Non-surgical artificial insemination using a GnRH analogue for ovulation induction during natural oestrus in African lions (*Panthera leo*)

Isabel Callealta,[1] Andre Ganswindt,[2] Martin Malan,[3] Imke Lueders.[2]

Highlights:

First thorough study focusing on a non-invasive approach for AI in wild felids

Single intramuscular dose of 20 μg burserelin-acetate induces ovulation in lionesses

Only one anaesthesia required per AI, minimizing animal distress and cost

Fresh-semen AI in lions is possible both, prior to and after ovulation

Roughly, 10-fold lower sperm dose required for intrauterine versus intravaginal insemination

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Running title: Non-surgical artificial insemination in African lionesses using GnRH

Abstract

Despite postulated potential for wildlife conservation, success of assisted reproduction techniques (ART) in *ex-situ* feline breeding remains <25%. The aim of this project was to develop a simplified, non-surgical artificial insemination (AI) protocol for African lions (*Panthera leo*), using an exogenous GnRH analogue to induce ovulation in females presenting natural oestrus, and minimizing manipulation of the animals. Four protocols were tested in five trained lionesses (3.5-8 years), for a total of 14 inseminations (2-4 per lioness). These protocols differed in the time lapse between GnRH injection and insemination, on days 4, 5, or 6 from onset of natural oestrus, determined by daily behavioural observation and vaginal cytology. Semen was collected from 8 different

males by urethral catheterization and electro-ejaculation, during full anaesthesia. Females were immediately immobilized for AI after semen collection. After transrectal ultrasound examination of the reproductive tract, insemination was performed either intravaginal or transcervical using a commercial dog urinary catheter (2.0 x 500 mm, Buster®, Krusse, South Africa) with a metal stylet. A single intramuscular dose of exogenous GnRH (20 μg burserelin-acetate, Receptal®, MSD, Intervet, South Africa) administered 30 or 48 hours before AI or during the AI procedure induced ovulation successfully, as all females entered either a non-pregnant luteal phase of 59.6 ± 0.95 days (n=10) or a pregnant luteal phase of 111.7 ± 0.33 days (n=3). However, the timespan between GnRH injection and end of behavioural and/or cytological oestrus differed widely (range: 0 to 120 hours). The final pregnancy success rate was 33.3%.

Key words: artificial insemination, African lion, ovulation induction, GnRH, fresh sperm, natural oestrus.

1. Introduction

At present, the red list of the International Union for the Conservation of Nature lists 25 of the 38 known cat species as Vulnerable or Endangered in at least some part of their habitat [1]. Therefore, captive management and *ex-situ* breeding programs are extremely important for the conservation of these species. However, many felids reproduce poorly, and most captive populations have limited genetic variation and a tendency for inbreeding, which leads to reproductive anomalies and an increased risk of extinction [2, 3]. *Ex-situ* breeding programs implemented with assisted reproduction techniques (ART), such as artificial insemination (AI), may help to improve the

reproductive success and genetic diversity of endangered species by introducing new genes into isolated populations [4, 5]. Unfortunately, overall ART success rate in non-domestic felids remains <25% to date [6]. For the last decades, successful AI trials in non-domestic cats have involved the African lion, *Panthera leo* [7, 8], Persian leopard, *Panthera pardus* [9], cheetah, *Acinonyx jubatus* [10], Siberian tiger, *Panthera tigris altaica* [11, 12], puma, *Puma concolor* [13], ocelot, *Leopardus pardalis* [14], snow leopard, *Panthera uncia* [15], clouded leopard, *Neofelis nebulosa* [16, 17], and Asiatic Golden cat, *Catopuma temminckii* [18]. Nevertheless, the majority of pregnancies achieved in prior studies relied on a laparoscopic approach. Despite being minimally-invasive, this surgical procedure may lead to complications and requires postoperative care. In addition, most AI protocols for felids include the use of exogenous gonadotropins to induce oestrus and ovulation [19]. However, repeated doses of these hormones (namely, eCG and hCG) trigger immunogenic responses and other side effects such as hyper-oestrogenism, superovulation, or luteal insufficiency, which may reduce fertility [20, 19].

The African lion population has declined by around 40% during the last two decades, and is currently listed as Vulnerable by the IUCN with less than 30 000 individuals and a decreasing population trend [21]. In South Africa, however, there is an increasing number of lions living in private and national reserves that breed successfully [21]. This species may represent an accessible baseline for studying the applicability of ART within the conservation breeding programs of large, non-domestic felids. Yet, the success of such techniques often relies on extensive prior investigation of the specific reproductive physiology of the targeted species [4]. Lion females are polyoestrous, and not affected by season or photoperiod [22]. The ovarian cycle of these felids lasts about 2-3 weeks,

with an oestrus duration of 2-9 days [23]. As other cats, they are induced ovulators, although may ovulate spontaneously [24]. The gestation length is around 110 days [23], and pseudopregnancy may appear after non-conceptive ovulation, ranging from 35 to 54 days in duration [22, 23].

