Theriogenology Wild 2 (2023) 100032

Contents lists available at ScienceDirect

Theriogenology Wild

journal homepage: www.journals.elsevier.com/theriogenology-wild

Periovulatory changes in behavior and fecal hormone metabolite concentrations but not vaginal cytology or vaginoscopy are indicative for the fertile period in female African wild dogs (*Lycaon pictus*)

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ARTICLE INFO

Keywords:
African wild dog
Artificial insemination
Estrus
Sexual behavior
Fecal estrogen metabolites
Fecal progesterone metabolites

ABSTRACT

Artificial insemination (AI) can aid conservation of African wild dogs (AWD), but methods to determine the appropriate timing of AI in females are not feasible without immobilization. This study determined whether certain behaviors coupled with fecal estrogen (fEM) and progestogen (fPM) metabolite concentrations, could be used as non-invasive parameters to predict the fertile period in female AWDs. Behavior was observed in three alpha females before, during and after the mating period, and feces analyzed for hormone metabolites. During the periovulatory period, females were immobilized 2–3 times to evaluate vulvar condition, blood hormone concentrations and vaginal cytology, and to conduct vaginoscopy. Late estrus (fertile period) could be distinguished from pro-estrus, early estrus, and diestrus using behavior; with a 2- to 5-fold higher rate of male-female affiliative behavior, sexual and non-sexual follow, alpha male initiating behavior, ride-up and copulation ($P \leq 0.05$). Sexual behaviors, and male-female resting patterns declined significantly or ceased the day after last mating. Two females showed a 2.5- to 3-fold increase in fPM concentrations during late estrus compared to the pro-estrus period ($P \leq 0.05$) and elevated fEM levels that rose in pro-estrus, declined to baseline by late estrus. The one anovulatory female showed no distinct pattern in fPM or fEM concentrations. Vaginal cytology and vaginoscopy could not discriminate between different phases. In summary, behavioral observations coupled with rising fPM and declining fEM can determine the fertile period in African wild dog females, whereas infrequent measurement of blood hormone concentrations, vaginal cytology and vaginoscopy are unreliable.

1. Introduction

African wild dogs (AWD; *Lycaon pictus*) are endangered with a total estimated number of ~6600 animals in fragmented populations predominantly scattered across East and Southern Africa [1]. Assisted breeding techniques, including sperm freezing and artificial insemination (AI), can contribute to respective conservation measures by improving distribution of genetics [2]. However, one of the major limitations for AI in wild canids is determining the female fertile period. Luckily, most canids studied to date have similar reproductive cycles [3] that include a mono-estrous cycle with a long pro-estrus and estrus (together classified as heat), a pregnant or non-pregnant (pseudopregnant) period of diestrus, and an obligatory period of anestrus [2–4]. In the extensively studied domestic dog, the most fertile period when AI is typically performed, occurs in late estrus [5,6].

The reproductive cycle of AWD females appears similar to that of

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https://doi.org/10.1016/j.therwi.2023.100032
Received 12 December 2022; Received in revised form 19 April 2023; Accepted 26 April 2023
Available online 28 April 2023

domestic dogs. Based on behavioral and clinical signs, the combined period of pro-estrus and estrus lasts 14–20 days [7]. Pro-estrus is characterized by increased estrogen concentrations, an increase in female proceptivity, vulvar lip swelling, sanguineous vaginal discharge, and a strengthened bond with the dominant male [7–9]. During estrus, intense sexual follow of the female by the dominant male has been observed [7]. Copulation occurs over a period of 3–7 days, when estrogen concentrations decline, and progestogen concentrations rise [9]. However, further information about reproductive physiology during the peri-ovulatory period in female AWDs is still missing.

Accurate estimation of the fertile period is crucial when performing AI, and even more so when using frozen semen, due to its reduced quality and longevity [10,11]. To estimate the fertile period in domestic dogs, quantification of progesterone in the peripheral blood is the most widely used technique. Progesterone is at baseline levels during pro-estrus and steadily increases during estrus. However, given individual variation exists, use of additional tools is advised. Vaginal cytology and endoscopy can both help determine the phase of the reproductive cycle as hormonal changes cause significant changes in the vaginal epithelium and mucosal folds [12]. Unfortunately, the use of invasive techniques is not possible without immobilization in wild canids, therefore precluding their utility as diagnostic tools for determining the timing of ovulation in AWDs. Behavioral observations coupled with non-invasive quantification of reproductive hormones or their metabolites could be a suitable alternative to time AI, as respective methods have already been established for AWDs [7–9] and adopted in reproductive research [13–15].

The aim of this study, therefore, was to determine whether certain behaviors, coupled with changes in fecal estrogen and progestogen metabolite levels, can be used to determine the fertile period in female AWDs. More specifically, we endeavored to benchmark these parameters against observed mating, clinical signs of estrus, serum estrogen and progesterone concentrations, vaginoscopy and vaginal cytology.

2. Materials and methods

2.1. Animals, husbandry and behavioral observations

This study included three alpha females from three different packs housed at Harnas Wildlife Foundation, Gobabis, Namibia (Brutus pack, BRU F1 ♀; Platform pack, PLA F1 ♀; San pack, SAN F1 ♀; Table 1). As described previously [16], packs were of mixed-sex and held in large enclosures (0.7 ha, 14.4 ha, and 3.6 ha respectively) of natural habitat consisting of dense trees, scrub and an artificial waterhole. However, the PLA pack was moved into four smaller adjacent enclosures (0.1 ha each) for the period of study (observation period; Table 1) to facilitate observations, fecal sample collection, and capture as described previously [16]. Animals were group-fed daily with donkey and horsemeat on the bone or intestines, occasionally replaced by dog pellets (Hill's Pet Nutrition, Kansas, United States), or goat, sheep, or wild game meat. Water was available *ad libitum*.

The alpha female within each pack was observed over a period of 21–32 days (observation period) for signs of heat during the 2015/2016 breeding season (defined as the time mating occurs; Table 1). While the breeding season tends to occur between February and May in Southern Africa, in captivity, births can occur at low frequency at every month of the year [2]. Thus, the timing of the observation period for each pack was informed by historical records of mating and births previously observed (Harnas staff, *pers. comm.*). During this time, their behavior was video recorded for several hours each day (daily observation time; Table 1). The PLA and SAN pack females were observed to mate in January and March respectively, which falls within the expected breeding season. The BRU pack female was observed to mate around October. Recordings were performed within enclosures from the top of a car, which was moved when necessary to maintain visual contact with the alpha female. Recordings were analyzed from 6–3 days before the first until 2–6 days after the last observed copulation (behavioral analysis period; Table 1). The entire footage was scanned each day to check for the presence of copulation, then detailed behavioral analysis was confined to a subset of times each alpha pair was sexually active, resulting in a daily behavioral analysis time (Table 1). Behavior was not observed on the day of immobilization and sampling of alpha females (this study) or males (performed during a different study [16]). Thus, behaviour was not monitored for 5 (BRU; Day −4, −1, +6, +9, +13; Fig. 3), 4 (PLA; Day +1, +4, +7, +12; Fig. 4) and 3 days (SAN pack; Day −2, −1, +8, Fig. 5) respectively.

Sexual behavior was defined according to the ethogram in Table 2. Non-sexual behaviors, defined previously [17], were also noted. In addition, resting relations (static lying, standing or eating) and their duration were noted, and grouped as close contact between alpha female and male exclusively; close contact between the alpha female and other pack members with or without the alpha male; or the alpha female alone. Hourly rates for each behavior were calculated for each day. The PLA pack males did not show a clear stable hierarchy [16], with different males exhibiting sexual behavior with the alpha female on different observation days. These males were considered 'alpha male' on those specific days.

