REPRODUCTIVE PHYSIOLOGY OF THE FEMALE AFRICAN LION (*PANTHERA LEO*), AND DEVELOPMENT OF ARTIFICIAL INSEMINATION PROTOCOLS

by

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Photo: Isabel Callealta

"As Aristotle once said, '*the whole is greater than the sum of its parts*'. That certainly rings true for endangered species conservation efforts. So, let's congratulate the ranger who apprehends the poacher, reward the community that restores the forest, support the politicians who pass wildlife preservation legislation, and celebrate the scientists achieving breakthroughs that advance ART. Any one of these efforts alone will fall short, but working in concert, they may prevail in saving a few imperilled species and minimizing the loss of biodiversity during the Earth's sixth great extinction event."

Roth, T. and Swanson, W. (2018).

Declaration of originality

I, Isabel Callealta Rodríguez, student number 17403074, hereby declare that this dissertation, "*Reproductive physiology of the female African lion (*Panthera *leo), and development of artificial insemination protocols*" is submitted in accordance with the requirements for the Philosophiae Doctor degree at University of Pretoria, is my own original work and has not previously been submitted to any other institution of higher learning. All sources cited or quoted in this research paper are indicated and acknowledged with a comprehensive list of references.

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Pretoria, 3 December 2019

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Abstract

At present, most felid species are listed as Vulnerable or Endangered by the International Union for the Conservation of Nature (IUCN). Assisted reproduction techniques (ART), such as artificial insemination (AI), are extremely important for their conservation. However, ART overall success in non-domestic felids remains <25%. Thus, research on the specific feline reproductive physiology, and development of a model for application of ART into the breeding conservation of these species is needed.

With that purpose, the African lion served as a large-felid-model species. Six lionesses were trained by positive reinforcement conditioning (PRCT) to voluntary allow frequent collection of blood samples and vaginal swabs. In parallel, their ovarian cycles were non-invasively monitored by behavioural observation and measurement of faecal steroid metabolite concentrations. This information was used to determine the optimum timing for AI in this species.

Routine sampling by PRCT was possible after 20 weeks of training, enabling collection of about 750 vaginal swabs and 650 blood samples over 18 months. Revealed plasma and faecal oestrogen and progestogen profiles allowed identification of and differentiation between pregnancy and pseudopregnancy, but resulted inconclusive for oestrus detection. For the first time, the ovarian cycle of the African lioness was described from a detailed cytological point of view, including the report of *Simonsiella* spp. during oestrus, and a remarkably high number of neutrophils during proestrus in all females. Lastly, non-surgical AI with fresh semen in lionesses presenting natural heat was possible both, prior to and after ovulation induction with GnRH, resulting in the birth of the first lion cubs ever conceived by AI.

Key Terms:

African lion; fresh semen; GnRH; natural oestrus; non-invasive monitoring; non-surgical artificial insemination; ovarian cycle; positive reinforcement; reproductive behaviour; reproductive endocrinology; reproductive physiology; vaginal cytology.

List of abbreviations

AI	Artificial insemination
AKW	Akwaaba Predator Park
ART	Assisted reproductive technology
ARTs	Assisted reproductive techniques
BOS	Boskoppie Lion and Tiger Reserve
CL	*Corpora lutea*
CLIA	Chemiluminescence immunoassay
CV	Coefficients of variation
eCG	Equine chorionic gonadotropin
EE	Electroejaculation
EEP	European Endangered Species Programme
e.g.	For example ("exempli gratia")
EIA	Enzyme immunoassay
ET	Embryo transfer
fEM	Faecal oestrogen metabolite
fPM	Faecal progestagen metabolite
FSH	Follicle stimulating hormone
g	Grams
GnRH	Gonadotropin-releasing hormone
h	Hours
hCG	Human chorionic gonadotropin
i.e.	That is ("id est")
im	Intramuscular
iNPLP	Induced non-pregnant luteal phase
IUCN	International Union for the Conservation of Nature
IVF	*In vitro* fertilization
KiSS	Kisspeptin
LH	Luteinizing hormone
ml	Millilitres
mg	Milligrams
MHz	Megahertz

ng/ml	Nanograms per millilitre
NPLP	Non-pregnant luteal phase
OR	Odds ratio
pFSH	Porcine follicle stimulating hormone
pLH	porcine luteinizing hormone
PLP	Pregnant luteal phase
PRC	Positive reinforcement conditioning
PMN	Polymorphonuclear neutrophils
RSA	Republic of South Africa
SAVC	South African Veterinary Council
SD	Standard deviation
sE	Serum oestrogen
SEM	Standard error of the mean
sNPLP	Spontaneous non-pregnant luteal phase
sP	Serum progestagen
SSP	Species Survival Plan
UCC	Ukutula Conservation Center
UC	Urethral catheterization
US	Ultrasound
µg/g DW	Micrograms per gram of dry weight
η	Canonical correlation coefficient

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1. CHAPTER ONE:

GENERAL INTRODUCTION

Photo: Jim Zuckerman

1.1 INTRODUCTION

At present, the International Union for the Conservation of Nature (IUCN) lists 25 of the 38 known cat species as “vulnerable” or “endangered” in, at least, some part of their natural habitat. Some large felid species, such as the Sumatran tiger (*Panthera tigris sumatrae*) or the Northern Chinese leopard (*Panthera pardus japonensis*), are critically endangered, with more animals living in captivity and isolated protected landscapes than in the wild (Linkie *et al.* 2008; Stein *et al.* 2016). Therefore, in addition to *in-situ* measures such as increasing the number and size of protected areas, captive management and *ex-situ* breeding programs play an important role in the conservation of these species (Swanson, 2006). However, many felids reproduce poorly in captivity and most captive populations have limited genetic diversity and a tendency for inbreeding, which may lead to reproductive anomalies and increased risk of extinction (Wildt *et al.* 1987; Lacy, 1997).

The implementation of assisted reproductive techniques (ARTs) into *ex-situ* breeding programs may help to improve the reproductive success and genetic diversity of endangered species by introducing new genes into isolated populations (Swanson, 2006; Lermen *et al.* 2009). However, to successfully apply ARTs within wildlife management and conservation, it is essential to understand the specific reproduction physiology of each species (Swanson, 2006).

1.1.1 The African lion

According to recent studies, there are two subspecies of African lions: *Panthera leo leo* (Linnaeus, 1758), distributed throughout India, and central and western Africa, and *Panthera leo melanochaita* (Hamilton-Smith, 1842), distributed throughout southern and eastern Africa (Kitchener *et al.* 2017). Lions tend to live at higher population densities than other felid species, ranging from 1.5 to 55 adults per 100 km^2 depending on the inhabited region (Bauer *et al.* 2016). As opposed to most non-domestic felids, which are usually solitary, African lions form groups of about four to six adults (Bauer *et al.* 2016). While females remain in prides with their cubs and usually one dominant adult male, sub-adults and non-dominant males wander off to form small male-only groups (Bauer *et al.* 2016).

Globally, the lion population has declined by around 40% during the last two decades (Fig. 1.1) (Bauer *et al.* 2016). Currently, it stands at less than 30 000 individuals in total, and there is a decreasing population trend (Bauer *et al.* 2016). Indiscriminate killing and prosecution (Lagendijk and Gusset, 2008), habitat loss, prey depletion (Hayward *et al.* 2007), disease (Munson *et al.* 2008), and poaching or trophy hunting (Packer *et al.* 2009) are the main causes of population decline. In addition, fragmentation and isolation of smaller subpopulations may cause genetic variability loss in both wild and captive populations (Bjorklund, 2003; Bertola *et al.* 2011). Therefore, this large cat is listed as Vulnerable by the IUCN, although most subpopulations all over its geographical range meet the criteria for Endangered (Bauer *et al.* 2016). In South Africa, however, it is categorized as Least Concern, since there is an increasing number of animals living in private and national reserves that reproduce quite well (Bauer *et al.* 2016). Furthermore, captive lion breeding in this particular country has increased in the last 10 years, with an estimated population of 8 000 animals kept in lion farms and fenced reserves (Bauer *et al.* 2018).

Figure 1.1: *Panthera leo* distribution map depicting historic and current range. From: *Panthera.org*

Therefore, in South Africa, the African lion represents an accessible model species for the study of the reproductive biology of large, non-domestic cats, and the applicability of ARTs within the conservation programs of these felids.

1.1.1.1 Reproductive biology

Lions reach puberty at approximately 80 kg of body weight, which generally corresponds with 1.1 years of age in captive lions, and 1.75 years in free-roaming lions (Putman *et al.* 2015). They become sexually mature at around 26 months of age (Smuts *et al.* 1980), and the average age at first reproduction seems to be 3.5-4.5 years (Smuts *et al.* 1978). Females are polyoestrous (*i.e.* they undergo multiple cycles throughout the year) and not affected by season or photoperiod (Brown, 2011). They are considered to be induced ovulators (*i.e.* ovulation is generally stimulated by mating), although may occasionally ovulate spontaneously (Schramm *et al.* 1994). Pseudopregnancy appears after each non-conceptive ovulation, and ranges from 35 to 54 days in duration (Brown, 2011; Putman *et al.* 2015). The gestation length is around 110 days (Putman *et al.* 2015). Lionesses are multiparous, with a mean litter size of 2.3 cubs, which are usually weaned at 5-8 months of age (Schaller, 1972). Generally, females that lose their litters resume cycling sooner (16.4 ± 4 days) than those that nurse their cubs (31.7 ± 5.9 days) (Putman *et al.* 2015). Sexually mature lions may reproduce throughout their lives (Steyn, 1951). However, aged lionesses seem to show lower numbers of ovarian follicles (Smuts *et al.* 1978), and it is not recommended to breed old animals, due to an increased risk of complications during pregnancy (Putman *et al.* 2015).

1.1.1.2 Ovarian cycle

The ovarian cycle of the female African lion ranges between 8-30 days (Putman *et al.* 2015), and as in most felid species, it is divided into three different phases (Fig 1.2):

a) anoestrus, associated to reproductive inactivity and characterized by basal oestrogen and progestagen concentrations (Brown *et al.* 2011). Physiological anoestrus may be "juvenile" (prepubertal female) or "lactational" (female after parturition, nursing cubs). Anoestrus may also be the result of pathological conditions (Andrews *et al.* 2019).

b) follicular phase, characterized by follicular waves and fluctuating oestrogen concentrations (Andrews *et al.* 2019). This phase includes three stages:

   i. Proestrus, associated with the presence of growing ovarian follicles and increasing concentrations of oestrogen (Brown *et al.* 2011). As in most felids, it normally lasts less than one day (Brown *et al.* 2011). During this stage, males are occasionally attracted by females, but females do not allow mating (Brown *et al.* 2011).
   ii. Oestrus, associated with advanced follicular development (formation of pre-ovulatory follicles) and peak concentrations of oestrogens (Brown *et al.* 2011). It normally lasts 2-9 days (Putman *et al.* 2015). During this stage, females allow mounting and copulation (Brown *et al.* 2011). As other felids, lionesses may also present typical oestrous behaviours such as increased urine marking, lordosis, rolling, back rubbing, and foot treading (Andrews *et al.* 2019). However, silent oestrus seems to be common in this species (Putman *et al.* 2015). If ovulation does not occur at the end of this stage, the ovarian follicles regress (Andrews *et al.* 2019).
   iii. Interoestrous interval, associated with low follicular activity, and basal oestrogens and progestagen concentrations (Andrews *et al.* 2019). This stage is the period in between follicular waves;

c) luteal phase (or dioestrus), associated with the presence of *corpora lutea* (CL) and elevated concentrations of progesterone (Andrews *et al.* 2019). It only occurs after ovulation. When conception takes place, this phase is denominated “pregnant luteal phase” (PLP, pregnancy, or gestation); if conception does not occur, this phase is referred to as “non-pregnant luteal phase” (NPLP, pseudopregnancy, or pseudogestation) (Andrews *et al.* 2019). Overall, pseudopregnancy in felids is about one-half to one-third shorter than pregnancy (Andrews *et al.* 2019).

