnant females are markedly lower than in pregnant females (Riveros *et al*., 2009; Marozzi *et al*., 2020). Thus, if progestagens levels in samples collected during the austral fall are significantly higher than the expected range for non-pregnant females (>45.4 ± 24.4 μg/g; Marozzi *et al*., 2020), the female can be considered pregnant. The proportion of early and late pregnant females in wild conditions was significantly variable among years. Although they were collected randomly, the consistent observation of a lower proportion of pregnant females at the end of gestation suggests that there are instances where gestation does not reach full term. Possibly, such differences could be due to spontaneous abortions caused by hormonal or metabolic variations (Fowler and Bravo, 2010), parasitic infections (Kreizinger *et al*., 2015) or to other factors such as climate or primary productivity, which may influence gestation success (Gittleman and Thompson, 1988; Hamel *et al*., 2010). Furthermore, abortions may impact birth rate and recruitment and, consequently, the species conservation (Creel *et al*., 2007; Cotterill *et al*., 2018; Vitikainen *et al*., 2019). Given that climate change influences nutritional resource availability and parasitic infection prevalence, it is relevant to consider the effect of abortion on population parameters in future research (Root *et al*., 2003; Dimac-Stohl *et al*., 2018).

Females of wild ungulates tend to favor survival over reproduction (Hamel *et al*., 2010; Anouk Simard *et al*., 2014). After conception, pregnancy success will be mainly determined by environmental conditions, such as heavy snowfalls that reduce access to vegetation and the possibility of accumulating nutrients (Anouk Simard *et al*., 2014). Consequently, females in poor body condition may not be physiologically able to sustain pregnancy (Kirkpatrick *et al*., 1993; Albon *et al*., 2017). Future research should focus on understanding the influence of nutrient availability and females' nutritional state during the gestational process (Russell *et al*., 1998). Wild ungulates are an extraordinarily diverse group of mammals with substantial variation in their reproductive biology, whether in their anatomy, behavior or seasonality (Sontakke, 2018). Therefore, acquiring basic knowledge of their reproduction poses a significant challenge to address before making management decisions that may influence this sensitive aspect of their life.

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Author Contributions

Experimental and field work design: A.M., M.F.P., R.O. and P.D.C. Funding acquisition: P.D.C. and A.M. Field sampling: A.M, R.O., A.P., F.M.G., P.G., F.P. Laboratory Analysis: V.I.C., M.F.P. Statistical analysis: A.M. Original draft writing: A.M. Manuscript revision: V.I.C, A.P., F.M.G., R.O., P.F.G., F.P., M.F.P., P.D.C. All authors have reviewed and approved the final manuscript.

Conflicts of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Data Availability

Data is available on reasonable request.

References

Albon SD, Irvine RJ, Halvorsen O, Langvatn R, Loe LE, Ropstad E, Veiberg V, van der Wal R, Bjørkvoll EM, Duff EI *et al.* (2017) Contrasting effects of summer and winter warming on body mass explain population dynamics in a food-limited Arctic herbivore. *Glob Chang Biol* 23: 1374–1389. https://doi.org/10.1111/gcb.13435.

Anouk Simard M, Huot J, De Bellefeuille S, Côté SD (2014) Linking conception and weaning success with environmental variation and female body condition in a northern ungulate. *J Mammal* 95: 311–327. https://doi.org/10.1644/13-MAMM-A-036.

Audige L, Wilson PR, Morris RS (1998) A body condition score system and its use for farmed red deer hinds. *New Zeal J Agric Res* 41: 545–553. https://doi.org/10.1080/00288233.1998.9513337.

Bolgeri MJ (2016) *Caracterización de Movimientos Migratorios En Guanacos (Lama Guanicoe) y Patrones de Depredación Por Pumas (Puma Concolor) En La Payunia, Mendoza*. Doctoral Thesis, Universidad Nacional del Comahue. http://rdi.uncoma.edu.ar/bitstream/handle/uncomaid/164/BOLGERI,%20tesis%20doctoral.pdf?sequence=1

Bravo PW, Fowler ME, Stabenfeldt GH, Lasley BL (1990) Ovarian follicular dynamics in the llama. *Biol Reprod* 43: 579–585. https://doi.org/10.1095/biolreprod43.4.579.

