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Patterns of fecal gonadal hormone metabolites in the maned wolf (*Chrysocyon brachyurus*)

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Abstract

Ex situ populations of maned wolves are not viable due to low reproductive efficiency. The objective of this study was to increase knowledge regarding the reproductive physiology of maned wolves to improve captive management. Fecal samples were collected 3–5 d/wk from 12 females of various reproductive age classes (young, prime breeding and aged) and reproductive histories (conceived and raised pups, conceived but lost pups, pseudo-pregnant and unpaired). Ovarian steroids were extracted from feces and assessed by enzyme immunoassay. Concentrations of estrogen metabolites gradually increased, beginning 2–5 d before breeding, and declined to baseline on the day of lordosis and copulation. Fecal progestin metabolite concentrations increased steadily during the periovulatory period, when sexual receptivity was observed, and remained elevated during pregnancy and pseudo-pregnancy. During the luteal phase, young and prime breeding-age females excreted larger amounts of progestins than those of older age classes. Furthermore, progestin concentrations were higher during the luteal phase of pregnant versus pseudo-pregnant bitches. Profiles of fecal progestin metabolites for three singleton females were unchanged throughout the breeding season, suggesting ovulation is induced in this species. However, this finding could be confounded by age, as these females were either young or aged.

Keywords: Maned wolf; Ovarian steroids; Estrogens; Progestins; Pregnancy; Pseudo-pregnancy

1. Introduction

The maned wolf (*Chrysocyon brachyurus*) is the largest canid in South America. The species is monotypic and found only in Brazil, Argentina, Bolivia, and Paraguay [1]. However, maned wolves in the wild are threatened with extinction due to habitat loss resulting from expanding agriculture [1]; it is estimated that only 2000 individuals remain in nature [2]. Therefore, maintaining viable ex situ populations is considered an important hedge against extinction, as well as providing a 'research resource' to understand the biology of this charismatic species. The North American captive population of maned wolves is not self-sustaining, mainly due to low pregnancy success and high neonatal mortality [3,4]. Increased knowledge of reproductive physiology of this species would improve captive breeding and management.

There is limited specific information about maned wolf reproductive biology. The species is monoestrus with a well-known and distinctive 3–5-mo breeding season in captivity, occurring from April to June in Latin America and October to February in North America [5]. Proestrus lasts for ∼2 wk and is characterized by vaginal swelling and secretions, as

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well as increases in social solicitatious behavior [6]. The duration of estrus ranges from 1 to 10 d. There are no data on the precise onset of sexual maturity; although zoo-held females have produced pups at 1.5 yr of age [3], the average age at first parturition is 4 yr. Data from the Maned Wolf Studbook suggest that bitches can produce pups at 10–12 yr of age, but this is uncommon [3]. The preponderance of breeding occurs from 3 to 8 yr of age [3], an interval generally considered prime for reproduction. Litter size is usually one to six pups (mean ± S.E.M., 2.6 ± 0.2), with a gestation period of approximately 65 d [1,5]. The typical lifespan of maned wolves maintained in captivity is 16 yr [3].

Reproductive hormone patterns in the maned wolf, as measured by radioimmunoassays, have been previously described [6,7]. In those studies, a preovulatory estrogen surge occurred 1–10 d before onset of mating. Like the domestic dog [8–12], progestins steadily rose during late proestrus and throughout estrus, and remained elevated throughout pregnancy or the non-pregnant luteal phase [6].

The objectives of the present study were to: (1) adapt more user-friendly enzyme immunoassay technology to monitoring reproductive hormones in maned wolves; and (2) characterize endocrine profiles of female maned wolves on the basis of age and reproductive status.

2. Materials and methods

2.1. Animals and samples collection

Fecal samples (~30 g) were collected three to five times/week during the breeding season (October through February) from 12 females maintained in six institutions in the United States. These institutions were located between 30°63′ (Yulee, Florida) and 41°19′ (Bridgeport, Connecticut). For pregnant females, fecal samples were obtained up to the last week of gestation. The wolves were divided into three reproductive age classes: (1) young (<3 yr of age, $n = 3$); (2) prime breeding (>3 to <8 yr of age, $n = 5$); and (3) aged (>8 yr of age, $n = 4$). Five females (2, 6, 6, 8, and 8 yr of age, respectively) conceived and successfully raised pups during the monitoring interval. Two (2 and 7 yr of age) were pregnant, but lost pups within a few days after whelping. Two females (10 yr of age) were paired, but did not conceive and three (2, 12 and 15 yr of age) were housed singly. Fecal samples were stored (−20 °C) at each holding facility until the end of the breeding season and were then transported on dry ice to the Conservation & Research Center for analysis.