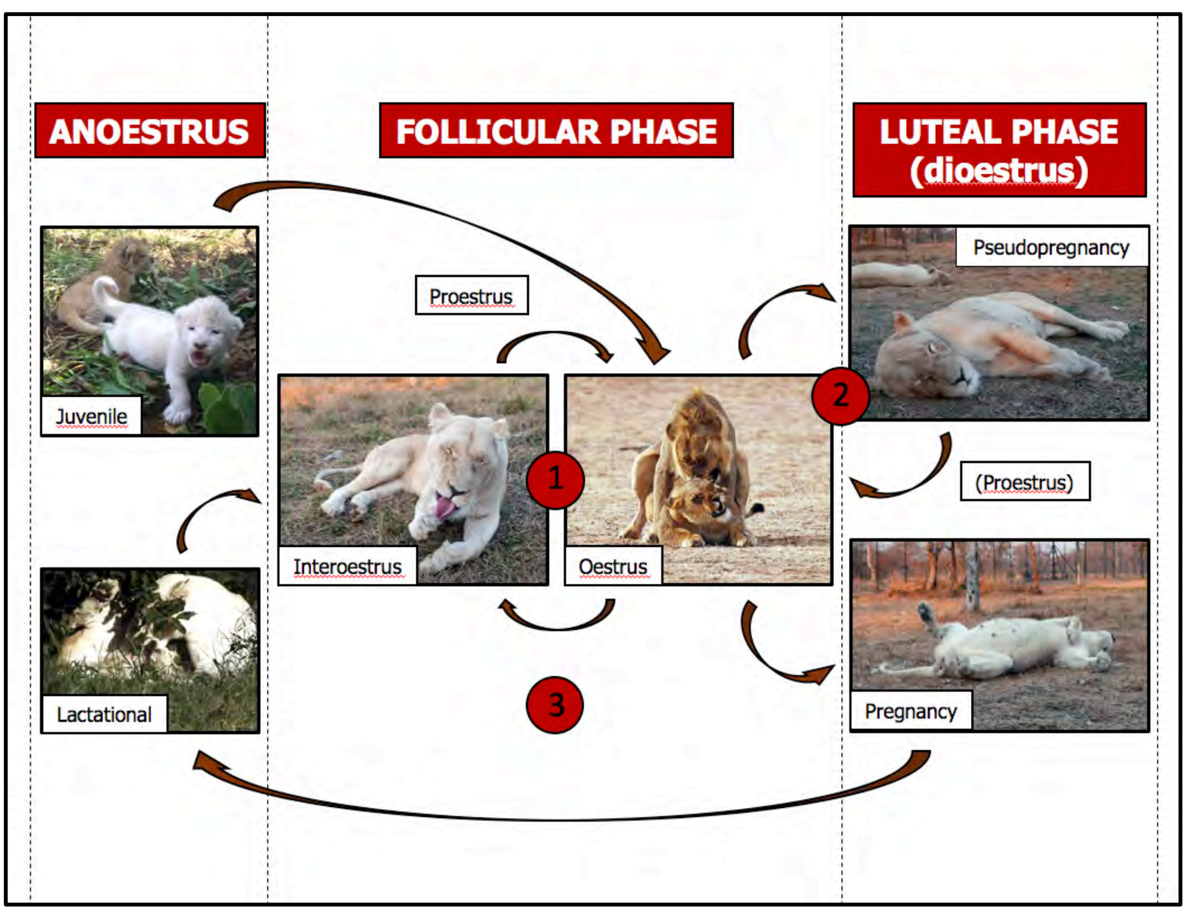


Figure 1.2: Flow diagram showing the different phases and reproductive stages of the African lioness during a 1) non-ovulatory cycle; 2) non-conceptive ovulatory cycle; and 3) conceptive ovulatory cycle.

1.1.1.3 Female reproductive endocrinology

In the last decades, reproductive steroid patterns have been described for a number of felid species, including lions (Schmidt *et al.* 1979; Putman *et al.* 2015), using primarily non-invasive methods (*i.e.* faecal hormone metabolite analyses) (Brown *et al.* 2011). However, the domestic cat remains the best studied species to date, and is often used as an example for other felids.

Overall, during proestrus, the pituitary gland, stimulated by the hypothalamic kisspeptin (KiSS) and gonadotropin-releasing (GnRH) hormones, releases follicle stimulating hormone (FSH), which in turn induces ovarian follicle development and recruitment (Bristol-Gould and Woodruff, 2006; Andrews *et al.* 2019). As the follicles grow, the ovarian granulosa cells start secreting oestradiol (Bristol-Gould and Woodruff, 2006) which works as an indirect positive feedback on the hypothalamic-pituitary-gonadal axis, up-regulating the activity of KiSS and GnRH (Andrews *et al.*

2019). Oestrogen increases have been positively associated with specific changes of behaviour in many felid species (Graham *et al*. 1995; Brown *et al.* 2006), and with cornification of the epithelial cells of the vaginal mucous membrane (Johnston *et al.* 2001). Therefore, the fluctuation of oestradiol seems to reflect the cyclic growth and regression of the ovarian follicles, and thus may enable distinction between oestrus and interoestrous intervals. Likewise, it is believed that most felid species need multiple copulations to mechanically stimulate the release of GnRH and subsequent luteinizing hormone (LH), by activation of the hypothalamic-pituitary axis (Shille *et al.* 1983). In domestic cats, surges of LH occur within minutes of coitus, and trigger final follicular maturation and ovulation at around 24-48 hours post-mating (Shille *et al.* 1983). One or two days after ovulation, the resultant CL start secreting progesterone, which increases rapidly and remains elevated for a variable length of time, depending on whether conception occurred or not (Wildt *et. al*. 1998). Progestagen concentrations appear to be similar in most felid species during both PLP and NPLP (Brown *et al.* 2011). In lions, however, it seems progestagen levels are higher during pregnancy (Putman *et al.* 2015). Oestrogens, on the other hand, fluctuate above basal levels throughout pregnancy, increasing steadily during the second half of gestation, and peaking about one week before parturition (Shille *et al.* 1983). In most felid species, prolactin (secreted by the pituitary) starts rising about mid-gestation (around day 35 in domestic cats) (Banks *et al.* 1983), while relaxin (secreted by the feto-placental unit) starts rising at the end of the first month of gestation (around day 20 in domestic cats) to abruptly decline around parturition (Stewart and Stabenfeldt, 1985). Prostaglandin F2α (produced by the feto-placental unit) seems to peak at the end of gestation, and facilitates parturition due to its luteolytic effect (Tsutsui and Stabenfeldt, 1993).

1.1.1.4 Anatomy of the female reproductive tract

The feline female reproductive system consists of two ovaries, two oviducts, a bicornuated uterus, cervix, vagina, *vestibulum vaginae*, and vulvar lips. In most felids, the ovaries are located caudal to the kidneys, covered by the ovarian bursa, and attached to the abdominal cavity by the suspensory ligament and the mesovaria (Brown, 2011). The lateral aspect of the ovaries and the oviducts are covered by the

mesosalpinx (Brown, 2011). The bicornuated uterus has a short uterine body, and remains attached to the abdomen by the mesometrium (Brown, 2011). During pregnancy, the foetuses occupy evenly both uterine horns, and the placenta seems to be endotheliochorial and zonary (Brown, 2011). The cervix is short and serves as a physical barrier during the anoestrus and luteal phase (Zambelli and Cunto, 2005). It forms an obtuse angle with the vagina, and the cervical opening is generally located above the fornix (*i.e.* ventral blind end of the vagina) (Brown, 2011). The vagina measures about 20 cm in length, and has two differentiated parts: the wider *vestibulum vaginae*, and the tighter, narrower, and longer cranial part. The vulva constitutes the outer part of the reproductive tract, and is located right below the anus (Brown, 2011).

1.1.2 Assisted Reproductive Technology

Ex-situ breeding programs have the capacity to sustain and improve the genetic diversity of threatened species (Swanson, 2006). Therefore, assisted reproductive technology (ART) becomes an essential tool when natural mating rates appear insufficient, or there is a need of intensive genetic management within controlled populations (Putman *et al.* 2015). To successfully apply ARTs, it is an essential prerequisite to thoroughly understand the basic reproductive biology and reproductive strategies of the targeted species (Swanson, 2006). Although extrapolation from similar species may be taken as a starting point, when species-specific information is scarce, it is highly recommended to use tools such as individual behavioural observation and hormonal monitoring, to improve the knowledge on the focal species before trying to develop and implement new breeding protocols (Pukazhenthi and Wildt, 2004).

1.1.2.1 Assessment of the reproductive cycle stage

There are different methods to study and assess the reproductive status of a non-domestic female, although generally a combination of these will provide the most accurate information (Silva *et al.* 2017). Since physical and chemical restraint methods may induce distress and physiological negative side effects, which in turn

may lead to reproductive failure, it is essential to utilize minimally-invasive techniques for the reproductive soundness evaluation of these generally intractable animals (Gilroy and DeYoung, 1986; Graham *et al.* 1995; Lambeth *et al.* 2006; Kersey and Dehnhard, 2014; Terio *et al.* 2014).

1.1.2.1.1 Behavioural monitoring

Behavioural monitoring is a non-invasive method, widely used in captive breeding programs. It is based on the fact that animals display different behaviours during different physiological states. Thus, observation of changes in/or expression of typical behaviours may help monitor the cyclicity of non-domestic females (Silva *et al.* 2017). However, besides the high frequency of silent oestrus reported in felids (Schmidt *et al.* 1979, 1993; Wildt *et al.* 1982; van Dorsser *et al.* 2007; Axnér *et al.* 2008; Putman *et al.* 2015), animals in captivity may show subtle or no oestrous signs at all. This may have several reasons such as species specific characteristics (*e.g.* cheetah, *Acinonyx jubatus*), the absence of a male counterpart, or presumably distress associated with intensive handling and/or disturbance of the habitat and social structure (Silva *et al.* 2017).

1.1.2.1.2 Reproductive hormone monitoring

Steroid and peptide hormone concentration monitoring may be a useful method to help delineate gonadal function, type of ovulation, timing of spermatogenesis and ovulation, and protocols to overcome infertility and assist breeding in non-domestic species (Pukazhenthi and Wildt, 2004; Brown, 2011).

Hormone concentration determination is typically performed by immunoassay, which involves the competition between one antigen (*i.e.* the hormone to be measured) and a labelled form of the same antigen, for binding the appropriate antibody (Kersey and Dehnhard, 2014). Depending on the type of tag used, the immunoassay may be called chemiluminescence immunoassay (CLIA; when the antigen is tagged with luminophores), enzyme immunoassay (EIA; when the antigen is tagged with one enzyme), or radio immunoassay (RIA; when the antigen is tagged with a radio-isotope) (Kersey and Dehnhard, 2014). The matrixes used to perform

this assays may be collected either invasively (*e.g.* blood samples) or non-invasively (*e.g.* faecal samples, urine, hair) (Ganswindt et al. 2012; Silva *et al.* 2017).