The aim of this study was to develop a non-surgical artificial insemination protocol for African lions using a GnRH analogue to induce ovulation in females presenting natural oestrus. This non-invasive methodology was preferred to the invasive AI approach in order to avoid potential complications associated with surgery. In addition, we chose exogenous GnRH for ovulation induction instead of gonadotropins to reduce the risk of side effects associated with the repetitive use of these drugs, and minimize the animal handling.

2. Materials and Methods

2.1 Study Animals

The subjects of this study were five female African lions held in captivity at a private conservation centre in South Africa. Three of the lionesses were mature adults (7 to 9 years) that had previously produced several litters of cubs. These three females were housed together in an 800 to 1200 m^2 outdoor enclosure with natural substrate, trees, and a shelter. The remaining two females were housed together under the same conditions, but were young adults (about 3.5 years). They presented normal oestrous cycles, but had never mated before. All lionesses were healthy and in good body condition, and remained within visual, auditory, and olfactory range to an adult male African lion lodged in an adjacent enclosure during the entire study period. The five females were trained by positive reinforcement conditioning to voluntarily allow

Table 1. Study animals. The UCC (Ukutula Conservation Center, Brits, South Africa) was the main research site where all artificial inseminations (AI) took place. AKW (Akwaaba Predator Park, Rustenburg, South Africa) and BOS (Boskoppie Lion and Tiger Reserve, Kronstaad, South Africa) were satellite facilities used for occasional semen collection. These facilities were located about 90 and 290 km from UCC, respectively. Thus, when M6 and M8 were used as sperm donors, time from collection to AI was longer than the usual 1.5-2.5 h (about 6 and 9 h, respectively).

Id	Gender	Age	Population Dinamics	Known Breeder	Location
F1	Female	7	With other females	Yes	UCC
F2	Female	3.5	With another female	No	UCC
F3	Female	8	With other females	Yes	UCC
F4	Female	9	With other females	Yes	UCC
F5	Female	3.5	With another female	No	UCC
M1	Male	6	With one female	Yes	UCC
M2	Male	10	Within pride	Yes	UCC
M3	Male	3.5	With other males	No	UCC
M4	Male	3.5	With other males	No	UCC
M5	Male	6	With other males	No	UCC
M6	Male	5.5	With another male	Yes	AKW
M7	Male	6	With other males	No	UCC
M8	Male	6.5	Within pride	Yes	BOS

collection of vaginal swabs, and drug administration by hand-syringe [25]. Eight unrelated adult males (3.5 to 10 years) housed at three different facilities within South Africa were used as semen donors. Specific data such as location, age, population dynamics, and breeding history of all animals are presented in Table 1. This study was conducted with the permission of the Animal Ethics, Use and Care, and Research Committees of the University of Pretoria, South Africa (V052-17).

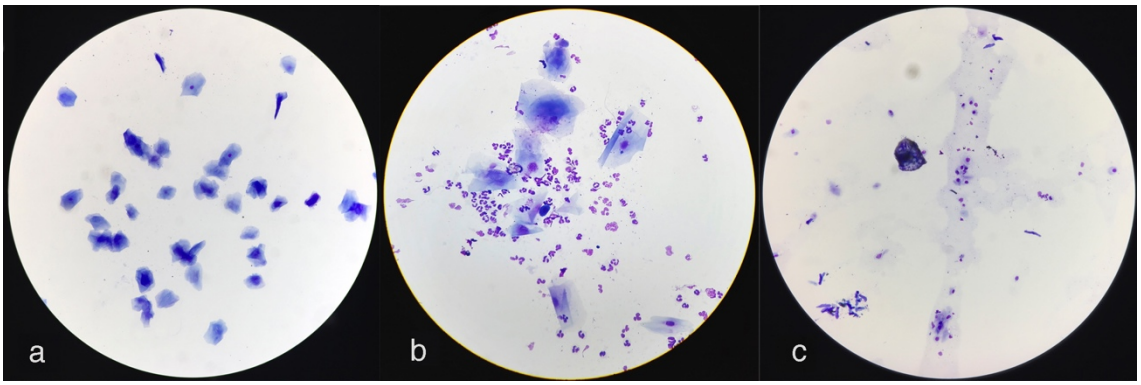
2.2 Anaesthesia

Before semen collection, each male was immobilized with a combination of 50 μg/kg medetomidine (Kyron Laboratories, South Africa) and 1.8-2.0 mg/kg ketamine (Kyron Laboratories, South Africa). After semen collection and preparation for evaluation, the female was anaesthetized using a combination of medetomidine, ketamine, and midazolam (6.5-14.0 μg/kg; Aspen, South Africa). All drugs were administered IM, via dart gun. At the end of each procedure, the animal was moved back to its enclosure and anaesthesia reversed with either atipamezole (2.0 mg/mg medetomidine used; Alphanil®, Wildlife Pharmaceuticals, South Africa) or 125 μg/kg yohimbine (Kyron Laboratories, South Africa), administered by hand-syringe, either IV or IM.

