Reproductive Analysis of Male and Female Captive Jaguars (*Panthera onca*) in a Colombian
Zoological Park

Santiago Jimenez Gonzalez,^{a,1} Jo Gayle Howard,^b Janine Brown,^b Henry Grajales,^a Jorge Pinzón,^a
Haydy Monsalve,^c María Angélica Moreno,^c Claudia Jimenez Escobar^a

Abstract

A reproductive analysis of a captive group of jaguars (*Panthera onca*) ($n = 6$) at the Santacruz Zoological Foundation in Cundinamarca, Colombia, was conducted by performing a longitudinal, noninvasive, hormonal analysis of estradiol and progestogens in females and of androgens in males. During four seasons, female jaguars confined in solitary were evaluated for ovarian activity and spontaneous ovulation, male jaguars for testicular activity. A second hormonal follow-up was conducted in the females after administration of gonadotropins. Hormones were extracted from fecal samples of three females ($n = 3$) and two males ($n = 2$). Estradiol measurements were obtained by radioimmunoassay (RIA) and progestogens by enzyme immunoassay (EIA). The linear mixed-effect regression (LMER) showed that there was a significant effect of seasons in the concentrations of estradiol (Chi square = 15.97, DF = 3, $p < 0.01$). Post hoc comparisons of all pairs of seasonal means were conducted according to Tukey's honest significant difference (HSD), revealing significant differences between seasons: Dry 1 vs. Rains 2 ($p < 0.01$), Rains 1 vs. Rains 2 ($p < 0.05$), and Dry 2 vs. Rains 2 ($p < 0.05$). Elevations of progestogens compatible with spontaneous ovulation occurred in three jaguars and the LMER showed that there was also a significant effect of seasons (Chi square = 28.56, DF = 3, $p < 0.01$). Post hoc comparisons showed significant differences only between seasons: Dry 2 vs. Rains 2 ($p < 0.01$). The season with the lowest average concentration was Rains 2 (October, November, and December). During this season, periods of anestrus were registered that lasted between 31 and 83 days. The three females presented estradiol peaks after the administration of eCG. A noninvasive longitudinal analysis for androgens was also made (Males 1 and 2) over the course of one year, and no significant differences were found between the different seasons. A seminal analysis of three adult male jaguars (*Panthera onca*) ($n = 3$) was also performed after electro-ejaculation under general anesthesia (Male 1 and 2) and by a postmortem epididymal wash (Male 3). The mean concentration of spermatozooids was $5.7 \times 10^6 \pm 1.1 \times 10^6$ spermatozoa/ml. The progressive motility + SD averaged 80 %. The percentage of normal spermatozooids obtained by electro-ejaculation was $80 \pm 2.8\%$, and the abnormalities found more frequently were head defects ($7 \pm 1.4\%$). The seminal fluid obtained by epididymal flush contained $35 \pm 1.4\%$ normal spermatozooids, and the most frequent abnormalities found corresponded to distal cytoplasmic droplets ($39 \pm 11.3\%$).

Key words: Jaguar, estradiol, progesterone, testosterone, spermatozoa, endocrinology

1.1 Introduction

The jaguar (*Panthera onca*) is the largest felid in the Americas. It is an emblematic animal for different cultures and an umbrella species that when protected provides protection to entire ecosystems. The jaguar is one of the most important top predators of Central and South America and has maintained the balance of tropical rain forest ecosystems for nearly three million years [1]. Nevertheless, this species has been classified as “near threatened” on the International Union for Conservation of Nature (IUCN) red list due to habitat reduction and fragmentation, illegal trafficking, and indiscriminate hunting [2]. Knowledge of the reproductive patterns of jaguars is a critical factor for predicting both the sustainability of their wild populations and the viability of reproductive programs in captivity [3].

Previous reproductive studies have focused on analyzing the seminal characteristics of male jaguars in Colombia [6], the analysis of the sperm production [10-12], and the interaction between endocrine and seminal level of jaguars in Brazil [7-9]. In female jaguars the estrous and ovarian activity was reported by Wildt et al. (1979) [4]. A more recent study by Barnes et al. (2015) characterized and manipulated the reproductive cycles in female jaguars showing spontaneous ovulation in this species, and multiple estrous cycles throughout the year and no notable seasonality. [5]. The hormonal characterization of the reproductive cycles of females has been conducted using noninvasive longitudinal monitoring techniques in feces of different species of felids in captivity, including the jaguar [4, 5], lions (*Panthera leo*), tigers (*Panthera tigris*), caracals (*Caracal caracal*), cheetahs (*Acinonyx jubatus*), and domestic cats (*Felis catus*) [13] to evaluate hypophysis-gonad function in lions roaming free in the Serengeti savannah and in the crater of the Ngorongoro [14, 15]. These noninvasive techniques permit learning about the physiological reproductive condition of animals found in zoological collections, with minimal human intervention, thus lowering animal stress and maintaining their well-being [16]. Some studies have determined the comparative aspects of hormonal metabolism and ovarian activity in leopard cats (*Felis bengalensis*), cheetahs, clouded leopards (*Neofelis nebulosa*) and snow leopards (*Uncia uncia*) [16].

