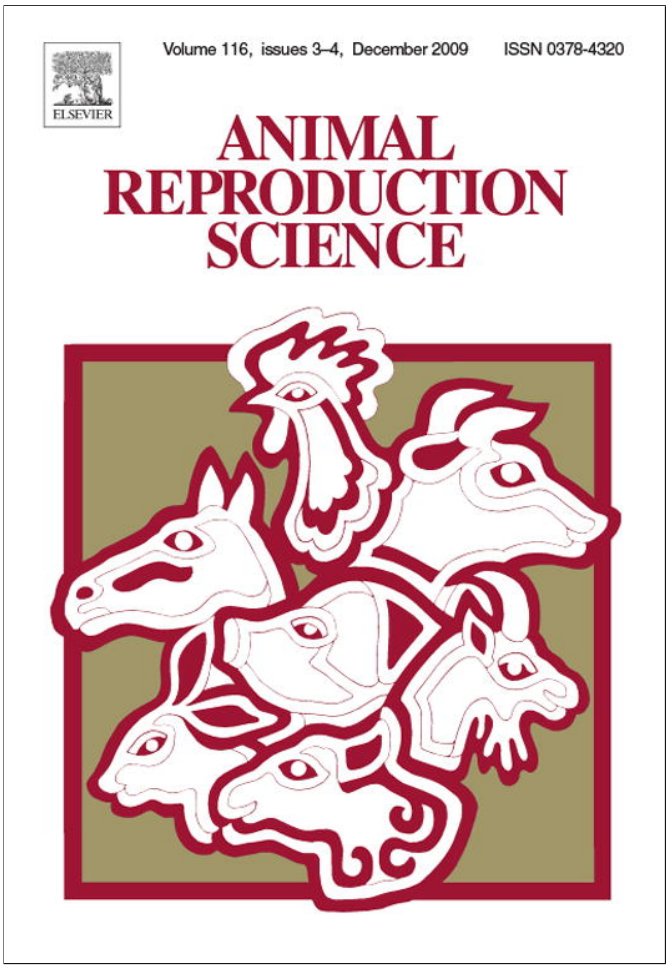

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Characterization of seasonal reproduction patterns in female pichis *Zaedyus pichiy* (Xenarthra: Dasypodidae) estimated by fecal sex steroid metabolites and ovarian histology

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ABSTRACT

Reproductive strategies vary considerably among species, but most studies have focused on a very limited number of mammalian species. Knowledge of the reproductive cycle and behavior is essential for developing and implementing *in situ* and *ex situ* conservation strategies for threatened and endangered species. This study aimed at characterizing the seasonal reproductive pattern of female pichis *Zaedyus pichiy*, a threatened small armadillo native to arid regions of Argentina and Chile, through direct observations, histological studies, and by measuring fecal immunoreactive estrogens, progestagens and glucocorticoids in 10 wild-born, captive pichis and in free-ranging individuals. Results suggest that pichis are seasonal breeders that give birth to one yearly litter of 1–2 offspring, which do not leave the burrow until they are weaned at approximately 37 days. Ovarian follicular growth seems to occur throughout the year. Fecal progestagen, estrogen and glucocorticoid concentrations were minimal during the first half of pregnancy, increased to peak concentrations of up to 3500, 200 and 200 ng/g dry feces, respectively, and decreased before parturition. Postpartum progestagen concentrations were greater in lactating females than females that aborted or did not raise their offspring ($p < 0.0001$), which is probably related to an elevated corticosteroid synthesis that contributes to maintain lactation, given that fecal glucocorticoid concentrations were of similar pattern. Observations of a second pregnancy after

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late abortion or death of the newborn litter and sustained follicular growth during pregnancy and lactation suggest that female pichis can become receptive briefly after having lost their litter. Fecal estrogen and progestagen concentrations of non-pregnant, non-lactating females did not have a well-defined hormonal cyclic pattern, and corpora lutea were only observed in pregnant females.

1. Introduction

The pichi *Zaedyus pichiy* (Xenarthra, Dasypodidae) is a small (approximately 1 kg body mass) armadillo native to arid and semi-arid regions of Argentina and Chile (Meritt and Benirschke, 1973; Wetzel, 1985; Superina, 2008). In Mendoza Province, in central-west Argentina, this species is constantly poached by subsistence and sports hunters. Although no past or recent census data are available, a decline in field observations and reduced sightings reported by people who reside locally suggest that wild populations have suffered considerable declines (Superina, personal observation). Recent studies have shed some light on the natural history of this poorly known species (Superina, 2008). Pichis are opportunistic omnivores that hibernate during winter and can enter torpor (i.e., reduce their metabolism and body temperature during brief periods) outside the hibernation season (Superina and Boily, 2007; Superina et al., 2009). They are seasonal breeders that mate soon after emerging from hibernation (Superina and Jahn, in press) and give birth to 1–3 offspring after a gestation period of approximately 60 days (Redford and Eisenberg, 1992), but no information is available about the steroid hormone concentrations related to reproduction during different stages of the reproductive activity of female pichis. Although serum or plasma are best suited for determining hormonal secretion patterns, serial blood collection in wildlife is usually difficult, very stressful for the animals, and can interfere with subsequent sampling. Determination of fecal hormone metabolites is a non-invasive method that has been widely used to characterize patterns of hormone secretion in wild animals, allowing for frequent collection over extended time spans, and reliable indirect measure of the reproductive status of several wild mammalian species (Czekala et al., 1994; Brown et al., 1997; Schwarzenberger et al., 1999; Graham et al., 2002; Monfort, 2003; Busso et al., 2005; Busso et al., 2007).