Cain SL, Higgs MD, Roffe TJ, Monfort SL, Berger J (2012) Using fecal progestagens and logistic regression to enhance pregnancy detection in wild ungulates: a bison case study. *Wildl Soc Bull* 36: 631–640. https://doi.org/10.1002/wsb.178.

Candino M, Donadio E, Pauli JN (2022) Phenological drivers of ungulate migration in South America: characterizing the movement and seasonal habitat use of guanacos. *Mov Ecol* 10: 1–14. https://doi.org/10.1186/s40462-022-00332-7.

Carmanchahi P, Funes M, Panebianco A, Gregorio P, Leggieri L, Marozzi A, Ovejero R (2022) Taxonomy, distribution, and conservation status of wild guanaco's populations. In: Carmanchahi PD, Lichtenstein G, eds. *Guanacos and People in Patagonia - A Social Ecological Approach to a Relationship of Conflicts and Opportunities*. Springer, Basel, https://doi.org/10.1007/978-3-031-06656-6_1.

Christensen A, Bentley GE, Cabrera R, Ortega HH, Perfito N, Wu TJ, Micevych P (2012) Hormonal regulation of female reproduction. *Horm Metab Res* 44: 587–591. https://doi.org/10.1055/s-0032-1306301.

Correa LM, Moreno RD, Riveros JL (2020) The effect of photoperiod and melatonin on plasma prolactin concentrations in female guanaco (Lama guanicoe) in captivity. *Reprod Domest Anim* 56: 680–683.

Cotterill GG, Cross PC, Middleton AD, Rogerson JD, Scurlock BM, du Toit JT (2018) Hidden cost of disease in a free-ranging ungulate: brucellosis reduces mid-winter pregnancy in elk. *Ecol Evol* 8: 10733–10742. https://doi.org/10.1002/ece3.4521.

Creel S, Christianson D, Liley S, Winnie JA (2007) Predation risk affects reproductive physiology and demography of elk. *Science* 315: 960. https://doi.org/10.1126/science.1135918.

Decesare NJ, Hebblewhite M, Bradley M, Smith KG, Hervieux D, Neufeld L (2012) Estimating ungulate recruitment and growth rates using age ratios. *J Wildl Manage* 76: 144–153. https://doi.org/10.1002/jwmg.244.

Dimac-Stohl KA, Davies CS, Grebe NM, Stonehill AC, Greene LK, Mitchell J, Clutton-Brock T, Drea CM (2018) Incidence and biomarkers of pregnancy, spontaneous abortion, and neonatal loss during an environmental stressor: implications for female reproductive suppression in the cooperatively breeding meerkat. *Physiol Behav* 193: 90–100. https://doi.org/10.1016/j.physbeh.2017.11.011.

Flacke GL, Schwarzenberger F, Penfold LM, Walker SL, Martin GB, Millar RP, Paris MCJ (2017) Characterizing the reproductive biology of the female pygmy hippopotamus (Choeropsis liberiensis) through non-invasive endocrine monitoring. *Theriogenology* 102: 126–138. https://doi.org/10.1016/j.theriogenology.2017.07.017.

Fowler ME, Bravo PW (2010) *Medicine and Surgery of Camelids. Medicine and Surgery of Camelids*. Wiley-Blackwell, Ames, Iowa, USA

Franklin WL (1983) Contrasting socioecologies of South America's wild camelids: the vicuña and the guanaco. *Adv study Mamm Behav* 7: 573–629.

Gaillard JM, Coulson T, Festa-Bianchet M (2008) Recruitment. In BD Fath, SE Jørgensen, eds, *Encyclopedia of Ecology*. Elsevier Inc., pp. 2982–2986

Garrott RA, Monfort SL, White PJ, Mashburn KL, Cook JG (1998) One-sample pregnancy diagnosis in elk using fecal steroid metabolites. *J Wildl Dis* 34: 126–131. https://doi.org/10.7589/0090-3558-34.1.126.