Techniques such as radioimmunoassay (RIA) and enzyme immunoassay (EIA) allows the detection of hormonal metabolites. Its use has been successful in other felids such as tigers, lions, and caracals [13]; snow leopards [17]; clouded leopards [18] [19]; cheetahs [20]; ocelots (*Leopardus pardalis*) [21], margays (*Leopardus wieddi*) [21, 22], and oncilla (*Leopardus tigrinus*) [21] and jaguars [5, 8, 9]. Other studies have been conducted to evaluate the action of equine chorionic gonadotropin (eCG) as a promoter of follicular growth and human chorionic gonadotropin (hCG) as

an inducer of ovulation in jaguars [5], clouded leopards [18, 19], domestic cats [13, 23-25], ocelots [26], cheetahs [19], snow leopards [27], and tigers [28, 29].

We conducted a one-year, noninvasive analysis through longitudinal hormonal monitoring for the reproductive hormones estradiol and progesterone in females and testosterone in males. We evaluated the action of equine chorionic gonadotropin (eCG) as a promoter of follicular growth and human chorionic gonadotropin (hCG) as an inducer of ovulation in jaguars. In addition, a seminal analysis was conducted, obtaining samples through electro-ejaculation protocols [30] and epididymal flush [31]. The objectives of this study were (1) to determine whether jaguars present seasonal changes in their reproductive hormonal activity, (2) to determine whether jaguars ovulate spontaneously, (3) to determine whether the species responds to the administration of exogenous gonadotropins, and (4) to describe seminal characteristics in captive male jaguars.

1.2 Materials and methods

1.2.1 Animals

Reproductive characterization was conducted with a group of jaguars in captivity ($n = 6$) composed of three females ($n = 3$) and three males ($n = 3$) for a one year period at the Fundación Zoológico Santacruz (Santacruz Zoo Foundation) in the San Antonio del Tequendama municipality in the department of Cundinamarca, Colombia (latitude $4^{\circ}35'0.65''\text{N}$, longitude $74^{\circ}20'1.25''\text{W}$).

According to the Jaguar Regional Studbook for Colombia, Females 1 and 3 were born in captivity and Female 2 was born in the wild. Age range of the females was from 10 to 15 years. The weight average was 45.6 ± 2.5 (Female 1 = 46 kg, Female 2 = 43 kg and Female 3 = 48 kg). Male 1 was born in captivity, Male 2 in the wild and the origin of Male 3 is unknown. The age range of the males was found to be between 10 and more than 20 years, with a weight average of 53 ± 2.6 (Male 1 = 54 kg, Male 2 = 55 kg, Male 3 = 50 kg). All of the animals included in this study were in good health as well as in good physical condition. The jaguars were kept in individual confinement, with a separation den, tree trunks, and a bed to enrich the environment. All confinement spaces were outdoors. Due to the equatorial location, there are 12 hours of daylight with no seasonal variation throughout the year. The animals had access to fresh water *ad libitum* and were fed five times per week with horse or cow meat, bones, and other organs. All the protocols were reviewed and approved by the bioethics committee of the veterinary medicine department of the National

University of Colombia. During the study, the oldest male (Male 3) died from natural causes and was excluded from the hormonal analysis but included in the seminal analysis.

1.2.2 Sample processing

Fecal samples were collected for a year from feces found in the enclosures of each jaguar; from two to seven samples (2 to 4 gr) per week were collected, depending on the frequency excreted. Plastic containers were employed for the collection of samples. Samples were frozen and stored at -20°C until the time of their processing. Fecal steroids were extracted from complete wet samples ($n = 204$) for males and ($n = 411$) for females, following the protocol described by Brown et al., 1994. Samples of 0.5 gr of feces were mixed by vortex with 5 ml of 90% ethanol for 3 minutes and then agitated in a Shaker Orbital-Thermo® for 3 hours at 37°C and then centrifuged in a Beckman Model J2-21 centrifuge at 3,000 rpm for 20 minutes. Supernatants were collected, dried, and suspended in 1 ml of methanol. A parallelism test of extracts of the feces was conducted, using a buffer (0.01 M PO_4 , 0.14 M NaCl, and 0.01% sodium azide Ph 7.0) to determine the proper dilutions for each immunoassay.

1.2.3 Analysis of females

Ovarian activity

The mean concentrations of estradiol and progesterone in the three female jaguars ($n = 3$) were compared to determine the seasonality of ovarian activity and to determine the presence of spontaneous ovulation through the presentation of non-pregnant luteal activity in four climatological seasons within the year as follows: (Dry 1: January, February, March; Rains 1: April, May, June; Dry 2: July, August, September; Rains 2: October, November, December) [32].

Induced ovarian activity

To induce ovarian activity, 300 IU of equine chorionic gonadotropin (eCG) (Folligon®) was administered intramuscularly (im), followed by 225 IU (im) of human chorionic gonadotropin (hCG; Chorulon®) 80 hours later. A daily noninvasive follow-up of hormones (estradiol and progesterone) was conducted from feces during a two-month period, as previously described, with the objective of assessing luteal activity [25].

Estradiol

Measurement of 17 β estradiol was conducted through radioimmunoassay, employing an ImmuChemTM Estradiol-17 β ¹²⁵I RIA kit, (ICN Pharmaceuticals, Inc., Costa Mesa, California, USA) (estradiol specificity 100%, sensitivity 95%. Cross reactivity estrone 6.2%, estriol 1.45%). To process the samples, a Beckman 5500 gamma counter was used. Data were processed using RIA PC software. The inter-assay coefficient of variation was 5%. The intra-assay coefficient of variation was < 10%, and the dilution used based on the parallelism test was 1:16.