The goal of the present research is to characterize the seasonal reproductive pattern in female pichis by determining fecal radioimmunoreactive estrogen, progestagen, and glucocorticoid concentrations. The hormone studies were complemented with direct observations on wild-born, captive individuals and histological studies.

2. Materials and methods

2.1. Animal use

This project was approved by the Institutional Animal Care and Use Committee of the University of New Orleans and the Dirección de Recursos Naturales Renovables of Mendoza Province, Argentina.

2.2. Sample collection

2.2.1. Fecal samples

Ten wild-caught female pichis (Table 1) from southern (36°S, 69°W; $n = 9$) or northern (32.5°S, 68.5°W; $n = 1$) Mendoza Province, Argentina were maintained in individual pens made of wire mesh (2 m × 1.5 m × 2.5 m). Soil to a depth of 2 m provided a natural substrate for digging, and the above ground border of the pens was covered with galvanized sheet metal, 50 cm high, to prevent the pichis from escaping by climbing. The complex of enclosures was located in Luján de Cuyo, Mendoza, Argentina (33.0°S, 68.9°W), within the pichis' native range, to ensure their exposure to natural light cycles and variations in ambient temperature. Adult, wild-born males of unknown reproductive

Table 1
Identification of captive-kept female *Zaedyus pichiy*, age at onset of fecal sample collection, and reproductive success during sampling seasons.

Female	Age at onset of the study	Year	Reproductive success
ZP99	Adult	2004	None
ZP104	Adult	2004	No emerged offspring[a]
ZP108	Adult	2005	Abortion, stress-related
		2006	Birth and lactation 2 females
ZP144	Yearling	2005	Birth and lactation 1 male, 1 female
		2006	Birth and lactation 2 males
ZP150	Yearling	2005	No pregnancy
		2006	Birth and lactation 1 female
ZP152	Yearling	2005	No pregnancy
		2006	Birth and lactation 1 male
ZP153	Yearling	2005	Birth and lactation 1 male
		2006	No emerged offspring[a], followed by birth and lactation 2 females
ZP154	Yearling	2005	Abortion; stung by spider
ZP162	Yearling	2005	No emerged offspring[a]
		2006	Birth and lactation 1 male, 1 female
ZP185	Yearling	2006	Birth and lactation 2 males

[a] Unknown whether these females aborted in the last trimester of gestation or gave birth but failed to raise their young. Fecal hormone concentrations had a pattern indicative of pregnancy followed by a sharp decline that suggested parturition or abortion near term; no offspring emerged from the den.

history were housed in contiguous pens and paired with the females during the estimated breeding season to allow mating, with the exception of one pair (female ZP162 and an adult male) that shared an enclosure during the entire hibernation season of 2006 and was not separated prior to the onset of the following reproductive season. All females were paired with males from the same area of origin (northern or southern Mendoza). The genetic relatedness of the pairs was not evaluated, but was assumed to be minimal because the study animals were wild-caught at different dates and in varying areas. All males, except one, were removed from the females' enclosures after approximately 50 days. Fresh feces were collected 1–4 times weekly for periods up to 17 months. Collection of deposited feces was not possible because pichis usually defecate in their burrows. Because pichis usually defecate as soon as they are caught, they were captured by hand when above ground and fecal samples collected into zip-lock plastic bags. Defecation during capture is usually a stress response, and a sampling method based on physical restraint of an animal could, therefore, potentially interfere with hormone cycle dynamics. Nevertheless, the sample collection method used in this study is considered to be much less stressful than blood extraction because the animals were restrained only for a few seconds and the animals were accustomed to being handled prior to initiation of sampling period. In addition, fecal hormone concentrations reflect the cumulative secretion over a period of several hours and are less affected by fluctuations, such as those caused by a brief stress episode (Touma and Palme, 2005). Samples were frozen within an hour of collection and stored at −20 °C. Additional samples were collected from 18 wild females. Pichis were chased on foot, captured by hand, and restrained manually to take morphometric measurements and to determine their reproductive status by visual inspection and palpation. Fecal samples were collected into zip-lock plastic bags, and the animals released at the capture site immediately after sampling. Samples were placed in liquid nitrogen within an hour of collection, transferred to the laboratory, and stored at −20 °C.

To estimate excretion lag time, five pichis were maintained temporarily in large metal boxes with paper bedding and were fed a natural dye (beetroot). All deposited feces were collected over a period of 72 h and inspected for a change in color or fragments of beetroot. The passage time varied between 48 and 52 h.

