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Subject: A predictive model to diagnose pregnancy using noninvasive methods in guanacos (Lama guanicoe)

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A predictive model to diagnose pregnancy using non-invasive methods in guanacos (*Lama guanicoe*)

A. Marozzi[1,*], V. I. Cantarelli[2], F. M. Gomez[3], A. Panebianco[1], L.R. Leggieri[1], P. Gregorio[1], M.F. Ponzio[2], P.D. Carmanchahi[1]

[1]Grupo de Investigación de Eco-Fisiología de Fauna Silvestre (GIEFAS), INIBIOMA, CONICET, AUSMA, UNCo, Pasaje de la Paz 235, 8370 San Martín de los Andes, Neuquén, Argentina. Authors' emails: antomarozzi@conicet.gob.ar; apanebianco@comahue-conicet.gob.ar; lrleggieri@gmail.com; pablogregorio@comahue-conicet.gob.ar; pablocarman@comahue-conicet.gob.ar.

[2]Instituto de Investigaciones en Ciencias de la Salud (CONICET – INICSA), Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Santa Rosa 1085, X5000ESU Córdoba, Argentina. Authors' emails: veronica.cantarelli@gmail.com; mponzio@mater.fcm.unc.edu.ar.

[3]Universidad Nacional del Comahue (CONICET- AUSMA-UNCo), Pasaje de la Paz 235, 8370 San Martín de los Andes, Neuquén, Argentina. Author's email: facugomez@agro.unlp.edu.ar.

[*]Corresponding author: Antonela Marozzi, Pasaje de la Paz 235, (Zip Code: 8370) San Martín de los Andes, Neuquén, Argentina, phone number: +54 9 351 2390774, email: antomarozzi@comahue-conicet.gob.ar.

A predictive model to diagnose pregnancy using non-invasive methods in guanacos (*Lama guanicoe*)

A. Marozzi[1,*], V. I. Cantarelli[2], F. M. Gomez[3], A. Panebianco[1], L.R. Leggieri[1], P. Gregorio[1], M.F. Ponzio[2], P.D. Carmanchahi[1]

Abstract:

Pregnancy status is usually not included in ecological studies due to the difficulty of its evaluation. The use of non-invasive methods is an opportunity to incorporate pregnancy on population studies as can be used avoiding physical restraint of the individuals. In this study, we evaluated sex steroid hormones in plasma and fecal samples on pregnant and non- pregnant females to develop a pregnancy predictive model on guanacos *(Lama guanicoe* Müller, 1776). Samples were obtained during the procedures for live shearing management (capture, shear, and release). Enzyme immunoassays were used to evaluate progesterone (P4) and estradiol (E2) concentrations in plasma and pregnanediol glucuronides (PdG) and conjugated estrogens (EC) on feces. Mean hormonal and fecal metabolites concentrations were significantly higher in pregnant females compared with non-pregnant females. A linear relationship was found between each hormone and its fecal metabolites. Finally, hormone data were combined with an independent source of pregnancy diagnosis such as abdominal ballottement to develop a logistic regression model which will be applicable to diagnose pregnancy in unhandled individuals. The use of predictive models and non-invasive methods might be suitable to incorporate pregnancy information in large scale population studies on guanaco and other free-ranging ungulates.

Keywords: fecal metabolites, logistic regression model, EIA, non-invasive methods, wildlife management, *Lama guanicoe*, guanaco

Introduction

Monitoring the reproductive status of ungulate populations is important for demographic studies and for management decisions (Kersey and Dehnhard 2014). Pregnancy assessment in free-ranging animals is key to estimate population dynamics such as birth rates, neonates survival and population recruitment rates (Altmann et al. 2004; Burgess et al. 2012). Traditionally, the evaluation of pregnancy diagnosis requires capture and physical restraint of the individuals which makes it difficult to apply to free-ranging wildlife populations. Some of the most commonly used methods to assess pregnancy include ultrasonography, fetal echocardiography, laparoscopy, abdominal ballottement, among others (Purohit 2010). During the last decades, non-invasive monitoring methods using fecal metabolites of sex hormones were developed (Bamberg et al. 1991; Schwarzenberger et al. 1991) contributing greatly to improve the knowledge of other disciplines such as ecology or behavior. Steroid hormones are resistant to degradation and as a consequence, their metabolites are stable and can be easily quantified in laboratory assays, even after long storage periods (Lasley and Kirkpatrick 1991; Kersey and Dehnhard 2014).