Progesterone

Measurement of progesterone was conducted using an EIA-Steroid-Progesterone kit (DSI Italy) (progesterone specificity 100%, sensitivity 0.5 nmol/l. Cross reactivity 17-OH-progesterone 2.9%). Incubation was conducted in a Stat Fax[®] Incubator; for washing, a Stat Fax 2600 Microplate Washer was used, and the reading was done with an Agra Quant Stat Fax[®] EIA Reader. The inter-assay coefficient of variation was 6.3%, the intra-assay coefficient of variation was <10%, and the dilution used based on the parallelism test was 1:100.

1.2.4 Analysis of males

Testicular hormonal activity

Mean concentrations of testosterone were compared to determine patterns of seasonality in Males 1 and 2. The mean concentrations of testosterone were compared in four seasons: (Rainy 1: April, May, June; Dry 2: July, August, September; Rainy 2: October, November, December; Dry 1: January, February, March) [32]. Measurement was conducted using an EIA-Steroid-Testosterone kit (DSI Italy). Incubation was conducted in a Stat Fax[®] Incubator. For the wash, the Stat Fax 2600 Microplate Washer was used, and the reading was done with an Agra Quant Stat Fax[®] EIA Reader. Testosterone specificity 100%, sensitivity 0.2 nmol/l. Cross reactivity dihydrotestosterone 4.8%, androstenedione 3.6%). The intra- and inter-assay coefficient of variation was < 10%, and the dilution used based on the parallelism test for testosterone was 1:16.

Seminal analysis

Seminal analysis from three male jaguars ($n = 3$) was conducted by means of electro-ejaculation in Males 1 and 2 and through an epididymal sperm flush *post mortem* in Male 3.

Anesthesia and electro-ejaculation

Males 1 and 2 were premedicated, using xylazine (AnaSed, 0.4 mg/kg, im), and the anesthesia was induced with ketamine hydrochloride (Imalgene, 4 mg/kg, im) [33]; maintenance was conducted with isoflurane (2% to 3%). A rectal probe (16 cm $\times$ 3 cm) and an electro-stimulator (Pulsator IV Auto AdjustTM Lane Mfg. Inc., Denver, CO) were used to collect the semen samples. The sequence regime for electro-ejaculation consisted of 80 stimuli divided into three series; the first and second series consisted of 30 stimuli, the third one of 20 stimuli, each of which fluctuated between 4 and 6 volts [30, 34]. Samples were collected in 2 ml Eppendorf sterile tubes. The testicular volume was calculated with the formula $4/3\pi ABC$, where A = half the thickness, B = half the width, C = half the length [12].

Epididymal flush

A postmortem seminal analysis was carried out through an epididymal flush in Male 3. The procedure was conducted according to that described by Spindler et al. (1999). The body and the tail of the epididymis were preserved. The tail was placed inside an Eppendorf tube containing 0.5 ml of Ham F10-supplemented media with 1 nM pyruvate, 2 mM glutamine, and 100 IU of penicillin/streptomycin. Incubation was conducted at 38 °C in 5% CO₂. After 30 minutes, the epididymal tissue was discarded, and the tube was centrifuged at 300 g for 8 minutes. The supernatant was removed, and the pellet was suspended in 50 μ l of Ham F10 media.

Seminal assessment

To determine sperm vitality, an eosin nigrosine stain was performed. Concentration of sperm cells was determined in a Neubauer camera in a 1:200 dilution of distilled water. Morphology was determined in 100 cell counts. Mobility and progression rate were estimated subjectively with the aid of a light microscope at X100 and was expressed in a percentage of mobile spermatozooids [35].

1.3 Statistical analysis

1.3.1 Experimental design

The data obtained from hormonal monitoring were analyzed using linear mixed-effect regression (LMER) with the lme4 package [36] in the R statistical software environment [37]. Using this approach, we controlled for the influence of different mean ratings between animals without the need for data aggregation, as is the case in a traditional, repeated-measures analysis of variance (ANOVA). Season (i.e., Dry 1, Rainy 1, etc.) was entered in the model as a fixed effect. Jaguars (i.e., Female 1, Female 2, Female 3), on the other hand, were entered into the model as a random effect. Given that it is desirable to generalize the results beyond this sample and that variance due to jaguar was expected to be idiosyncratic, analysis according to LMER is appropriate to account for such variability. Significance testing was performed by Likelihood Ratio Tests of the full model, including the fixed effect of season, against a null model that did not include season. Post hoc pairwise comparisons of mean hormonal concentrations between seasons were conducted according to Tukey's HSD to control for Type I error rates. The full model was estimated as

$$y_{ij} = \beta_0 + \beta_{1j}Season_j + v_j + \varepsilon_{ij}$$

where:

y_{ij} is the concentration of the hormone measured in season j of i jaguar;

β_0 is the general mean;

β_{1j} is the estimate of the fixed effect of season j ;

v_j is the variance of the random intercepts, $v_j \sim \text{NID}(0, \tau_{00}^2)$; and

ε_{ij} is the random error among jaguars and $\varepsilon_{ij} \sim \text{NID}(0, \sigma^2)$.

1.3.2 Descriptive statistics

The peak values were considered significant for elevations above the baseline +2 standard deviation (SD). The baseline values for the three hormones for each jaguar were calculated through an iterative process where high values that exceeded the mean +2 SD were excluded and removed temporarily from the database; the average of the remaining values was re-calculated, and the process of data removal was repeated until no value would exceed the mean +2 SD [25, 29, 38]. The remaining hormonal values were considered baseline concentrations. Progesterone peaks higher

than the mean +2 SDs were considered significant and, in conjunction with other elevations above baseline concentrations, was interpreted as luteal activity. Estradiol peaks higher than the mean +2 SD was considered significant, and other elevations of estradiol above baseline concentrations (associated with estrous behavior) were interpreted as follicular activity.