2.2.2. Tissue samples

Necropsies and visual inspections for external signs of reproductive status were performed on 79 road kill specimens, dead females that had been confiscated from poachers by law enforcement agencies, and captive pichis that died during the study period. Seven females originated from northern Mendoza and the remaining animals were from southern Mendoza. Most samples were collected in February and March. This sampling schedule depended on the confiscations made by inspectors and rangers. Poaching activity is greatest between the end of the pichi's reproductive season and the start of the hibernation season (i.e., between February and April). The animals were classified as juveniles, yearlings or adults based on morphological signs, such as carapace length and width, and the presence or absence of scars. Juveniles ($n = 12$) and animals that could not be assigned to an age class ($n = 2$) were excluded from the analyses. Ovaries were collected and their maximum length and width were measured with a caliper. Organ volume was calculated with the formula for the volume (V) of an ellipsoid: $V = 4/3 \cdot \pi a b^2$, where $a = 1/2$ maximum length and $b = 1/2$ maximum width. Tissues were stored in 10% formalin, fixed in Bouin's fluid, desiccated, and embedded in paraffin wax. A section of 5 μm was cut from the equatorial region and stained with hematoxylin and eosin. Ovaries of nine females could not be analyzed histologically due to advanced autolysis. Thus, a total of 56 samples was analyzed histologically: 3 lactating yearlings (sampled in February and March); 21 lactating adults (February, March, and November); 1 adult female that aborted days before death (November); 1 adult pregnant female (October); 15 non-pregnant, non-lactating yearlings (February and March); and 15 non-pregnant, non-lactating adults (February to May).

2.3. Sample analysis

Fecal samples were lyophilized and pulverized, then 0.20 g was solubilized in 5 ml of 90% ethanol: distilled water, shaken during 30 min, and centrifuged at $500 \times g$ for 20 min at room temperature. The supernatant was recovered and the pellet re-suspended in 5 ml of 90% ethanol, shaken during 1 min, and re-centrifuged. Both supernatants were combined, dried completely, and re-dissolved in 1 ml methanol. Samples were vortexed, diluted in 4 ml PBS buffer, and kept at −20 °C until their analysis. A solid-phase radioimmunoassay (Coat-A-Count Progesterone, Diagnostic Products Corporation, Los Angeles, CA, USA) was used to quantify fecal progestagens. According to the manufacturer, cross-reactivity of the progesterone antibody is as follows: progesterone = 100%, corticosterone = 0.9%, cortisol = 0.03%, danazol = 0.006%, 11-deoxycorticosterone = 2.2%, 11-deoxycortisol = 0.01%, DHEA-SO_4 = 0.002%, 20α-dihydroprogesterone = 0.2%, 17α-hydroxyprogesterone = 3.4%, medroxyprogesterone = 0.3%, 5β-pregnan-3α-ol-20-one = 0.05%, 5α-pregnan-3,20-dione = 9.0%, 5β-pregnan-3,20-dione = 3.2%, pregnenolone = 0.1%, 5-pregnen-3β-ol-20-one-sulfate = 0.05%. There is no detectable cross-reactivity with androstenediol, estradiol, and pregnane. The results, thus, reflect the amount of intact progesterone excreted into feces. Although the amount of excreted unmetabolised progesterone may be a very small proportion of the circulating hormone, the pattern of degradation to inactive metabolites occurring in this species is unknown, and a significant increase in progesterone was observed during pregnancy validating the assay's capacity to detect well-established biological variations in hormonal concentrations. Determination of the pattern of steroid hormone degradation to inactive metabolites usually involves injections of the radioactive precursor and determination of the excreted metabolites by HPLC (Brown et al., 1994; Busso et al., 2007). This was technically unfeasible in the present work because pichis are a threatened species and the study animals live in open enclosures.

Fecal estrogen metabolite concentrations were determined by means of a liquid-phase radioimmunoassay (Ultra-sensitive estradiol RIA, Diagnostic Systems Laboratories, Inc., Webster, TX, USA). This kit was used because radio-metabolism studies have shown that in feces, estrogens are present as estradiol and/or estrone and thus can be determined by using assays that are specific for estradiol or total estrogen assays (Schwarzenberger, 2007). Cross-reactivity of the estradiol antiserum against other compounds is indicated by the manufacturer as follows: estrone = 2.40%, estrone-β-D-glucuronide = 0.20%, estrone-3-sulfate = 0.01%, equilin = 0.34%, D-equilenin = 3.40%, 17α-estradiol = 0.21%, 16-keto-estradiol = 0.21%, 17-β-estradiol-3-glucuronide = 2.56%, estradiol-3-

SO_4 = 0.17%, estriol = 0.64%. There is no detectable cross-reactivity with testosterone, DHEA, diethyl stilbestrol, and 17βE_2-17-glucuronide.

Fecal glucocorticoid metabolites were analyzed by means of a corticosterone radioimmunoassay developed by Jahn et al. (1995) because preliminary evaluations showed that mean corticosterone concentrations in pichi feces were more than an order of magnitude greater than cortisol concentrations. The antisera were raised in rabbits against corticosterone-6-CMO-bovine serum albumin. Assay sensitivity was less than 70 fmol/tube.