Measurements of reproductive hormones concentrations in blood such as Estradiol (E2) and Progesterone (P4) might be a valuable tool to evaluate the number of reproductive individuals in a population, the age of reproductive maturation, the timing of breeding and breeding success (Kumar et al. 2013; Kersey and Dehnhard 2014; McCormick and Romero 2017). These hormones can be also used as indicators of pregnancy state. In general, P4 and E2 levels increase during pregnancy and drop after parturition (Schwarzenberger et al. 1991; Schwarzenberger et al. 1996; Graham et al. 2001; Kersey and Dehnhard 2014), yet their concentration and release patterns during pregnancy are distinctive in each species.

Fecal metabolites of estrogens consist mainly of estrone and estradiol-17α and - 17β. Progesterone is extensively metabolized prior to fecal excretion and its fecal metabolites consist of several 5 α and 5 β pregnanes (Bamberg et al. 1991; Schwarzenberger et al. 1991; Schwarzenberger et al. 1996; Schwarzenberger et al. 1997).

Most of wild mammal's studies on pregnancy diagnosis using non-invasive methods compare the mean concentration of pregnanediol glucuronides (PdG) and conjugated estrogens (EC) on pregnant and non-pregnant females (Schwarzenberger et al. 1994; Garcia Pereira et al. 2006; Burgess et al. 2012; Krepschi et al. 2013; Kumar et al. 2013; Valenzuela-Molina et al. 2018). An alternative to evaluate pregnancy assessment in wild populations is to develop a predictive model under controlled conditions (Cain et al. 2012). Hence, this approach allows the inclusion of pregnancy rates in demographic studies and therefore increasing the understanding of population dynamics. Furthermore, pregnancy rate estimation in free-ranging ungulates is important to plan management strategies such as game hunting, population control, species conservation actions, sustainable use, among others (Morden et al. 2011; Mithileshwari et al. 2016).

In South America, the guanaco (*Lama guanicoe* Müller, 1776) is a widely distributed wild ungulate and it is subject to handling with the aim of obtaining its high-quality wool by capture, shear and release of wild individuals (live shearing, Carmanchahi et al. 2011). The guanaco is an induced ovulator, therefore, E2 peaks during the follicular recruitment but P4 only rises after copulation (Riveros et al. 2010; Fowler 2011; Vaughan 2011; Brown 2018). Several studies on the guanaco reproductive physiology were performed using invasive methods in captive animals (Riveros et al. 2009; Riveros et al. 2010; Tibary 2018). However, to extend reproductive studies to wild populations is necessary to apply non-invasive methods.

In this work, we determined P4 and E2 plasma concentrations in guanaco females and we compared our results with reference values (Riveros et al. 2009). Then we evaluated differences between mean concentration of pregnant and non-pregnant females diagnosed by abdominal ballottement. Finally, we used this information to evaluate parallel fluctuations in fecal metabolite concentrations (PdG and EC) in order to develop a predictive model of non-invasive pregnancy diagnosis that can be applied in wild populations.

Material and methods

Ethical statement

All studies performed with animals were carried out in accordance with the Good Practices Protocol of Wild Guanaco (*Lama guanicoe*) Management (Carmanchahi and Marull 2012) which is consistent with the Guide for the Care and Use of Laboratory Animals of the Canadian Council on Animal Care (Council 2009).

Study area

This study was performed in two different field sites: (1) La Payunia Provincial Reserve (Mendoza province, Argentina; 36º00' S 68º34 W'). It has a surface of 4 500 km^2 approximately. Average temperatures range between 6ºC in winter and 20ºC in summer, with a mean 255 mm annual precipitation (Martínez Carretero 2004). The area has a transition volcanic environment between Patagonian steppe and Monte (Martínez Carretero 2004). Vegetation is flat and with mid coverage (Candia et al. 1993). La Payunia Reserve contains one of the few remaining migratory guanaco populations in Argentina (Bolgeri 2016) which is also the biggest wild guanaco population on this region with 26 000 individuals in spring (Schroeder et al. 2014). A small portion of this population (<600