2.3 Oestrus monitoring and timing of AI

For 18 months, the behaviour of the five females under study was monitored twice a day (at sunrise and dusk), 5-7 days per week, in sessions of 15-60 minutes. A high frequency of specific reproductive signs such as purring, flirting run, lordosis, allowing mount by other female, and rolling, enabled detection of natural oestrus [26]. Behavioural oestrus was confirmed by the presence of a predominant proportion of

Figure 1. Microscopic images of Diff-Quik® stained vaginal smears of lionesses presenting different stages of the cycle. Vaginal cytologies were typically classified as a) oestrus smear: predominant proportion of superficial cornified epithelial cells associated to a moderate-to-high number of bacteria, and clearing of the background (magnification x200); b) post-oestrus smear: moderate-to-high number of neutrophils associated to the superficial cornified epithelial cells (x400), and c) dioestrus smear: predominant proportion of parabasal and intermediate epithelial cells associated, or not, with neutrophils, and a dirty background (x200).



superficial cornified epithelial cells associated to a moderate-to-high number of bacteria and clearing of the background in the vaginal cytology [27] (Fig 1a). Day 1 of oestrus was defined as the first day a female showed behavioural and cytological oestrous signs after a resting period (where none of these signs were observed). AI timing was considered appropriate when the female presented both, behavioural and cytological oestrous signs, as well as medium-to-large ovarian follicles and/or *corpora lutea* (CL) at the time of insemination.

2.4 Ovulation induction

Once oestrus was detected, the focal female received a single intramuscular dose of the GnRH analogue burserelin-acetate (20 μg; 5 mL Receptal®, Intervet, South Africa) by hand-syringe to induce ovulation at the end of the natural oestrus, as dominant follicles seem to be more sensitive to ovulation-inducing hormones at this stage of the feline ovarian cycle [18]. We tested four protocols that differed in the time lapse between GnRH injection and the actual AI, in relation to Day 1 of oestrus: in *protocol 1* (n=2), GnRH was injected on Day 6 of oestrus, during the AI procedure; in *protocol 2* (n=4), GnRH was injected on Day 5 of oestrus, also during the AI procedure; in *protocol 3* (n=4), GnRH was injected on Day 4 of oestrus, and the AI was performed on Day 6, about 48 hours after the injection; in *protocol 4* (n=4), GnRH was injected on Day 5 of oestrus, and the AI was performed on Day 6, about 30 hours after the injection.

2.5 Semen collection and analysis

Semen collection took place each time right before the AI procedure. Two collection methods were applied in each individual. Firstly, we employed the urethral

catheterization (UC) method previously described for lions by Lueders *et al* [28], by inserting a sterile commercial 2.6 x 500 mm dog urinary catheter (Buster®, Krusse, South Africa) in the urethra, after extruding and cleaning the penis. Afterwards, in order to increase the total number of sperm available for AI, an additional semen sample was collected from the same male through electro-ejaculation (EE). Here, we applied three sets of 10 electrical pulses (2 Volts), transrectally over the prostate and along the urethra, using a portable battery driven system (El Toro 2, Electronic Research Group, Johannesburg, South Africa). All semen samples were deposited in 1.5-5.0 mL capped Eppendorf vials, diluted 2-3 times in prewarmed 37°C cell culture medium (Medium 199, Sigma-Aldrich®, Germany), and stored at room temperature (26°C) until AI. A small aliquot (about 0.01 mL) of each semen sample was immediately examined for sperm motility, and another one diluted in distilled water (1:80) for further evaluation of sperm concentration, using the Neubauer haemocytometer method. Additionally, two smears were prepared to examine sperm morphology, plasma membrane integrity, and presence of foreign cells as previously described by Barth & Oko [29].

2.6 AI

After semen collection and preparation for evaluation, the oestrous female was immobilized. The time span from collection until insemination was in most cases between 1.5 and 2.5 hours. The immobilized female received a rectal enema as a preliminary step for ultrasound (US) examination. Evaluation of the reproductive tract and assessment of follicle development at the time of insemination was performed using a Mindray® DP-10 ultrasound scanner (Mindray Bio-medical Electronics, Shenzhen, China) with a 5-10 MHz linear rectal probe. After US evaluation, the lioness

was placed in sternal recumbency with her hind quarters slightly lifted (Fig 2a) in an attempt to mimic the posture she would acquire during natural mating (Fig 2b). Also, this position is believed to help stabilize the cervix, which may facilitate the passage of the AI catheter through it [18]. After cleaning the perineal region, a commercial 2.0 x 500 mm dog urinary catheter (Buster®, Krusse, South Africa) with a metal stylet was introduced in the vagina and followed by transrectal ultrasound up until the cervix, located about 20 cm cranial to the vulva. In the cases where the catheter passed through the cervix, the fraction of semen collected by UC was deposited into the uterine body lumen, and the fraction collected by EE was inseminated into the most cranial part of the vagina, right caudal to the cervix. When the catheter could not pass through the cervix, the fraction collected by UC was inseminated into the most cranial part of the vagina, right at the entrance of the cervix, and the fraction collected by EE was deposited along the vagina while the catheter moved out of it. The mean volume of diluted semen inseminated into the uterus was 0.68 ± 0.13 ml (range: 0.4-1 ml), and into the vagina 2.6 ± 0.46 ml (range: 0.94-5.2 ml). After insemination, the female was left in sternal position with the hind quarters lifted for 5-10 minutes to avoid semen reflux. Meanwhile, a member of the staff grabbed the lioness firmly by the scruff of the neck, another massaged the hind quarters, and the outer vagina was mechanically stimulated to mimic all regular stimuli that occur during natural mating. Then, the lioness was repositioned into dorsal recumbency for five more minutes (Fig 2c), with the objective of mimicking the rolling behaviour shown by these animals right after natural mating (Fig 2d). Finally, the female was returned to the enclosure for recovery, and the anaesthesia was reversed as described above.