The interval in days between estradiol with no luteal activity was calculated and interpreted as anovulatory cycles. Intervals between estradiol peaks with luteal activity were considered ovulatory cycles. In addition, for males, the variables analyzed for spermatid morphology were: normal spermatozoa, detached heads, proximal coiled tails, distal coiled tails, proximal cytoplasmic droplets, distal cytoplasmic droplets, and head defects [39].

1.4 Results

1.4.1 Female analysis

Natural ovarian activity

For estradiol the mean baseline concentrations found was 1.35 ± 0.74 ng/gr (Female 1 = 1.08 ng/gr, Female 2 = 0.79 ng/gr, and Female 3 = 2.19 ng/gr). The total range was 0.13 to 313.80 ng/gr. The mean concentration of natural peaks for Female 1 was 28.13 ± 34.04 ng/gr, for Female 2 was 23.04 ± 38.15 ng/gr, and for Female 3 was 74.39 ± 72.17 ng/gr.

In the three females, non-pregnant luteal activity (spontaneous ovulation) was evidenced by the increase in peaks of progesterone. For progestogens the mean baseline concentrations was 17.76 ± 6.03 ng/gr (Female 1 = 15 ng/gr, Female 2 = 24.68 ng/gr, and Female 3 = 13.60 ng/gr). The mean concentration of natural peaks of progestogens for female 1 was 49.19 ± 19.77 ng/gr, for female 2 was 87.55 ± 27.71 ng/gr, and for female 3 was 77.73 ± 30.90 ng/gr. Non-pregnant luteal phases ranged between 14 and 67 days. Anestrous periods ranged between 31 and 83 days (Figure 1).

Induced ovarian activity

All animals responded to the administration of eCG as a promotor of follicular activity. The effect of gonadotropins was determined in the animals by the behavioral changes related to estrous (e.g., vocalizations, lordosis postures, etc.), which were registered on the days subsequent to the administration of gonadotropins. Estradiol peaks took place 7.30 ± 0.58 days after the application of the eCG, the mean concentration of induced peaks of estradiol $\pm$ SD for female 1 was 93.17 ± 129.02 ng/gr, for female 2 was 76.04 ± 144.65 ng/gr, and for female 3 was 94.48 ± 145.59 ng/gr. The average length of estrous was 2.67 ± 0.57 days. The highest peak reached during stimulation

with eCG for Female 1 was 534.6 ng/g, for Female 2 was 737.1 ng/g, and for Female 3 was 699.9 ng/g; the total average found was $655.2 \text{ ng/g} \pm 105.6$ (Figure 1).

The mean concentration of progestogens found during the trial using gonadotropins for female 1 was $48.10 \pm 16.82 \text{ ng/gr}$, for female 2 was $88.03 \pm 16.77 \text{ ng/gr}$, and for female 3 was $58.57 \pm 30.35 \text{ ng/gr}$. In the three females, luteal activity was found before and after the administration of gonadotropins; In Female 1 before the administration of the gonadotropins a peak of estradiol was observed followed by luteal activity with progestogen peaks that exceeded the mean $+2\text{SD}$, suggesting that possible spontaneous ovulation occurred not triggered by the gonadotropin protocol but for a possible resumption of activity after a period of inactivity considered an anestrus phase.

Seasonality

The mean concentration of estradiol ($\text{ng/g} \pm \text{SEM}$) for the season Dry 1 was 38.49 ± 8.70 , for Rains 1 was 29.60 ± 6.44 , for Dry 2 was 29.97 ± 6.30 , and for Rains 2 was 8.18 ± 2.19 . The LMER showed that there was a significant effect of seasons (Chi square = 15.97, $\text{DF} = 3$, $p < 0.01$). Post hoc comparisons of all pairs of seasonal means was conducted according to Tukey's HSD, revealing significant differences between seasons: Dry 1 vs. Rains 2 ($p < 0.01$), Rains 1 vs. Rains 2 ($p < 0.05$), and Dry 2 vs. Rains 2 ($p < 0.05$). The season with the lowest average concentration was Rains 2 (October, November, and December). The mean concentration of progestogens ($\text{ng/g} \pm \text{SEM}$) for the season Dry 1 was 39.22 ± 6.48 , for Rains 1 was 40.49 ± 5.06 , for Dry 2 was 52.38 ± 3.95 , and for Rains 2 was 25.39 ± 2.30 . The LMER showed that there was a significant effect of seasons (Chi square = 28.56, $\text{DF} = 3$, $p < 0.01$). Post hoc comparisons of all pairs of seasonal means were conducted according to Tukey's HSD, revealing significant differences between seasons: Dry 2 vs. Rains 2 ($p < 0.01$). No significant differences were found between other pairs of seasons. The season with the lowest average concentration was Rains 2 (October, November, and December) (Figure 2).

1.4.2 Male analysis

Testicular activity

Mean baseline concentrations of testosterone for Male 1 were $6.3 \pm 0.9 \text{ ng/g}$ with a 4.0 to 59.3 ng/g range. For Male 2, mean baseline concentrations of testosterone were $6.30 \pm 0.57 \text{ ng/g}$; the range was 5.2 to 21.8 ng/g. Average values found for testosterone concentrations within a one-year period for the two jaguars were $8.6 \pm 5.7 \text{ ng/g}$. In Male 1, the levels of testosterone remained below baseline levels during the months of September and October; then increased over baseline levels for

two weeks with another period of inactivity between the months of November and December, corresponding to the second rainy season (Rains 2). No significant differences were found between the mean concentrations of testosterone for each of the different seasons.