2.2. Fecal sample collection and hormone metabolite analysis

Gut transit time in African wild dogs is approximately 24 h [18] thus, fecal hormone values are indicative of pooled blood hormone values over the preceding 24 h in this species. Fecal samples were collected opportunistically within a few minutes after each alpha female (n = 3) was seen defecating during the observation period, which occurred infrequently for SAN F1 ♀. Fecal collection was suspended for an additional 2 days during the periovulatory period when pack males were sedated for a parallel study [16]. Approximately 23 fecal samples

Table 1
Pack composition, age, observation and analysis time, and reproductive history of the African wild dog (*Lycaon pictus*) alpha females (n = 3) sampled in this study.

Pack	*Composition, age*	*Observation period*	*Daily observation time (h; mean ± SEM)*	*Behavioral analysis period*	*Daily behavioral analysis time (h; mean ± SEM)*	*Reproductive history α ♀*
BRU	**Total 4 ♂, 3 ♀** α ♂ & ♀: est. > 7 y (unknown age) Offspring 3 ♂, 2 ♀: 1.8 y	32 days (15 Sep - 16 Oct 2015)	4.1 ± 0.3	23 days (23 Sep - 15 Oct 2015)	1.6 ± 0.1	≥ 1 litter
PLA	**Total 13 ♂, 4 ♀** All siblings different litters incl. α ♀: est. 3–5 y (unknown age)	28 days (7 Jan - 3 Feb 2016)	3.1 ± 0.3	17 days (18 Jan - 3 Feb 2016)	2.3 ± 0.1	unknown
SAN	**Total 4 ♂, 6 ♀** Siblings 2 ♂, 3 ♀ incl. α pair: 5 y Offspring 2 ♂, 3 ♀: 1.7 y	21 days (12 Mar - 1 Apr 2016)	2.3 ± 0.3	15 days (16 Mar - 30 Mar 2016)	1.8 ± 0.1	≥ 1 litter

BRU, Brutus pack; est., estimated; PLA, Platform pack; SAN, San pack.

Table 2
African wild dog (*Lycaon pictus*) ethogram characterizing sexual behavior used for analysis (modified from Vlamings, 2011).

Behavior	*Description*
Sexual follow	male follows female closely, with his neck straight and nose in a sniffing attitude.
Ride-up	male mounts female; this is accompanied by pelvic thrusting but without intromission.
Copulation	act of mating, resulting in a copulatory tie.
Present, this includes:	
Present reactive	female directs her anogenital region to male (sometimes averts her tail) as a reaction to advances of the male.
Present active	as above, but characterized by an active backwards approach, pushing towards male.
Mate guarding	all behaviors where male prevents other pack members from approaching female (intervention by approach, chase away).
Initiating behavior from male directed at the female, this includes:	
Genital lick	self-explanatory.
Head under	male pushes with his head towards the ventro-lateral side of female, occasionally lifting, with his head, her back quarters from the ground.
Pass under head	male passes from a lateral side close under the head of female, usually in a somewhat crouching manner; often a short nose-chin contact with female is evident.
Push side/scruff	male pushes with his nose on the side or the scruff of female.
Tail position	male stands behind female with his head directed towards her ano-genital region.
Fur licking	self-explanatory.

Fig. 1. Measurement of vulva thickness (a) and height (b) using callipers in female African wild dogs, and (c) aspect of swollen vulva and vestibule of PLA F1 ♀ (white arrow).

were also collected from n = 7 subordinate behaviorally anestrus females (mean 3.3 feces/female) from each of the 3 packs during their respective observation period. Collected samples were processed as previously described [16], and resulting extracts measured for immunoreactive fecal progestogen (fPM) and estrogen (fEM) metabolite concentrations using enzyme-immunoassay techniques established for AWDs [17]. Assay characteristics including antibody cross-reactivities are described in [19] for the fPM assay and [20] for the fEM assay. EIA sensitivity was 20 ng/g dry weight (DW) (fPM) and 0.5 ng/g DW (fEM), respectively. Intra-assay coefficients of variation (CV), determined by repeated measurements of high- and low-value quality controls, were 4.0 % and 6.6 % (fPM) and 4.8 % and 6.7 % (fEM), respectively. Similarly obtained inter-assay CVs were 5.5 % and 10.1 % (fPM) and 11.4 % and 14.2 % (fEM), respectively. All EIAs were performed at the Endocrine Research Laboratory, University of Pretoria as described by [21].

2.3. Animal sedation and timing of examination procedures

To more precisely link behavior and fecal hormone metabolite levels with physiological events associated with estrus and ovulation, each of the three alpha females were immobilized on two or three occasions at different stages in and around the estrus period and blood collection, vaginal cytology and vaginal endoscopy were performed. For each female, estrus was considered to begin from the first day (day 0) to the last day (day +11, day +10, and day +4 for BRU F1 ♀, PLA F1 ♀, and