individuals) is subjected to live shearing management with conservation purposes through sustainable use by the local community (Carmanchahi et al. 2011; Carmanchahi et al. 2015). (2) Los Peucos farm (Neuquén province, Argentina; 39°43´ S 71°03´W) is located in the Patagonian steppe (108 km^2 surface). Average temperatures are lower than 10ºC with a mean 800 mm annual precipitation (León et al. 1998; Bran et al. 2002). The landscape is mainly formed by hills and the soil is predominantly constituted by volcanic ashes. The vegetation is characterized by a gramineous steppe (Gonzalez-Polo et al. 2015) and by native forest and exotic pine (*Pinus ponderosa;* Douglas ex P. Lawson & C. Lawson) plantations. The farm contains 400 guanacos in captive conditions which are annually enclosed and sheared for fiber production.

Pregnancy assessment

We evaluated the pregnancy status in 69 female guanacos during live shearing management, using abdomen ballottement (in both study sites). A female was considered pregnant if we could detect a fetus in its abdomen by applying gentle pressure and manual palpation maneuvers (Pratt and Hopkins 1975; Purohit 2010). Live shearing activities are usually performed during the spring season, when the fetuses probably have between nine to ten months of gestation (Fowler 2011). At this point, the fetuses are easily detected using the abdomen ballottement method. Blood and fecal samples were then collected from individual females to measure hormonal concentrations. As live shearing activities are performed under a strict animal welfare protocol, and the time of handling is required to occur during a period of 12 minutes or less on each animal (Carmanchahi and Marull 2012), we could obtain fecal samples and assess pregnancy by abdomen ballottement on 69 individuals. From these 69 guanacos, only 31were also sampled for blood tests. Blood samples were centrifuged in the field and the plasma was separated; plasma and fecal

samples were stored in liquid nitrogen in the field and thereafter, samples were kept at -20ºC, until hormonal analysis.

E2 and P4 determinations in plasma

Total steroids were extracted from 1 mL of plasma using 5 mL of diethyl ether. The procedure for extraction was performed twice. The organic phases were then pooled, evaporated and resuspended in 1mL of phosphate buffered saline (PBS, pH 7.4, Ovejero et al. 2013). E2 and P4 plasma levels were determined using commercial EIA kits (EIA1561, DRG Progesterone ELISA, DRG, Marburg; EIA2693, DRG Estradiol ELISA, DRG, Marburg). Cross reactivity reported for E2 the EIA kit are: Estradiol-17ß 100% 11-Deoxycortisol 0%; Androstenedione 0%; 21-Deoxycortisol 0%; Androsterone 0% Dihydrotestosterone 0%; Corticsterone 0%, Dihydroepiandrosterone 0%; Cortisone 0%; 20-Dihydroprogesterone 0%; Epiandrosterone 0%; 11-Hydroxyprogesterone 0%; 16-Epiestriol 0%; 17α-Hydroxyprogesterone 0%; Estradiol-3-sulfate 0%; 17α-Pregnenolone 0%; Estradiol-3-glucoronide 0%; 17α-Progesterone 0%; Estradiol-17α 0%; Pregnanediol 0%; Estriol 0.05%; Pregnanetriol 0%; Estriol-16-glucoronide 0%; Pregnenolone 0%; Estrone 0.2%; Progesterone 0%; Estrone-3-sulfate 0%; Testosterone 0%; Dehydroepiandrosterone 0%. E2 EIA kit sensitivity: 10.6 pg/mL. Cross reactivity reported for the P4 EIA kit are: Progeterone 100%; 11 -Desoxycorticosterone 1.1%; Pregnenolone 0.35%; 17α OH Progesterone 0.3%; Corticosterone 0.2%; Estriol < 0.1%; Estradiol 17β < 0.1%; Testosterone < 0.1%; Desoxycortisol 0.1%; Cortison < 0.1% DHEA-S < 0.02%; Cortisol < 0.02%;. P4 EIA sensitivity: 0.045 ng/mL. Intra-assay coefficient of variation (CV) was < 7% for both hormones. As only one kit was used for plasma hormonal analysis, not inter assay CV was calculated.