Seminal analysis

Ejaculations from Male 1 and from Male 2 were obtained through electro-ejaculation and from Male 3 through an epididymal flush, with one sample for each animal. The volume obtained was between 1.5 and 2.5 ml; the mean concentration of spermatozoids was $5.7 \times 10^6 \pm 1.1 \times 10^6$ spermatozoa per ml. As for the percentage of progressive motility of the two semen samples obtained, an average of 80% was found.

The percentage of normal spermatozoids from the samples obtained by electro-ejaculation was $80 \pm 2.8\%$. Abnormalities most frequently found corresponded to head defects $7 \pm 1.4\%$, distal coiled tails $4.5 \pm 4.9\%$, proximal coiled tails $3.5 \pm 2.1\%$, detached heads $2.5 \pm 0.7\%$, proximal cytoplasmic droplets $1.5 \pm 0.7\%$, and distal cytoplasmic droplets $1 \pm 1.4\%$.

Epididymal flush

The seminal fluid obtained by epididymal flush contained $35 \pm 1.4\%$ normal spermatozoids. Most frequent abnormalities found corresponded to distal cytoplasmic droplets $39 \pm 11.3\%$, distal coiled tails 7%, proximal coiled tails $5 \pm 3.5\%$, proximal cytoplasmic droplets $5.5 \pm 4.9\%$, detached heads $3.5 \pm 2.1\%$, and head defects $1 \pm 1.4\%$.

Discussion

To understand the reproductive physiology of the jaguar, we performed a series of analyses to determine whether jaguars present seasonal changes in their reproductive hormonal activity, if ovulation could be spontaneous without the presence of the male, to determine whether the species responds to the administration of exogenous gonadotropins, and to describe seminal characteristics in male jaguars.

This is the first study that describes seasonal differences for both estradiol and progesterone through the year of 2010 in female jaguars and contrast with Barnes et al. (2015) where no seasonal patterns were detected. Data obtained from the noninvasive analysis of female jaguars' reproductive hormones revealed significant differences between the seasons; the lowest ovarian activity was observed between October and December. During this season, artificially induced ovarian activity

would be more likely to succeed because the ovaries are in a resting phase. Between January and March, the ovarian activity is moderate, and the highest activity occurred between April and September, this season being most favorable for natural controlled reproductions if the population management plan for the species in captivity requires it.

The other striking finding was the presence of an anestrus period in the three females studied; this anestrus period was present in the fourth season, also between October and December. During this period, a decrease in peak tendency was observed. Other researchers have found that despite the fact that in tropical conditions the photoperiod is constant, Johnston et al. (1994) found that the anestrus period is a very important component in the annual reproductive cycles and is affected by seasons more than the relation of light hours during the day. In countries with seasons, anestrus has been reported in several species of felids such as tigers [29] and cheetahs [20] during winter (November to January). Data in this study also suggests a seasonal pattern for jaguars in tropical environments and that hormonal variations mostly depend on idiosyncratic aspects of each jaguar.

In addition, we found evidence of luteal activity during the year of study. The concentration of progesterone found in EIA tests confirmed the presentation of non-pregnant luteal activity for the three females confined alone, suggesting that spontaneous ovulations occurred in this way, it was proven that female jaguars may present multiple spontaneous ovulations during a year, similar to the findings that Barnes et al. (2015) reported in their study of female jaguars. The results of this study contrast with the results published by Wildt et al. (1979), where no spontaneous ovulations were detected in a female jaguar, it was reported that ovulations were triggered only by the administration of hCG or the luteinizing hormone (LH). Due to these findings, we can infer that female jaguars can present estrus cycles either with or without spontaneous ovulations that correspond to ovulatory or anovulatory cycles, respectively. Spontaneous ovulations have been also reported in studies conducted in clouded leopards [18, 19].

We also found evidence of ovarian activity during the protocols with gonadotropins. Estradiol peaks were found after the administration of eCG to the three females, suggesting follicular growth and activity. Longitudinal analysis by RIA showed that changes in fecal concentrations of estradiol were related with estrus presentation or after the administration of exogenous gonadotropins. This study proved the efficacy of eCG as follicular growth agent in the three females, evidenced in the presentation of estradiol peaks. We were able to induce follicular activity at a lower dose (300 IU) than the one reported by Barnes et al. (2015) (600 IU). With this new information we can conclude that a range between 300 and 600 IU of eCG is effective to promote follicular development in female jaguars. It is important to consider that the doses to induce follicular development and

ovulation are specific for each species; for example, in ocelots, the doses used were higher relative to other species [26]. Results found in clouded leopards [19] and Siberian tigers (*Panthera tigris altaica*) [28] showed that eCG worked as an analogue of the FSH as a growth promoter and follicular developer similar to our findings and the reports of Barnes et al. (2015) in jaguars. As for ovulation induction, it was not clear if the females responded to hCG as an analogue of the LH as demonstrated in studies in other felid species including the jaguar [5, 16, 18-20, 24, 26-29]. It was not fully determined because progesterone peaks were found before the administration of the gonadotropins. It was unclear whether the progestogens elevation found corresponded to a previous natural spontaneous ovulation or the action of the gonadotropin. To avoid this possibility, other researchers had used an inhibitor of ovarian activity such as a progestin implant at least two weeks before starting a protocol (Howard personal communication, 2009). After removing the implant, progesterone levels will decrease, and the ovaries will be prepared for the action of the follicular stimulant hormone (FSH) or eCG as follicular development promoters. On the other hand, it is important to point out that the effect of cumulative previous progestin protocols may be a predisposing factor to cystic endometrial hyperplasia that could directly affect implantation [35].