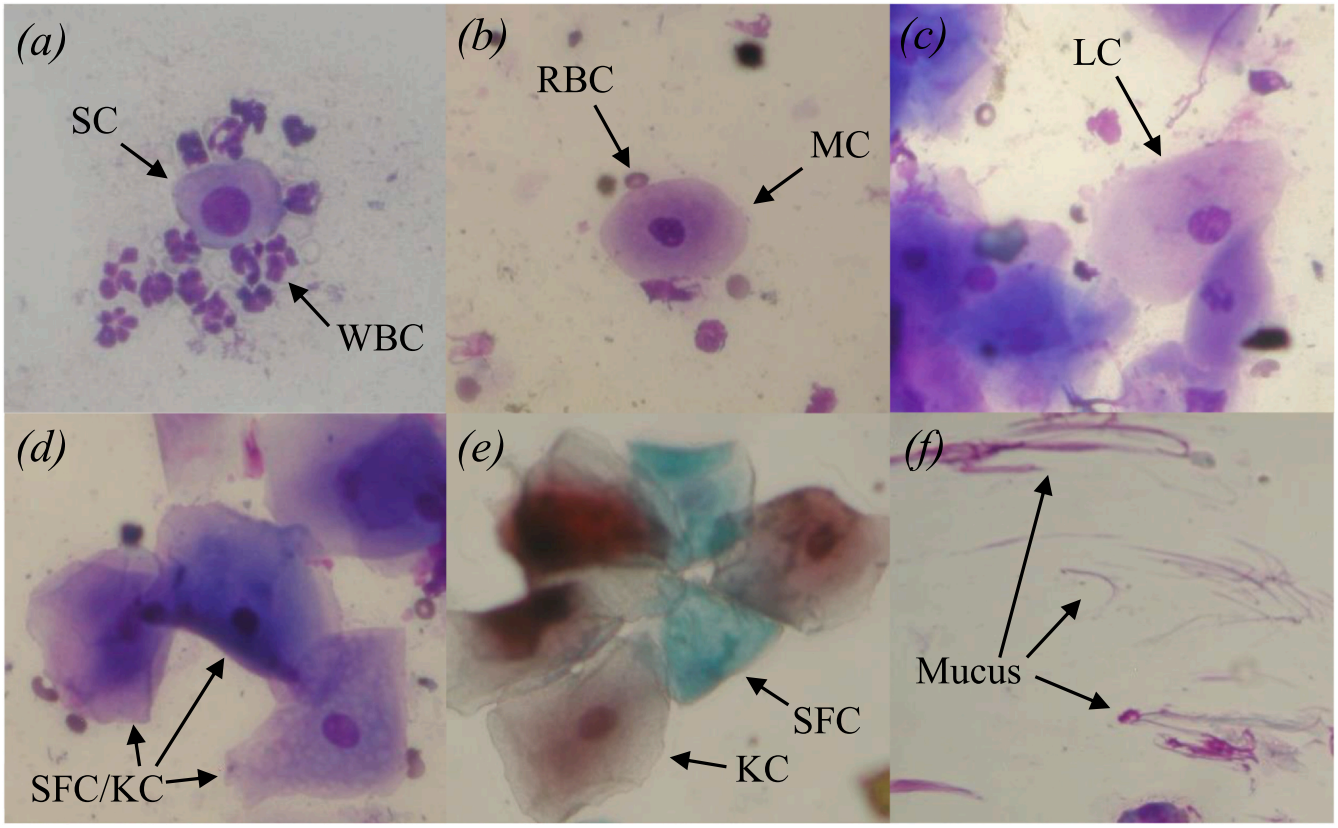

Fig. 2. Classification of African wild dog vaginal epithelial cells stained with Diff-quick (a-d, f) or Harris-Shorr (e). (a) SC, small epithelial cell (rounded, parabasal and small intermediary cells with large nucleus/cytoplasm ratio); (b) MC, medium epithelial cell (rounded, medium nucleus/cytoplasm ratio); (c) LC, large epithelial cell (rounded, very low nucleus/cytoplasm ratio); (d) SFC, superficial epithelial cell/KC, keratinized cell (both polygonal and very large, nucleus often pyknotic or absent); (e) blue-green SFC and red-orange KC distinguished by Harris-Shorr stain; (f) mucus secretion. 400 X magnification; RBC, red blood cell; WBC, white blood cell (neutrophil).

SAN F1 ♀, respectively) in which copulation was observed. To collect physiological measurements spanning late pro-estrus, estrus, and early diestrus, BRU F1 ♀ was immobilized on days −1, +6 and +9; PLA F1 ♀ on days +1, +4 and +7; and SAN F1 ♀ on days +4 and +8 (Table 5). In addition, one lower ranking PLA pack female not displaying signs of estrus, was also immobilized (PLA F2 ♀; anestrus control), 2 days after the estrus period of PLA F1 ♀ ceased. Immobilization mainly occurred in the morning using a combination of 5.3–12.2 mg/kg ketamine (Anesketin®, Albrecht GmbH, Aulendorf, Germany) and 1.4–2.3 mg/kg xylazinehydrochloride (Rompun® TS, Bayer Vital GmbH, Leverkusen, Germany) administered IM using a CO_2 dart gun, after which the animal was transported to the clinic for examination. Following examination, anesthesia was reversed and 10 mL of dog appeasing pheromone (DAP) was applied between the shoulders and on the base of the tail to facilitate reintroduction into the pack [17].

2.4. Vaginal examination

The vulva was inspected for lesions, swelling and mucosal color. Vulvar thickness (W; Fig. 1a) and height (H; Fig. 1b) were measured using callipers (Kinchrome Australia Pty Ltd, Scoresby, VIC, Australia) and vulvar size calculated as W x H then adjusted for bodyweight. A swab was taken from the cranial vagina, its color noted, and gently rolled onto several glass slides. Slides were stained with Diff-Quick (*Kyro-Quick stain*; Kyron Laboratories, Benrose, South Africa) and a modified Harris-Shorr stain for vaginal cytology. Diff-quick-stained slides were first examined for cellularity at X 100 magnification under brightfield microscopy. Thereafter, individual epithelial cells (> 200 cells) were analyzed at X 400 magnification and, according to their nucleus/cytoplasm ratio and form, classified as small, medium, large or superficial/keratinized cells (Fig. 2). The difference between superficial and keratinized cells could not be distinguished using Diff-Quick stain, as both have a polygonal aspect (Fig. 2d). Therefore, the proportion of keratinized cells was determined using Harris-Shorr stain, in which keratinized cells stain red-orange while superficial non-keratinized and all other cells stain blue-green (Fig. 2e).

Deep vaginal endoscopy was performed using a Single Channel Ureteroscope A2940 (Olympus, Shinjuku, Tokyo, Japan), and color of the vaginal mucosa, presence and color (hemorrhagic, serosanguineous, serologic) of uterine secretions, and amount of vaginal edema were noted. The latter was scored as 0 – no edema; 1 – presence of primary folds (edema + + +); 2 – appearance of secondary folds (edema + +); 3 – secondary folds (edema +); 4 – start angulation/crenulation (edema ±); 5 – angulation (edema –).

2.5. Blood sampling and reproductive hormone analysis

Following endoscopy, ten mL of blood was collected from the cephalic or saphenous veins. After 30 min, blood was centrifuged at 3000*g* for 15 min. Serum was frozen at −18 °C and transported to the University of Namibia (Windhoek, Namibia) on dry ice where they were thawed. Subsequently, 2.5 mL diethyl ether was added to 0.5 mL serum and vortexed for 5 min. After keeping the solution for 5 min at RT it was frozen in a dry ice/ethanol bath. Approximately 2 mL of the top-layer was removed and air-dried for 5–6 h. Dried extracts were transported to the Endocrine Research Laboratory, University of Pretoria, where they were reconstituted in assay buffer and analyzed for estrogen and progestogen concentrations using the EIAs mentioned in Section 2.2. Assay sensitivity was 0.64 ng/mL and 16 pg/mL serum for the progestogen and estrogen EIA, respectively.

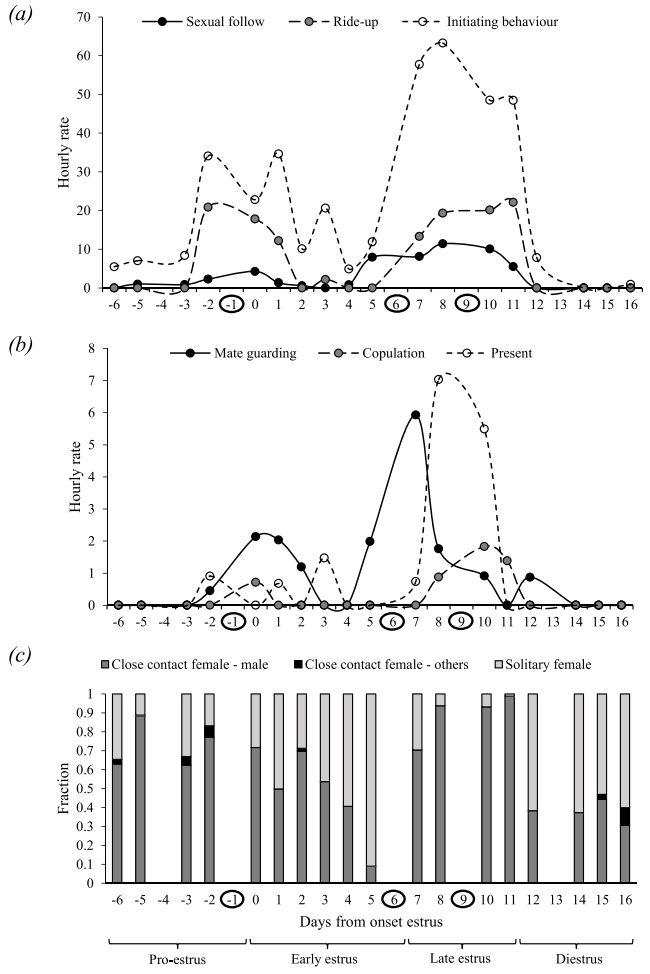

Fig. 3. (a, b) Hourly rates of sexual behavior and (c) resting relationships (expressed as fraction of total resting time) relative to the onset of estrus (D0; first day of observed copulation) for the alpha African wild dog female BRU F1 ♀. Circles indicate days of immobilization.