EC and PdG metabolites in feces

In the laboratory, steroid metabolites were extracted according to the protocol of Palme (2005) with slight modifications: briefly 5mL of 80% methanol in water was added to falcon tubes containing 0.5g (dry weight) of each fecal sample. Fecal suspensions were incubated in an orbital shaker at room temperature (21°C) for 120 min. Then, samples were vortexed for 10 seconds and finally centrifuged at approximately 500g for 20 min, in order to separate the debris. The supernatant containing the steroid metabolites was recovered and stored at -20°C until hormone dosage.

Validation of the non-invasive technique for detecting EC and PdG in fecal samples

Validation of fecal sexual hormone metabolites analysis in guanacos was done by the demonstration of: (1) parallelism between dilutions of pooled fecal extracts and the respective standard curve, for the detection of immunological similarities between the standard and the sample hormone; (2) efficiency of the extraction protocol applied, evaluated through the recovery of a known amount of exogenous EC and PdG added to a pool of wet feces before the extraction procedure (amount observed/amount expected*100%); (3) recovery of exogenous hormone in the range of the standard curve added to the fecal extracts, in order to examine possible interference of components in feces or the solvent with antibody binding (matrix interference). Parallelism data analysis was performed using Infostat statistical software (Di Rienzo et al. 2012). A regression analysis with auxiliary variables (dummy) was used to compare if the curves obtained in the assay of serial dilutions of pooled fecal extracts and the corresponding standard curve were parallel.

EC and PdG determination in feces

EC and PdG concentrations in the fecal extracts were determined by enzyme immunoassay with polyclonal antibodies and their corresponding horseradish peroxidase conjugate (Anti

EC R522-2 and Anti-PdG R13904, Department of Population Health and Reproduction, Coralie Munro, UC Davis, CA, USA). Prior to the assay and according to parallelism results, fecal extracts were diluted in EIA buffer (0.1 mM sodium phosphate buffer, pH 7.0, containing 9 g of NaCl and 1 g of BSA per liter; final dilution, EC: 1:20 for non-pregnant females and 1:200 for pregnant females; PdG: 1:20 for pregnant and non-pregnant females), and assayed in duplicate. The EIA was performed according to Munro and Lasley (1988). Briefly, flat-bottom microtiters plates (Nunc Maxisorp, VWR, Mississauga, ON, Canada) were first coated with 50µL of antibody diluted in coating buffer solution (50mM bicarbonate buffer, pH 9.6, final dilution EC=1:13000, PdG=1:10000)covered with acetate sealers to prevent evaporation and incubated overnight at 4°C. After 16-24 hours, the plates were washed in order to remove unbound antibody molecules, with a a wash solution (0.05% Tween 20 in water, 0.15 M NaCl) using an automatic microplate washer (Bio-Tek EL 40 VR, Bio-Tek Instruments, Winooski, VT). Immediately after washing, 50µL of the fecal extracts, standards and controls were added in duplicate, followed by 50 µL of the corresponding horseradish peroxidase conjugate diluted in EIA buffer (final dilution EC = 1:37500, PdG = 1:21500). The plates were then covered and incubated at room temperature (21°C) for 2 h in an orbital shaker. Immediately after incubation, the plates were washed and blotted dry, followed by the addition to each well of 100µL of the substrate solution (50 mM citrate, 1.6 mM hydrogen peroxide and 0.4 mM 2, 20-azino-di-(3- ethylbenzothiazoline sulfonic acid) diammonium salt, pH 4.0; Munro et al. 1991). Finally, absorbance was measured at 405 nm using a microplate reader (Thermo Electron Corporation, USA).

Cross-reactivity reported for EC are: 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 are: 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 sensitivity of the assay for EC and PdG was 0.0078 and 1.954 ng/mL, respectively. The intra-assay coefficient of variation was <12% for both hormones and the inter-assay was 14.8% for EC and 11.4% for PdG.