Gittleman JL, Thompson SD (1988) Energy allocation in mammalian reproduction. *Am Zool* 28: 863–875. https://doi.org/10.1093/icb/28.3.863.

Hadfield J (2021) MCMC Generalised Linear Mixed Models.

Hamel S, Côté SD, Festa-bianchet M (2010) Maternal characteristics and environment affect the costs of reproduction in female mountain goats published by : Ecological Society of America Linked references are available on JSTOR for this article : your use of the JSTOR archive indicates your accept. *Ecology* 91: 2034–2043. https://doi.org/10.1890/09-1311.1.

Hodges JK, Brown JL, Heistermann M (2010) Endocrine monitoring of reproduction and stress. In D Kleiman, KV Thompson, C Baer, eds, *Wild Mammals in Captivity: Principles and Techniques for Zoo Management*. The University of Chicago Press, Chicago, pp. 447–467

Hundertmark KJ, Stephenson TR, Schwartz CC (2000) Evaluation and testing of techniques for ungulate management. In *Evaluation and Testing Techniques for Ungulate Management*. Alaska Department of Fish and Game, Division of Wildlife Conservation, pp. 1–11. https://www.adfg.alaska.gov/static/home/library/pdfs/wildlife/research_pdfs/1.45_00.pdf.

Kersey DC, Dehnhard M (2014) The use of noninvasive and minimally invasive methods in endocrinology for threatened mammalian species conservation. *Gen Comp Endocrinol* 203: 296–306. https://doi.org/10.1016/j.ygcen.2014.04.022.

Kirkpatrick JF, Gudermuth DF, Flagan RL, McCarthy JC, Lasley BL (1993) Remote monitoring of ovulation and pregnancy of Yellowstone bison. *J Wildl Manage* 57: 407. https://doi.org/10.2307/3809441.

Knott KK, Roberts BM, Maly MA, Vance CK, DeBeachaump J, Majors J, Riger P, DeCaluwe H, Kouba AJ (2013) Fecal estrogen, progestagen and glucocorticoid metabolites during the estrous cycle and pregnancy in the giant anteater (Myrmecophaga tridactyla): evidence for delayed implantation. *Reprod Biol Endocrinol* 11: 83. https://doi.org/10.1186/1477-7827-11-83.

Kreizinger Z, Szeredi L, Bacsadi Á, Nemes C, Sugár L, Varga T, Sulyok KM, Szigeti A, Ács K, Tóbiás E *et al.* (2015) Occurrence of Coxiella burnetii and Chlamydiales species in abortions of domestic ruminants and in wild ruminants in Hungary, Central Europe. *J Vet Diagnostic Investig* 27: 206–210. https://doi.org/10.1177/1040638714563566.

Lasley BL, Kirkpatrick JF (1991) Monitoring ovarian function in captive and free-ranging wildlife by means of urinary and fecal steroids. *J Zoo Wildl Med* 22: 23–31.

Marozzi A, Cantarelli VI, Gomez FM, Panebianco A, Leggieri LR, Gregorio P, Ponzio MF, Carmanchahi PD (2020) A predictive model to diagnose pregnancy in guanacos (Lama guanicoe) using non-invasive methods. *Can J Zool* 98: 13–20. https://doi.org/10.1139/cjz-2019-0070.

Martínez Carretero E (2004) La provincia fitogeográfica de la Payunia. *Boletín la Soc Argentina Botánica* 39: 195–226.

Mastromonaco GF, Cantarelli VI, Galeano MG, Bourguignon NS, Gilman C, Ponzio MF (2015) Non-invasive endocrine monitoring of ovarian and adrenal activity in chinchilla (Chinchilla lanigera) females during pregnancy, parturition and early post-partum period. *Gen Comp Endocrinol* 213: 81–89. https://doi.org/10.1016/j.ygcen.2015.02.006.

Miller A, Jentz E, Duncan C, Merriman D (2021) Progestogen metabolites for use in pregnancy monitoring of 13-lined ground squirrels (Ictidomys tridecemlineatus). *Reprod Fertil* 2: 81–88. https://doi.org/10.1530/RAF-20-0071.