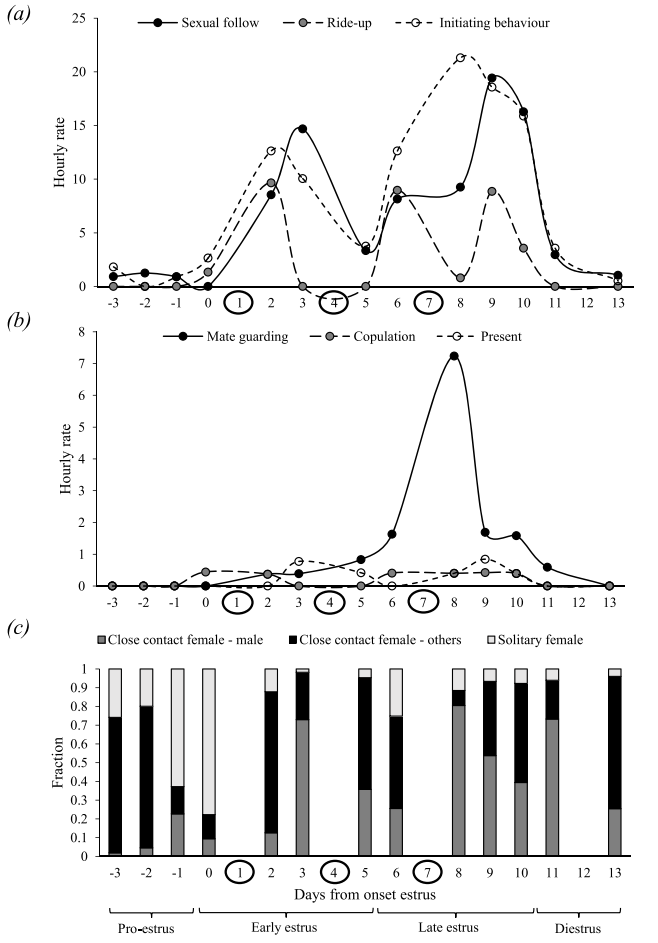

Fig. 4. (a, b) Hourly rates of sexual behavior and (c) resting relationships (expressed as fraction of total resting time) relative to the onset of estrus (D0; first day of observed copulation) for the alpha African wild dog female PLA F1 ♀. Circles indicate days of immobilization.

2.6. Statistical analysis

Based on observed sexual behaviors, the stages of behavioral analysis could be divided into pro-estrus (prior to first copulation), early estrus (first half of copulation period), late estrus (fertile period, second half of copulation period), and diestrus phases (after last copulation) for each of the three alpha females. Hourly rates of sexual and non-sexual behaviors, and resting patterns (expressed as fraction of total resting time) for the three females were obtained each day and grouped by reproductive phase, with data tested for normality by Shapiro-Wilk test and histograms. Differences in hourly rates of these behaviors between phases were evaluated using Kruskal-Wallis and post-hoc Mann-Whitney U tests with Bonferroni adjustment; except for male-female and solitary female resting patterns (normally distributed), which were analyzed by one-way ANOVA and post-hoc Tukey test. Fecal hormone metabolite concentrations during the different reproductive phases were compared by one-way ANOVA with post-hoc Tukey test for BRU F1 ♀ and PLA ♀ F1. $P \leq 0.05$ was significant. Statistical analyses were performed with SPSS Statistics 23 (IBM® SPSS® Statistics 23, SPSS Inc., IBM, Armonk, New York, USA).

3. Results

3.1. Behavior

In the BRU pack, sexual behavior including copulation attempts (ride-up) started on day −2, but copulation was only seen on days +0, +8, +10 and +11 (Fig. 3a and b). For the PLA pack there was no ride-up prior to the first day of observed copulation, which occurred on days 0, +2, +6, +8, +9 and +10 (Fig. 4a and b). The estrus period in the SAN pack alpha female only lasted for 5 days, with first ride-up coinciding with the first day of observed copulation, which occurred on days +0, +2, +3 and +4 (Fig. 5a and b). However, observations could not be performed on days −1 and −2 in the SAN pack due to immobilization of males on those days for a separate study [16]. As such, it is possible that the onset of early estrus in this female may have begun on day −1 or −2. All three alpha females showed a clear biphasic increase in most sexual behaviors and a monophasic increase in female-male resting behavior that typically extended from a few days before estrus and ended abruptly on the last day of copulation (Figs. 3, 4 and 5). The hourly rate of several of these behaviors was generally higher during the second phase of estrus (classified as late estrus).

The behavioral period observed was subdivided into four distinct reproductive phases: (1) pro-estrus (days −6 to −1 for BRU F1 ♀, days −3 to −1 for PLA F1 ♀ and days −5 to −3 for SAN F1 ♀); (2) early estrus (days +0 to +6 for BRU F1 ♀, days +0 to +5 for PLA F1 ♀, and days −2 to +2 for SAN F1 ♀); (3) late estrus (days +7 to +11 for BRU F1 ♀, days +6 to +10 for PLA F1 ♀, and days +3 to +4 for SAN F1 ♀); and (4) diestrus (days +12 to +16 for BRU F1 ♀, days +11 to +13 for PLA F1 ♀, and days +5 to +9 for SAN F1 ♀) (Figs. 3, 4 & 5).

When data was analyzed by reproductive phase, there was no difference in any sexual or non-sexual behaviors nor resting patterns between pro-estrus and early estrus ($P > 0.05$; Table 3). Hourly rates of

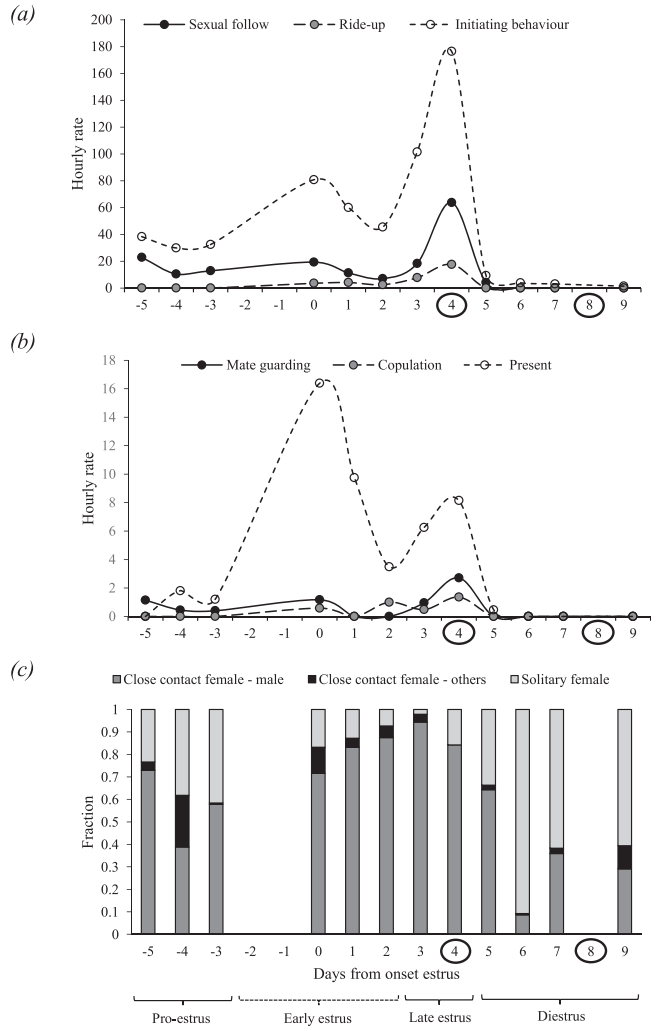

Fig. 5. (a, b) Hourly rates of sexual behavior and (c) resting relationships (expressed as fraction of total resting time) relative to the onset of estrus (D0; first day of observed copulation) for the alpha African wild dog female SAN F1 ♀. Circles indicate days of immobilization. Dashed bracket, putative early estrus period.

sexual follow, ride-up, copulation and initiating behavior increased over three-fold, and alpha male-female affiliative behavior and non-sexual follow increased over two-fold from early to late estrus ($P \leq 0.05$). Sexual follow and initiating behavior were observed to occur at particularly high frequency (Table 3). Apart from sexual follow, these behaviors were higher during late estrus than any other reproductive phase ($P \leq 0.05$), and all dropped abruptly or ceased completely on the day after last copulation (Figs. 3, 4 & 5, Table 3); clearly indicating the first day of diestrus. While hourly rates of alpha female-male resting patterns tended to increase and solitary female resting patterns tended to decrease during late estrus, hourly rates of these patterns as well as present and marking behavior only differed at diestrus ($P \leq 0.05$). Mate guarding was higher during the entire estrus period but tended to be highest in late estrus (Table 3).