For all analyses, samples were thawed, vortexed, centrifuged for 5 min at 8500 × *g* at 4 °C, and assayed in duplicate. Extraction efficiencies for hormones added to fecal samples were 95% at 8 ng progesterone and 70% at 10 ng estradiol. Serial dilutions of fecal extracts were parallel to standard curves. The intra-assay coefficients of variation at 40% binding were <10% for all hormones. To reduce errors due to inter-assay variation, all samples obtained from a female during one reproductive season were analyzed at the same time. Inter-assay coefficients of variation were 19% for progestagens, 9% for estradiol, and 10% for corticosterone. Average water content of the fresh feces was 52 ± 15% (n = 466).

Ovaries were examined by means of light microscopy to determine the presence or absence of early secondary follicles, late secondary follicles, tertiary follicles, corpora lutea, and polyovular follicles.

2.4. Data analysis

An approximation was necessary to define parturition date (day 0), as the exact date of birth could not be determined through direct observation because pichis give birth inside their burrow and the offspring do not emerge until they are at least a month old. Pregnancy diagnosis is difficult in armadillos (Superina, 2000). No signs of imminent birth or early post-parturition could be detected in spite of intensive efforts to observe behavioral changes or a variation in abdomen or mammary gland size. Abdominal palpation is very difficult in unsedated pichis and could, therefore, not be used to determine parturition date. It is possible that no changes in mammary gland size could be seen because the females nursed their neonates before emerging. Parturition day was defined based on the sharp decrease in circulating progesterone that occurs a few hours before or during parturition in most mammalian species (Challis and Lye, 1994). In addition, one pregnant female (ZP144) was paired on September 15, 2005, closed the burrow entrance on November 20, and did not emerge for 2 days. The emergence of two offspring 41 days later led to the conclusion that female ZP144 had given birth inside the burrow around November 20. This female had minimal hormone concentrations in the last fecal sample collected prior to delivery (i.e., on November 20), suggesting that in pichis the decrease in fecal progestagen concentrations may precede parturition by at least 1 day. Similarly, female ZP162 shared an enclosure with a male during the hibernation season of 2006. She did not emerge at all on October 9, 2006, which is uncommon for captive pichis outside hibernation season, and was observed to have enlarged mammary glands a few days later. On November 30, two healthy offspring were seen for the first time inside ZP162's enclosure. This female's fecal progestagen concentrations were greater before October 9 than in subsequent samples. These observations were decisive for setting day 0 in other females as the lowest progestagen measurement after a peak >400 ng/g dry feces, although the effective timing of parturition may vary 1–2 days from the estimated day 0. The same estimation for day 0 was used for females that lost their offspring.

Hormone concentrations were grouped into intervals of 5 days each from day −40 to +40 and, due to the small sample size in some periods, into intervals of 10 days from days −60 to −40, and +40 to +60. Two-way analyses of variance (ANOVA) with Bonferroni post hoc tests were used to compare hormone concentrations over time of females that successfully raised their offspring and of females that lost their offspring shortly before or after delivery. When the variances were not homogeneous, values were log-transformed for statistical analysis. Fecal estrogen and progestagen concentrations outside pregnancy were tabulated and graphed to screen for cyclic patterns as indicators of ovulation occurrence and luteal phases. Independent-samples t-tests were used to compare hormone levels of captive and wild juvenile pichis.

Statistical analyses were performed using SPSS (Version 11.0, SPSS Inc., Chicago, IL, USA) and GraphPad Prism (Version 3.0a for Macintosh, GraphPad Software Inc., San Diego, CA, USA). Results are presented as mean ± SEM. p-Values below 0.05 were considered statistically significant. Statistical

tests were not performed to compare hormone concentrations of wild and captive adult pichis due to the small number of samples from wild females per reproductive status, and because visual inspection and palpation did not allow identification of wild females in early pregnancy or differentiation between females in early or late lactation. No statistical analyses were performed on the histological findings of ovaries.

3. Results

Because all hormone results reflect fecal hormone concentrations, the terms "fecal estrogens", "fecal progestagens", and "fecal glucocorticoids" will be used when referring to fecal radioimmunoreactive estrogen, progesterone, and corticosterone metabolites, respectively. Information in Table 1 provides the reproductive history of females included in the study. Most captive pichis reached sexual maturity at 9–10 months of age, but some females did not reproduce until their second year. Mounting attempts only occurred between September and December. Seven captive females included in this study gave birth and successfully raised their offspring during the study period; litters consisted of up to two young of the same or of different sex. Two females (ZP144 and ZP153) delivered offspring twice in two consecutive reproductive seasons (Table 1). Five females aborted or did not raise their offspring. One of them, ZP153, lost her first litter in 2006, became pregnant again immediately thereafter and gave birth to two offspring, which were raised successfully in the presence of the male. In 2006, two females gave birth 58 and 59 days, respectively, after being paired with a male (ZP144) or after having lost the first litter (ZP153). If gestation length in pichis is approximately 60 days (Redford and Eisenberg, 1992), successful mating must have occurred shortly after pairing in these females, and 7–18 days after pairing in other females.

