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Seasonal reproduction in male pichis *Zaedyus pichiy* (Xenarthra: Dasypodidae) estimated by fecal androgen metabolites and testicular histology

Mariella Superina [a,*], Graciela A. Jahn [b]

[a] *University of New Orleans, Department of Biological Sciences, New Orleans, LA 70148-0001, USA*

[b] *Laboratorio de Reproducción y Lactancia, IMBECU, CRICYT-CONICET, Casilla de Correos 855, 5500 Mendoza, Argentina*

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Abstract

Poaching poses a threat to a wide variety of wildlife, and basic information about the biology of hunted species needs to be collected before their populations decline to the extent that requires drastic human intervention. As the survival of a species is related to its ability to reproduce, data on its reproductive cycle are necessary for the development of management strategies. The hypothesis was tested that the reproductive season of pichis (*Zaedyus pichiy*), small hibernating armadillos that inhabit arid environments in Argentina and Chile, is limited to spring months. Gonadal competence of semi-captive and wild-caught male pichis of Mendoza Province, Argentina was studied, by measuring fecal immunoreactive testosterone concentrations and evaluating spermatogenic activity. Results suggest that *Z. pichiy* is a seasonal breeder that regulates reproduction through photoperiodic cues. Gonadal competence was limited to a period of 3–5 months in spring and early summer and was reflected in enlarged testes, increased spermatogenesis, and significantly elevated fecal immunoreactive testosterone concentrations. The reproductive season for males from southern Mendoza was almost 6 weeks shorter than in the north. This fact, along with significant morphological differences between both groups, suggests that northern and southern pichis belong to two distinct populations. It is concluded that prolonged breeding seasons and more favorable environmental

* Corresponding author. Present address: Laboratorio de Reproducción y Lactancia, IMBECU, CRICYT-CONICET, Casilla de Correos 855, 5500 Mendoza, Argentina. Tel.: +54 261 5244150/4155; fax: +54 261 5244001.
E-mail address: mesuperi@uno.edu (M. Superina).

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conditions in northern Mendoza favor a prolongation of the reproductive season that may allow pichis to breed later in the year, thus maximizing reproductive opportunities.

Keywords: Pichis—Argentina; Fecal hormones; Fecal testosterone metabolites; Spermatogenesis

1. Introduction

Argentina's diverse terrestrial fauna has endured subsistence hunting for centuries. In Mendoza Province, in central-west Argentina, hunting, capturing or disturbing native fauna is prohibited. Nevertheless, a wide range of mammalian species is constantly poached by subsistence and sport hunters. Basic information about the biology of these species needs to be collected before their populations decline to the extent that requires drastic human intervention, such as capturing the last individuals from the wild to breed them in captivity (Holt et al., 2003). Baseline data are not only helpful for evaluating the impact of hunting on wild populations; more importantly, they are essential pre-requisites for planning *in situ* and *ex situ* conservation strategies. As the survival of a species is related to its ability to reproduce, data on its reproductive cycle are necessary for the development of management strategies (Dixson et al., 2003).

The family Dasypodidae includes 21 extant armadillo species that show a remarkable range in size and reproductive strategies (Redford, 1985; Fonseca and Aguiar, 2004). While *Dasypus* exhibits delayed implantation and polyembryony, the other genera, including *Zaedyus*, do not seem to share this pattern (Loughry et al., 1998). Differences among species of armadillos also include variations in seasonality. For example, *Euphractus sexcinctus* is suspected to breed all year long, while other species seem to be seasonal breeders (Superina, 2000). Most armadillo species probably have only one litter per year, although it has been suggested that *Chaetophractus villosus* can give birth to several litters per year (Parera, 1996). Up to three yearly litters have been produced by captive *C. villosus* (Ratajszczak and Trzesowska, 1997).

The pichi (*Zaedyus pichiy*) is a small (approximately 1 kg body mass) armadillo that inhabits arid and semi-arid environments in central and southern Argentina and Chile, at altitudes up to 2500 m (Meritt and Benirschke, 1973; Wetzel, 1985; Superina, 2008). Pichis have opportunistic omnivorous food habits (Superina, 2007). They are the only known armadillos that hibernate during winter and can enter daily torpor outside the hibernation season (Superina and Boily, 2007). Hibernation is interrupted by periodic emergences that have been observed in the field and in captive conditions (personal observations). With a gestation length of 60 days and approximately 50 days of lactation (Superina, 2007), pichis must reproduce shortly after emerging from torpor to maximize the chances of survival of their young. Offspring must grow to sub-adult size and put on enough fat reserves to survive their first winter, and females must wean their offspring several weeks before hibernation to be able to recover from the energetically challenging lactation period. The hypothesis was tested that gonadal competence of male pichis is limited to spring months, indicating seasonal reproduction. Because environmental conditions vary along a latitudinal gradient, we compared the reproductive season length of pichis originating from two different latitudes. Gonadal competence was studied by measuring fecal immunoreactive testosterone and evaluating spermatogenetic activity. Determination of fecal hormone metabolites is a non-invasive method that has been widely used to characterize patterns of hormone secretion in wild animals, allows frequent sample collection over extended time spans, and is a reliable indirect measure of the reproductive status in various species (Czekala et al., 1994; Brown et