2.2. Fecal extraction and analysis

Fecal extraction was performed as previously described [6]. Briefly, frozen feces were lyophilized, and fecal powder was stored at −20 °C until steroid extraction. A 0.2 g aliquot of well-mixed fecal powder was boiled for 20 min in 90% ethanol:10% distilled water. Each sample was centrifuged at 500 × g for 20 min, the supernatant recovered and the pellet re-dissolved in 5 mL of 90% ethanol and re-centrifuged at 500 × g for 15 min. The supernatant was recovered and pooled with the previous one, dried and re-dissolved in 1 mL methanol (100%). In this study, average fecal extraction efficiency was 80% (CV < 10%).

Estrogen and progestin metabolites were quantified by enzyme immunoassay [13]. Antibodies for estrogen (polyclonal estrone sulfate R583; 1:40,000 dilution) and progestin (monoclonal pregnane CL425 1:10,000 dilution) metabolite analysis were obtained from Coralie Munro (University of California, Davis, CA, USA). The R583 cross reacts with estrone-3-sulphate (100%), estrone-3-glucoronide (70%), estrone (269%), estradiol-17β (9.8%), estrodiol-3-glucoronide (94.0%), and estrodiol-3-sulphate (4.8%)[14]. The CL425 cross reacts with various progesterone metabolites, including 4-pregnen-3,20-dione (100%), 4-pregnen-3α-ol-20-one (188%), 4-pregnen-3β-ol-20-one (172%), 4-pregnen-11α-ol-3,20-dione (147%), 5α-pregnan-3β-ol-20-one (94%), 5α-pregnan-3β,20-dione (64%), 5α-pregnan-3,20-dione (55%), 5β-pregnan-3β-ol-20-one (12.5%), 5-pregnan-3,20-dione (8.0%), 4-pregnen-11β-ol-3,20-dione (2.7%), and 5β-pregnan-3α-ol-20-one (2.5%) [15]. Before analysis, fecal extracts were diluted in phosphate-buffered saline (1:150 for estrogen and 1:1000 to 1:60,000 for progestins). Serial dilutions of pooled fecal extracts produced displacement curves parallel to those of the appropriate standard. Inter- and intra-assay CVs were <10%. Assay sensitivities were 1.95 and 0.78 pg/well for the estrogen and progestin EIA, respectively.

2.3. Behavioral observations

Behavioral observations were recorded for females ($n = 9$) paired with males using an established ethogram developed by the American Zoo and Aquarium Association's Maned Wolf Species Survival Plan. Wolves were observed for 30 min/session, with three to five sessions/wk for solitary, social and sexual (i.e., mounting, solicitation, copulatory) behaviors. Observations relevant to solicitous behavior, sexual receptivity, copulation and parturition were superimposed on endocrine profiles.

2.4. Longitudinal profiles and data analysis

Longitudinal profiles of estrogen and progestin metabolites were aligned to the day of the estrogen peak (Day 0). In females that lacked a distinctive estrogen peak, Day 0 was identified as the first day that estrogen rose above nadir concentrations by two standard deviations (S.D.). Baseline values for each individual were calculated by an iterative process, whereby high values (exceeding the mean plus two S.D.) were excluded. Each time the average was recalculated, and the elimination process repeated until no values exceeded the mean plus two S.D. Because daily fecal samples were not always available, fecal hormone levels across an individual's profile were pooled into 3-d means.

Data are reported as means ± S.E.M. Data were transformed by application of a logarithm (base 10) and tested for normality. Longitudinal steroid metabolite profiles were divided into three reproductive stages using modified criteria of those described by Velloso et al. [6] and Walker et al. [16]: (1) preovulatory phase or basal phase (days −30 to −10), (2) periovulatory phase (days −9 to 9), and (3) luteal phase (days 10–70). Differences in fecal steroid patterns among age categories and among females of different reproductive status within the same reproductive stage were determined by averaging individual means, followed by comparisons by analysis of variance (ANOVA, SigmaStat 3.0, SPSS Inc., Chicago, IL, USA). Comparison of steroid concentrations within reproductive status groups across reproductive stages (preovulatory versus periovulatory versus luteal phase) was also conducted using ANOVA. Differences were considered significant at $P < 0.05$.