Comparison of plasma hormones and fecal metabolites in pregnant and non-pregnant females

Plasma hormones and fecal metabolite concentrations are presented as mean concentration ± Standard Deviation (SD). Statistical analysis was performed using the R software (version 3.5.1). We confronted our plasma results with reference values (Riveros et al. 2009). Furthermore, we compared plasma hormone and fecal metabolite concentrations on pregnant and non-pregnant females (diagnosed by abdominal ballottement) using a Bayesian approach for ANOVA. This statistical analysis evaluates the probability that mean hormonal concentrations are different in pregnant and non-pregnant females (Stanton 2017). To perform this test we used the BayesFactor package (https://cran.r-project.org/web/packages/BayesFactor/index.html) which includes the concept of Bayes Factor (BF) on the statistical results. BF is an odds ratio obtained of comparing two statistical models (the null hypothesis, i.e.: no differences in hormone concentrations between pregnant and non-pregnant females; and the alternative hypothesis, i.e.: differences in hormone concentrations between pregnant and non-pregnant females). We also calculated the lower and upper bounds of the 95% highest density interval (HDI; Stanton 2017). The relationship between plasma hormone concentrations and fecal

metabolites was estimated using a Bayesian regression model with the package JAGS UI (https://cran.r-project.org/web/packages/jagsUI/index.html; Kellner 2015). To evaluate the adjustment of the model, we considered the HDI ranges, the Markov Chain Monte Carlo convergence and the Gelman-Rubin convergence statistics (Rhat, <1.1). We also calculated the effective sample size (n.eff) to estimate the number of independent draws from the posterior distribution of the parameter. In both analyses, we used uninformative priors to estimate the parameters because we wanted that the posterior distributions were mainly determined by our data. E2 and EC concentrations were $\log_{10}$ transformed for both analysis and P4 and PdG were $\log_{10}$ transformed for the regression analysis to obtain a better adjustment of the variables.

Pregnancy predictive model

We developed a predictive model that could allow us to evaluate pregnancy in non- handled individuals using a Bayesian approach. For this, we performed a Bayesian logistic regression (Gelman and Hill 2006) using the package JAGS UI (https://cran.r-project.org/web/packages/jagsUI/index.html; Kellner 2015). The response variable "Pregnancy state" (y) has a Bernoulli distribution:

$$y \sim Bernoulli(p_i) \qquad \text{(Eq.1)}$$

The probability of being pregnant (p_i) was modeled as a linear function of the PdG and EC concentrations (Gelman and Hill 2006):

$$Logit(p_i) = \beta_0 + \beta_1 \times PdG + \beta_2 \times EC \qquad \text{(Eq.2)}$$

$$p_i = log\left(\frac{p_i}{1-p_i}\right) \qquad \text{(Eq.3)}$$

The odds of being pregnant were defined as the probability of being pregnant divided by the probability of not being pregnant. To develop this model, prior distribution for the parameters were:

$$\beta_0 \sim U(0,1) \quad \text{(Eq. 4)}$$

$$\beta_1 \sim N(0,10000) \quad \text{(Eq. 5)}$$

$$\beta_2 \sim N(0,10000) \quad \text{(Eq. 6)}$$

We chose a uniform prior to the intercept (β_0) because we wanted that the predictions were mainly regulated by the slopes of our model (β_1 and β_2; i.e.: fecal metabolites concentrations). Furthermore, uninformative priors were selected for β_1 *and* β_2 because we wanted that our results were explained principally for our data. The posterior distribution of each parameter was obtained. We used the mean of each parameter to estimate the logit of the probability of being pregnant and then we calculated the probability of being pregnant according to equation 2 and 3 (Gelman and Hill 2006). To test the model assessment to predict pregnancy, we introduced one by one the pregnant and non-pregnant female data in the model and calculated the probability of being pregnant. Finally, we compared the results obtained from the model with our field diagnostic. Our model assumed independent observations and a linear relationship between the log odds of pregnancy and the explanatory variables. Despite different sites could present differences in productivity which could influence hormone excretion due to variation in nutritional components, several studies have shown that fecal output of hormone metabolites was not affected (Rabiee et al. 2002; Schwarzenberger 2007; Morden et al. 2011). Therefore, for the predictive model construction, we assumed that there were no differences in fecal metabolites among sites. To evaluate the adjustment of the model, we considered the HDI ranges, the Markov Chain Monte Carlo convergence and the Gelman-Rubin convergence

statics (Rhat, <1.1). We also calculated the effective sample size (n.eff) to estimate the number of independent draws from the posterior distribution of the estimand.