al., 1997; Schwarzenberger et al., 1999; Graham et al., 2002; Monfort, 2003; Busso et al., 2005, 2007).

2. Materials and methods

2.1. Sample collection

Eight male pichis were captured in the wild in southern Mendoza Province, Argentina (36° S, 69° W) on 5 November 2004 (one yearling and two adults), 27 February 2005 (two adults), 1 May 2005 (one yearling), 18 June 2005 (one adult), and 28 August 2005 (one yearling). Three wild males were caught in northern Mendoza (32.5° S, 68.5° W) on 4 September 2004 (two adults) and 9 March 2005 (one adult). Northern and southern pichis live in different habitat types and are exposed to different environmental conditions. The northern and eastern parts of the province belong to the Low Monte ecoregion (Olson et al., 2001) and are hotter and drier (average rainfall 150 mm/year) than the southern part, which represents the northernmost tip of the Patagonian steppe (Olson et al., 2001) and has an average yearly precipitation rate of 300 mm and occasional snowfall in winter.

All animals were transported to the common study site (see below) on the day of capture, quarantined, and finally transferred to individual, open 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 aboveground 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. Females were housed in contiguous pens and paired with males during the estimated breeding season to allow mating. Collection of deposited feces was not possible because pichis usually defecate in their burrows. However, because pichis usually defecate as soon as they are caught, they were manually captured when above ground and fecal samples collected into zip-lock plastic bags. Fresh feces were collected once weekly for periods up to 16 months. Pichis hibernated from the end of April until the end of July. During hibernation they emerged at irregular intervals (every 4–14 days) and were sampled whenever possible. Samples were frozen within an hour of collection and stored at −20 ° C. Wild males ($n = 22$) were manually captured in southern Mendoza Province, Argentina. Their reproductive status was determined by visual inspection and palpation, fecal samples were collected into zip-lock bags, and the pichis were released at the capture site immediately after sampling. Samples were placed in liquid nitrogen within an hour of collection and later stored at −20 °C.

Testes were collected from roadkills and dead males that had been confiscated from poachers by law enforcement agencies. Additional samples were obtained from captive pichis that died during the study period. Morphometric measurements, such as carapace length and width, and the presence or absence of scars were used to classify the animals as juveniles, yearlings or adults. Animals that could not be assigned to an age class were excluded from further analyses. The maximum length and width of testes were measured with a caliper. Organ volume was calculated with the formula for the volume (V) of an ellipsoid: $V = (4/3)\pi ab^2$, where $a = 1/2$ maximum length and $b = 1/2$ maximum width. Testes then were preserved in 10% formalin. They were 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–eosin.

2.2. 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 × *g* for 20 min at room temperature. The supernatant was recovered and the pellet resuspended in 5 ml of 90% ethanol, shaken during 1 min, and recentrifuged. Both supernatants were combined, dried completely, and redissolved 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 Total Testosterone, Diagnostic Products Corporation, Los Angeles, CA, USA) was used to quantify immunoreactive fecal testosterone. Samples were thawed, vortexed, centrifuged for 5 min at 8500 × *g* at 4 °C, and analyzed in duplicate. Extraction efficiency for testosterone added to a fecal sample was 73% at 40 ng testosterone. Serial dilutions of fecal extracts were parallel with the standard curve. The intra-assay coefficient of variation at 40% binding was <10%. To reduce errors due to inter-assay variation, all samples obtained from an individual during one reproductive season were analyzed in the same assay. The inter-assay coefficient of variation based on three identical samples included in each assay was 16%. Average water content of the fresh feces was 52 ± 13% ($n = 363$).