The use of non-invasive hormone monitoring by faecal sample analyses in non-domestic cats provides some benefits over the invasive methods (Pukazhenthi and Wildt, 2004). It allows sequential sampling without immobilization of the animal, which may provide accurate longitudinal hormonal profiles minimizing stressful handling (Pukazhenthi and Wildt, 2004). However, different variables, such as the period of time between voiding and sample collection, environmental exposure of the sample, or the sample volume, consistency, and homogeneity can significantly affect the steroid metabolite concentrations, limiting the sensibility to detect specific reproductive events (Touma and Palme, 2005; Wielebnowski and Watters, 2007; Brown, 2011). Additionally, this approach is not suitable for the analyses of peptide hormones such as LH or FSH, which are extensively degraded prior excretion (Pukazhenthi and Wildt, 2004). However, at present, faecal steroid hormone metabolite monitoring appears the most common approach for reproductive endocrine studies in wildlife species, including non-domestic felids (Silva *et al.* 2017), since performing routine blood collection in these animals entails great difficulty (Graham *et al.* 1995; Kersey and Dehnhard, 2014), and repeated anaesthetic procedures may increase the probability of deleterious side effects (Gilroy and DeYoung, 1986). However, when samples are available, blood tests generally provide faster results in the laboratory, and allow accurate assessment of a larger number of hormones (Silva *et al.* 2017).

1.1.2.1.3 Vaginal cytology

Vaginal cytology is an easy, inexpensive, and practical technique to monitor the ovarian cycle in domestic species (Silva *et al.* 2017). Variations in the concentration of circulating oestrogen cause changes in the epithelial cells of the mucous membrane of the reproductive tract (Johnston *et al.* 2001). Thus, the evaluation of serial vaginal smears may serve as an indirect method to monitor oestradiol fluctuations, and therefore, progression of the reproductive cycle, as well as presence of endocrine and reproductive pathologies (von Heimendahl and England, 2010). It may also help predict the ideal time for natural mating and artificial

insemination (Johnston *et al.* 2001). This method has potential to be applied into the breeding management of some wild species (Silva *et al.* 2017). However, vaginal cytology is not commonly used for monitoring reproductive events in non-domestic felines, due to the general intractability of these animals. This simple swabbing technique requires close contact to the animal, which is unproblematic in domestic cats and dogs, but would involve full immobilization or physical restraint in untrained non-domestic felids. Therefore, it is generally not applicable for longitudinal monitoring in the latter, due to distress and costs involved (Silva *et al.* 2017).

1.1.2.1.4 External reproductive features

Observation and scoring of distinct external reproductive features such as genital appearance (*i.e.* vulvar swelling and colour) and/or vaginal discharge may also help identify oestrus in some wild species, such as the steppe polecat (*Mustela eversmanni*; Mead *et al.* 1990), the maned wolf (*Chrysocyon brachyurus*; Weinhardt and Rodden, 1995), the Fennec fox (*Vulpes zerda*; Valdespino *et al.* 2002), the giant panda (*Ailuropoda melanoleuca*; Durrant *et al.* 2003), the bush dog (*Speothos venaticus*; DeMatteo *et al.* 2006), and the collared peccary (*Pecari tajacu*; Maia *et al.* 2014). However, these changes seem to be mild or difficult to detect in non-domestic felids (Silva *et al.* 2017).

1.1.2.1.5 Ultrasound evaluation

Despite requiring experience and certain amount of restraint, ultrasonography is a minimally-invasive, practical tool to evaluate the reproductive status of non-domestic animals (Silva *et al.* 2017). This method is based on the creation of digital images of the scanned tissues, using high-frequency sound waves produced by a transducer. As different organic tissues have different densities, the emitted sound waves either penetrate the encountered tissues or are reflected by these. The transducer then captures the reflected sound waves, and transforms them into electric signals that are displayed as an image on a screen (Lutz and Soldner, 2011). Due to the anatomy of the feline female reproductive tract, two different approaches may be used to scan felids: a) transabdominal and b) transrectal. The

transabdominal approach is less invasive, and seems to be more common in non-domestic felids (Kirberger *et al.* 2011; Painer *et al.* 2014; Schulman *et al.* 2015). However, the transrectal approach allows visualisation of the full reproductive system, and may provide a better image quality, especially in larger felid species, because of the proximity of the targeted organ to the rectal wall (Gonçalves, 2019). Ultrasound evaluation allows monitoring of the ovarian activity by providing information about the size and shape of the ovaries and functional structures, such as ovarian follicles and CL, and also helps with the diagnosis of reproductive tract pathologies (Silva *et al.* 2017). Thus, it is considered a highly valuable tool when performing ARTs such as oocyte or embryo retrieval, artificial insemination (AI), or embryo transfer (ET) (Silva *et al.* 2017).

1.1.3 Assisted Reproductive Techniques

To date, sperm collection and cryopreservation, AI, and *in vitro* fertilization (IVF) are the most widely applied ARTs in wildlife species (Jewgenow *at al.* 2017). In non-domestic felids, different protocols for sperm collection (Lueders *et al.* 2012) and cryopreservation (Swanson *et al.* 2007, Luther *et al.* 2017), ovulation induction (Pelican *et al.* 2006), artificial insemination (Swanson, 2012; Lueders *et al.* 2014), *in vitro* fertilization (Herrick *et al.* 2010; Swanson, 2012), and embryo transfer (Pope *et al.* 2006) have been tested in the last two decades. However, these techniques are not sufficiently advanced yet in feline species, and overall success rate of ARTs in wild felids still remains below 25% (Swanson, 2006).

1.1.3.1 Artificial insemination

AI may contribute to conservation and *ex-situ* breeding programs by solving some of the problems related to the management and breeding of non-domestic species, such as lack of genetic variability, translocation of large animals with breeding purposes, disease transmission, and sexual or behavioural incompatibilities (Pukazhenthi and Wildt, 2004). Up to date, successful AI trials in non-domestic cats have involved the African lion (*Panthera leo*, Bowen *et al.* 1982; Goeritz *et al.* 2012), Persian leopard (*Panthera pardus,* Dresser *et al.* 1982),

cheetah (Howard *et al.* 1992), Siberian tiger (*Panthera tigris altaica,* Donoghue *et al.* 1996; Silva *et al.* 2000), puma (*Puma concolor*, Barone *et al.* 1994), ocelot (*Leopardus pardalis,* Swanson *et al.* 1996), snow leopard (*Panthera uncia,* Roth *et al.* 1996), clouded leopard (*Neofelis nebulosa,* Howard *et al.* 1996; Tipkantha *et al.* 2017), and Asiatic Golden cat (*Catopuma temminckii,* Lueders *et al.* 2014). However, although conception was confirmed in a number of the above-cited reports, live birth has seldom been achieved. Despite extensive investigation of the species-specific reproductive cycle, and the development of precise protocols for ovulation induction in these species, the overall success rate for AI in small and large wild felids, such as tigrinas (*Leopardus tigrina*), ocelots, clouded leopards, snow leopards, pumas (*Felis concolor*), and Siberian tigers, is around 10% (Swanson, 2006; Howard and Wildt, 2009).

1.1.3.2 Ovarian stimulation and ovulation induction

To date, most AI protocols for felids include the use of exogenous gonadotropins to induce oestrus and ovulation (Pelican *et al.* 2006; Thongphakdee *et al.* 2018). According to these protocols, the ovaries must be prepared before follicular maturation and ovulation induction, by inhibiting prior underlying follicular development using progestins (Pelican *et al.* 2006). In this regard, pre-treatments with levenorgestrel have been tested in the domestic cat (Pelican *et al.* 2003), the cheetah, and the fishing cat (*Prionailurus viverrinus*) with good results, showing fresh ovulation sites with minimal numbers of unovulated follicles after gonadotropin stimulation (Pukazhenthi and Wildt, 2004). Equine chorionic gonadotropin (eCG) is generally used to stimulate folliculogenesis, while human chorionic gonadotropin (hCG) induces follicular maturation and ovulation (Pelican *et al.* 2006). Follicle rupture and ovulation usually takes place within 24-48 hours after exogenous hormone administration (Pelican *et al.* 2006; Brown, 2011). Nevertheless, it is extremely important to closely monitor the ovarian cycle of female felids to avoid the presence of CL before initiating the endocrine stimulation via exogenous hormone administration (Pukazhenthi and Wildt, 2004).

Overall, the current protocols for ovarian stimulation and ovulation before AI have only succeeded in approximately one-third of all cat species (Pelican *et al.*

2006). This could be due to the lack of association between the female body mass and the dose of chorionic gonadotropins required to induce an appropriated and predictable ovulatory response (Howard *et al.* 1997; Pelican *et al.* 2006), but also to the immunogenic responses and reproductive side effects associated with frequent, repeated doses of gonadotropins, such as hyper-oestrogenism, superovulation, and luteal insufficiency (Swanson *et al.* 1995; Pukazhenthi and Wildt, 2004; Pelican *et al.* 2006). Fortunately, there are other exogenous hormones that could be applied to induce oestrus and ovulation in felids (Pelican *et al.* 2006). The porcine FSH (pFSH) and porcine LH (pLH) have been used in some felids to initiate folliculogenesis, and stimulate follicular maturation and ovulation, respectively (Pelican *et al.* 2006; Thongphakdee *et al.* 2018). However, these exogenous hormones also require multiple injections for a few consecutive days (which may be stressful for the animals), and seemed to produce oocyte maturation failure and erratic progesterone patterns in Siberian tigers (Crichton *et al.* 2003). Furthermore, these gonadotropins are not always available and are rather expensive. Exogenous GnRH and GnRH agonists, such as leuprolide acetate and buserelin-acetate are produced synthetically and readily available. They have been tested in clouded leopards (Pelican *et al.* 2001), domestic cats (Swanson *et al.* 2001), and Asiatic golden cats (Lueders *et al.* 2014) with variable, but promising results. Therefore, there is still a need to develop new hormonal protocols, that induce safe and consistent ovarian responses in felids (Pelican *et al.* 2006).

1.1.3.3 Semen collection and deposition during AI

Semen collection is an essential part of basically every assisted reproduction protocol. In addition, semen may be collected for research purposes, genome banking and cryopreservation, and male fertility assessments (Lueders *et al.* 2012).

In felids, there are three different approaches for semen collection in live animals: a) electro-ejaculation (Platz and Seager, 1978) and b) urethral catheterization (Zambelli *et al.* 2008), during full anaesthesia; and c) artificial vagina, using trained males and females in oestrus (Dooley *et al.* 1991). Sperm may also be collected from the epididymis or vas deferens after castration and post mortem (Hay and Goodrowe, 1993). For a long time, electro-ejaculation (*i.e.* induction of

ejaculation by transrectal low voltage stimulation of the prostate) was the preferred method for semen collection in both, domestic and non-domestic cats (Lueders *et al.* 2012). However, within the last decade, urethral catheterization has proved to be an economic, field-friendly technique for felids, providing excellent quality samples with small volumes, but high sperm concentration, which makes it ideal for AI and cryopreservation (Lueders *et al.* 2012).