3. Results

Of the nine paired females, seven conceived after displaying behaviors consistent with estrus and increased estrogen metabolite excretions. Four of nine paired females (age range, 2–8 yr) experienced copulatory ties. Dates of estrogen peak, breeding (exhibiting estrous behavior with or without copulatory ties) and pup delivery in pregnant females are shown in Table 1. The copulatory ties could be observed from 2 d before to 9 d after the estrogen peak. Copulatory ties were observed on more than one occasion in two of the four females (2 and 5 d after the estrogen peak in one female and 2 d before and 7 and 9 d after the estrogen peak in another female). Of the seven females that conceived, breeding or estrous behavior was observed from October to January. The duration of gestation of the seven pregnant females (successfully raised and failed to raise pups) ranged from 62 to 69 d (average, 64 d). Births occurred from December to March.

Longitudinal profiles of fecal estrogen and progestin metabolites in a representative pregnant maned wolf are shown in Fig. 1. Estrogen metabolite concentrations gradually increased beginning about 2 wk before breeding and declined to baseline on the day of lordosis and copulation (Figs. 1, 2A and B). Fecal progestin metabolite concentration rose steadily during the time that sexual receptivity was observed and remained elevated during pregnancy (Figs. 1, 3A and B). Sexual behaviors were not observed in the paired non-pregnant females, although we cannot exclude the possibility that mating occurred. In two paired females (10 yr of age) that did not conceive, longitudinal profiles revealed a distinct estrogen peak followed by a rise in progestins. Progestin metabolite concentration remained high until

Table 1
Dates of estrogen peak, breeding and pup delivery of seven pregnant maned wolf females

Studbook no.	Date of estrogen peak (d/m/y)	Copulatory ties	Breeding dates (d/m/y)[a]	Birth date (d/m/y)	Gestation (d)	Raised pups
1615	22 November 2002	No	28 November, 2002	29 January 2003	62	Yes
1672	06 December 2002	No	11 December 2002	15 February 2003	65	No
1818	01 October 2002	Yes	06 October 2002	10 December 2002	65	Yes
1821	N/A[b]	Yes	23 December 2002	25 February 2003	64	Yes
2009	23 November 2002	Yes	20 November 2002, 01 December 2002, 02 December 2002	04 February 2003	63–64[c]	Yes
2260	17 January 2003	No	22 January 2003	27 March 2003	64	No
2347	03 January 2003	Yes	05 January 2003, 08 January 2003	13 March 2003	67–69	Yes

[a] Days that copulatory tie was observed or days that wolves exhibited estrous behaviors without experiencing copulatory tie.
[b] Fecal samples were obtained after breeding was observed.
[c] Gestation was calculated based on the last two observed copulatory ties (1 and 2 December 2002).

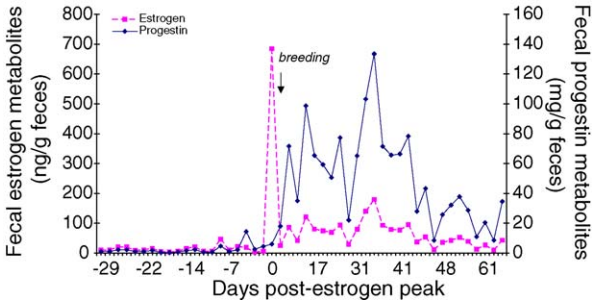

Fig. 1. Longitudinal profiles of fecal estrogen and progestin metabolites for a pregnant maned wolf from 14 d before the estrogen peak to 2 wk before parturition.

the end of the monitoring period (Figs. 2C and 3C). These females were classified as pseudo-pregnant.

Because it was difficult to observe sexual behaviors in the absence of males, behavioral data were not available for the three unpaired females. Hormonal profiles of these bitches revealed the absence of detectable estrogen increases or an estrogen metabolite surge (Fig. 2D). Progestin metabolite concentrations also remained at baseline in maned wolf females maintained as singletons (aged 2, 12 and 15 yr old, Fig. 3D).