Results

E2 and P4 in plasma

At approximately 300 days of gestation, plasma hormone concentrations were significantly higher in pregnant than in non-pregnant females. (Figure 1; P4 = 17.3 ± 6.1 nmol/L; E2 = 580.9 ± 330.2 pmol/L for pregnant females; P4 = 8.1 ± 6.8nmol/L; E2 = 193.0 ± 265.5 pmol/L in non-pregnant females). Bayes factor indicated a strong evidence in favor of the alternative hypothesis (BF= 168.6 %, HDI= 10.7; 15.6 for P4 concentrations and BF= 39.5%; HDI= 2.1; 2.5 for E2 concentrations).

Validation of the non-invasive technique for detecting EC and PdG in fecal samples

To establish the pharmacological validity of the technique in guanacos, the immunoassay was performed with a serially diluted pool of fecal extracts that showed parallel displacement to the EC (R^2 = 0.96; p = 0.13; Figure 2a) and the PdG (R^2 = 0.96; p = 0.47; Figure 2b), proving immunological similarities between the sample solution and the standard.

Recovery of exogenous hormone in the range of the standard curve (EC: 0.0078 - 2 ng/ml; PdG: 1.954 - 500 ng/ml) added to a pool of fecal extracts indicated a low interference of components in the feces with the antibody binding (EC: 91.43 ± 5.13 %; y = 1.0001x + 0.0077; R^2 = 0.99, P < 0.01; PdG: 81.9 ± 2.71 %; y = 0.8186x + 12.7622; R^2 = 0.99; P < 0.01; Figure 3 a and b). Efficiency of the entire fecal extraction procedure with the solvent was 85.74 ± 3.99 % and 88.35 ± 4.88%, for EC and PdG respectively.

EC and PdG metabolites determinations in feces

From the females sampled for fecal analysis (*n*=69), 35 were pregnant and 34 were non-pregnant females (diagnosed by abdominal ballottement). Fecal metabolite concentrations were significantly higher in pregnant than in non-pregnant females. We found differences in EC and PdG concentrations (EC=485.6±412.0 ng/g ; PdG=81.9 ± 58.5µg/g; for pregnant females; EC=76.2 ± 47.9 ng/g; PdG= 45.4±24.4 µg/g in non-pregnant females). Bayes factor indicated a strong evidence in favor of the alternative hypothesis (BF= $6{,}2x10^{10}$ % HDI=52.9; 74.5 for EC ; BF=24.9%; HDI=52.8; 74.6 for PdG;; Figure 4).

Plasma hormone concentrations and their relationship with fecal metabolites

E2 and P4 plasma concentrations showed a linear relationship with EC and PdG, respectively (Figure 5). The adjustment of the parameters is presented on Table 1.

Pregnancy predictive model

The estimated logistic regression equation for the predictive model was:

$$logit(p[i]) = 0.495 - 1.697 \times \left[ln\left(pdg_i\ \right)\right] + 3.827 \times [ln(ec_i)] \qquad \text{(Eq. 6)}$$

$$p_i = \frac{exp\left\{0.495-1.697\times\left[ln\left(pdg_i\ \right)\right]+3.827\times[ln(ec_i)]\right\}}{1+exp\left\{0.495-1.697\times\left[ln\left(pdg_i\ \right)\right]+3.827\times[ln(ec_i)]\right\}} \qquad \text{(Eq.7)}$$

Eq. 6 and 7 contain the mean values of the posterior distribution of the parameters. HDI intervals, Rhat and n.eff are reported in Table 2. The threshold value for determining pregnant versus non-pregnant status that resulted in the best predictive success for this model was 0.4 (Figure 6). Our model accurately predicted 88.57% of the pregnant females and 94% the non-pregnant females. In very few cases, borderline fecal metabolite levels drove to false positive or false negative prediction of pregnancy (Table 3).

Discussion

This is the first study in which a pregnancy predictive model is developed using non-invasive methods in female guanacos. To develop this model, we used hormone fecal metabolites as indicators of pregnancy status. Our results indicated that EC and PdG levels were higher in pregnant than in non-pregnant females of guanacos during late gestation (i.e: 9 or 10 months of gestation). As a consequence, they are good indicators for pregnancy diagnosis and could be used at a population scale. Since this is the first study that develops the use of EC and PdG in guanaco, it was necessary to corroborate their direct relationship with the circulating hormones, estradiol (E2) and progesterone (P4). Plasma hormone levels and their corresponding fecal metabolite concentrations showed a strong association supporting the use of these metabolites as indicators of hormone fluctuations (Table 1, Figure 5).