AI in felids can be performed with either fresh, refrigerated, or frozen semen, providing a minimum number of 10 $x10^6$ motile sperm per trial (Swanson *et al.* 2007). However, semen evaluation and assessment of sperm functionality (at least, progressive motility and acrosome condition, but preferably volume, sperm concentration, motility, morphology, and acrosome integrity) right after collection, as well as after rewarming/thawing is essential before insemination (Howard *et al.* 1986; Swanson *et al.* 2007).

Semen collection and AI in untrained non-domestic cats require previous chemical immobilization. However, some anaesthetic drugs may interfere with sperm transport (hindering uterus contractility) and ovulation (inhibiting ovum release) (Howard *et al.* 1992). Thus, post-ovulatory, intrauterine sperm deposition is frequently recommended in most AI protocols (Pukazhenthi and Wildt, 2004; Pelican *et al.* 2006). However, this inconvenience may be avoided using drugs such as the α2-agonists (*e.g.* medetomidine) and its antagonists (*e.g.* atipamezole). In the last two decades, pre-ovulatory sperm deposition led to successful non-surgical AI in Asiatic golden cats and a Persian leopard (*Panthera pardus saxicolor*) with ovulation induced by GnRH during the same AI procedure (Lueders *et al.* 2014; Lueders *et al.* 2015); and intravaginal deposition of sperm resulted in successful AI trials in different felids, requiring very high concentrations of sperm (up to $x10^8$) (Silva *et al.* 2000; Tanaka *et al.* 2000). To date, most successful AI trials in felids were achieved by direct deposition of the semen into the uterus or into the oviduct using laparoscopy or laparotomy (Swanson, 2006). Despite being minimally-invasive, these surgical procedures may lead to complications and require postoperative care. Nonetheless, successful non-surgical, transcervical, intrauterine AI has been reported in both domestic and non-domestic cats (Dresser *et al.* 1982; Chatdarong *et al.* 2007; Lueders *et al.* 2014; Lueders *et al.* 2015; Zambelli *et al.* 2015).

1.2 PROBLEM STATEMENT

The African lion is one of the iconic "Big Five", and possesses an enormous economic and touristic value in South Africa, for both wild (Lindsey *et al.* 2007) and captive populations (van der Merwe *et al.* 2017). However, over the past 20 years, more than 40% of the lions have vanished across Africa (Bauer *et al.* 2016).

A suitable breeding management is key for any managed wildlife population, including non-domestic felids such as lions. This is especially relevant in the situation of South Africa, where privately owned, fenced game reserves comprise about three times the size of the state protected areas (Els, 2016). Healthy populations need genetic diversity and thus, breeding management must be included in wildlife conservation programs (Pukazhenthi and Wildt, 2004). In addition to more traditional measures such as monitoring of threatened populations, release projects, relocation of individuals, and exchange of breeders, ARTs are advocated as new potent tools in wildlife conservation breeding (Swanson, 2006). The application of these techniques could provide a faster diversification and distribution of the genetics, the independence from translocating animals for breeding purposes, and a reduction of disease transmission (Pukazhenthi and Wildt, 2004). Especially in the light of the IUCN One-Plan-Approach, which regards *in-situ* and *ex-situ* populations of threatened species as one entity (Byers *et al.* 2013), the transfer of genetics could be achieved by sperm or embryos rather than live animals. Although these benefits would enhance our efforts in wildlife conservation, to date, relatively little has been researched into this field.

The proposed project targets the management, breeding, and conservation of wild felids in South Africa, by researching into the reproductive physiology of the lion, as a model species for large cats, and the applicability of new biotechnologies within this sector. Thus, through research into modern conservation breeding and assisted reproduction efforts, this project focuses on filling knowledge gaps in the reproduction physiology of African lions, as well as on developing a suitable non-surgical artificial insemination protocol for this species.

1.3 AIMS OF THE RESEARCH

The two main goals of this study were:

a) to describe female reproductive cornerstones for the African lion, such as oestrous behaviour, ovarian endocrine activity, and vaginal cytology
b) to develop a non-surgical artificial insemination protocol for lionesses during natural oestrus based on the insights gained under a).

In this respect, the study aimed to accomplish the following objectives:

i. To observe and describe the behavioural signs of oestrus and related reproductive behaviours of lionesses in a captive setting.
ii. To test the feasibility of implementing positive reinforcement conditioning (PRC) training in captive lionesses to allow frequent minimally-invasive collection of samples, such as blood and vaginal swabs.
iii. To measure and compare longitudinal profiles of serum steroid hormones (*i.e.* oestrogens and progesterone) and respective faecal hormone metabolite concentrations during the reproductive cycle of the lioness.
iv. To describe cyclic changes in the vaginal cytology of female African lions, and investigate the use of this technique to predict reproductive stages, such as oestrus or pregnancy.
v. To investigate the response of lionesses in natural oestrus to a single dose of the exogenous GnRH analogue buserelin-acetate to induce ovulation.
vi. To develop a non-surgical artificial insemination protocol for African lions using buserelin-acetate to induce ovulation in females presenting natural oestrus.

1.4 RESEARCH QUESTIONS UNDER INVESTIGATION

The initial hypotheses proposed by this study were:

H1) African lions can be trained by positive reinforcement conditioning to allow collection of blood samples.

H2) African lions can be trained by positive reinforcement conditioning to allow collection of vaginal swabs.

H3) Behavioural monitoring enables detection of oestrus in African lionesses.

H4) Behavioural monitoring enables differentiation between oestrus, interoestrus, and dioestrus in African lionesses.

H5) The pattern of faecal oestrogen metabolite concentrations relates to that of serum oestrogen concentrations throughout the ovarian cycle of the African lioness.

H6) The pattern of faecal progestagen metabolite concentrations relate to that of serum progestagen concentrations throughout the ovarian cycle of the African lioness.

H7) Female African lions show overt signs of oestrous behaviour (*i.e.* vocalizing, lordosis, rolling…) when oestrogen concentration reaches the highest levels within an ovarian cycle.

H8) Female African lions present occasional silent oestrus (*i.e.* not all oestrogen peaks correlate with overt oestrous behaviour).

H9) Monitoring of progestagen concentrations in combination with behavioural observations allow pregnancy diagnosis in lionesses.

H10) Vaginal cytology in lionesses allows detection of oestrus and dioestrus.

H11) Vaginal cytology in lionesses does not allow differentiation between transitional reproductive stages, such as proestrus and interoestrus.

H12) One single dose of buserelin-acetate induces ovulation in African lionesses in natural oestrus.

H13) Non-surgical transcervical artificial insemination can be successfully performed in African lionesses.

1.5 PURPOSE AND VALUE OF THE RESEARCH

Although the conservation value of captive animals is often questioned, non-domestic felids in captive and semi-captive settings constitute educational tools and genetic resources that play an important role for the conservation of the species (Swanson, 2003). Captive non-domestic felids have facilitated the investigation of specific reproductive physiology characteristics in numerous occasions, providing valuable information applicable to both *ex-situ* and *in-situ* conservation programs (Jewgenow *et al.* 2017; Andrews *et al.* 2019). However, to be successful, the study of the species-specific reproduction physiology and ART application needs to be performed while there is still a viable population, and not only when few individuals of the focal species are left.

The ultimate purpose of this study was to prove the feasibility of implementing ARTs into the African lion breeding practices. The results of this study will open new opportunities to improve breeding of captive and free-ranging lion populations, and thereby assist conservation efforts on this species, within the IUCN One-Plan-Approach. Using the African lion as a model species for the study of large non-domestic felids, the results of this study will also potentially serve as a baseline for other threatened felid species. In addition, by supporting management and conservation efforts, this study will indirectly help the wildlife industry, as the lion represents the African natural heritage, and has great economic value as a key species for wildlife tourism and farming in South Africa (Els, 2016).

Expected outputs of this study are:

a) A better understanding of the fundamental aspects of female African lion reproduction by an in-depth investigation of the ovarian cycle, combining minimally-invasive techniques such as behavioural observation, longitudinal hormone monitoring, and vaginal cytology.

b) To implement basic ARTs, such as ovulation induction and artificial insemination, as part of *ex-situ* conservation breeding programs, such as the European Endangered Species Programme (EEP) for lions or the Lion Species Survival Plan (SSP).

c) To develop a model for the application of ARTs in African lions that could provide a basis for other threatened large felid species, such as Asiatic lions (*Pathera leo persicus*), leopards (*Panthera pardus spp*), or tigers (*Panthera tigris spp*).

1.6 RESEARCH DESIGN AND APPROACH

1.6.1 Field site

All data collection and reproductive assays in this study were performed at Ukutula Conservation Center (UCC), a private research facility and genetic biobank located at Ukutula Game Reserve (Brits, North-West Province, South Africa; -25° 30' 55.23", 27° 40' 24.55"). This bushveld farm of 260 hectares hosts not only lions, but also other felid species such as cheetah, caracal (*Caracal caracal*) and black-footed cat (*Felis nigripes*), as well as hyenas, antelope, giraffes, zebras, and numerous bird species. The UCC boasts state-of-the-art laboratory facilities where all animal procedures (*e.g.* anaesthesia maintenance, animal examination, semen collection, artificial insemination) were conducted with full South African Veterinary Council (SAVC) accreditation and authorised veterinary supervision.

1.6.2 Targeted population

In total, 14 African lions (6 females and 8 unrelated males) were used in this study. Two of the females were juvenile at the start of the project (2.5 years), and cohabited in an 800 to 1200 m^2 outdoor enclosure with natural substrate, trees, and a shelter. The remaining four lionesses were fully grown adults (7-9 years), and cohabited with an adult male (6 years) in another enclosure under the same environmental conditions. All animals remained within visual, auditory, and olfactory range. Based on veterinary assessment, all lions were healthy and in good body condition. Before the start of the ovulation induction and artificial insemination experiments, one of the adult females was excluded from the study due to suspected uterine pathology, according to ultrasound imaging, and was relocated with the male into an adjacent similar enclosure. Out of the seven remaining males (3.5-10 years), five were housed together in a male-only group, under the same

conditions as the females, while the other two males were located at two different facilities within South Africa (Table 1.1). Animals were fed every 7-10 days and water was accessible *ad libitum*. Food items consisted mainly of full cow and horse carcasses, supplemented with game meat and farmed chicken.

Table 1.1: Study animals. The UCC (Ukutula Conservation Center, Brits, South Africa) was the main research site of the study. 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. F6 was excluded from the ovulation induction and insemination experiments due to suspected uterine pathology, and relocated with M1 into another enclosure.

Id	Gender	Age	Population dynamics	Proven 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
F6	Female	9	With one male	Yes	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

1.6.3 Experimental plan

Briefly, six African lionesses housed at UCC and identifiable by microchips and unique exterior features, were trained by clicker and basic targeting principles to voluntarily allow blood collection from the coccygeal vein, and vaginal swabbing.

Behavioural monitoring and faecal sample collection started and were carried out in parallel with PRC training, as well as with blood and vaginal swab sampling for a period of time.

Faecal and blood samples were analysed for oestrogen and progestagen concentrations by EIA at the Endocrine Research Lab of the University of Pretoria (South Africa), and reproductive steroid hormone levels were correlated with behavioural data.