A comparison of fecal steroid data during the preovulatory (days −30 to −10), periovulatory (days −9 to 9) and luteal (days 10–70) phases among females of different age classes is shown in Table 2. Three females maintained as singletons and failing to ovulate were excluded from this analysis. Mean estrogen metabolite concentration was higher ($P < 0.05$) in aged females than young and prime-breeding age classes during the preovulatory phase, and was higher ($P < 0.05$) than the young age class during the periovulatory phase. Concentrations of progestin metabolites during the preovulatory and periovulatory periods were similar ($P > 0.05$) among age groups. However, young females and those of prime breeding age excreted more ($P < 0.05$) progestins during the luteal phase than aged counterparts.

Fecal estrogen and progestin concentrations across reproductive phases in females with various pregnancy outcomes are shown in Table 3. Mean estrogen

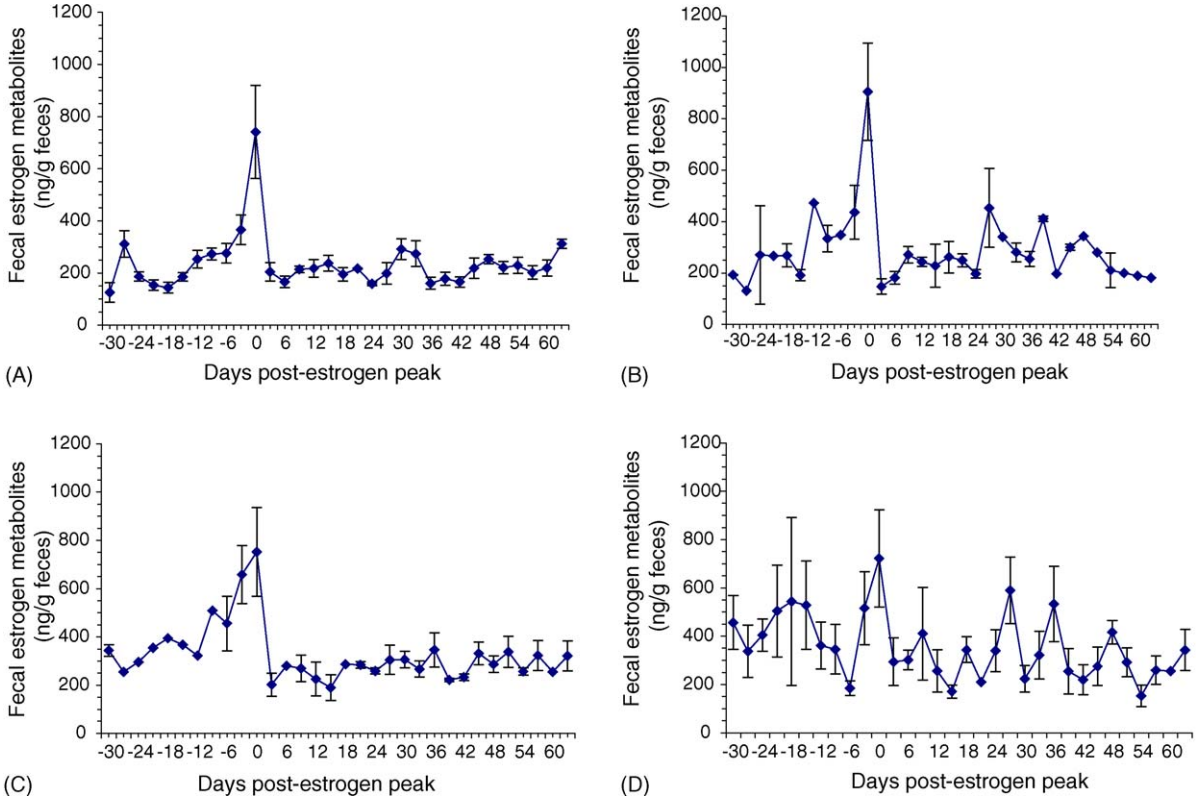

Fig. 2. Mean (±S.E.M.) longitudinal profiles of fecal estrogen metabolites for female maned wolves that: (A) conceived and successfully raised pups ($n = 5$); (B) conceived but failed to raise pups ($n = 2$); (C) became pseudo-pregnant ($n = 2$); and (D) were unpaired ($n = 3$). Each data point is a pool of 3-d mean.