In all females studied, fecal progestagen concentrations remained minimal during the first half of pregnancy, increased about ten-fold to peak concentrations of as much as 3500 ng/g dry feces, and then decreased to baseline concentrations (Fig. 1a). Fecal estrogen concentrations were of a similar pattern: remained minimal for the first month, peaked at concentrations of about 200 ng/g before returning to baseline concentrations at approximately the same time as fecal progestagens (Fig. 1b). During pregnancy, fecal progestagen concentrations of females that subsequently raised their offspring successfully were not significantly different from those of pichis that lost their litters ($p > 0.05$), while varying significantly over time ($p < 0.0001$). Fecal estrogen concentrations varied between groups ($p < 0.05$) and over time ($p < 0.0001$), with the females that subsequently nursed their offspring having lesser concentrations on day −10 ($p < 0.05$) and a tendency to lesser concentrations on day −5 compared with females that aborted during late pregnancy or did not raise their young. In at least two of these cases, day 0 would represent day of abortion, while the day of parturition would have occurred at an unknown period of time later. Hence, the greater fecal estrogen concentrations in females that did not raise their offspring probably do not reflect a physiological difference leading to the loss of the litter, but instead are related to the uncertainty in defining day 0. Fecal glucocorticoids showed a pattern similar to that of progestagens, with a marked increase during pregnancy ($p < 0.0001$). The concentrations of pregnant females that subsequently raised their offspring successfully were not significantly different from those of pichis that lost their litters ($p > 0.05$).

Postpartum progestagen and glucocorticoid concentrations were greater in lactating pichis than in non-lactating females ($p < 0.0001$) and varied over time ($p < 0.001$). For progestagens, a difference was observed from day 5 to 40 ($p < 0.05$ on day 5, $p < 0.01$ from day 10 to 40), while for glucocorticoids differences were observed on day 5 ($p < 0.05$), day 15 ($p < 0.01$), and day 20 ($p < 0.05$). Postpartum fecal estrogen concentrations were similar in both groups ($p > 0.05$). Both hormone patterns of lactating and non-lactating females were observed consecutively in the female ZP153, which apparently had two consecutive pregnancies in 2006 (Fig. 2). Elevated fecal progestagen, estrogen, and glucocorticoid concentrations indicative of a pregnancy were followed by hormone concentrations around baseline, suggesting the absence of lactation due to loss of the litter. Fecal progestagen and glucocorticoid concentrations increased again at about day 30, declined to reach a nadir around day 60, and remained clearly above baseline until the end of the study period, suggesting that parturition was followed by a successful lactation. Indeed, two healthy offspring emerged about a month later. This pattern makes