In the case of the males, our analysis detected the presence of testosterone metabolites by EIA test, confirming testicular activity in lower levels than those detected by RIA in other studies reported for jaguars in Brazil [8, 9]. The results of the seasonality analysis of the males' testosterone were similar to the results found in Brazil through RIA. In both studies, no significant differences ($p > 0.05$) in endocrine-testicular function during seasons were found. However, Morato et al. (2004) reported that fecal androgen concentration differed ($p < 0.05$) between dry and wet seasons. To understand this difference, more research is suggested, preferably including more individuals in future investigations.

The seminal evaluation results contrast with the findings in the other trials in Colombian captive jaguars [6] and in the Brazilian as well [7]. In this study, seminal volume obtained through electro-ejaculation and the average concentrations of sperm per ml were lower than the values reported for the species by Oyuela et al. (2004) and by Morato et al. (2001). Higher percentage levels in progressive motility 80% were found when compared to other reported values such as $48 \pm 23\%$ [6], $64 \pm 2.4\%$ [7], and $57.0 \pm 4.5\%$ [9]. A lower percentage in sperm abnormalities were also found: 20 ± 2.8 vs 38.9 ± 12 [6] and 30 ± 0.9 [7]. This low abnormality percentage contrasts with high values of pleiomorphisms found in other felid species such as cheetahs [39-41], clouded leopards [34, 42], snow leopards [43-45], bobcats (*Lynx rufus*) [46], ocelots, margays (*Leopardus wiedi*), and oncillas (*Leopardus tigrina*) [21, 47]. It was also observed that the epididymal flush of Male 3 presented the

highest levels of abnormalities, with distal cytoplasmic droplets the most frequent abnormality. This secondary abnormality [35] has been previously reported in analyses of sperm obtained of domestic cats' epididymis and has been associated with their sperm aging process observed in serial ejaculates, in which the presence of distal cytoplasmic droplets was significantly reduced in the second ejaculate, 5 to 10 min after the first [48]. However, similar assays on cheetahs, when the seminal test was performed at a 48-hour interval, did not show differences in the sperm abnormality proportion, which suggests the existence of other factors that may directly affect the sperm morphology related to the genetic diversity levels present in the *Acinonyxinae* family [49]. Based on this, we can infer that the high level of normal spermatozoa found in jaguars is a good indication of their genetic diversity.

As a summary, the present study established a baseline knowledge of jaguars' physiology of reproduction as the pillars of a reproductive management program based on population management and genetic analysis. The results of this study open doors to future investigations in multiple fields such as artificial insemination, in vitro fertilization, embryo transfers, and gamete cryopreservation. Studies of this nature can facilitate and contribute to the maintenance of genetic variability of captive populations while decreasing movement of live animals between institutions. The success of these techniques presents a serious alternative to ex situ conservation for jaguars.

1.5 Conclusions

This study, conducted in a Colombian zoological park, found that female jaguars presented seasonal differences in ovarian activity during the year of 2010.

During the year of this study, multiple non-pregnant luteal phases were found in female jaguars isolated from males, suggesting that multiple spontaneous ovulations occurred.

The females responded to the administration of eCG as a follicular promoter and it was unclear if ovulation was triggered by the action of hCG.

The anesthetic and electro-ejaculation protocols worked properly to obtain the seminal samples from captive jaguars and may be used for the establishment of gamete banks.

Testicular activity occurs regularly during the year, and low levels of abnormal spermatozoa were found compared with other felid species.