3.2. Fecal steroid hormone metabolites

There were differences in the fecal hormone metabolite profiles of the three alpha females. For BRU F1 ♀, fPM levels were at baseline (8.1 ± 1.1 µg/g DW) until day −6 then rose over two-fold by day −1 and continued to rise during estrus (24.9 ± 3.4 µg/g DW; Fig. 6a); with levels in early and late estrus significantly higher compared to pro-estrus (Table 4). Baseline levels of fEM could be observed from days −14 to −10 (0.18 ± 0.10 µg/g DW) and a distinct peak on day −7 (1.28 µg/g DW), after which levels decreased again over early estrus (0.54 ± 0.08 µg/g DW) and declined to baseline during late estrus (0.15 ± 0.06 µg/g DW; Fig. 6a). However, no significant differences could be seen in fEM levels between the different reproductive phases (Table 4).

For PLA F1 ♀, fPM levels did not rise from baseline levels during the estrus period (Fig. 6b; Table 4) and were similar to levels observed in anestrus females (Table 4), possibly indicating an anovulatory cycle in this female. Although fEM levels on day −10 were slightly higher compared to the other pro-estrus samples of this female, a distinct peak in fEM levels of the same magnitude as observed for BRU F1 ♀ was absent. Fecal samples could not be collected on days −9 to −4, but a minor increase (0.35 µg/g DW) in fEM levels could be seen at day −2 (Fig. 6b). Levels of fEM did not change across the reproductive phases (Table 4), suggesting anovulation in this female.

Fewer fecal samples were collected for SAN F1 ♀ making statistical comparison by reproductive phase impossible (Table 4). However, the steroid hormone patterns showed similar trends to that observed for BRU F1 ♀. Baseline fPM levels were seen on days −8 to −6 (11.3 ± 2.5 µg/g DW), after which they rose on day −5 and continued to rise into diestrus at day +8 (63.3 µg/g DW; Fig. 6c). As for BRU F1 ♀, levels in late estrus were approximately two and a half-fold higher than baseline. Baseline fEM levels (day −8 to −6; 0.20 ± 0.14 µg/g DW) rose to a peak on day −5 (1.93 µg/g DW) and dropped back to baseline levels in late estrus on day +4 (0.09 µg/g DW).

3.3. Vaginal examination

The vulva had a swollen aspect in all cases except for PLA F2 ♀ (anestrus control) and SAN F1 ♀ (last day estrus and early diestrus; Table 5 and Fig. 1c). When standardized to body weight, vulvar size was small in anestrus (0.4 cm^2/kg), showed more than a 2-fold increase by the end of pro-estrus (0.9 cm^2/kg), after which it declined again during estrus (0.6–0.7 cm^2/kg), reaching a size of 0.5 cm^2/kg by the end of estrus and diestrus (Table 5). Visible vulvar discharge was only present at the first sedation of PLA F1 ♀, however vaginal swab color showed the presence of sanguineous secretions (PLA F1 ♀ and BRU F1 ♀) into early estrus (Table 5).

In anestrus, few epithelial cells were present, all of which were small (parabasal and small intermediary), with a few old neutrophils and mucus (Table 5; Fig. 7a and b). At the end of pro-estrus, epithelial cells had increased and transitioned to similar proportions of large, superficial, and keratinized cells. No neutrophils or mucus were present, while bacteria were abundant (Table 5; Fig. 7c and d). By mid-estrus, the number of keratinized cells increased in BRU F1 ♀ and reached a maximum (84 %) at day +6 (Table 5; Fig. 7e and f), but declined again toward late estrus (Table 5; Fig. 7g and h). A similar trend was observed for PLA F1 ♀, where the number of keratinized cells decreased from 59 % in early estrus to 33 % during late estrus, and mucus and neutrophils reappeared. On the last day of estrus (SAN F1 ♀ day +4), the dominant epithelial cell population consisted of medium cells, and the fraction of keratinized cells decreased to 2 % (Table 5; Fig. 7i and j). In early diestrus, the size of epithelial cells continued to decline, and neutrophils were abundant (Table 5; Fig. 7k and l).

Vaginoscopy revealed a pink, dry mucosa with flat mucosal folds and no edema during anestrus. By the end of pro-estrus (BRU F1 ♀ day −1; Table 5), the mucosa was thickened, white and edematous with presence of hemorrhagic secretions (score 1). By early estrus (PLA F1 ♀ day +1), mucosal folds shrank slightly, causing secondary folds to appear (score 2). The mucosa still had a white aspect and secretions were now serosanguinous (Table 5). The mucosal folds only shrank slightly further over the remainder of the estrus period, maintaining the appearance of secondary folds (score 3; Table 5). Secretions progressively changed to serologic and then disappeared, while the mucosa maintained a pale aspect (Table 5). Angulation (score 4) was not

Table 3
Mean (± SEM) hourly rates of sexual and non-sexual behavior and fractional resting patterns in n = 3 African wild dog (*Lycaon pictus*) alpha females during different phases of the reproductive cycle.

	Pro-estrus	*Early estrus*	*Late estrus*	*Diestrus*	*P-value**
Sexual behavior					
Sexual follow of f1 by m1	5.3 ± 2.4^{ab}	4.9 ± 1.4^{b}	17.0 ± 5.4^{a}	0.8 ± 0.5^{b}	< 0.001
Mate guarding	0.2 ± 0.1^{b}	0.8 ± 0.2^{ab}	2.4 ± 0.7^{a}	0.1 ± 0.1^{b}	0.001
Ride-up	2.1 ± 2.1^{bc}	3.9 ± 1.6^{b}	12.2 ± 2.3^{a}	0.0 ± 0.0^{c}	< 0.001
Copulation	0.0 ± 0.0^{b}	0.2 ± 0.1^{b}	0.8 ± 0.2^{a}	0.0 ± 0.0^{b}	< 0.001
Present	0.4 ± 0.2^{ab}	0.3 ± 0.1^{ab}	2.9 ± 1.1^{a}	0.0 ± 0.0^{b}	0.011
Initiating behavior	15.8 ± 5.0^{bc}	11.7 ± 2.4^{b}	56.4 ± 15.9^{a}	3.1 ± 1.0^{c}	< 0.001
Non-sexual behavior					
Non-sexual follow of f1 by m1	3.7 ± 1.0^{b}	4.3 ± 1.0^{b}	8.7 ± 1.8^{a}	4.3 ± 2.1^{b}	0.037
Marking behavior	3.1 ± 0.9^{ab}	5.6 ± 1.4^{ab}	5.3 ± 0.8^{a}	1.6 ± 0.8^{b}	0.013
Interactions f1 - others	2.7 ± 0.5	1.9 ± 0.5	2.5 ± 1.1	3.4 ± 0.9	0.480
Affiliative behavior m1 - f1	1.0 ± 0.3^{b}	3.6 ± 0.9^{b}	8.4 ± 1.5^{a}	1.4 ± 0.5^{b}	< 0.001
Dominant behavior f1 to m1	1.0 ± 0.6	0.4 ± 0.2	2.2 ± 0.8	1.3 ± 0.8	0.135
Submissive behavior f1 to m1	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.609
Dominant behavior m1 to f1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.623
Submissive behavior m1 to f1	0.2 ± 0.2	0.0 ± 0.0	0.1 ± 0.1	0.3 ± 0.2	0.211
Resting pattern					
Close contact female - male	0.49 ± 0.10^{ab}	0.48 ± 0.08^{ab}	0.73 ± 0.08^{a}	0.39 ± 0.06^{b}	0.031
Close contact female - others	0.20 ± 0.09	0.16 ± 0.07	0.15 ± 0.07	0.12 ± 0.07	0.512
Solitary female	0.31 ± 0.05^{ab}	0.36 ± 0.08^{ab}	0.11 ± 0.03^{b}	0.49 ± 0.09^{a}	0.005