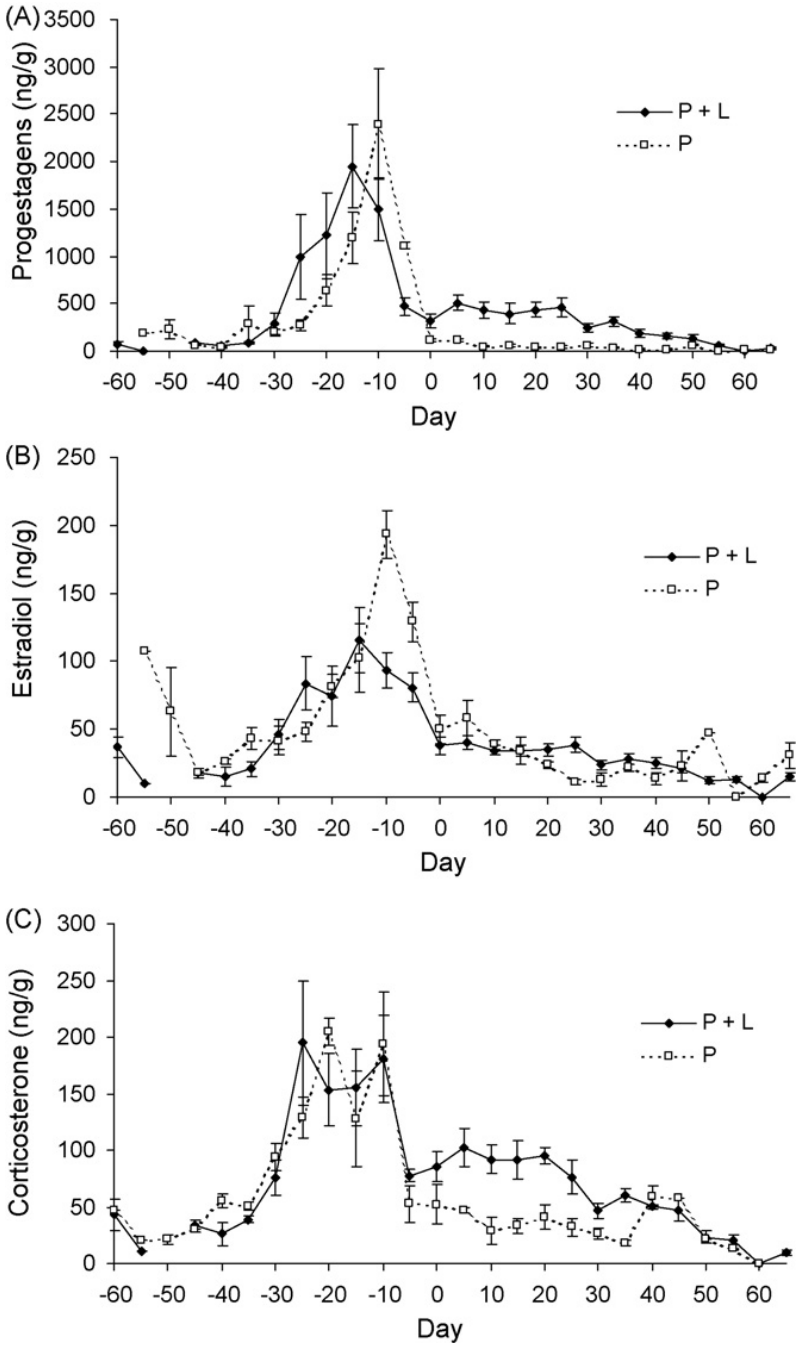

Fig. 1. Average (a) fecal progestagen, (b) estrogen, and (c) glucocorticoid concentrations in female pichis *Zaedyus pichiy*. P + L: females that were pregnant and successfully raised their offspring; P: females that aborted or did not raise their offspring. Day 0 = estimated day of parturition or abortion, defined as the lowest fecal progestagen level after the first peak of >400 ng/g.

it very probable that, soon after losing her first pregnancy or litter, this female had an estrus period, mated, and became pregnant again in the same (2006) season.

In all cases, the offspring remained in their burrows during the first 37 ± 3 days after the estimated birth date. Females were observed carrying food to their burrow several days prior to the first emergence of their offspring. At first emergence, the young foraged for solid food either in proximity of the female or by themselves. The weaning period coincided with the return of progestagen and glucocorticoid concentrations to baseline.

Non-pregnant, non-lactating females did not have any well-defined peaks in estrogen concentrations followed by increases in progestagen indicating ovulation and luteal phase, respectively. Instead, fecal estrogens and progestagens fluctuated independent of each other and no clear cyclic pattern

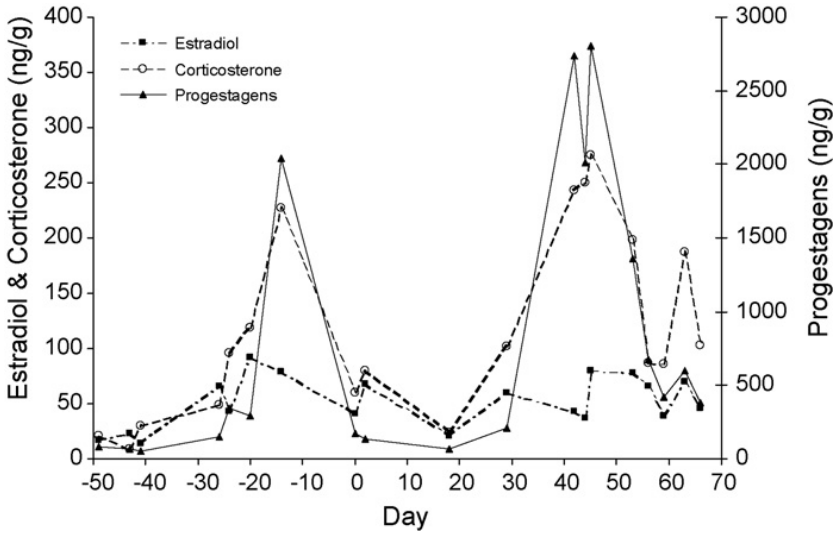

Fig. 2. Fecal progestagen, estrogen, and glucocorticoid concentrations in a female pichi *Zaedyus pichiy* that lost its first litter, conceived again, and raised her second litter. Day 0 = day of first parturition or abortion, defined as the least fecal progestagen concentration after the first peak of >400 ng/g.

Table 2
Number of lactating, non-lactating, and pregnant *Zaedyus pichiy* carrying follicles of different stages or corpora lutea. Only yearlings and adults are included.

Reproductive status	*n*	Follicles[a]			Corpora lutea[a]	Mean ovary volume (mm^3)
		Early secondary	Late secondary	Tertiary		
Lactating	24	22	16	1	0	36.0 ± 2.5
Not lactating	30	22	16	3	0	33.2 ± 2.0
Pregnant[b]	2	0	2	2	2	33.0 ($n = 1$)

[a] Indicates number of females in which a certain structure was observed.
[b] One female aborted days before death.

could be established (data not shown). Hormone concentrations of captive and wild juvenile females were not significantly different ($p > 0.05$), and hormones of adult wild females were within range of captive females (data not shown).

Ovaries of lactating and non-lactating females, and of one pregnant pichi were of similar size (Table 2). Almost all lactating females and two thirds of all non-lactating yearlings or adults presented early secondary follicles (Table 2). Late secondary follicles (Fig. 3b) were present in two thirds of the lactating females, half of the non-lactating females, and in the two pichis that were pregnant or had recently aborted (Table 2). Tertiary follicles were found in only a few lactating and non-lactating individuals, in the pregnant female, and in the pichi that had recently aborted. Corpora lutea were rare. They were only found in histological preparations from the pregnant adult and the female that had aborted a few days before death. Polyovular follicles (Fig. 3d) were present in 5 of 16 yearlings and 7 of 36 adults.