Vaginal swabs were collected and evaluated on a daily basis to assess and monitor the cycle of each female in real time, as well as to help with the timing of the AI trials.

Once the ovarian cycle was defined by behavioural, endocrine, and cytological results in at least four lionesses, the final part of the project started.

Different protocols for non-surgical artificial insemination of lionesses in natural behavioural oestrus, using fresh semen collected from unrelated males, were tested in five out of six females. Overall, 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.

For semen collection, the designated donor males underwent anaesthesia prior to the female procedures, and sperm was collected by urethral catheterization and electro-ejaculation.

Pregnancy was determined by a combination of behavioural monitoring, physical signs such as abdomen and nipple enlargement, and vaginal cytology.

Lionesses that did not conceive after AI were inseminated again on the second next natural heat. All females that conceived were followed up by retrospective endocrine analyses, vaginal cytology, and transabdominal ultrasound when possible. After parturition, cubs were generally pulled away at the age of three weeks for hand-rearing, as per UCC animal management policies.

1.6.4 Experimental procedures

1.6.4.1 Behavioural observations

A standardized ethogram for felids (Stanton *et al.* 2015) was used to define targeted state and event behaviours, with special focus on sexual and oestrous behaviour. All animals were observed twice a day, five days per week, over a period of 12 months. Behavioural monitoring sessions took place around sunrise and dusk, and lasted 30-120 minutes in total. Focal animal observations were conducted for

all adult females. General behaviour was measured by scan sampling every five minutes, while specific reproductive behaviour was recorded *ad-libitum* in an attempt to identify females in oestrus based on changes in/or expression of typical behaviours. Additionally, daily records from the animal keepers and tour guides were added to the behavioural information to ensure that no behavioural oestrus was overlooked.

1.6.4.2 Faecal sample collection

Faecal sample search and collection was performed daily for nine months, as part of routine animal care, overlapping behavioural observations. Three 20 g fractions of fresh faeces (*i.e.* within 24 hours of being voided) were collected with gloved hands, and deposited in a double-layer Ziploc plastic bag. All collected material was immediately labelled and stored at -20°C to minimize microbial alteration of the steroid metabolites before analyses.

1.6.4.3 Blood sampling

Blood samples were collected from trained lionesses 1-7 times per week over a period of 18 months, and from animals under general anaesthesia during AI procedures. Approximately 5 ml whole blood were collected from the dorsal or lateral coccygeal veins using a 21G butterfly needle attached to a 10 ml syringe. Samples were deposited in clot-activator tubes and then centrifuged for 15 minutes at 1 000 g. The resulting serum was placed in cryovials, labelled and stored at -80°C until analyses.

1.6.4.4 Vaginal cytology and ultrasound

Vaginal samples were collected every 1-3 days from females in oestrus and interoestrus, and every 3-7 days from females in dioestrus, during training. After separating the labia with a gloved hand, a cotton-tipped swab was carefully introduced about 4 cm dorsally into the vagina to avoid the urethral orifice. The swab was gently rotated against the vaginal walls, and rolled twice onto a clean glass

microscope slide. The prepared slide was then air-dried at room temperature (26°C), and fixed and stained according to the modified Wright-Giemsa method (Rapidiff Fixative®, Clinical Sciences Diagnostics CC, South Africa). Once mounted, every slide was scanned under the microscope at x40 and x200 magnification to assess clearing of the background and clumping degree. Then, 200 epithelial cells were counted at x1000 magnification, and classified into groups (*i.e.* basal, parabasal, intermediate, superficial nucleated, and superficial enucleated) as described for other species (Johnston *et al.* 2001).

Additional vaginal cytologies were collected from anaesthetized lionesses during the AI procedures, and transrectal ultrasound scans were performed using a Mindray® DP-10 device (Mindray Bio-medical Electronics, Shenzhen, China) with a 5-10 MHz linear rectal probe mounted to a straight, 50 cm-long PVC pipe to evaluate the reproductive tract, as well as presence, growth, and/or regression of ovarian follicles and CL.

1.6.4.5 Hormone monitoring

Frozen faecal and serum samples were transferred for endocrine analysis to the Endocrine Research Laboratory at the University of Pretoria (South Africa). Based on Webster *et al.* (2018), 0.050 – 0.055 g faecal powder was extracted using 3 ml of 80% ethanol in water. The suspension was vortexed for 15 minutes and, after centrifugation for 10 minutes at 1500 g, the supernatant was decanted into 1.5 ml Eppendorf microtubes, and kept stored at -20°C until analyses. Oestrogen and progestogen concentrations were determined in both, faecal and serum samples, by EIA as previously described by Palme (1993) and Schwarzenberger *et al.* (1996), respectively, utilizing antibodies against 17β-oestradiol-17-HS:BSA (oestrogen EIA) and 5β-pregnane-3α-ol-20-one-3HS:BSA (progestagen EIA). All assays were validated for lions by demonstrating biological relevance of the results, and parallelism between serial dilutions of the samples and respective standard curves.

1.6.4.6 Ovulation induction

A single intramuscular dose of the GnRH analogue buserelin-acetate (20 μg; 5 mL Receptal®, Intervet, South Africa) was administered by hand-syringe during training or anaesthesia (depending on the protocol tested), to induce an LH peak and subsequent ovulation in lionesses showing natural oestrous signs. 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*, GnRH was injected on Day 6 of oestrus, during the AI procedure; in *protocol 2*, GnRH was injected on Day 5 of oestrus, also during the AI procedure; in *protocol 3* , 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*, GnRH was injected on Day 5 of oestrus, and the AI was performed on Day 6, about 30 hours after the injection.

1.6.4.7 Semen collection and evaluation

Before each AI trial, semen was collected from one unrelated male under general anaesthesia. For each collection, the urethral catheterization (UC) method previously described for lions by Lueders *et al.* (2012) was employed, 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, an additional semen sample was collected from the same male through electro-ejaculation (EE). Here, three sets of 10 electrical pulses (2 Volts), were applied transrectally over the prostate and along the urethra, using a portable battery driven system (El Toro 2, Electronic Research Group, Johannesburg, South Africa) equipped with a small ruminant probe of 2 cm in diameter. 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 and Oko (1989).

1.6.4.8 Artificial insemination

All lionesses were anaesthetized prior to AI. After evaluation of the reproductive tract and assessment of follicle development, the lioness was placed in sternal recumbency with her hind quarters slightly elevated in an attempt to mimic the posture she would acquire during natural mating. Then, a commercial 2.0 x 500 mm dog urinary catheter (Buster®, Kruuse, South Africa) with a metal stylet was introduced in the vagina and followed by transrectal ultrasound up until the cervix. 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, and the fraction collected by EE was deposited along the vagina while the catheter moved out of it. 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.

1.7 DATA COLLECTION AND ANALYSIS

All data, including behavioural, endocrine and cytological findings, as well as the results of the different ovarian stimulation and artificial insemination experiments, were processed and tested statistically.

Initially, and when possible, descriptive statistics, including medians and interquartile ranges, were calculated. Frequency of occurrence of the different base and state behaviours was estimated, and individual and general baselines for hormone concentrations were determined as previously described by Moreira *et al.* (2001). Then, endocrine data were plotted against time and compared to data resulting from behavioural monitoring to correlate oestrogen peaks with behavioural oestrous events, and clusters of elevated progesterone concentration with behavioural luteal phases. Likewise, cytological findings were also compared with resulting behavioural data to assess the vaginal epithelial changes throughout the

different phases of the ovarian cycle, and the different protocols used for ovulation induction were compared to evaluate respective derived success rates. All statistical analyses were performed using the statistical computing software R, and using a significance level of $P<0.05$. Overall, differences between two groups were tested with Mann–Whitney, while differences between more than two groups were tested with the Kruskal–Wallis test, and we used the canonical correlation coefficient (η) to estimate effect sizes. All variables were previously tested for normality using Shapiro-Wilk's normality test, and for equality of variances using Levene's and Fligner-Killeen's tests. Nevertheless, specific tests performed to analyse further aspects of each particular objective (such as the association between faecal metabolite and blood hormone results, or comparison between our dataset and previous studies) are described in detail in the respective chapters.

1.8 METHODS TAKEN TO ENSURE VALIDITY AND RELIABILITY

To ensure validity of the methods applied to monitor the lionesses' behaviour, targeted state and event behaviours were defined using a standardized ethogram for felids (Stanton *et al.* 2015). Behavioural monitoring sessions took place around sunrise and dusk, presuming the nocturnal and crepuscular activity pattern of lions (Hayward and Hayward, 2007), and two different observers videoed focal individuals from different angles during each session. All behavioural data were subsequently logged in capture sheets and scored by one single investigator to avoid inter-observer bias and ensure reliability of the results obtained, as suggested by Martin *et al.* (1993). The oestrogen and progestagen enzyme-immunoassays applied in this study were previously established by Palme (1993) and Schwarzenberger *et al.* (1996), respectively. Reliability of endocrine results was given by parallelism, sensitivity, and intra- and inter-assay coefficients of variation of high- and low-quality controls, as well as by biological validation. All veterinary procedures conducted during this study followed comparable published protocols, such as those of Broder *et al.* (2008) for PRC in captive felids, Johnston *et al.* (2001) for vaginal swabbing and cytology assessment, Lueders *et al.* (2012) for semen collection, and Lueders *et al.* (2014) for ovulation induction and AI.

1.9 ETHICAL CONSIDERATIONS

All lions participating in this study, either for the purpose of sampling, behavioural monitoring, semen donation, ovulation induction assays, or artificial insemination, are property of the Ukutula Game Reserve, the Akwaaba Predator Park, and the Boskoppie Lion and Tiger Reserve, and were housed and cared for at these enclosed facilities during the entire project duration.

All procedures were classified into three different stress-related conditions: a) unrestraint lions (*e.g.* behavioural observation, faecal collection), b) behaviourally restraint lions (*e. g.* blood collection, vaginal swabbing, hand-injection of drugs), and c) chemically restraint lions (*e.g.* semen collection, transrectal ultrasound, artificial insemination). Behavioural monitoring and faecal sample collection were performed from an appropriate distance to not disturb the natural behaviour of the animals. In addition, the risk of distress produced by these procedures was considered minimal, since the lions available for this study were hand-raised and habituated to human presence. The procedures performed under trained conditions were also considered to cause minimal or no distress, since the animal could always leave if not willing to cooperate. All other procedures, although considered minimally-invasive, were performed under anaesthesia and with the supervision of at least one SAVC accredited and authorised veterinarian.

Anaesthesia via dart gun was applied to minimize the difficulties and potential danger associated to the handling of adult lions, but always kept to a minimum duration (generally, around 40-90 minutes). In any case, midazolam was generally included within the anaesthetic protocol, due to its anxiolytic effect and associated retrograde amnesia in felids, to reduce the possible distress occasioned by the darting.

Painkillers were not used in this study, as all procedures performed under anaesthesia could have been tolerated in an awake animal, or caused mild discomfort only at the time of application, with no further pain expected once the procedures were terminated (*e.g*. electro-ejaculation).

All procedures involved in this study were safe, repeatable, and yielded no pain or discomfort beyond ethical considerations. This study had the approval of the Animal Ethics, Use and Care, and Research Committees (V052-17) of the University of Pretoria, South Africa.