Figure 2. Above, artificial insemination (AI) positioning: (a) female in sternal recumbency during AI, (c) female after semen deposition in dorsal recumbency; both positions tried to artificially mimic the behaviours typically presented by females during natural mating. Below, natural reproductive behaviour: (b) lions mating and (d) right after copulation.

2.7 Data analysis

Total length of oestrus for females whose ovulation was pharmacologically induced was calculated (in days) by adding the number of hours from GnRH administration to first day of dioestrus, divided by 24, to the number of days from start of oestrous signs to GnRH administration. First day of dioestrus was considered to be the first day the females did not show behavioural and/or cytological oestrous signs after an oestrus phase. Length of dioestrus (in days) was estimated by subtracting the number of hours from GnRH administration to the first day of dioestrus, divided by 24, to the number of days from GnRH administration to the first day of oestrus (of the following cycle).

Statistical analyses were conducted using the R version 3.4.4 (The R Foundation for Statistical Computing, Vienna, Austria). The R RcmdrMisc package was used to calculate descriptive statistics for each variable (*i.e.* oestrus duration, timespan between GnRH administration and end of oestrus, timespan between GnRH administration and beginning of dioestrus, pseudopregnancy and pregnancy duration, semen concentration and volume, sperm motility and morphological abnormalities, number and diameter of ovarian follicles and *corpora lutea*, and AI success in relation to each protocol and type of insemination). Basic results appear as untransformed mean ± standard error of the mean (SEM), unless stated otherwise. Differences between groups (created according to the protocol applied, or the day of GnRH injection) were tested using the Mann–Whitney test (when 2 groups were considered) or the Kruskal–Wallis test (when more than 2 groups were considered), using the R coin package in all cases. When needed, respective data sets were tested for normality using the Shapiro-Wilk's normality test, and for equality of variance using the Levene's test and the Fligner-Killeen's test. Comparison with previous results (such as those from Putman *et al* [23]) were

confronted using the Student´s t-test for two independent samples and unequal variances and assuming normality of the data to compare. AI success in relation to the type of insemination was evaluated by the odds ratio and the Fisher's exact test. Significances were determined at the $p<0.05$ α level, and results double-checked by Monte Carlo re-sampling approximation methods.

3. Results

3.1 Oestrus monitoring and AI timing

Oestrus lasted on average 6.84 $\pm$ 1.62 days (n=25 oestrus events, range: 4-10 days), and the difference observed between this duration and that reported by Putman *et al* [23] (4.4 $\pm$ 0.2 days; n=57; range: 2-9 days) was not significant (2-sample t-test; t=1.495; df=25; n=25, 57 trials; p=0.148). The vaginal smears of the two females inseminated on Day 6, following *protocol 1,* presented a moderate-to-high number of neutrophils associated to the superficial cornified epithelial cells, indicative of post-oestrus and, thus, inappropriate timing for AI (Fig 1b). In all remaining trials, the vaginal samples collected at the time of AI provided the classic oestrus image described in 2.3 (Fig 1a).

3.2 Ovulation induction

The timespan between GnRH injection and end of behavioural and cytological oestrus ranged between 0-120 hours throughout all trials (n=14). The two females receiving the GnRH analogue on Day 6 (*protocol 1*) were considered to start the dioestrus phase on the same day of injection. The lionesses that received the GnRH analogue on Day 5 (*protocols 2* and *4*) terminated oestrus 75 $\pm$ 11.5 hours after the injection (n=8; range: 36-120 hours). The females that received the GnRH analogue on Day 4 (*protocol 3*)

Table 2. Burserelin-acetate (GnRH) ovulation induction and artificial insemination (AI) trials performed in five different lionesses. This table shows the type of AI according to semen deposition, as well as the timespan between GnRH injection and end of oestrus, and subsequent duration of induced non-pregnant luteal phase (NPLP). *This female suffered an abortion during the last third of gestation.

Protocol no.	Trial no.	Female	GnRH injection	AI	Timespan to dioestrus (hours from GnRH)	NPLP length (days from GnRH)
1	1	F1	Day 6	Intrauterine	0	56
	2	F5	Day 6	Intravaginal	0	56
2	3	F1	Day 5	Intravaginal	48	58
	4	F3	Day 5	Intrauterine	84	Pregnant
	5	F5	Day 5	Intravaginal	84	65
	6	F4	Day 5	Intravaginal	60	59
3	7	F4	Day 4	Intravaginal	96	59
	8	F2	Day 4	Intravaginal	84	61
	9	F1	Day 4	Intrauterine	120	Pregnant
	10	F3	Day 4	Intrauterine	120	64
4	11	F5	Day 5	Intravaginal	48	85* (Pregnant)
	12	F4	Day 5	Intravaginal	36	59
	13	F2	Day 5	Intravaginal	120	Pregnant
	14	F1	Day 5	Intravaginal	120	59