4. Discussion

The present findings reinforce previous observations that pichis are seasonal breeders (Superina and Jahn, in press) because all births occurred between October and January. Wild juvenile pichis were only recorded between January and March (Superina, 2008), suggesting that births occurred no earlier than November. Furthermore, a pattern typical of pregnancy was observed in the fecal excretion of progestagens and estrogens only during the months of September to January.

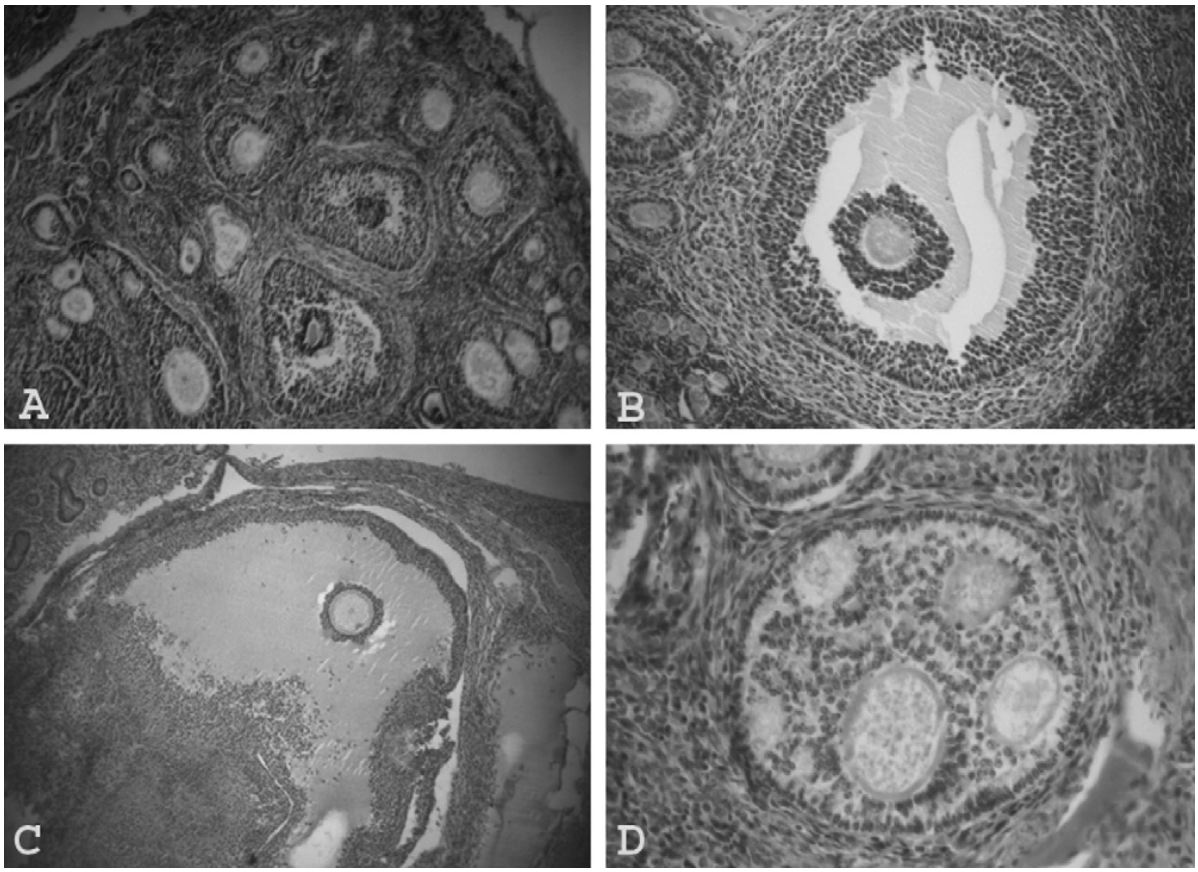

Fig. 3. (a) Overview of an active ovary of *Zaedyus pichiy* with different stages of ovarian follicular growth and several polyovular follicles (×40); (b) late secondary follicle (×100); (c) tertiary follicle (×40); (d) polyovular follicle with four oocytes (×100).

During the remainder of the year, fecal hormone analyses indicated minimal values and revealed no pattern in variations of progestagen and estrogen concentrations that indicate ovarian cyclic patterns. Although the methods used may have prevented detection of cyclical variations in these hormones, the possibility that pichis are induced ovulators cannot be excluded. Further studies are under way to investigate this possibility.