1.6 Acknowledgments

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413 **References**

- 414 [1] Johnson WE, Eizirik E, Pecon-Slattery J, Murphy WJ, Antunes A, Teeling E, et al. The late
415 Miocene radiation of modern Felidae: a genetic assessment. *Science*. 2006;311:73-7.
- 416 [2] IUCN [International Union for Conservation of Nature and Natural Resources]. IUCN Red List
417 of Threatened Species. IUCN G, Switzerland. IUCN official website (www.redlist.org) 2011.
- 418 [3] Comizzoli P, Crosier AE, Songsasen N, Gunther MS, Howard JG, Wildt DE. Advances in
419 reproductive science for wild carnivore conservation. *Reprod Domest Anim*. 2009;44 Suppl 2:47-
420 52.
- 421 [4] Wildt DE, Platz CC, Chakraborty PK, Seager SW. Oestrous and ovarian activity in a female
422 jaguar (*Panthera onca*). *J Reprod Fertil*. 1979;56:555-8.
- 423 [5] Barnes SA, Andrew Teare J, Staaden S, Metrione L, Penfold LM. Characterization and
424 manipulation of reproductive cycles in the jaguar (*Panthera onca*). *Gen Comp Endocrinol*.
425 2016;225:95-103.
- 426 [6] Oyuela A KP, Gaviria M, Arias L. Recolección, Evaluación y Criopreservación del Semen de
427 Jaguares (*Panthera onca*). Tesis, Facultad de Medicina Veterinaria, Universidad de la Salle. 2004.
- 428 [7] Morato RG, Conforti VA, Azevedo FC, Jacomo AT, Silveira L, Sana D, et al. Comparative
429 analyses of semen and endocrine characteristics of free-living versus captive jaguars (*Panthera*
430 *onca*). *Reproduction*. 2001;122:745-51.
- 431 [8] Morato RG, Bueno MG, Malmheister P, Verreschi IT, Barnabe RC. Changes in the fecal
432 concentrations of cortisol and androgen metabolites in captive male jaguars (*Panthera onca*) in
433 response to stress. *Braz J Med Biol Res*. 2004;37:1903-7.
- 434 [9] Morato RG, Verreschi IT, Guimaraes MA, Cassaro K, Pessuti C, Barnabe RC. Seasonal
435 variation in the endocrine-testicular function of captive jaguars (*Panthera onca*). *Theriogenology*.
436 2004;61:1273-81.
- 437 [10] Azevedo MH, Paula TA, Balarini MK, Matta SL, Peixoto JV, Guiao Leite FL, et al.
438 Organization and quantification of the elements in the intertubular space in the adult jaguar testis
439 (*Panthera onca*, LINNAEUS, 1758). *Micron*. 2008;39:1166-70.
- 440 [11] Costa GM, Chiarini-Garcia H, Morato RG, Alvarenga RL, Franca LR. Duration of
441 spermatogenesis and daily sperm production in the jaguar (*Panthera onca*). *Theriogenology*.
442 2008;70:1136-46.
- 443 [12] de Azevedo MH, de Paula TA, Matta SL, Fonseca CC, da Costa EP, Costa DS, et al. Cell
444 population indexes of spermatogenic yield and testicular sperm reserves in adult jaguars (*Panthera*
445 *onca*). *Anim Reprod Sci*. 2010;118:83-8.
- 446 [13] Graham LH, Raeside JI, Goodrowe KL, Liptrap RM. Measurements of faecal oestradiol and
447 progesterone in non-pregnant and pregnant domestic and exotic cats. *J Reprod Fertil Suppl*.
448 1993;47:119-20.
- 449 [14] Brown JL, Bush M, Packer C, Pusey AE, Monfort SL, O'Brien SJ, et al. Developmental
450 changes in pituitary-gonadal function in free-ranging lions (*Panthera leo leo*) of the Serengeti
451 Plains and Ngorongoro Crater. *J Reprod Fertil*. 1991;91:29-40.
- 452 [15] Brown JL, Bush M, Packer C, Pusey AE, Monfort SL, O'Brien SJ, et al. Hormonal
453 characteristics of free-ranging female lions (*Panthera leo*) of the Serengeti Plains and Ngorongoro
454 Crater. *J Reprod Fertil*. 1993;97:107-14.
- 455 [16] Brown JL, Wasser SK, Wildt DE, Graham LH. Comparative aspects of steroid hormone
456 metabolism and ovarian activity in felids, measured noninvasively in feces. *Biol Reprod*.
457 1994;51:776-86.
- 458 [17] Schmidt AM, Hess DL, Schmidt MJ, Lewis CR. Serum concentrations of oestradiol and
459 progesterone and frequency of sexual behaviour during the normal oestrous cycle in the snow
460 leopard (*Panthera uncia*). *J Reprod Fertil*. 1993;98:91-5.

- [18] Brown JL, Wildt DE, Graham LH, Byers AP, Collins L, Barrett S, et al. Natural versus chorionic gonadotropin-induced ovarian responses in the clouded leopard (*Neofelis nebulosa*) assessed by fecal steroid analysis. *Biol Reprod.* 1995;53:93-102.
- [19] Howard JG, Roth TL, Byers AP, Swanson WF, Wildt DE. Sensitivity to exogenous gonadotropins for ovulation induction and laparoscopic artificial insemination in the cheetah and clouded leopard. *Biol Reprod.* 1997;56:1059-68.
- [20] Brown JL, Wildt DE, Wielebnowski N, Goodrowe KL, Graham LH, Wells S, et al. Reproductive activity in captive female cheetahs (*Acinonyx jubatus*) assessed by faecal steroids. *J Reprod Fertil.* 1996;106:337-46.
- [21] Morais RN, Mucciolo RG, Gomes ML, Lacerda O, Moraes W, Moreira N, et al. Seasonal analysis of semen characteristics, serum testosterone and fecal androgens in the ocelot (*Leopardus pardalis*), margay (*L. wiedii*) and tigrina (*L. tigrinus*). *Theriogenology.* 2002;57:2027-41.
- [22] N. M. Reproduction in small female felids: Iowa State University Press; 2001.
- [23] Swanson WF, Horohov DW, Godke RA. Production of exogenous gonadotrophin-neutralizing immunoglobulins in cats after repeated eCG-hCG treatment and relevance for assisted reproduction in felids. *J Reprod Fertil.* 1995;105:35-41.
- [24] Swanson WF, Wolfe BA, Brown JL, Martin-Jimenez T, Riviere JE, Roth TL, et al. Pharmacokinetics and ovarian-stimulatory effects of equine and human chorionic gonadotropins administered singly and in combination in the domestic cat. *Biol Reprod.* 1997;57:295-302.
- [25] Graham LH, Swanson WF, Brown JL. Chorionic gonadotropin administration in domestic cats causes an abnormal endocrine environment that disrupts oviductal embryo transport. *Theriogenology.* 2000;54:1117-31.
- [26] Swanson WF, Howard JG, Roth TL, Brown JL, Alvarado T, Burton M, et al. Responsiveness of ovaries to exogenous gonadotrophins and laparoscopic artificial insemination with frozen-thawed spermatozoa in ocelots (*Felis pardalis*). *J Reprod Fertil.* 1996;106:87-94.
- [27] Roth TL, Armstrong DL, Barrie MT, Wildt DE. Seasonal effects on ovarian responsiveness to exogenous gonadotrophins and successful artificial insemination in the snow leopard (*Uncia uncia*). *Reprod Fertil Dev.* 1997;9:285-95.
- [28] Crichton EG, Bedows E, Miller-Lindholm AK, Baldwin DM, Armstrong DL, Graham LH, et al. Efficacy of porcine gonadotropins for repeated stimulation of ovarian activity for oocyte retrieval and in vitro embryo production and cryopreservation in Siberian tigers (*Panthera tigris altaica*). *Biol Reprod.* 2003;68:105-13.
- [29] Graham LH, Byers AP, Armstrong DL, Loskutoff NM, Swanson WF, Wildt DE, et al. Natural and gonadotropin-induced ovarian activity in tigers (*Panthera tigris*) assessed by fecal steroid analyses. *Gen Comp Endocrinol.* 2006;147:362-70.
- [30] Wildt DE, Bush M, Howard JG, O'Brien SJ, Meltzer D, Van Dyk A, et al. Unique seminal quality in the South African cheetah and a comparative evaluation in the domestic cat. *Biol Reprod.* 1983;29:1019-25.
- [31] Spindler RE, Wildt DE. Circannual variations in intraovarian oocyte but not epididymal sperm quality in the domestic Cat. *Biol Reprod.* 1999;61:188-94.
- [32] IDEAM. Instituto de Hidrología, Meteorología y Estudios Ambientales. Boletín informativo sobre el monitoreo del fenómeno de "el niño y la niña". 2010;No. 7-24.
- [33] Jiménez-González S MW. Manejo y anestesia en cinco especies de félidos en cautiverio en parques zoológicos de Colombia. *Revista UDCA, Actualidad y Divulgación Científica.* 2004;6:73-83.
- [34] Wildt DE, Howard JG, Hall LL, Bush M. Reproductive physiology of the clouded leopard: I. Electroejaculates contain high proportions of pleomorphic spermatozoa throughout the year. *Biol Reprod.* 1986;34:937-47.
- [35] Johnston DJ KM, Olson P. Breeding management, artificial insemination, in vitro fertilization and embryo transfer in the queen. In *Canine and Feline Theriogenology*. Ed. Saunders. Philadelphia. United States. 2001:406-13.