Histological analysis of testes was performed by evaluating spermatogenic activity in 10 seminiferous tubules per animal. The spermatogenic index (SI) developed by Grocock and Clarke (1974) was used as a semi-quantitative measure of reproductive competence. In addition, presence or absence of spermatocytes in the epididymis was recorded. SI scores were assigned from 0 to 5 according to the following criteria (cf. Grocock and Clarke, 1974): SI 0 = very small tubules containing only Sertoli cells and spermatogonia; SI 1 = small tubules containing only Sertoli cells, spermatogonia, and primary spermatocytes; SI 2 = seminiferous tubules with Sertoli cells, spermatogonia, primary spermatocytes and round spermatids, but no elongated spermatids; SI 3 = tubules showing presence of spermatozoa and spermatids, but in fewer numbers than in the greater categories; SI 4 = relatively large seminiferous tubules showing complete spermatogenesis, but with less abundant elongated spermatids and spermatozoa than in the greatest category; SI 5 = large seminiferous tubules that display complete spermatogenesis.

2.3. Data analysis

The reproductive season of male pichis from southern and northern Mendoza was calculated separately. For each geographic group, individual values were combined to obtain one mean value per week. The onset of reproductive season was defined as the first increase above the upper limit of the 95% confidence interval (CI) of all fecal samples collected from animals of the same geographic area. The end of the reproductive season was defined as the first value below the upper limit of the 95% CI. Off-season baseline values were calculated for each geographic group as the average of all testosterone values measured outside the reproductive season. Independent-samples *t*-tests were used to compare hormone concentrations of captive pichis during the reproductive season and off-season, to compare off-season hormone concentrations of northern and southern pichis, and to test for significant differences between hormone levels of captive and wild pichis both during the reproductive season and off-season. Statistical analyses were performed using SPSS (Version 11.0, SPSS, Inc., Chicago, IL, USA), and *p*-values below 0.05 were considered statistically significant. Unless otherwise stated, results are presented as mean ± S.E.M. The sample size and distribution over time did not allow statistical analyses on the size or the histological analysis of testes extracted from dead pichis. Photoperiod was calculated from data provided by the Servicio de Hidrografía Naval, Armada Argentina (http://www.hidro.gov.ar). Climate data were obtained from the Servicio

Meteorológico Nacional, Fuerza Aérea Argentina, Comando de Regiones Aéreas, Argentina. This project was approved by the Institutional Animal Care and Use Committee of the University of New Orleans (IACUC Protocol No. 079) and the Dirección de Recursos Naturales Renovables of Mendoza Province, Argentina.

3. Results

Male pichis reached sexual maturity at the age of approximately 9 months. They had enlarged testes and significantly elevated fecal immunoreactive testosterone concentrations ($p < 0.001$, Fig. 1), indicative of gonadal competence, during a period of 3–5 months starting in late winter until early summer. These physiological and morphological changes were accompanied by increased aggressiveness. Males kept in neighboring enclosures grunted and reached through the mesh fence with their forelegs in an attempt to fight with their neighbor. Aggressive behavior, as well as injuries attributable to fights with conspecifics, was also observed in gonadally competent wild males. During this period, chasing sequences and mounting attempts occurred immediately after pairing of males and females. Mating could not be observed although 10 females gave birth and successfully raised their offspring and 5 females aborted or lost their newborn during the study period. No mating behavior or aggressiveness was observed outside the estimated reproductive season.

Reproductive season length, as estimated by fecal immunoreactive testosterone concentrations, varied between males from different latitudes. In animals from northern Mendoza province, fecal immunoreactive testosterone concentrations remained above the upper limit of the 95% CI from week 32 to week 2 (beginning of August to mid-January). In individuals from southern