1.10 RESEARCH STRUCTURE

To assure a well-structured research report in which the content flows in a logical order, and in which the research aims and questions are fully addressed, this dissertation consists of a general introduction (Chapter 1), five peer-reviewed published, accepted, or submitted articles (Chapters 2-6), and a summarizing discussion (Chapter 7):

Chapter 1: General introduction

This chapter includes a thorough literature review about the reproductive biology and physiology of felids, and the current status or ARTs within these species. It also states the aims, objectives, and main materials and methods utilized in this study.

Chapter 2: Positive reinforcement conditioning as a tool for frequent minimally invasive blood and vaginal swab sampling in African lions (*Panthera leo*). – *published as a "Research Report" in the "Journal of Applied Animal Welfare Science" (2019; doi: 10.1080/10888705.2019.1709066. Epub 2019 Dec 29).*

This chapter focuses on the importance of applying non-invasive and minimally invasive sampling techniques to improve current research on and management of non-domestic animals, and describes all steps involved in the development of a training routine for captive lions.

Chapter 3: Blood and faecal reproductive steroid concentration patterns during the ovarian cycle of African lions (*Panthera leo*), and response to ovulation induction with a GnRH analogue. – *submitted to the journal "Hormones and Behavior" as a "Research Paper" manuscript on the 17th of November 2019.*

In this chapter, the focus is on the basic reproductive endocrinology of the female African lion. It includes a precise comparison of the longitudinal profiles of serum steroid concentrations and respective faecal metabolites for lionesses, and it reflects upon the use of buserelin-acetate to induce ovulation in this species.

Chapter 4: Reproductive cycle stage assessment using vaginal cytology evaluation in African lions (*Panthera leo*). – *published an "Original Research Paper" in the journal "Animal Reproduction Science" (2020 Feb; 213, 106260. doi: 10.1016/j.anireprosci.2019.106260. Epub 2019 Dec 2019).*
This chapter provides a detailed assessment of the changes observed in the vaginal epithelium of the African lioness throughout the different stages of the ovarian cycle as classified by animal behaviour.

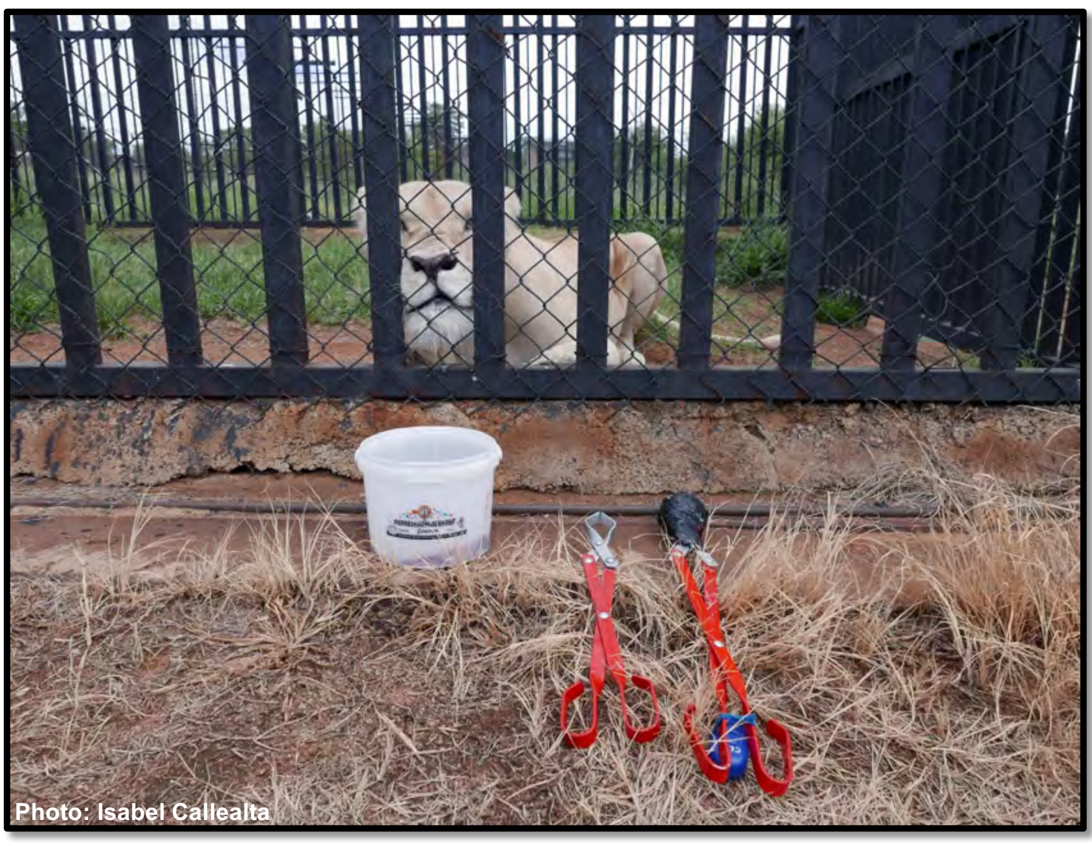
Chapter 5: Detection of *Simonsiella* spp. in the vagina of lions and leopard in oestrus. – *published as a "Short Communication" in the journal "Reproduction of Domestic Animals" (2018 Dec; 53(6): 1605-1608. doi: 10.1111/rda.13298. Epub 2018 Sep 1).*
This chapter focuses on the finding of a micro-organism unfrequently observed in the vaginal flora of any mammal, that may help identify oestrus by vaginal cytology.

Chapter 6: Non-surgical artificial insemination using a GnRH analogue for ovulation induction during natural oestrus in African lions (*Panthera leo*). – *published as an "Original Research Paper" in the journal "Theriogenology" (2019 Nov; 139: 28-35. doi: 10.1016/j.theriogenology.2019.07.022. Epub 2019 Jul 22).*
The main focus of this chapter is the assessment of four protocols for ovulation induction and artificial insemination in African lionesses, that differed in the time lapse between the administration of exogenous GnRH and the actual insemination.

Chapter 7: Findings and recommendations.
This chapter informs the reader of what was discovered during the research and relates to the aims and research questions on which the findings and recommendations are based.

2. CHAPTER TWO:

POSITIVE REINFORCEMENT CONDITIONING AS A TOOL FOR FREQUENT MINIMALLY INVASIVE BLOOD AND VAGINAL SWAB SAMPLING IN AFRICAN LIONS (*PANTHERA LEO*)



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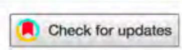

Positive Reinforcement Conditioning as a Tool for Frequent Minimally Invasive Blood and Vaginal Swab Sampling in African Lions (*Panthera Leo*)

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2.1 ABSTRACT

Information regarding the physiology of African lions is scarce, mainly due to challenges associated with essential routine research procedures. The aim of this experiment was to test the possibility of training six captive lionesses by positive reinforcement conditioning (PRC) to voluntarily allow the collection of vaginal swabs and blood samples. This was done with the final goal of avoiding frequent anaesthesia, and potential stressful management during reproduction research. All lionesses mastered basic clicker and targeting principles within two weeks. Routine sampling was possible after 20 weeks of training, enabling the collection of about 750 vaginal swabs and 650 blood samples over 18 months. The animals remained calm and cooperative during all sessions, and demonstrated curiosity in the training. PRC training of captive lionesses proved to be a suitable, minimally invasive method for repeated collection of vaginal swabs and blood. Additionally, PRC may serve as behavioural enrichment for African lions in captive settings. Compared to chemical or physical restraining methods, this non-invasive management approach may reduce distress and physiological negative side effects, thus opening up new avenues for feline research.

2.2 KEYWORDS

African lion; behavioural training; blood collection; operant conditioning; vaginal swab collection.

2.3 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 part of their natural habitat (IUCN, 2018). Therefore, captive population management and breeding constitutes an extremely important tool for the preservation of feline species, as part of inclusive conservation programs. To be effective, these programs require a thorough understanding of feline ecology and behaviour, but also of feline physiology. In order to gather additional physiological information, researchers must apply capture and handling methods in line with the codes of ethics and welfare guidelines published by professional societies and involved countries (Proulx *et al.* 2012).

Given the global decline of around 40% in the last two decades, and the continued decreasing population trend, less than 30 000 individual lions (*Panthera leo*) remain to date (Bauer *et al.* 2016). However, since they breed exceptionally well in captivity (Bauer *et al,* 2016), African lions represent an accessible model species for studying the biology and reproductive physiology of large, non-domestic cats.

Information regarding physiological parameters, such as normal ranges for haematology and blood chemistry, or endocrine mechanisms is currently scarce for large felids such as the African lion. This is mainly due to the general intractability of these animals, since routine research, and diagnostic or therapeutic procedures may be challenging in both wild and captive individuals. Physical restraint, such as the use of squeeze cages, may pose risk of injury to both the non-domestic animal and personnel, while chemical restraint often requires the use of expensive drugs in large doses coupled with a lengthy latent period before taking effect (Pistey and Wright, 1961). Additionally, these techniques may affect homeostasis, altering certain blood parameters such as cortisol levels, haematology or serum chemistry, increasing the variability of data, and the number of subjects needed to achieve statistically significant results in research (Brockway *et al.* 1993).

Positive reinforcement conditioning (PRC) training, originally implemented in laboratory animals (Phillippi-Falkenstein and Clarke, 1992; Bloomsmith *et al.* 1998; Laule *et al.* 2003; Gillis *et al.* 2012), is becoming an increasingly common practice in zoos, as part of the daily behavioural enrichment of the animals, and husbandry

routines (Phillips *et al.* 1998; Savastano *et al.* 2003; Broder *et al.* 2008). The PRC approach facilitates the frequent performance of non-invasive or minimally invasive techniques such as oral, intramuscular or subcutaneous drug administration and blood collection. Also, ultrasound scans become possible in non-sedated animals, reducing the stress associated with physical restraint, and the possible physiological effects of anaesthesia (*e.g.* hypoventilation, hypotension) (Gilroy and DeYoung, 1986; Lambeth *et al*. 2006). PRC training has been known to reduce stress, as evidenced by cortisol response and defensive reactions in rhesus macaque (*Macaca mulatta*), and by haematology and serum chemistry profiles in captive chimpanzees (*Pan troglodytes*) (Reinhardt, 2003; Lambeth *et al*. 2006). In addition to refining the research methodology through controlling the undesirable variable of stress, voluntary cooperation by the study subjects during sample collection proved to diminish the handler's risk of injury (Reinhardt, 2003). Despite the occasional application of PRC for blood collection and vaccination of tigers and lions at various zoological institutions, the published literature on this topic remains scarce at the time of writing. Overall, protocols and training guidelines for felid species are rarely accessible, if they exist at all. Only a few reports show the use of training by positive reinforcement for welfare applications in non-domestic feline species such as cheetah (*Acinonyx* jabatus; Bergman and Janssen, 2005), snow leopard (*Uncia uncia;* Broder *et al.* 2008), and Bengal tiger (*Panthera tigris tigris*; Lin and Wang, 2018).

Therefore, we hypothesized PRC training would enable frequent blood sampling and vaginal swabbing in large felids. The final goal of this study was to confirm that PRC may be used as an easy, cost-effective, minimally invasive tool to advance physiological research and improve husbandry of captive African lions.

The objectives of this study were: a) to train six African lionesses by PRC to allow frequent, routine collection of vaginal swabs and blood from the lateral coccygeal vein, without any additional physical or chemical restraint, and b) to record all steps involved in order to create an accessible, practical protocol that may be used by others implicated in research and management of captive large felids.