Due to the pichis' burrowing habits, it was impossible to observe parturition and thus determine its exact date. However, a rapid decrease in progesterone occurs immediately before or after parturition in all mammal species where assessments have been performed (Challis and Lye, 1994), which led to the estimation of the approximate date of parturition as the time when fecal progestagen concentrations decreased to baseline after having remained elevated for more than 20 days (Fig. 1a). Importantly, fecal steroid concentrations reflect the average circulating values present in the previous 40–60 h if one considers an excretion lag time of 48–52 h. Thus, the actual parturition may have occurred 48–72 h prior to the observed minimum progestagen values. Although the exact timing of mating is not known, the intervals between pairing and the nadirs in fecal progestagens indicate a duration of pregnancy of approximately 60 days, which is in accordance with the gestation length suggested by Redford and Eisenberg (1992). Fecal estrogen concentrations increased and decreased at the same time as progestagens (Fig. 1b). Increased circulating estrogens during pregnancy have also been described for goats, guinea pigs, Barbary sheep, and humans (De Hertogh, 1973; Heap et al., 1973; Hamon and Heap, 1990; Sawada et al., 1995). Corticosterone values also increased during the second half of pregnancy, with a pattern very similar to that of progestagens. Increases in circulating glucocorticoids during mammalian pregnancy are well-documented and are essential for the normal development of the fetus and for regulating the onset of parturition (Barlow et al., 1973; Pepe and Albrecht, 1995; Keller-Wood and Wood, 2001).

Postpartum fecal progestagen concentrations were greater in lactating females than females that aborted or did not raise their offspring (Fig. 1a). Greater serum progesterone concentrations have also

been reported in lactating nine-banded armadillos *Dasypus novemcinctus* (Peppler and Stone, 1980). Lactation is maintained through a species-dependent, complex interaction of prolactin, growth hormone, corticosteroids, thyroid hormones, insulin, and parathyroid hormone (Tucker, 1988; Neville et al., 2002). Adrenal glands are enlarged in wild lactating pichis, suggesting an increased corticosteroid production during lactation (Superina, unpublished data). Indeed, postpartum fecal corticosterone concentrations were also greater in lactating pichis than females that lost their offspring (Fig. 1c), which may indicate that the elevated lactational progestagens are of adrenal origin. Presumably, because progesterone and pregnenolone are the precursors of all other steroid hormones (Sanderson, 2006), an elevated corticosteroid synthesis during lactation may result in a concomitant increase in progesterone concentrations. The increased release of adrenocorticotropin after a suckling stimulus (Walker et al., 1992) may, therefore, not only induce the release of glucocorticoids for lactogenesis, but also cause the observed increased fecal progestagen concentrations in lactating pichis. The absence of functional or atretic corpora lutea in the ovaries of lactating females (Table 2) also indicates spontaneous postpartum ovulations do not occur and the increased circulating progesterone during this stage of reproduction is not of ovarian origin. Fecal progestagen and glucocorticoid concentrations decreased sharply around the first emergence of the offspring. Weaning was initiated several days before first emergence of offspring from the burrow with the females carrying food into the burrows, and juveniles were observed foraging and ingesting solid food when first above ground. The decreases in fecal progestagen and glucocorticoids, therefore, appear to be related to a reduction in suckling stimuli and milk production, supporting the hypothesis that increased fecal progestagen concentrations during lactation are of adrenal origin and a by-product of corticosteroid production.

Early and late secondary ovarian follicles were found in a large proportion of the females (Table 2). Tertiary follicles were less common and occurred during and outside the breeding season and independent of the female's reproductive status. Late secondary and pre-antral follicles were present in the ovaries of two females that were pregnant at time of death (ZP97) or aborted only a few days before death (ZP154). It is possible that production of tertiary follicles during pregnancy and lactation bears an ecological advantage. A high risk of abortion or neonatal mortality would increase the probability that females need to mate and ovulate a second time during the breeding season. Stress-induced abortions, as well as abandonment or injuries of neonates are common in captive armadillos (Superina et al., 2008). In the wild, environmental stress, such as prolonged drought periods or intense summer rains, or stress related to predator attacks may lead to loss of the offspring and result in a second mating during the breeding season. The offspring must be born as early as possible to maximize chances of growing and putting on sufficient fat reserves before the hibernation season. Having a "backup follicle" in an advanced stage of development (i.e., late secondary or tertiary follicle) is beneficial for the female because it allows her to conceive soon after losing her offspring. This hypothesis is supported by observations on one of the captive females (ZP153), which in 2006 aborted or lost her offspring shortly after birth and gave birth to two healthy offspring 60 days later (Fig. 2). This inter-birth interval corresponds to the gestation length. Late secondary or tertiary follicles must, therefore, have been present when the first litter was lost for mating to have occurred and pregnancy be initiated shortly after loss of the litter.

Polyovular development seems to be a natural polymorphism. Polyovular follicles were found in one third of the yearlings and one fifth of all adults. Follicles containing more than one oocyte have been identified in many species; among others, they are relatively common in the domestic bitch (Telfer and Gosden, 1987) and have been observed in armadillos *Chaetophractus vellerosus* and *Z. pichiy* (Codón and Casanave, 2000). Consistent with earlier reports (Bodemer and Warnick, 1961; Telfer and Gosden, 1987), these were more frequent in immature ovaries than in the ovaries of adult pichis.

In conclusion, pichis are seasonal breeders that produce one yearly litter of 1–2 offspring. As observed in other mammalian species, pregnancy was characterized by increased estrogen, progestagen and glucocorticoid levels, while steroid hormones presumably of adrenal origin were elevated during lactation. The histological and hormonal analyses, as well as observations of the behavior of captive pichis, suggest that this species can have a second pregnancy after late abortion or death of the newborn litter, and that ovarian follicular growth is sustained during pregnancy and lactation.

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