Values with different letters for a particular behavior indicate a significant difference between reproductive phases. m1, alpha male; f1, alpha female. **P*-value of Kruskal-Wallis/one-way ANOVA analysis.

present during estrus in any AWD females, but appeared later during early diestrus (SAN F1 ♀ day +8; Table 5).

3.4. Blood reproductive hormones

Blood progesterone levels were comparatively lower during anestrus and through pro-estrus to early estrus, after which they slowly started rising for BRU F1 ♀ and SAN F1 ♀, but not in PLA F1 ♀, through end-estrus to diestrus (Table 5). No clear pattern in blood estrogen levels was evident during the periovulatory period in all three AWD females (Table 5).

4. Discussion

Behavioral observations coupled with non-invasive monitoring of reproductive hormones in feces has great potential to aid wildlife captive and assisted breeding, conservation and management. This study demonstrated that a two- to three-fold increase in the frequency of several key behaviors (male-female affiliative behavior, male initiating behavior, sexual and non-sexual follow, ride-up and copulation) coupled with significant rises in fPM concentrations can discriminate late estrus (the fertile period) from other reproductive phases during the periovulatory period in AWD females. The ability to non-invasively distinguish late estrus from early estrus and other reproductive phases is crucial for timing of AI in the AWD, as this phase corresponds to the attainment of oocyte maturity and window for fertilization in canids; which typically ovulate immature oocytes that require 48–60 h to mature in the oviduct. Other invasive diagnostic procedures performed under anesthesia, such as vaginal cytology and vaginal endoscopy, were insufficient to clearly distinguish late estrus from early estrus or pro-estrus, presumably due to infrequent sampling and species-specific differences compared to that observed in domestic dogs.

The variable duration of the estrus period between females in this study, demonstrates that performing AI at a fixed time interval after the onset of pro-estrus or estrus, or after a rise in fPM levels, as has been suggested for the red wolf [22], is unlikely to be successful. The most consistent parameter to determine the moment of late estrus in this study was behavior. Four types of sexual (sexual follow, initiating behavior, ride-up and copulation) and two non-sexual behaviors (male-female affiliative behavior and non-sexual follow) were significantly higher in late estrus compared to early estrus. Initiating behavior and sexual follow where particularly high, as the alpha male was following and interacting with the female continually. Since such behaviors were already occurring in pro-estrus and early estrus, some quantification is necessary to detect the 3.4- to 4.8-fold increase during late estrus. These results are consistent with other studies [7–9,23]. However, our study is the first to describe clear differences between early and late estrus, which is critical to proper timing of artificial insemination. Van Heerden and Kuhn [7] described an incessant follow of the alpha female, intense and frequent urine marking, and mate guarding by the alpha male during estrus. However, behavior in this study was descriptive, so differences between pro-estrus and estrus were not quantified.

The goal for conservation of African wild dogs by AI (in captive and wild metapopulation management) is to augment the genetics of the alpha male, rather than completely replace them. As such vasectomisation of a highly endangered alpha male is both undesirable and unwarranted. This allows the male to naturally mate the female in parallel with AI, theoretically resulting in mixed paternity litters. By so doing, foreign genes of value (e.g. disease resistance) could be introduced by AI into the pack without completely replacing other important locally-derived survival genes naturally introduced by the alpha male – thus increasing the chance that some offspring will survive if conditions change adversely one way or another. Mixed paternity co-sired by beta and lower ranking males has been reported to occur naturally in 10 % of African wild dog litters [24–26]; increasing our confidence that such a hybrid AI approach will support successful rearing of litters by the whole pack.

Fecal steroid profiles were variable between females. The PLA pack alpha female did not show an increase in either fecal or serum progesterone levels during estrus, indicating an anovulatory cycle [27]. However, fPM and fEM profiles of the two other females were similar to that seen in domestic dogs [5,6], as well as profiles previous described in female AWDs [7,9,23]. Important here, is that in late estrus, fEM levels tended to return to baseline levels, while fPM levels showed a significant two and a half- to three-fold increase. These results need to be interpreted with caution as they are only derived from two females, one of which had a limited number of fecal samples analyzed. In addition, previous studies show diverse results. Creel et al. [8] did not observe differences in fPM concentrations between anestrus and estrus periods, after which they increased during gestation, while estrogens increased during estrus. However, all estrus samples were grouped