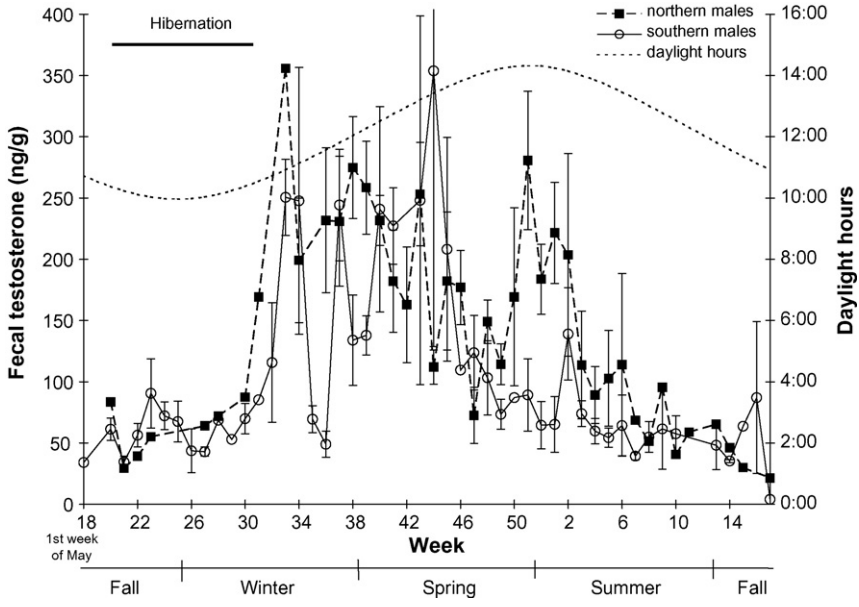

Fig. 1. Correlation of weekly average fecal immunoreactive testosterone concentrations of wild-born, captive-kept *Zaedyus pichiy* from northern ($n = 3$, solid line) and southern ($n = 8$, dashed line) Mendoza Province, Argentina, and variation in daylight time (dotted line) at the study site. The estimated duration of the hibernation season is based on observations in the field and on captive individuals.

Mendoza the concentrations were elevated from week 33 to week 47 (mid-August to end of November, Fig. 1). In the pichis from southern Mendoza and the single male from northern Mendoza that could be sampled in spring, hormone concentrations started increasing after winter solstice and reached the upper limit of the 95% CI at a photoperiod of 10 h and 45 min. Pichis from northern Mendoza returned to this limit at a photoperiod of 14 h and 15 min after summer solstice, while their conspecifics from southern Mendoza did so at 14 h and 15 min of daylight before summer solstice. Off-season concentrations were similar in pichis of different geographic origin ($p > 0.05$). Hormone concentrations of wild males from southern Mendoza were not significantly different from those of captive pichis, neither during the reproductive season (captive males: 199.6 ± 23.1 ng/g; wild males: 231.2 ± 48.8 ng/g; $p > 0.05$) nor off-season (captive males: 68.7 ± 4.1 ng/g; wild males: 83.6 ± 14.2 ng/g; $p > 0.05$).

When captive males were paired with sexually mature females, mounting attempts occurred between August and December. Births could not be observed directly because they occurred inside the burrows. According to fecal ovarian hormone concentrations of the females, the interval between first pairing and parturition averaged 67 days (range 58–78 days), with births of one to two offspring occurring between the beginning of October and mid-January. The offspring remained inside the burrow until they were weaned at approximately 35 days of age (Superina, 2007). Wild juvenile pichis were only recorded between January and March (Superina, 2008), suggesting that births occur no earlier than November. None of the captive females that shared an enclosure with a male during parturition and lactation or which were paired during the lactation period conceived again in the same season. Histological and hormonal analyses, as well as observations on one captive female, however, suggest that females that abort or lose their offspring during early lactation, can become pregnant almost immediately after losing their first litter and successfully raise their second one (Superina, 2007).

Testes from 65 wild-born males were collected from roadkills, dead animals confiscated by law enforcement agencies or from individuals that died in captivity. Thirteen samples had advanced autolysis and were excluded from further analyses. Eight samples originated from northern Mendoza and the remaining samples were from southern Mendoza. The large majority of samples (85%) were collected in February and March because poaching activity is greatest between the end of the reproductive season and the start of the hibernation season of pichis, i.e., between February and April. As a consequence, confiscations by inspectors and rangers occur mainly during these months. Spermatogenic indices (SIs) and testis volumes were least in juveniles and greatest in adults, with yearlings having intermediate concentrations (Table 1). In adults, SI scores were consistently greater in November and showed large variations in animals sampled in February and March. Spermatozoa were only present in the epididymides of nine adults that were captured in November, February and March (Table 1).

4. Discussion

Results suggest that *Z. pichiy* is a seasonal breeder that regulates reproduction through photoperiodic cues. The reproductive season for male pichis from southern Mendoza ended almost 6 weeks earlier than that in northern Mendoza and this difference persisted during the 2 years of sampling although all animals were kept at the same location. Fecal androgen metabolite concentrations of southern pichis started increasing around mid-August and returned to baseline around the end of November, while testosterone concentrations of northern pichis were elevated from the beginning of August until mid-January (Fig. 1). It should be noted that only one male from the northern population could be sampled from week 22 to week 33. The exact start of the

Table 1
Histological analysis of testes of wild confiscated pichis *Zaedyus pichiy* of different age classes (SI = spermatogenic index)