- [36] Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. 2015. 2015;67:48.
- [37] Team RDC. R: A language and environment for statistical computing. R Foundation for Statistical Computing; 2015.
- [38] Santymire RM, Brown JL, Stewart RA, Santymire RC, Wildt DE, Howard J. Reproductive gonadal steroidogenic activity in the fishing cat (*Prionailurus viverrinus*) assessed by fecal steroid analyses. Anim Reprod Sci. 2011;128:60-72.
- [39] Crosier AE, Henghali JN, Howard J, Pukazhenthir BS, Terrell KA, Marker LL, et al. Improved quality of cryopreserved cheetah (*Acinonyx jubatus*) spermatozoa after centrifugation through Accudenz. J Androl. 2009;30:298-308.
- [40] Wildt DE, O'Brien SJ, Howard JG, Caro TM, Roelke ME, Brown JL, et al. Similarity in ejaculate-endocrine characteristics in captive versus free-ranging cheetahs of two subspecies. Biol Reprod. 1987;36:351-60.
- [41] Terrell KA, Wildt DE, Anthony NM, Bavister BD, Leibo SP, Penfold LM, et al. Evidence for compromised metabolic function and limited glucose uptake in spermatozoa from the teratospermic domestic cat (*Felis catus*) and cheetah (*Acinonyx jubatus*). Biol Reprod. 2010;83:833-41.
- [42] Howard JG, Wildt DE. Approaches and efficacy of artificial insemination in felids and mustelids. Theriogenology. 2009;71:130-48.
- [43] Johnston LA, Armstrong DL, Brown JL. Seasonal effects on seminal and endocrine traits in the captive snow leopard (*Panthera uncia*). J Reprod Fertil. 1994;102:229-36.
- [44] Roth TL, Howard JG, Donoghue AM, Swanson WF, Wildt DE. Function and culture requirements of snow leopard (*Panthera uncia*) spermatozoa in vitro. J Reprod Fertil. 1994;101:563-9.
- [45] Roth TL, Swanson WF, Collins D, Burton M, Garell DM, Wildt DE. Snow leopard (*Panthera uncia*) spermatozoa are sensitive to alkaline pH, but motility in vitro is not influenced by protein or energy supplements. J Androl. 1996;17:558-66.
- [46] Gañán N, González R, Sestelo A, Garde JJ, Sánchez I, Aguilar JM, et al. Male reproductive traits, semen cryopreservation, and heterologous in vitro fertilization in the bobcat (*Lynx rufus*). Theriogenology. 2009;72:341-52.
- [47] Swanson WF, Brown JL. International training programs in reproductive sciences for conservation of Latin American felids. Anim Reprod Sci. 2004;82-83:21-34.
- [48] Axner E, Strom B, Linde-Forsberg C. Sperm morphology is better in the second ejaculate than in the first in domestic cats electroejaculated twice during the same period of anesthesia. Theriogenology. 1997;47:929-34.
- [49] Pukazhenthir BS, Neubauer K, Jewgenow K, Howard J, Wildt DE. The impact and potential etiology of teratospermia in the domestic cat and its wild relatives. Theriogenology. 2006;66:112-21.