This study was prerequisite for a research project focused on the reproductive physiology of African lions and the applicability of assisted reproduction techniques into large felids' conservation programs. The final results of the aforementioned

project were not the purpose of this study, and are reported elsewhere (Callealta, *et al*. 2018; Callealta *et al*. 2019; Callealta *et al*. 2020; Callealta *et al*. submitted).

2.4 MATERIALS AND METHODS

2.4.1 Study animals

Six, captive-born female African lions held at a private conservation centre near Brits (North West province, South Africa; -25° 30' 55.23", 27° 40' 24.55") were chosen for this study. Two females were juvenile at the start of the training (2.5 years), and the remaining four females were fully grown adults (7-9 years). Although all individuals were hand-raised at the same facility, they had not been in direct contact with humans after six months of age. Animals were healthy and in good body condition and had not had previous experience with operant conditioning training. For research purposes, study animals were kept in three adjacent outdoor enclosures: enclosure A housed a group of three adult females; enclosure B housed one adult male and one adult female; and enclosure C housed two juvenile females. All animals remained within the visual, auditory, and olfactory range of each other. Each mesh wire enclosure was between 800 and 1200 m^2. Enclosures contained trees, shelter, and a separation compartment; substrate was natural (*i.e.* grass, soil). Animals were fed every 7-10 days and water was accessible *ad libitum*. Food items consisted mainly of full cow and horse carcasses, and were supplemented with game meat and farmed chicken. Animals remained at the private conservation centre upon completion of the study.

This study was conducted with the permission of the Animal Ethics, Use and Care, and Research Committees (V052-17) of the University of Pretoria, South Africa.

2.4.2 Training enclosure description

Training took place in the separation compartment attached to each enclosure. Fully enclosed compartments measured 5 x 5 m in size, were fenced off by 3 m-high, 50 mm reinforced square tubing, and surrounded by 5 x 5 cm fully galvanized diamond mesh wiring (Fig 2.1). Compartments were connected to the main enclosure by a reinforced square tubing sliding gate. To facilitate training

development and sampling procedures, four features were implemented in each separation compartment: (a) a concrete platform of 55 x 160 cm along the fence, indicating the location where the lionesses should lie down during the training session (Fig 2.1); (b) five 60 cm-high wooden poles of 10 cm diameter located along the concrete platform, opposite the cage fence - these poles created a passage that allowed the lioness to walk into the platform, but prevented her from turning over, *i.e.* she had to walk forward to exit the platfor m (Fig 2.1); (c) a 10 x 10 cm access window located at the front of the concrete platform that the trainer used to introduce food rewards (henceforth *window a*; Fig 2.2); and (d) a mechanical safety lock opening of 13 x 55 cm located on the fence along the concrete platform which was used by the researcher to access animals and collect the required samples (henceforth *window b*; Fig 2.1 and 2.2). The passage created by the wooden poles, as well as the two openings, allowed the trainer to interact with the animals, and the researcher to access them with a minimum risk of injury. Before training implementation, individuals accessed the separation compartment only when fed a whole carcass or if veterinary intervention was necessary. Thus, they generally showed no interest in this area, or even tried to avoid it, unless they could see a carcass inside. To address this, lions were given free access to the compartment at night to facilitate acclimatization to the new training enclosure. In addition, care-takers left portions of meat in this compartment before the start of each session, until the lionesses started entering the enclosure voluntarily.

Figure 2.1: Training enclosure. Fencing and mesh wiring may be noticed. Concrete platform is indicated by red arrows; wooden poles are indicated by stars. *Window b* can also be observed, closed, on the left.

Figure 2.2: ***Window a*** **(left) was used by the trainer to safely introduce the food rewards in the cage.** ***Window b*** **(right) was used by the researcher to safely access the animal and collect the samples needed. Both windows are indicated by red arrows.**

2.4.3 Conditioned training methodology

A combination of classical and operant conditioning was used to train these lions. The first phase was to introduce basic clicker training. For one week, the sound of a clicker (conditioned stimulus) was repeatedly paired with the taste of a food reward (unconditioned stimulus). Initially, the trainer activated a clicker (Fig 2.3) every 15-30 seconds in front of the lioness. Subsequently, using 35 cm-long barbeque tongs (Fig 2.3) for safety reasons, a 50-75 g red meat reward (positive reinforcement) was given immediately after the "click" sound. This exercise was repeated 10 times per session, twice a day, five days per week. After the 10th successful response, the session was finished by a voice reward ("good!"), and the trainer clapped his hands in front of the lioness, who could then exit the training enclosure. Once she reached the housing enclosure, the lioness received a chicken as a final reward for her good work.

Once animals were habituated to the "click" sound and understood it was followed by a reward, clicking was subsequently used as a continuous conditioned reinforcement (or bridging stimulus) to achieve the next desired behaviours. During the following training sessions, food rewards were offered using a variable interval to stay unpredictable and keep the lionesses' attention high, and after completion of every intended training step.

The second phase of training development involved the implementation of basic targeting. For this, a handmade target made from 35 cm-long barbeque tongs

and a tennis ball wrapped in duct tape (Fig 2.3) was presented against the fence of the training enclosure. The trainer encouraged each animal to approach the target and touch it with the nose. Every time the lioness moved closer to the target or accidentally touched it, the clicker was activated (conditioned reinforcement) and a food reward was immediately offered (positive reinforcement).

Once all animals learnt to voluntarily touch the stationary target and lie in front of it, the trainer started to present the target at different locations along the fence, encouraging the lioness to follow and touch it at each location. When a lioness displayed the desired behaviour, the action was reinforced with a “click” and a food reward. When unwanted behaviours, such as trying to claw or bite the target instead of touching it with the nose were displayed, a session time-out of 30 seconds to 5 minutes was established. During a time-out, the lioness was ignored and the target removed from the fence until she stopped these undesirable behaviours. If, however, after 5 minutes from the beginning of the training session (or session time-out), the subject did not show any interest in the activity (*e.g.* not paying attention to the target or pacing along the training enclosure), the trainer allowed her to exit the enclosure, and considered the session terminated, offering the lioness no reward.

By following the moving target and increasing the duration of interaction with the target, individuals were trained to step onto the concrete platform, lie down, and wait for the 5-20 minutes it took the researcher to complete the required sampling procedures (Fig 2.4). During this time, the lioness was not required to continuously touch the target, but at least pay attention to it. The “click” sound was used as a

Figure 2.3: Training tools. From top to bottom: feeding tongs, clicker, handmade target, and tail-handling hook.

bridging signal every 10-15 seconds, and a food reward was given every 2-3 clicks to keep the lioness' attention high. The target training was divided into sessions of 10 food rewards. Each session was performed twice a day, five days per week, for about 10 weeks, until all lionesses mastered the basic intended behaviours, and were ready for the next, more practical, stage of the training.

All red meat and chicken rewards used during the training sessions were consistently weighed and formed part of the animals' weekly nutritional intake.

Figure 2.4: Training set-up. The picture shows a lioness laying on the concrete platform, waiting by the target. On the left, the researcher and assistant handle the tail through *window b*. On the right, the trainer holds the target on his right hand and the clicker on his left hand, while the feeder holds a bucket with the food rewards and tongs.

2.4.4 Touch desensitization and sampling

Once the lionesses voluntarily waited on the concrete platform near the target for five minutes, touch desensitization was implemented in the training sessions. During the five minutes, while the trainer provided conditioned reinforcement every 10-15 seconds, and offered positive reinforcement every 30 seconds, the researcher used a 50 cm-long snake-handling hook (Fig 2.3) to approach the lioness' tail through window *b*. Once the hook came into contact with the individuals tail, the stimulus bridge was activated, and a food reward was given. This routine was repeated until the individual stopped moving the tail away after noticing the touch of the hook. The next step was to place the hook on the tail, providing positive reinforcement only if the lioness tolerated the contact for at least three seconds. Once the subjects were habituated to the touch of the hook to the tail, attempts were made to hold, palpate, and handle the tail manually. As before, the trainer would provide positive reinforcement only when the subject showed no resistance to the

researcher's handling. In this way, all lionesses learnt to tolerate palpation of the hind-quarters, perineum, vulvar lips, and entrance to the vagina.

After six weeks of touch desensitization, the collection of the first samples was possible. Insertion of a cotton swab to about 2 cm-deep into the vagina and gentle rotation against the mucosal wall enabled the researcher to obtain vaginal swabs (Fig 2.5). Conditioned and positive reinforcement rewards were given to the subject as soon as the cotton swab was introduced in the vagina, and right after collection of the sample. The collection of approximately 5 ml whole blood samples from the dorsal or lateral coccygeal veins was performed using a 21G butterfly needle attached to a 10 ml syringe (Fig 2.5). Conditioned and positive reinforcement rewards were provided as soon as the needle was inserted through the skin, and right after the blood sample was collected. The trainer had to ensure that the animal was completely focused on the target before the researcher could attempt any sample collection. This active communication routine decreased the risk of accidents during the handling and sampling procedures. Sample collection by positive-reinforced clicker training took place 1-3 times per week, depending on the stage of the reproductive cycle the female presented.

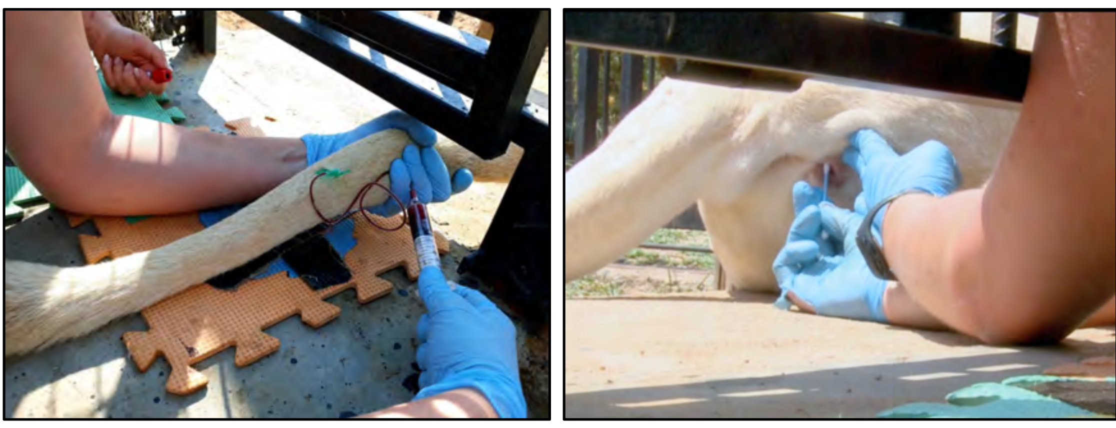

Figure 2.5: Blood sample (left) and vaginal swab (right) collection through *window b*.

By the end of the study, each lioness received (per session) one food reward when she: a) touched the target for the first time once in the training enclosure, b) laid on the concrete platform, c) allowed vaginal swab, and d) allowed blood collection. The remaining six rewards were given following an unpredictable routine to keep the lioness' interest in the activity high.