terminated oestrus 105 ± 9 hours after the injection (n=4; range: 84-120 hours). The median time interval between burserelin injection and first day of dioestrus for females treated on Day 4 (108 hours) and Day 5 (72 hours) differed by 36 hours (Table 2). However, this difference was statistically non-significant (Mann-Whitney, U=7; W=35; n= 4 8 AI trials; p=0.079; effect size r=0.45). After GnRH administration, all females entered either a non-pregnant luteal phase (NPLP, 10 out of 14) or pregnant luteal phase (PLP, 4 out of 14), regardless of the protocol applied. During these phases, the females did not present oestrous signs, and the vaginal cytologies provided a classic dioestrus image (*i.e.* predominant proportion of parabasal and intermediate epithelial cells associated, or not, with neutrophils, and a dirty background, Fig 1c). The median length of the induced NPLP was 59 days (n=10; range: 56-65 days), and appeared to be shorter on average when the GnRH analogue was injected later in time (on Day 4: 61 days; on Day 5: 59 days; on Day 6: 56 days), although these differences between groups resulted in non-significance (Kruskal-Wallis; H=0.68; n= 3 5 2 AI trials; p=0.747).

3.3 Semen evaluation

Semen collection using the UC method resulted in smaller volumes (0.32 ± 0.05 mL; n=13; range: 0.1-0.7 mL) and higher sperm concentrations (2006.0 ± 242.0 $x10^6$ sperm/mL; n=12; range: 237.0-3038.0 $x10^6$ sperm/mL) compared to the EE method (semen volume: 0.9 ± 0.29 mL, n=16, range: 0.04-5.0 mL; sperm concentration: 157.8 ± 59.0 $x10^6$ sperm/mL, n=16, range: 4.5-956.0 sperm/mL; see Table 3). The average percentage of progressively motile spermatozoa in the samples used for AI was 71.9 ± 3.47 (n=13; range: 45-90%) and 57.8 ± 4.18 (n=16; range: 20-85%) for semen collected by UC and EE, respectively. The average total number of sperm used in successful AI

Table 3. Characteristics of the semen samples collected by urethral catheterization (UC) and electroejaculation (EE), and used in 14 artificial insemination (AI) trials in five different lionesses. *This sample was divided in two equal aliquots in order to inseminate two females simultaneously. **Only trial no. 13 was successful. [1st] This value corresponds to the first fraction of the ejaculate. [2nd] This value corresponds to the second fraction of the ejaculate.

Trial no.	Male	Collection method	Colour	Consistency	Volume (ml)	Concentration ($x10^6$/ml)	Progressive Motility (%)	Vitality (%)	Morphological Abnormalities (%)	Pregnancy achieved
1	M1	UC	White	Thick milky	0.1	2078	65	82	37	No
		EE	Grey	Thin milky	0.8 [1st]	251	45	78	43	
2	M2	UC	White	Thick milky	0.11	2527.5	75	71	28	No
		EE	Grey	Watery	0.04	42.5	55	62	23	
3	M3	UC	White	Thick milky	0.35	N/A	90	N/A	13	No
		EE	Transparent	Watery	0.075	19	20	16	53	
4	M1	UC	Grey	Watery	0.2	237	75	82	36	Yes
		EE	Grey	Thin milky	0.15	348	55	69	46	
5	M7	UC	White	Thick milky	0.45	2075	80	71	54	No
		EE	Grey	Watery	1 [1st]	60	45	60	48	
6	M8	UC	White	Thin milky	0.45	1450	55	76	43	No
		EE	Grey	Watery	0.15 [1st]	135	65	72	46	
		EE	Grey	Watery	1.4 [2nd]	115	65	63	41	
7	M3	UC	White	Thick milky	0.15	2883.5	65	84	37	No
		EE	White	Thin milky	0.65	130	55	71	33	
8	M4	UC	White	Thick milky	0.4	1642	85	67	32	No
		EE	Grey	Watery	1.3	40	60	50	35	
9	M3	UC	White	Thick milky	0.25	2777	75	72	39	Yes

10, 13	M6	EE	Grey	Watery	0.5	61.5	40	41	55	Yes**
		UC	White	Thick milky	0.3*	2530	80	76	18	
		EE	Transparent	Watery	1.1*	25.3	55	54	30	
11	M5	UC	White	Thick milky	0.7	1918	65	76	47	Yes
		UC	White	Thin milky	0.35	926	45	68	49	
12	M1	EE	Grey	Watery	5.0	30.5	85	37	60	No
		EE	White	Thick milky	0.3 1st	956	80	83	54	
		EE	White	Thick milky	0.6 2nd	273	55	80	48	
14	M3	UC	White	Thick milky	0.4	3038	80	81	62	No
		EE	Grey	Watery	0.9 1st	34	85	66	47	
		EE	Transparent	Watery	0.4 2nd	4.5	60	N/A	N/A	
				Mean	0.64	950.26	64.14	66.96	41.32	
				SEM	0.17	205.77	3.04	3.06	2.28	

procedures was 857.7 ± 712.2 x 10^6 (n=4 pregnancies; range: 99.6-1819.2 x10^6 sperm/mL) when UC and EE samples were combined.