Miragaya MH, Aba MA, Capdevielle EF, Ferrer MS, Chaves MG, Rutter B, Agüero A (2004) Follicular activity and hormonal secretory profile in vicuna (Vicugna vicugna). *Theriogenology* 61: 663–671. https://doi.org/10.1016/S0093-691X(03)00238-3.

Mithileshwari C, Srivastava T, Kumar V, Kumar A, Umapathy G (2016) Non-invasive assessment of fecal progestagens and pregnancy detection in Himalayan musk deer (Moschus chrysogaster). *Theriogenology* 85: 216–223. https://doi.org/10.1016/j.theriogenology.2015.09.009.

Nagl A, Kneidinger N, Kiik K, Lindeberg H, Maran T, Schwarzenberger F (2015) Noninvasive monitoring of female reproductive hormone metabolites in the endangered European mink (Mustela lutreola). *Theriogenology* 84: 1472–1481. https://doi.org/10.1016/j.theriogenology.2015.07.023.

Nystrand M, Dowling DK (2020) Effects of immune challenge on expression of life-history and immune trait expression in sexually reproducing metazoans - a meta-analysis. *BMC Biol* 18: 1–17. https://doi.org/10.1186/s12915-020-00856-7.

Palme R (2005) Measuring fecal steroids: guidelines for practical application. *Ann N Y Acad Sci* 1046: 75–80. https://doi.org/10.1196/annals.1343.007.

Palme R, Rettenbacher S, Touma C, El-Bahr SM, Möstl E (2005) Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples. *Ann N Y Acad Sci* 1040: 162–171. https://doi.org/10.1196/annals.1327.021.

Pereira RJG, Polegato BF, De Souza S, Negrão JA, Duarte JMB (2006) Monitoring ovarian cycles and pregnancy in brown brocket deer (Mazama gouazoubira) by measurement of fecal progesterone metabolites. *Theriogenology* 65: 387–399. https://doi.org/10.1016/j.theriogenology.2005.02.019.

Piasecke JR, Bender LC, Schmitt SM (2009) Factors affecting pregnancy in free-ranging elk, Cervus elaphus nelsoni, in Michigan. *Can Field-Naturalist* 123: 230–235. https://doi.org/10.22621/cfn.v123i3.969.

R Core Team (2020) *R: A Language and Environment for Statistical Computing R Foundation for Statistical Computing*, Vienna, Austria. https://www.R-project.org/.

Riveros JL, Schuler G, Bonacic C, Hoffmann B, Chaves MG, Urquieta B (2010) Ovarian follicular dynamics and hormonal secretory profiles in guanacos (Lama guanicoe). *Anim Reprod Sci* 119: 63–67. https://doi.org/10.1016/j.anireprosci.2009.11.005.

Riveros JL, Schuler G, Urquieta B, Hoffmann B, Bonacic C (2015) Ovarian follicular activity during late gestation and postpartum in guanaco (Lama guanicoe). *Reprod Domest Anim* 50: 129–134. https://doi.org/10.1111/rda.12462.

Riveros JL, Urquieta B, Bonacic C, Hoffmann B, Bas F, Schuler G (2009) Endocrine changes during pregnancy, parturition and post-partum in guanacos (Lama guanicoe). *Anim Reprod Sci* 116: 318–325. https://doi.org/10.1016/j.anireprosci.2009.02.005.

Root TL, Price JT, Hall KR, Schneider SH, Rosenzweig C, Pounds JA (2003) Fingerprints of global warming on wild animals and plants. *Nature* 421: 57–60. https://doi.org/10.1038/nature01333.

Russell DE, Gerhart KL, White RG, Van De Wetering D (1998) Detection of early pregnancy in caribou: evidence for embryonic mortality. *J Wildl Manage* 62: 1066. https://doi.org/10.2307/3802559.