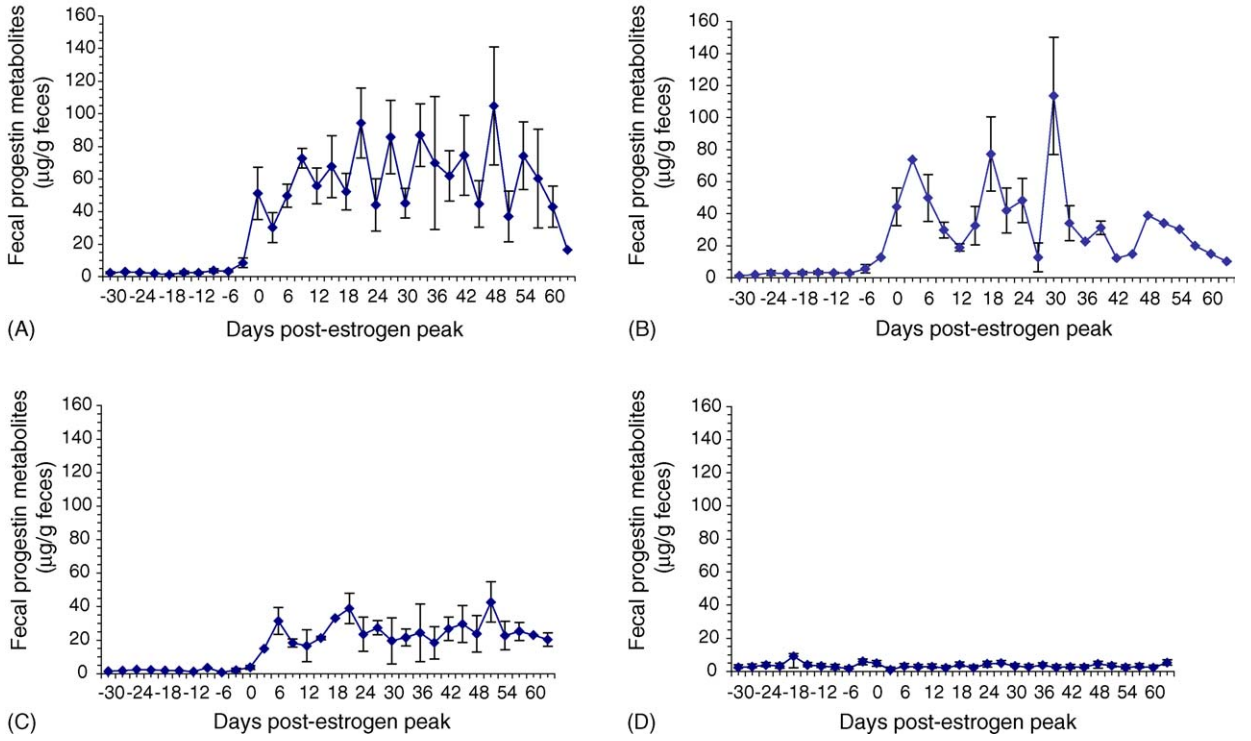

Fig. 3. Mean (±S.E.M.) Longitudinal profiles of fecal progesterone metabolites for female maned wolves that: (A) conceived and successfully raised pups ($n = 5$); (B) conceived but failed to raise pups ($n = 2$); (C) became pseudo-pregnant ($n = 2$); and (D) were unpaired ($n = 3$). Each data point is a pool of 3-d mean.

metabolite concentration during the preovulatory and luteal phases in pregnant females that successfully raised pups was lower ($P < 0.05$) than that of other groups. Mean progestin concentrations during the preovulatory interval did not vary ($P > 0.05$) among groups; however, during the periovulatory period, pregnant maned wolves had higher ($P < 0.05$) progestin concentrations compared to pseudo-pregnant and unpaired females. During the luteal phase, the highest mean progestin metabolite concentration was found in females that raised pups, followed by pregnant females that lost pups and then pseudo-pregnant females. Progestin metabolite concentrations in singleton females during the luteal phase was ($P < 0.05$) lower than those of other groups. The difference in post-ovulatory progestins between pregnant females that raised or lost pups was the result of a decline in concentrations during the second half of gestation in wolves that lost pups (Fig. 3A and B). Fecal progestin metabolite concentrations in the singleton females remained at baseline throughout the breeding season (mean ± S.E.M.; 3.2 ± 0.5 µg/g of feces; Fig. 2D), lower ($P < 0.05$) than all other females post-ovulation.