3.4 Ultrasound scans and AI

The number of antral ovarian follicles detected in both ovaries by transrectal ultrasonography at the time of AI was 4.67 ± 0.69 (n=12 complete examinations; range: 1-8 follicles). The mean size of the largest ovarian follicle observed at the time of AI was 10.5 ± 0.88 mm (n=14 examinations; range: 5.55-17.1 mm). However, follicle size differed between protocols (Fig 3, Table 4). When US was performed on Day 6, before GnRH administration (*protocol 1*), the median size of the largest follicles detected was 9.85 mm, compared to 11.6 mm (when US was performed on Day 5, before GnRH administration, *protocol 2*), to 10.1 mm (about 48 hours after GnRH, *protocol 3*), and to 7.53 mm (about 30 hours after GnRH, *protocol 4*). No CL were detected in the ovaries of the females inseminated on Days 6 or 5, prior to GnRH injection (*protocols 1* and *2*). On average, 1.0 ± 0.33 CL (n=6 complete examinations; range: 0-2 CL) were observed in the ovaries of the females inseminated according to *protocols 3* and *4* at the time of AI. The CL observed on Day 6, about 48 hours after GnRH injection (*protocol 3*), had a median diameter of 9.0 mm, whereas the ones observed on Day 6, about 30 hours after injection (*protocol 4*), had a median of 11.1 mm. During the non-invasive AI procedures, we managed to pass the catheter through the cervix in four out of 14 trials (28.6%). Thus, in those four trials (1, 4, 9, and 10) the insemination was intrauterine, whereas in the remaining ten trials the semen was placed in the vagina (Table 2).

3.5 Pregnancy rate

Figure 3. Ultrasonographic images of lion ovaries: a) ovarian follicles observed on Day 6 of oestrus, prior to GnRH administration and artificial insemination (typically observed with *protocol 1*). Arrow indicates a regressing dominant follicle (with hyperechoic content); stars show two subordinate follicles; b) ovarian follicle observed on Day 5 of oestrus, before GnRH administration and artificial insemination (typically observed with *protocol 2*), distended and hypoechoic; c) ovary observed on Day 6, 30-48 hours after GnRH administration (typically observed with *protocols 3* and *4*). Arrows indicate two luteinizing follicles; star shows one subordinate follicle. White bar = 1 cm.

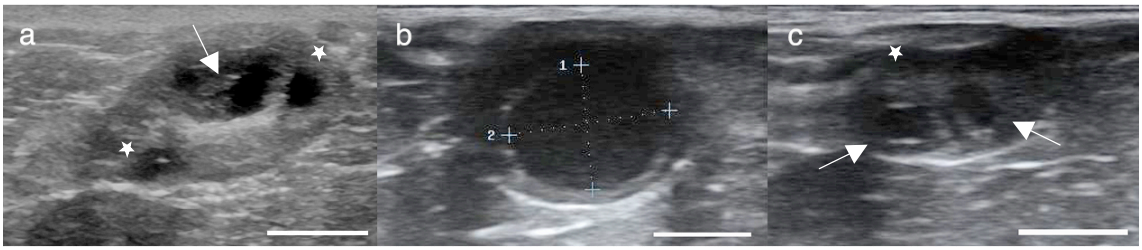


Table 4. Number and size of ovarian follicles and *corpora lutea* (CL) observed during ultrasound examination at the time of artificial insemination, in relation to day of GnRH injection and oestrus onset (Day 1). RO = right ovary; LO = left ovary.

Protocol no.	Trial no.	Female	GnRH injection	Ultrasound	n. of follicles		Diameter of largest follicle (mm)		n. of CL		Diameter of largest CL (mm)	
					RO	LO	RO	LO	RO	LO	RO	LO
1	1	F1	Day 6	Day 6	4	4	8.90	7.35	0	0	N/A	N/A
	2	F5	Day 6	Day 6	2	1	10.20	10.80	0	0	N/A	N/A
2	3	F1	Day 5	Day 5	N/A	3	N/A	10.30	0	0	N/A	N/A
	4	F3	Day 5	Day 5	3	3	17.07	6.55	0	0	N/A	N/A
	5	F5	Day 5	Day 5	1	1	10.65	11.20	0	0	N/A	N/A
	6	F4	Day 5	Day 5	3	2	9.20	12.00	0	0	N/A	N/A
3	7	F4	Day 4	Day 6	1	1	N/A	N/A	N/A	N/A	N/A	N/A
	8	F2	Day 4	Day 6	4	3	8.80	6.90	0	0	N/A	N/A
	9	F1	Day 4	Day 6	1	0	14.90	0	0	2	N/A	8.75
	10	F3	Day 4	Day 6	1	6	7.85	10.05	2	0	9.25	N/A
4	11	F5	Day 5	Day 6	2	1	5.70	4.65	0	1	N/A	12.50
	12	F4	Day 5	Day 6	1	N/A	5.55	N/A	2	N/A	9.75	N/A
	13	F2	Day 5	Day 6	2	3	N/A	9.35	1	0	N/A	N/A
	14	F1	Day 5	Day 6	3	4	9.45	11.40	0	0	N/A	N/A