Previous studies in guanaco showing the variation of E2 levels during the reproductive cycle indicated that E2 levels in pregnant females were twice higher than in unmated females (Riveros et al. 2009; Riveros et al. 2010). In accordance with those results, we found that mean E2 levels were threefold higher in pregnant compared with non-pregnant females. However, we should consider that the secretory profile of E2 also reflects the follicular waves (Riveros et al. 2010) and thus, the determination of E2 alone is a weak indicator of pregnancy status (Figure 2). On the other hand, P4 levels remain low during follicular recruitment, increase after mating and continue rising until parturition (Riveros et al. 2009; Riveros et al. 2010). Hence, the use of P4 or the combination of E2 and P4 are better indicators of pregnancy state in this species. The same patterns were also observed in several other species (Schwarzenberger et al. 1996; Schwarzenberger et al. 1997; Morden et al. 2011; Mastromonaco et al. 2015; Nagl et al. 2015; Ahuja-Aguirre et al. 2017).

The data reported in this study clearly demonstrated that the polyclonal antisera and sexual hormones metabolites assays used here can detect variations in the metabolites excreted via feces in guanacos and give us the possibility of non-invasive hormone monitoring of female reproductive status, the variation of fecal metabolites throughout the year during gestation and on immature females avoiding physical restraint of guanacos.

Estimating pregnancy rates is a useful parameter for the study and management of wild ungulate populations that are rarely considered in demographic models (Cain et al. 2012). Indeed, the use of capture methods to assess pregnancy could cause stress and damage to the females and the fetus (Carmanchahi et al. 2011; Carmanchahi and Marull 2012). In addition, non-invasive methods allow to collect samples during the whole year, are easy to perform, an average value of hormonal concentration is obtained avoiding bias caused by daytime fluctuations and samples can be easily stored at -20ºC for a long term without degradation (Schwarzenberger and Brown 2013; Kersey and Dehnhard 2014;). Thus, the non-invasive approach proposed here will be useful to assess the state of wild guanaco populations as well as to take management decisions in protected areas, minimizing disturbance to animals, increasing safety for both animal and researchers, and avoiding potential data bias due to animals handling (Pauli et al. 2009; Kersey and Dehnhard 2014; Valenzuela-Molina et al. 2018).

Our pregnancy predictive model based on the measurement of two different sexual hormone metabolites will facilitate the work of wildlife managers by providing an effective and useful method for estimating pregnancy in free-ranging guanacos. Due to the easiness of computational processing of logistic regression models, a large number of samples can be evaluated instead of comparing each sample hormonal concentration with references values. In addition, this method makes easier to extrapolate individual data to large

ungulates populations. While our model is directly applicable to guanaco, its approach could be considered for other mammals species. This procedure will facilitate the prediction of pregnancy at large scale and long-term studies, and will simplify its inclusion in population dynamics researches.

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Figure Captions

Figure 1: Box plot showing differences between pregnant and non-pregnant females of *Lama guanicoe* (Müller, 1776) in plasma hormones concentrations. (a):P4; (b):E2.

Figure 2: Displacement parallels curves (a) EC, (b) PdG of *Lama guanicoe* (Müller, 1776).

Figure 3: Recovery of exogenous hormone from fecal extracts pool compared to amount added of the corresponding hormone of *Lama guanicoe* (Müller, 1776). (a) PdG, (b) EC.

Figure 4: Box plot showing differences between pregnant and non-pregnant *Lama guanicoe* (Müller, 1776) females in fecal metabolites concentrations. (a) PdG, (b) EC.

Figure 5: Linear relationship between plasma hormones and their fecal metabolites of *Lama guanicoe* (Müller, 1776). (a) PdG vs. P4, (b) EC vs. P2.

Figure 6: Assigned probabilities to the logistic regression model. Logit p according to the model fitted vs. *Lama guanicoe* (Müller, 1776) probability of pregnancy predicted by the model. Cut off probability: 0.4. Different kind of circles represent field diagnosis (filled circles: pregnant; open circles: non-pregnant).