4. Discussion

This is the first study to examine the relationship of gonadal hormone metabolite excretion in female maned wolves of various adult ages, with varying reproductive success. Furthermore, all data were generated using enzyme immunoassay technology, which avoids the use of radioisotopes (required by radioimmunoassay). We showed that the maned wolf was similar to the domestic dog in terms of: (1) temporal and kinetic profiles of gonadal steroids, especially during the periovulatory interval; and (2) pregnancy-specific differences in fecal progestin metabolite concentration post-ovulation. We also found that females that lost their pups soon after giving birth had lower progestin concentrations during the second half of gestation. There also were preliminary indications that a female may fail to ovulate in the absence of a male, an observation that warrants further study.

The general hormone patterns of female maned wolves observed in the present study were similar to those reported by Velloso et al. [6] using radio-immunoassay technology. This confirms the validity of the enzyme immunoassay procedures for monitoring

Table 2
Mean ± S.E.M. concentrations of ovarian steroid metabolites during the preovulatory (days −30 to −10), periovulatory (days −9 to 9) and luteal (days 10–70) phases of reproductively young ($n = 2$), prime breeding ($n = 5$) and aged maned wolves ($n = 2$)

Age classes[1]	Mean concentrations of estrogen metabolites (ng/g feces)	Mean concentration of progestin metabolites (μg/g feces)
Preovulatory		
Young	160.1 ± 22.9[a]	2.7 ± 0.3
Prime breeding	212.6 ± 13.9[a]	2.4 ± 0.2
Aged	334.6 ± 16.0[b]	1.7 ± 0.2
Periovulatory		
Young	317.5 ± 56.7[a]	26.1 ± 7.5
Prime breeding	337.5 ± 39.4[a,b]	27.7 ± 4.7
Aged	498.5 ± 74.3[b]	9.9 ± 3.5
Luteal		
Young	220.1 ± 15.3	46.3 ± 7.6[b]
Prime breeding	244.5 ± 10.2	60.6 ± 4.3[b]
Aged	211.7 ± 15.9	25.5 ± 2.0[a]

Means ± S.E.M. were calculated from raw data of hormone metabolites during each reproductive cycle for each age class.
[a,b]For each steroid, different letters within the same reproductive phases indicate differences ($P < 0.05$).
[1] Females that did not show estrual changes in fecal estrogens and did not ovulate (one young, two aged) excluded.

Table 3
Mean ± S.E.M. concentrations of ovarian steroid metabolites during the preovulatory (days −30 to −10), periovulatory (days −9 to 9) and luteal (days 10–70) phases of females that conceived and successfully raised pups ($n = 5$), conceived but lost pups ($n = 2$), became pseudo-pregnant ($n = 2$) or were unpaired ($n = 3$)

Reproductive outcomes	Mean concentrations of estrogen metabolites (ng/g feces)	Mean concentration of progestin metabolites (μg/g feces)
Preovulatory		
Pregnant, raised pups	189.6 ± 12.7[a]	2.4 ± 0.2
Pregnant, lost pups	246.1 ± 19.6[a,b]	2.6 ± 0.3
Pseudo-pregnant	334.6 ± 15.9[b]	1.7 ± 0.2
Unpaired	428.5 ± 48.6[b]	2.9 ± 0.3
Periovulatory		
Pregnant, raised pups	321.4 ± 38.8	24.7 ± 5.5[b]
Pregnant, lost pups	352.9 ± 58.4	28.4 ± 8.1[b]
Pseudo-pregnant	498.5 ± 74.3	9.9 ± 4.1[a]
Unpaired	416.8 ± 51.9	3.1 ± 0.5[a]
Luteal		
Pregnant, raised pups	219.5 ± 7.7[a]	68.9 ± 5.9[c]
Pregnant, lost pups	279.0 ± 21.2[b]	39.0 ± 6.3[b,c]
Pseudo-pregnant	274.1 ± 11.2[b]	11.3 ± 1.8[b]
Unpaired	313.9 ± 25.4[b]	3.3 ± 0.3[a]