Four pregnancies resulted from this study, entailing a total success rate of 28.6% (4 out of 14). However, the final pregnancy success rate achieved was 33.3% (4 out of 12) when considering the trials where AI timing was completely appropriate (12 out of 14). Three of these pregnancies (trials no. 4, 9, and 13) were successfully maintained to term, and whelping occurred on average 111.7 ± 0.33 days (n=3; range 111-112) after GnRH administration. In total, eight lion cubs were born, posing a mean litter size of 2.67 ± 0.33 (n=3; range: 2-3). The fourth pregnancy (trial no. 9) ended with a stillbirth at around Day 85 from GnRH administration (Day 84 from AI), and the cause of this abortion remains unknown. The number of successful pregnancies achieved by transcervical intrauterine insemination (n=2) and intravaginal insemination (n=2) was the same (50% of the total, each). However, the number of transcervical AI trials was considerably lower than the number of intravaginal AI trials (n=4 *vs.* n=10, respectively), which may implicate a higher success rate of intrauterine insemination (50%, 2 out of 4) compared to intravaginal AI (20%, 2 out of 10). *Protocol 4* led to 50% of pregnancies achieved (Table 2).

4. Discussion

In this study, we were able to determine a suitable timing for AI during natural oestrus in trained lionesses, and to demonstrate that ovulation may be induced by a single hormone injection. We also confirmed that successful AI with fresh-semen is possible both prior to and after ovulation, although semen deposition shortly after ovulation may positively affect the success rate of the procedure. Consequently, we report here the birth of the first African lion cubs ever conceived by AI. In addition, we describe in detail physiological events previously unknown for African lions, such as vaginal cytological

findings, pre-ovulatory follicle size, and the different sperm doses required for both, intrauterine and intravaginal insemination.

4.1 Use of GnRH analogue as an ovulation-inductor in lionesses

Felids present exceptionally variable ovarian cycles [6]. Even within species, reports about cycle duration vary distinctively, depending on the number of animals and cycles observed, as well as the methodology used [6]. The non-significant difference detected between the oestrus length determined in the current study (by behaviour and vaginal cytology), and that previously reported by Putman *et al* [23] (by faecal oestrogen metabolite measurement) supports the validity of our method for oestrus detection in lions.

All females entered either a NPLP or a PLP after receiving one dose of burserelin-acetate at the end of the oestrous period. Despite regular collection of vaginal samples, only 19% of the oestrous cycles in which GnRH was not administered (4 out of 21) resulted in spontaneous ovulation and NPLP. If the mechanical stimulation derived from frequent vaginal swabbing were enough to induce ovulation alone, the number of spontaneous ovulations observed would have been higher than the rates previously reported for lions (20-26%) where no ovulation induction, either with exogenous hormones or vaginal stimulation, was performed [see 23, 24]. We, therefore, support burserelin-acetate was the main trigger of ovulation in this study. The current results dissent previous studies where GnRH agonists were not recommended to induce follicular maturation and ovulation in felids, due to the unreliable effect of this drug in some species, such as domestic cats and clouded leopards [19, 30, 31]. In the current study, exogenous GnRH administration successfully induced ovarian stimulation and ovulation in all cases.

However, the timespan between GnRH injection and end of behavioural and/or cytological oestrus differed widely (from 0 to 120 hours), regardless of the time of injection. The ultrasound data described in 3.4 suggest that ovulation generally started within 30 hours after GnRH administration, as CL were detected at the time of AI in some of the treated animals. Even though, in many species, most ovulations occur between 24 and 60 hours after an LH surge, some follicles may take longer to rupture (up to 96 hours), which would explain the variable occurrence of oestrous signs observed after GnRH injection in this study [32, 33]. Overall, all females receiving GnRH on Days 4 or 5 ceased oestrous signs around Day 8 from onset of oestrus, regardless of the protocol used. Additionally, in at least three of the four spontaneous ovulations observed during this study, oestrous signs also stopped at around Day 8 from oestrus onset. This finding may imply a programmed mechanism for ovarian follicle development and ovulation, but further investigation would be needed to confirm this hypothesis.

NPLP induced-by-GnRH was 13 days longer than the spontaneous NPLP and NPLP induced-by-mating reported in previous studies [23]. However, further investigation would be required to confirm whether this difference was related to the use of burserelin-acetate as an ovulation inductor, the distinct methods applied for length determination, or only a bias due to our small sample size.

4.2 Non-invasive approach for AI in lionesses

To the authors' knowledge, this is the first thorough study focusing on a non-invasive approach for AI in wild felids. To date, the majority of successful AI cases in non-domestic cats required a surgical approach, and just a few isolated reports on successful non-surgical AI can be found in the literature [9, 12, 18]. Only two prior studies described

the achievement of successful non-surgical AI in African lions [7, 8]. Despite reporting a success rate of up to 50%, all embryos obtained in these experiments were retrieved and used for other purposes. Thus, none of the pregnancies achieved was indeed maintained to term. In addition, Goeritz *et al* [8] required three full anaesthesias to prepare and inseminate each lioness, as well as hormonal treatment for up to six days before the actual AI. In comparison, with the here-described methodology, only one anaesthesia was required per animal and AI, and the logistical challenges associated with drug administration in these animals were minimized. Overall, this new non-invasive approach for AI was less harmful for the lionesses, and posed additional economic value.