Schoenecker KA, Lyda RO, Kirkpatrick J (2004) Comparison of three fecal steroid metabolites for pregnancy detection used with single sampling in bighorn sheep (Ovis canadensis). *J Wildl Dis* 40: 273–281. https://doi.org/10.7589/0090-3558-40.2.273.

Schwarzenberger F (2007) The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. *Int Zoo Yearb* 41: 52–74. https://doi.org/10.1111/j.1748-1090.2007.00017.x.

Schwarzenberger F, Brown JL (2013) Hormone monitoring: an important tool for the breeding management of wildlife species. *Wien Tierarztl Monatsschr* 100: 209–225.

Schwarzenberger F, Speckbacher G, Bamberg E, Speckbacherl G, Barnberg E, Universitat V, Bahngasse L (1995) Plasma and fecal progestagen evaluations during and after the breeding season of the female vicuna (vicuna vicuna). *Theriogenology* 43: 625–634. https://doi.org/10.1016/0093-691X(94)00068-6.

Solberg EJ, Heim M, Arnemo JM, Sæther B, Øystein O (2003) Does rectal palpation of pregnant moose cows affect pre- and neo-natal mortality of their calves? *Alces* 39: 65–78.

Sontakke SD (2018) Monitoring and controlling ovarian activities in wild ungulates. *Theriogenology* 109: 31–41. https://doi.org/10.1016/j.theriogenology.2017.12.008.

Sumar JB (1994) Reproduction in llamas and alpacas: a review. *Theriogenology* 41: 573–592. https://doi.org/10.1016/0093-691X(94)90169-J.

Taraborelli P, Mosca Torres ME, Gregorio PF, Moreno P, Rago V, Panebianco A, Schroeder NM, Ovejero R, Carmanchahi P (2017) Different responses of free-ranging wild guanacos (Lama guanicoe) to shearing operations: implications for better management practices in wildlife exploitation. *Anim Welf* 26: 49–58. https://doi.org/10.7120/09627286.26.1.049.

Urivola García AP, Riveros JL (2017) Modulating factors of reproductive seasonality in ungulates. *Rev Investig Altoandinas* 19: 319–336.

Valenzuela-Molina M, Atkinson S, Mashburn K, Gendron D, Brownell RL Jr (2018) Fecal steroid hormones reveal reproductive state in female blue whales sampled in the Gulf of California, Mexico. *Gen Comp Endocrinol* 261: 127–135. https://doi.org/10.1016/j.ygcen.2018.02.015.

Vaughan JL, Tibary A (2006) Reproduction in female South American camelids: a review and clinical observations. *Small Rumin Res* 61: 259–281. https://doi.org/10.1016/j.smallrumres.2005.07.015.

Vitikainen EIK, Inzani E, Marshall HH, Thompson FJ, Kalema-Zikusoka G, Cant MA (2019) Spontaneous abortion as a response to reproductive conflict in the banded mongoose. *Biol Lett* 15: 20190529. https://doi.org/10.1098/rsbl.2019.0529.

Watson EM, Kurth KA, Metts DL, Moorey SE, Miller BF, Gerhold RW, Muller LI (2023) Evaluating the efficacy of noninvasive fecal sampling for pregnancy detection in elk (Cervus canadensis). *J Wildl Dis* 59: 0–000. https://doi.org/10.7589/JWD-D-22-00041.

White PJ, Garrott RA, Kirkpatrick JF, Berkeley EV (1995) Diagnosing pregnancy in free-ranging elk using fecal steroid metabolites. *J Wildl Dis* 31: 514–522. https://doi.org/10.7589/0090-3558-31.4.514.

Young JK, Franklin WL (2004) Territorial fidelity of male guanacos in the Patagonia of southern Chile. *J Mammal* 85: 72–78. https://doi.org/10.1644/1545-1542(2004)085<0072:TFOMGI>2.0.CO;2.

Schroeder NM, Ovejero R, Moreno PG, Gregorio P, Taraborelli P, Matteucci SD, Carmanchahi PD (2013) Including species interactions in resource selection of guanacos and livestock in northern Patagonia. *J Zool* 291: 213–225. https://doi.org/10.1111/jzo.12065.