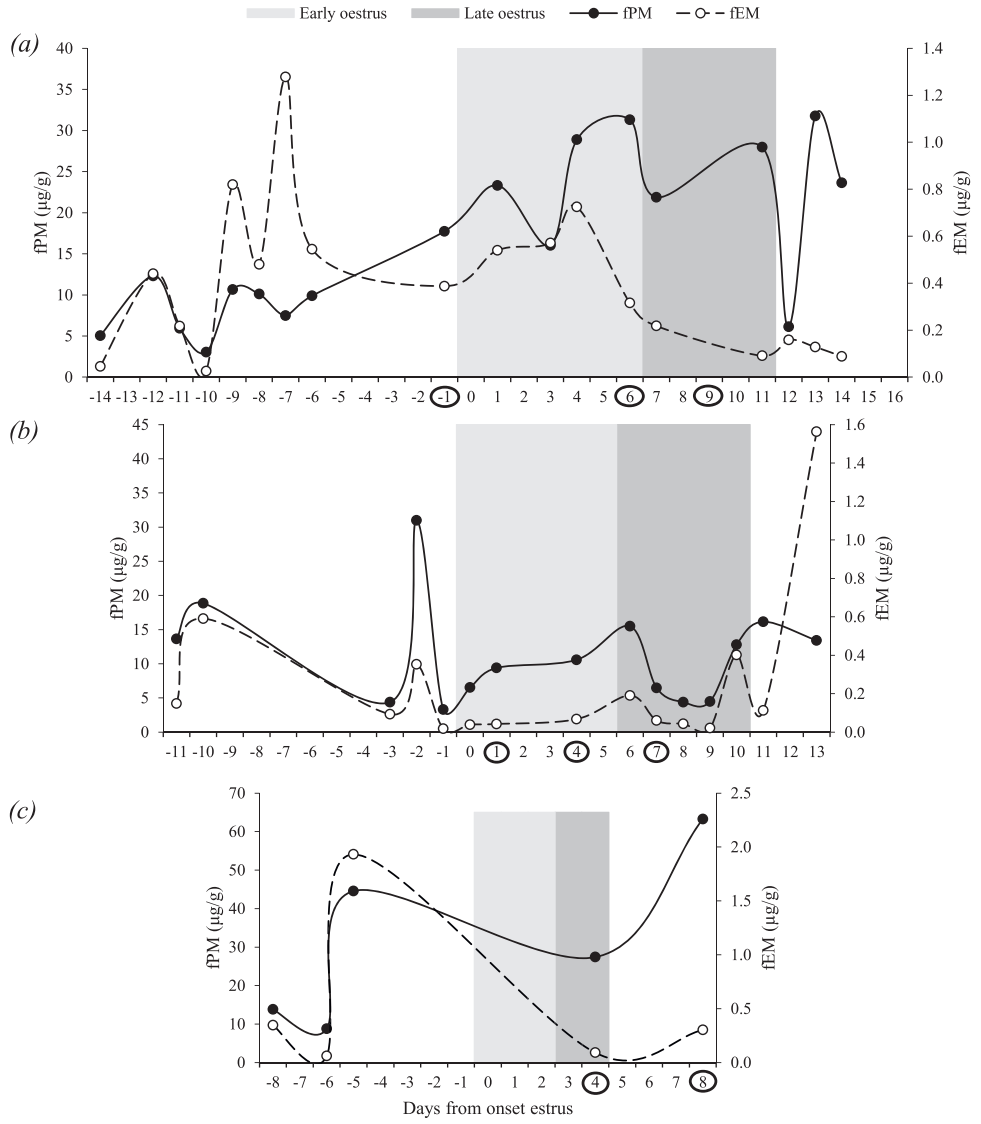

Fig. 6. Fecal progesterone (fPM) and estrogen (fEM) metabolite concentrations relative to the onset of estrus (D0; first day of observed copulation) for the alpha African wild dog female (a) BRU F1 ♀, (b) PLA F1 ♀, and (c) SAN F1 ♀. Circles indicate days of immobilization.

Table 4
Mean ± SEM fecal progesterone and estrogen metabolite concentrations in n = 3 African wild dog (*Lycaon pictus*) alpha females during different phases of the reproductive cycle.

Female	*Fecal hormone*	*Anestrus*	*Pro-estrus*	*Early estrus*	*Late estrus*	*Diestrus*	*P-value*
BRU F1 ♀	*fPM (µg/g DW)*	-	9.1 ± 1.5[b]	24.9 ± 3.4[a]	24.9 ± 3.0[a]	20.5 ± 7.6[ab]	0.005
	fEM (µg/g DW)	-	0.5 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.224
PLA F1 ♀	*fPM (µg/g DW)*	-	14.2 ± 5.1	8.8 ± 1.2	8.7 ± 2.3	14.8 ± 1.4	0.581
	fEM (µg/g DW)	-	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.8 ± 0.7	0.132
SAN F1 ♀	*fPM (µg/g DW)*	-	22.4 ± 11.2	-	27.4	63.3	-
	fEM (µg/g DW)	-	0.8 ± 0.6	-	0.1	0.3	-
Anestrus control (n = 7)	*fPM (µg/g DW)*	7.0 ± 0.9	-	-	-	-	-
	fEM (µg/g DW)	0.6 ± 0.3	-	-	-	-	-

Values with different letters for a particular hormone indicate a significant difference between reproductive phases. BRU, Brutus pack; DW, dry weight; fEM, fecal estrogen metabolite; fPM, fecal progestogen metabolite; PLA, Platform pack; SAN, San pack.

together without a clear separation between pro-estrus and estrus, or without taking longitudinal profiles or individual differences into account, which might explain this discrepancy. Van der Weyde et al. [23] saw a similar trend to our study, but with a slightly lower (less than two-fold) increase in fPM levels from baseline to estrus. Their use of wet instead of dry fecal samples and a different assay (RIA) might explain this difference. Blood progesterone levels seemed to follow a similar pattern as fPM concentrations. Blood estrogen levels did not exhibit a clear pattern in our study, probably caused by infrequent sampling. However, in other canids estrogen concentrations are known to be less reliable indicators of ovulation and the fertile period [12].

Interestingly, observed patterns of vaginal cytology and

Table 5
Other reproductive parameters in female African wild dogs (*Lycaon pictus*) during different phases of the reproductive cycle.

	Anestrus	***End pro-estrus***	***Early estrus***			***Late estrus***			***Early diestrus***
	PLA F2 ♀	***BRU F1 ♀ day −1***	***PLA F1 ♀ day+1***	***PLA F1 ♀ day+4***	***BRU F1 ♀ day+6***	***PLA F1 ♀ day+7***	***BRU F1 ♀ day+9***	***SAN F1 ♀ day+4***	***SAN F1 ♀ day +8***
Body weight (kg)	25.2	29.5	24.4	-	-	-	-	24.0	-
Pairs of nipples	5	6	6	-	-	-	-	7	-
Vulvar discharge	no	no	red-yellow	no	no	no	no	no	no
Vulva size (H x W; cm^2)	9.9	25.7	15.9	16.4	19.1	15.5	20.4	13.0	11.8
Vulva size adjusted for bodyweight (cm^2/kg)	0.39	0.87	0.65	0.67	0.65	0.64	0.69	0.54	0.49
Vaginal swab color	white	red	red	light red	white	light yellow	white	white	white
Vaginal cytology									
Small cells (%)	100	1	0	1	0	2	2	1.5	51
Medium cells (%)	0	3	2	5	0	17	8	48	36
Large cells (%)	0	31	17	31	7	32	16	20.5	9.5
Superficial cells (%)	0	31	22.5	18	9	17	24	3.5	1.5
Keratinized cells (%)	0	34	58.5	45	84	33	50	2	2
Bacteria	+	+	+ +	+ +	-	+ +	-	-	-
Mucus	+	-	-	+ +	+	+ +	+	-	+ +
Neutrophils	few	no	no	few	few	yes	few	yes	yes
Vaginoscopy									
Mucosa color	pink	white	white	white	white	light pink	white	white	pink
Secretions	no	hemorrhagic	serosanguinous	serosanguinous	no	serologic	no	serologic	no
Edema	0	1	2	3	3	3	3	2	4
Blood progesterone (ng/mL)	0.4	0.8	0.9	0.8	1.6	0.6	1.9	10.5	16.6
Blood estrogen (pg/mL)	12.6	104.8	10.1	56.8	54.0	7.2	9.0	8.2	98.8

BRU, Brutus pack; PLA, Platform pack; SAN, San pack.