The quality of the semen obtained during this study resembled prior results [34, 35]. Despite the total low volume used, and the moderate occurrence of teratoid sperm (typically observed in many felid species [6]), one female in the current study got pregnant with no more than 99.6 $x10^6$ spermatozoa (trial no. 4), from which only 48.42 $x10^6$ were morphologically normal and had intact membranes, according to semen evaluation. This was the minimum dose required in our study for intrauterine insemination. On the other hand, the minimum sperm dose required for successful intravaginal insemination in this study was 786.83 $x10^6$ spermatozoa, from which only 496.39 $x10^6$ were normal. These values confirmed that a rough 10-fold higher dose is needed for AI when sperm is deposited into the vagina instead of into the uterine body. This matches previous results obtained for both, domestic [36, 37, 38] and wild felids [39, 40].

GnRH stimulates both FSH and LH secretion [41]. Thus, burserelin-acetate may induce follicular growth and/or ovulation, depending on the follicle size at time of injection. The

current study showed that the type and size of the ovarian follicles at time of AI differed between protocols**.** In general, with *protocol 1*, no CL were detected, but only medium-size (6.5-9.5 cm) deflated follicles with hyperechoic content, which were presumed to be predominant follicles already regressing under no ovulatory stimuli. The vaginal smears in these two cases confirmed post-oestrus at time of AI, and the females ceased oestrous signs the same day of GnRH injection/AI. None of these trials was successful (Table 2), and we concluded that Day 6, without previous ovulation induction was too late for insemination. Most probably, burserelin-acetate induced final growth and rupture of some follicles in these cases, but the high number of polymorphonuclear neutrophils in the vagina impeded the passage of sperm towards the fallopian tubes, preventing fertilization of the oocytes. With *protocol 2*, no CL were detected, but mostly large-size (>9.5 cm) dominant, pre-ovulatory follicles. With *protocol 3*, we commonly found CL in one ovary (confirming ovulation had started to take place), alongside medium-to-large-size, pre-ovulatory follicles in the other ovary. With *protocol 4*, we usually found CL together with small-to-medium-size (<6.5 cm) subordinate follicles, which most probably were already regressing. These ultrasound images confirmed that all ovulations occurred within 48 hours after hormonal stimulation with the GnRH analogue. Ovulation failure due to anaesthesia as seen in previous studies [42] was not observed.

Blind cervical penetration with the catheter was one of the biggest challenges encountered during AI. We were able to do it on only four occasions, in two different females that had previously given birth (see Tables 1 & 2). Mechanic manipulation of the cervix through the rectal wall, as described for domestic [43] and small wild cats [18], was not possible, due to the far cranial position of this structure. Thus, adapted AI

catheters and endoscopic equipment will probably be necessary in the future to achieve better results.

The duration of the full-term pregnancies in our study (111-112 days) was quite similar to that previously reported for lions (105-114 days) [23]. Likewise, the resulting litter size (2-3 cubs) is comparable with the numbers reported elsewhere for litters born in the wild and in captivity (2 and 3 cubs, respectively) [44]. In this study, the final pregnancy success rate achieved by non-surgical AI in lionesses presenting natural oestrus, after administration of burserelin-acetate to induce ovulation, was 33.3%. While still low, this rate is higher than both, the 10% reported for overall AI in small wild felids [4], and the 28.6% achieved with intrauterine laparoscopic insemination in other large cats [10].

5. Conclusions

The establishment of an AI protocol with fresh semen is the first step towards the application of ART to maintain and improve genetic diversity in wild felids, which together with the protection of their habitats, should be the long-term goal of their conservation programs. In this regard, burserelin-acetate seemed to be a suitable drug to induce ovulation in African lions. We recommend the use of one single intramuscular dose (20 μg) on Day 5 from oestrus onset, about 24 hours before AI, and the deposition of at least 100 x10^6 sperm cells in the uterine body. Close monitoring of the reproductive cycle is highly endorsed to refine the AI timing. Our non-surgical approach proved to be a valid method for AI in this species, independent of whether the semen deposition was transcervical or intravaginal, and occurred prior or post ovulation. We support it has potential to be applied to other large felid species.

Acknowledgements

The authors thank Mr & Mrs Jacobs of the Ukutula Conservation Center (UCC, Brits, RSA), for hosting this project and making their animals available; Pierre Grobler and Willie Jr Jacobs, for their assistance during animal training and sample collection; Dr Gerhardus Scheepers, Dr James Roxburgh, Dr Adriaan Tordiffe, Dr Ilse Luther, Mr Mario Smuts, and Professor Javier Callealta for sharing their knowledge and time. Mr Nazeer Cadjee (from the Akwaaba Predator Park, Rustenburg, RSA) and Mr Pieter Swart (from the Boskoppie Lion and Tiger Reserve, Kronstaad, RSA) for making some of their animals available for this study. This work was supported by the South African National Research Foundation (grant number SFH150721128779, UID108947), the South African Veterinary Foundation, and the Zebra Foundation for Veterinary Zoological Education.

Declarations of interest: none.

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