Age class	Month	*n*	Median SI (range)	Number of males with spermatozoa in epididymis	Median testicular volume in cm^3 (range)
Juvenile	January	1	0	0	0.06
	February	2	0.5 (0–0.9)	0	0.25 (0.14–0.35)
	May	1	0.1	0	0.25
Yearling	February	5	0.3 (0–1.2)	0	0.27(0.10–1.25)
	March	9	1.2 (0.1–2.9)	0	0.78 (0.16–1.78)
Adult	January	1	0.1	0	0.50
	February	12	1.0 (0.1–4.1)	2	1.46 (0.32–2.88)
	March	16	2.1 (0.1–3.8)	4	1.45 (0.38–2.15)
	April	1	0.9	0	0.51
	October	1	0.9	0	Not measured
	November	3	4.0 (3.9–4.8)	3	4.14 ($n = 1$)

reproductive season of northern pichis could, therefore, vary considerably from the reported date (beginning of August).

Northern pichis concluded their reproductive season after summer solstice while the southern ones did so before summer solstice, but, interestingly, at the same day length of 14 h and 45 min. In accordance with observations in other mammals (Kriegsfeld and Nelson, 1999; Trainor et al., 2006), photoperiod seems to be an important cue regulating reproduction in pichis. Photoperiod reliably predicts environmental changes (Kriegsfeld and Nelson, 1999; Trainor et al., 2006) and allows the animals to synchronize the energetically demanding reproductive period with appropriate environmental conditions. The observation that the hormone concentrations of northern pichis fell below baseline during decreasing day length while southern ones did so during increasing day length may suggest that the two populations differ in their response to photoperiodic cues. The fact that this difference persisted although both groups of pichis were sampled at the same location over a period of up to 2 years, may indicate a subtle difference in the interaction between photoperiod and the central neuroendocrine structures that regulate reproduction, which may be suggestive of underlying genetic variations between the populations (Bronson and Heidemann, 1994). Variation in reproductive season length along a latitudinal gradient has been observed in mice of the genus *Peromyscus* (Lynch et al., 1981), in deer of the genus *Odocoileus*, and in lagomorphs (Bronson, 1985). In these three groups, as well as in pichis, longer reproductive seasons occur for populations living at lower latitudes. Morphometric measurements showed significant differences between the geographically isolated populations from northern and southern Mendoza (Superina, 2008), with pichis from northern Mendoza being larger and having a proportionally wider head than individuals from southern Mendoza. The morphological and reproductive season length differences lend support to our hypothesis that the animals sampled for this study indeed belong to two separate populations that diverged by selective adaptation to different environmental conditions.

Changes in size and weight can be observed in the testes and ovaries of seasonally breeding mammals, because the reproductive organs are larger and heavier during the reproductive season (Janse van Rensburg et al., 2002). Adult males of many species of seasonally breeding mammals, such as roe deer (*Capreolus capreolus*) or fallow deer (*Dama dama*), show circa-annual cycles of testicular involution and recrudescence, accompanied by arrest or activation of spermatogenesis,

respectively (Gosch and Fischer, 1989; Goeritz et al., 2003). As expected, largest testes and greatest SI scores were observed in November (Table 1). Although males characteristically achieve gonadal competence earlier and terminate it later than females (Bronson, 1985), it was surprising to find active spermatogenesis and mature spermatocytes in the epididymides of some males sampled in February and March. Presumably, the lesser testosterone concentrations at this time of year precludes sexual activity in spite of the greater SI scores and mature epididymal spermatocytes. The beginning of the reproductive season could not be histologically confirmed because no dead males could be sampled between May and September in spite of intensive efforts to obtain animals killed by road traffic or confiscated dead males. Poaching activity is less and animals killed by road traffic are infrequent from May to August because wild pichis emerge only rarely during hibernation season (Superina, unpublished data). In addition, poachers are seldom active and confiscations are, therefore, rare during the reproductive season of wild pichis because of the common belief that eating reproductively active pichis causes indigestion. The lack of samples during this period is a limitation in the present study, but the sampling method was the only ethically justifiable and legally acceptable means to collect organs from this endangered species.

In conclusion, observations in the present study reveal that pichis are seasonal breeders that are gonadally competent and sexually active in spring and early summer and show latitudinal differences in reproductive season length as estimated by fecal androgen concentrations. The prolonged breeding season and more favorable environmental conditions in northern Mendoza favor a prolongation of the reproductive season that may allow pichis to breed later in the year, thus maximizing reproductive opportunities. Results of the present study indicate that pichis can only produce one yearly litter, suggesting that mortality due to heavy poaching activity may be difficult, if not impossible, to compensate by the current birth rates.

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