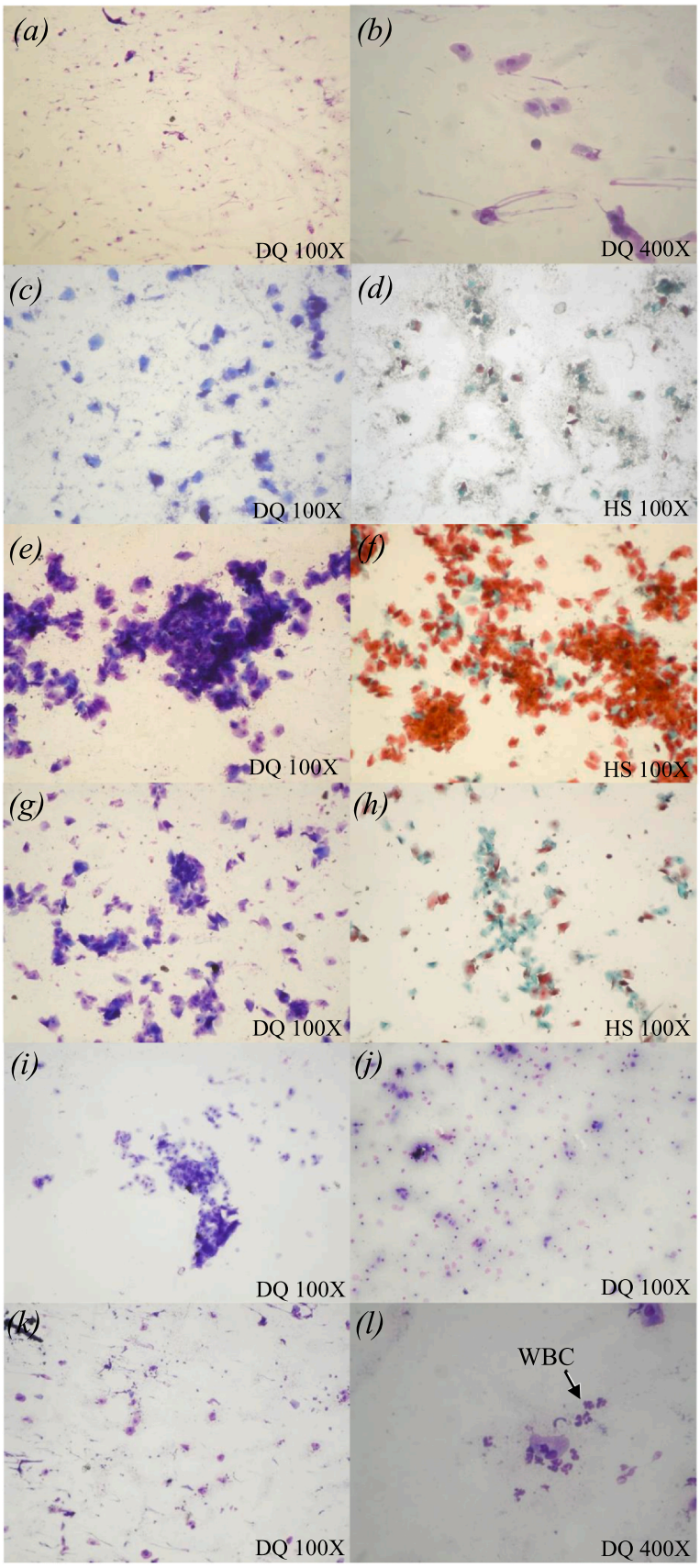

Fig. 7. Vaginal cytology in African wild dog females during (a, b) anestrus (PLA F2 ♀); (c, d) late pro-estrus (BRU F1 ♀ day −1); (e, f) early estrus (BRU F1 ♀ day +6); (g, h) late estrus (BRU F1 ♀ day +9); (i, j) late estrus (last day estrus, SAN F1 ♀ day +4); (k, l) early diestrus (SAN F1 ♀ day +8). DQ, Diff-Quick stain; HS, Harris-Shorr stain; WBC, white blood cell (neutrophil).

vaginoscopy in this study differed to that of domestic dogs, in which increasing estrogen levels during pro-estrus cause mitotic division of vaginal epithelia. This leads to an increase in cell layers and keratinization of the superficial layers. Maximum keratinization (98–100 %) is typically seen throughout the estrus phase, followed by an extensive rapid desquamation on the first day of diestrus [5]. Using a Harris-Shorr stain, in our study, maximum keratinization of 84 % was observed during mid-estrus, after which underlying non-keratinized cells reappeared again. As a result, the cytological profile in late estrus was similar to pro-estrus, making this technique insufficient to discriminate estrus in African wild dogs. Vaginal cytology has previously been reported in AWD females, but serial evaluations were not performed [14]. Moreover, our blood estrogen levels were inconsistent with some patterns of vaginal cytology observed in this study, with peak estrogen occurring on Day + 8 for the SAN alpha female while keratinization was low (2 %) at this time (Table 5).

Vaginal endoscopy is a very useful method in domestic dogs to pinpoint the fertile period [12]. During anestrus, vaginal mucosa is pink without edema. In pro-estrus, due to rising estrogen levels, increasing edema is visible with the formation of rounded longitudinal mucosal folds. The mucosal wall shows a paler aspect due to increased cell layers coinciding with keratinization. At the time of the LH surge, there is a decrease in estrogen levels together with an increase in progesterone. This results in a reduced tissue edema causing a shrunken and wrinkled appearance (angulation) around the time of ovulation [12]. In this study, persistently higher levels of vaginal edema were seen in AWD females compared to domestic dogs, without obvious changes during the estrus period. In addition, angulation was not observed until diestrus. Therefore, the results of these techniques should be interpreted with care, when used to confirm the fertile period during AI trials in AWDs.

The SAN pack alpha female successfully produced a litter approximately 2 months after observed mating (~3rd June 2016), confirming this was a fertile cycle. No puppies were subsequently observed to emerge from the den after mating in the BRU pack, however we suspect the pregnancy or litter was lost through stress-related infanticide by the alpha female; a documented phenomenon in this species [28]. It is unclear why the PLA pack alpha female did not ovulate in this study. However, this female was reported by Harnas staff to produce a litter of puppies much later than predicted (at the time 4–5 weeks old in July 2016). This suggests that this alpha female was in heat again around mid-March (approximately 1.5 months after the anovulatory cycle observed in this study); confirming anovulation and implicating a 'split-heat' as seen on occasion in domestic dogs [27,29]. It can be argued that ovulation in this female was inhibited by stress associated with multiple capture events, which in domestic dogs may decrease plasma LH levels and thus inhibit ovulation [30]. Measurement of glucocorticoid metabolites in the same fecal samples (fGCM) however, did not show any obvious peaks related to time of capture or anesthesia (unpublished data). Interestingly, the PLA alpha female was the only dog sedated on the day of potential ovulation (D + 1; Fig. 4), which may explain anovulation in this animal due to the anesthesia itself as reported in felids [31]. It is possible that the failure of ovulation influenced vaginal cytology and edema from this female. However, the appearance of both cornified cells and edema is influenced by increasing estrogen levels, and crenulation is due to decreasing estrogen:progesterone ratio [5,29]. In addition, domestic dogs with anovulatory cycles usually have vaginal cytology that resembles estrus (cornified cells) [27]. In this female, behavioral signs were not altered by anovulation, indicating the importance of measuring fPM concentration to confirm ovulation, as for domestic dogs [27].

In conclusion, a two- to five-fold increase in particular behaviors such as affiliative behavior, sexual and non-sexual follow, male initiating behavior, ride-up and copulation; a two and a half- to three-fold increase above baseline in fPM concentrations; and fEM levels that decline to baseline, appear suitable criteria to determine the fertile period in AWD females, and help guide the timing of artificial insemination attempts. However, without frequent highly-invasive sampling, blood steroid hormone concentrations, vaginal cytology and vaginoscopy do not have diagnostic value. Further research is needed to refine the non-invasive detection of the fertile period in this species.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Damien Paris, Monique Paris, Femke Van den Berghe reports financial support was provided by Morris Animal Foundation. Damien Paris, Monique Paris, Femke Van den Berghe reports financial support was provided by Roger William Park Zoo. Damien Paris, Monique Paris, Femke Van den Berghe reports financial support was provided by Fresno Chaffee Zoo. Harnas Wildlife Foundation for access to animals - DP, MP, FVdB, University of Namibia for access to laboratory space and equipment - DP, MP, FVdB, Olympus, IMV Technologies, E.I. Medical and Roche for the donation of equipment and supplies - DP, MP, FVdB.

Acknowledgements

This work was supported by the Morris Animal Foundation (United States) [grant number D15ZO-053], Roger William Park Zoo (United States), Fresno Chaffee Zoo (United States), James Cook University (Australia), and IBREAM (UK). The authors thank Harnas Wildlife Foundation for access to animals; University of Namibia for access to laboratory space and equipment; Olympus, IMV Technologies, E.I. Medical and Roche for the donation of equipment and supplies; and Prof Wenche Farstad for proofreading the manuscript.

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