Means ± S.E.M. were calculated from raw data of hormone metabolites during each reproductive cycle for each class of reproductive status.
[a–c]For each steroid, different letters within the same reproductive phase indicate differences ($P < 0.05$).

reproductive ovarian activity in the maned wolf. Similar to the domestic dog [8–10], African wild dog [17], gray wolf [18], and red wolf [16], sexual receptivity and ovulation in the maned wolf occurred in the presence of declining estrogen and rapidly rising progestins, reproductive characteristics that are highly conserved among Canidae. The preovulatory progestin rise was most likely due to early follicular luteinization [11].

In agreement with Velloso et al. [6], we noted that pseudo-pregnant females excreted overall lower concentrations of progestins than their pregnant counterparts. It remains to be determined if the monitoring of longitudinal profiles would be useful as a diagnostic tool for pregnancy in this species. In domestic dogs, it has been shown that there is a quantitative pregnancy-specific difference in fecal progestin metabolite concentrations [10]. Additionally, we demonstrated for the first time that mean progestin concentrations during the second half of gestation in pregnant females that successfully raised pups were higher than those of females that lost their pups within a few days after delivery. So far, the causes underlying low progestins in females experiencing neonatal loss have not been elucidated. However, exogenous corticosteroids reduce plasma progestin concentrations in the horse [19], and adrenocorticotrophic hormone decreases progestin content in placenta-uterine complexes of the deer mouse [20]. Perhaps reduced progestin excretion in maned wolves losing pups may be related to an adrenal/stress response, possibly due to suboptimal management. The number of animals studied was too small to make definitive conclusions; however, this observation warrants further studies on a larger scale.

Mean estrogen metabolite concentrations prior to ovulation in age females were higher than those in young and prime breeding age females. However, prime breeding age females produced more progestins than older counterparts post-estrus. It was not clear if this was due to a presumed increased ovulation number in the prime breeders or simply to more efficient luteal activity. In the domestic dog, the ovary is the major source for progestin production throughout gestation [8,9]. Thus far, there is no information regarding the association between numbers of corpora lutea and progestin concentration in canids. However, it has been documented in the goat and pig that there is a positive correlation between numbers of corpora lutea and progestin production [21,22]. Elevated progestins in two of three young and two of four aged bitches suggested that ovulation can occur in maned wolf females at these rather extreme ends of their life span.

This perhaps should not be surprising, given that studbook records reveal that female maned wolves are capable of producing pups from as young as 1.5 yr of age to at least 12 yr of age (albeit uncommonly [3]).

Of the three singleton females in the present study, all failed to ovulate, as determined by continuously low progestin concentrations. Our observations supported the earlier study of Velloso et al. [6] who also reported anovulation in two unpaired maned wolf females. However, two paired females in that study also failed to ovulate (defined as low progestins throughout the observational period), although it was not determined whether females actually had been bred. Taken together, these data raised questions regarding whether the presence of a male is required to provoke estrus and/or ovulation in the maned wolf. This intriguing question also is being addressed by investigators studying the Island fox (*Urocyon littoralis*), a species that also does not appear to ovulate without immediate access to a male (Cheryl Asa, personal communication). Unfortunately our data on the maned wolf was confounded in two ways, first by the small sample size and secondly because the three individuals that did not ovulate were either young or aged. However, this question can be addressed by appropriate endocrine monitoring of singly housed females of prime breeding age.

In summary, we have expanded the endocrine database on the reproductive biology of the threatened maned wolf. Longitudinal fecal steroid monitoring through enzyme immunoassay assessments revealed a temporal similarity to the dog in gonadal hormone profiles during the peri-estrual and pregnancy or pseudo-pregnancy intervals. Therefore, it is likely that hormonal mechanisms driving reproductive success in the domestic dog are quite similar to the maned wolf. Additional observations suggest that other high research priorities should include understanding: (1) the value of fecal progestin metabolite monitoring as a diagnostic tool for pregnancy assessment; (2) the biological relevance of reduced progestins in bitches that lose pups early post-partum; and (3) if there is a true biological effect of male presence on induction of estrus and/or ovulation in the conspecific female.

Acknowledgments

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