2.4.5 Data collection

At the beginning of each training session, meteorology data such as temperature, humidity, wind speed, and rain probability were recorded. For each individual session, the researcher captured starting and ending times in a data-sheet, and gave every attempted training step a score of either "1" (successful) or "0" (unsuccessful). Table 2.1 shows the complete training routine the lionesses followed along this study. The lionesses were considered to correctly perform one intended behaviour after three or more days scoring "1" for that specific training step (Table 2.2). All relevant behaviours observed during training (*e.g.* "does not approach training enclosure", "tries to claw/bite target", "presents hind-quarters after palpation of perineum") were also noted down.

2.5 RESULTS

2.5.1 Entering the training enclosure, and basic clicker training

Despite previous avoidance, all females (n=6) became confident around the separation compartment within the first week of training. After a few days, they started to voluntarily approach this compartment as soon as the trainer and researcher began to prepare the training setup. The lionesses needed 2 ± 0.68 (mean ± SEM) sessions (range: 1-5) to voluntarily enter the enclosure. All females showed some kind of aversion to the clicker sound the first time they heard it. In general, they would get a mild fright and suddenly jump back. To overcome this problem, the trainer made the "click" sound softer by hiding the clicker either inside a pocket or behind his back. The females rapidly got habituated, and there was no need to hide the clicker anymore from that first session forth.

Table 2.1: Positive reinforcement conditioning training steps.

Classic conditioning	Pairing conditioned stimulus with unconditioned stimulus
Basic clicker training	Establishing conditioned-primary reinforcer relationship
Target training	Touching stationary target
	Lying down by stationary target
	Following moving target
	Lying down on concrete platform Increasing holding time
Touch desensitization and sample collection	Allowing tail contact with handling hook
	Allowing tail rubbing with handling hook
	Allowing tail pressure with handling hook
	Allowing tail contact with hands
	Allowing tail rubbing with hands
	Allowing tail holding with hands
	Allowing palpation of hind-quarters with hands
	Allowing pressure of hind-quarters with fingers
	Allowing palpation of perineum with hands
	Allowing palpation of vulvar lips
	Allowing vaginal swap collection
	Allowing location of coccygeal vein by palpation
	Allowing alcohol pouring on the targeted injection site
	Allowing pressure of the targeted injection site with capped butterfly needle
	Allowing blood sample collection

Table 2.2: Individual training effort. This table shows the number of training sessions each lioness needed to correctly perform the main intended behaviours.

	Training effort					
Training steps	*Lioness 1*	*Lioness 2*	*Lioness 3*	*Lioness 4*	*Lioness 5*	*Lioness 6*
Classic conditioning	1	1	1	1	1	1
Entering training enclosure	3	5	1	1	1	1
Touching stationary target	2	0	1	1	3	1
Lying in front of target	1	2	1	4	2	1
Following moving target to concrete platform	7	8	2	3	3	1
Tail touch desensitization	5	21	27	3	17	5
Allowing vaginal swabbing	5	3	7	3	0	1
Allowing blood collection	4	2	1	3	1	0

2.5.2 Targeting

Three out of six lionesses showed signs of mild aggression towards the target the first time they saw it (*e.g.* they hissed at it, and/or tried to claw or bite it). However, these behaviours disappeared quickly, and all six females learnt to voluntarily search and touch the stationary target within 1.33 ± 0.42 sessions (range: 1-3). It took them an average of 1.83 ± 0.48 sessions (range: 1-4) to learn to lie down in front of the target and touch it to get the reward. Exercises with a moving target along the fence were repeated for 10 weeks until all new features were implemented in the separation compartment, and the training enclosures were ready. All subjects learnt to approach the concrete platform, lie down, and touch the target in 4.0 ± 1.15 sessions (range: 1-8) on average.

2.5.3 Touch desensitization

All females (n=6) needed on average 13.0 ± 4.10 sessions (range: 3-27) to allow the researcher to firmly hold their tails, externally locate the coccygeal vein, and to pour a small volume of alcohol on the targeted injection site, as well as to access the entrance of the vagina. Handling of the tail was the most challenging step of the training. In general, during the first sessions this step was attempted, all females refused contact moving the tail away, despite remaining alert to the target on the platform. Only one lioness (Lioness 5), that had suffered a tail fracture as a cub, abandoned the platform and show mild aggression towards the researcher (*e.g.* hissing or growling) during these first sessions.

2.5.4 Vaginal swab and blood sampling

Once the subjects were successfully touch-desensitized, the lionesses needed on average 3.17 ± 1.05 (range: 0-7) and 1.83 ± 0.60 (range: 0-4) sessions to allow collection of vaginal and blood samples, respectively. During oestrus, some females appeared more receptive at the time of vaginal swab sampling than during pro-oestrus, when females were usually more restless and numerous attempts were needed for successful sampling.

2.5.5 Exceptions

One of the females (Lioness 5) needed special training to meet the targeted goals in time. This particular female required reinforcement more frequently than the other lionesses: a “click” sound (bridging stimulus) every five seconds, and food reward (positive reinforcement) every 10-15 seconds while lying on the platform, waiting near the target. This lioness responded better and was more attentive to the target when the food rewards consisted of chicken instead of red meat. To prevent this individual from quickly losing interest in the target and leaving the concrete platform, the trainer allowed her to lick the food reward while the sampling procedures were taking place.

2.5.6 Overall summary

Routine sampling from all six lionesses was possible after 20 weeks of training (1-2 sessions per day, five days per week), which facilitated collection of about 750 vaginal swabs and 650 blood samples over the total course of the underlying research project (18 months). These samples served to describe in detail the African lioness' ovarian cycle by combination of behavioural observations, longitudinal steroid hormone monitoring, and vaginal cytology. They also helped establish the ideal time for ovulation induction and artificial insemination of lionesses presenting natural oestrus. These results have been reported elsewhere (Callealta *et al.* 2018; Callealta *et al.* 2019; Callealta *et al.* 2020; Callealta *et al.* submitted).

2.6 DISCUSSION

In this study, we conditioned six African lionesses by positive reinforcement training to allow the collection of vaginal swabs and blood samples. This is the first time, to our knowledge, that the training in parallel of such a number of large felids has been documented in the scientific literature, compared to common previous reports of single cases (*e.g.* Bergman and Janssen, 2005; Broder *et al.* 2008; Lin and Wang, 2018). Our results support the benefit of positive reinforcement training on captive African lion welfare, handling, and research methodology, and we suggest this practice may be beneficial for other captive felid species. Routine sample collection was possible after 20 weeks; however, all trained animals were hand-reared. Even though hand-rearing might still be common in some breeding facilities, this practice is currently in decline. As training of only hand-reared lions may be a practical limitation of this study, further research would be needed in the future to investigate the differences in learning speed and training performance of hand-reared and parent-reared animals under the same conditions.

Despite minor inter-individual differences in their performances, all lionesses mastered basic clicker training and targeting principles in two weeks, matching previous results described by Gillis *et al.* (2012) for squirrel monkeys (*Saimiri boliviensis*). The final training goal that enabled routine vaginal and blood sample collection was achieved in approximately five months. This was slightly longer than the time-frame reported by Broder *et al.* (2008), who needed about four months to

perform a transabdominal ultrasound on one unanaesthetized snow leopard (*Uncia uncia*). Nevertheless, one must be careful to compare results from different studies, due to the great variety of possible training conditions (*e.g.* rewarding technique, staff experience, training facilities), and individual experiences both within and between species. In this case, the lionesses had to perform basic targeting exercises for 10 weeks before starting with touch desensitization. The original architecture of the training enclosures impeded direct contact with the lionesses and, as a result, building modifications to and around the separation compartments were necessary before new training routines could be implemented. If the training enclosures had been ready before the start of the study, this time could have been shorter.

It may be interesting to note that, even though the complete training and sampling routine was implemented and mastered by all six females, specific situations seemed to affect the performance of some of the lionesses. For example, most lionesses did not show interest in the training the day they were given their normal food portion, nor the day right after a regular meal. On the other hand, probably due to increased hunger as their normal feeding day approached, all individuals demonstrated behavioural signs of boredom or impatience, such us pacing along the fence of the training enclosure, digging in front of the food reward’s bucket, clawing at the fence where food rewards were given, or leaving the concrete platform before the end of the session. Nevertheless, these behaviours as well as those observed during oestrus and proestrus, or those related to meat preference, are only initial observations noted by the trainer and researcher during the training development. Thus, further investigation to support these observations and improve the training efficiency would be needed, and is recommended for future studies.

The lionesses demonstrated increased curiosity and interest in shaping new tasks in exchange for food rewards. Further, the time required to allow partially invasive procedures such as vaginal swabs or blood sample collection once touch-desensitized, was minimal, basically entailing no extra challenge for them. These results lead us to conclude that it could be relatively simple to implement new training routines that are typically required in basic animal care and handling manoeuvres. Should this approach be chosen, drug injection or oral administration, superficial wound treatment, antiparasitic medication spraying, or pregnancy

monitoring via ultrasound scanning would be possible after an initial period of training.

Prior to the start of this study, the lionesses avoided entering the separation compartment unless a carcass was present. At the end of this study, all females would approach the separation compartment as soon as the trainer and researcher began to prepare the setup for the new session. Once the lionesses understood the association between the training enclosure, the bridging stimulus, and the food rewards, they seemed stimulated by the training sessions. On occasion, the subjects would wait on the concrete platform, or in front of the training tools and food rewards for the trainer to start the session. Since PRC training implementation, previous tedious, time-consuming tasks such as moving the animals to the separation compartment to clean the main enclosure, became easy, rapid, and non-stressful for both animals and personnel.

"Working for food" appeared to be a positive activity for these animals, as seen before in primates (Reinhardt, 2003), livestock (Hemsworth, 2003), cats (Broder *et al.* 2008), and even reptiles (Hellmuth *et al.* 2012). The ultimate goal of this study was to empirically demonstrate that PRC training may serve to improve research, vet care, and husbandry of captive lions, and not to test whether this approach was enriching. Nevertheless, the observed positive responses suggest that this activity is in fact stimulating for large felids, where environmental enrichment is especially challenging (AZA, 2012). This study therefore lends support to the idea that a well-orchestrated training program may be utilized as another tool for environmental enrichment for this species, as long as it gives the animals control over their environment, allows them to choose, and teaches them to deal with new challenges, as previously supported by Mellen and Shepherdson (1997), Melfi (2013), and Westlund (2014).

2.7 CONCLUSIONS

In summary, the PRC training of captive African lionesses resulted in a minimally-invasive, suitable, repeatable and cost effective method for the collection of vaginal swabs and blood samples. Based on the positive responses displayed by six out of six trained lionesses, our results indicate that this kind of training is feasible

for both physiological studies and veterinary treatment. Our sampling methodology by PRC training may reduce the psychological stress component associated with traditional physical and chemical restraint techniques, and avoids physiological effects associated with anaesthesia. The use of PRC would be beneficial to handlers and management as routines and behaviours previously warranting negative reinforcement or anaesthesia, can now be progressively shaped. In addition to opening new avenues of physiological research and veterinary treatment options, this approach appeared to be stimulating for these animals, and thus could be potentially considered a form of behavioural enrichment for African lions in captive settings.

2.8 DECLARATIONS OF INTEREST

None.

2.9 ACKNOWLEDGEMENTS

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3. CHAPTER THREE:

BLOOD AND FAECAL REPRODUCTIVE STEROID CONCENTRATION PATTERNS DURING THE OVARIAN CYCLE OF AFRICAN LIONS (*PANTHERA LEO*), AND RESPONSE TO OVULATION INDUCTION WITH A GnRH ANALOGUE

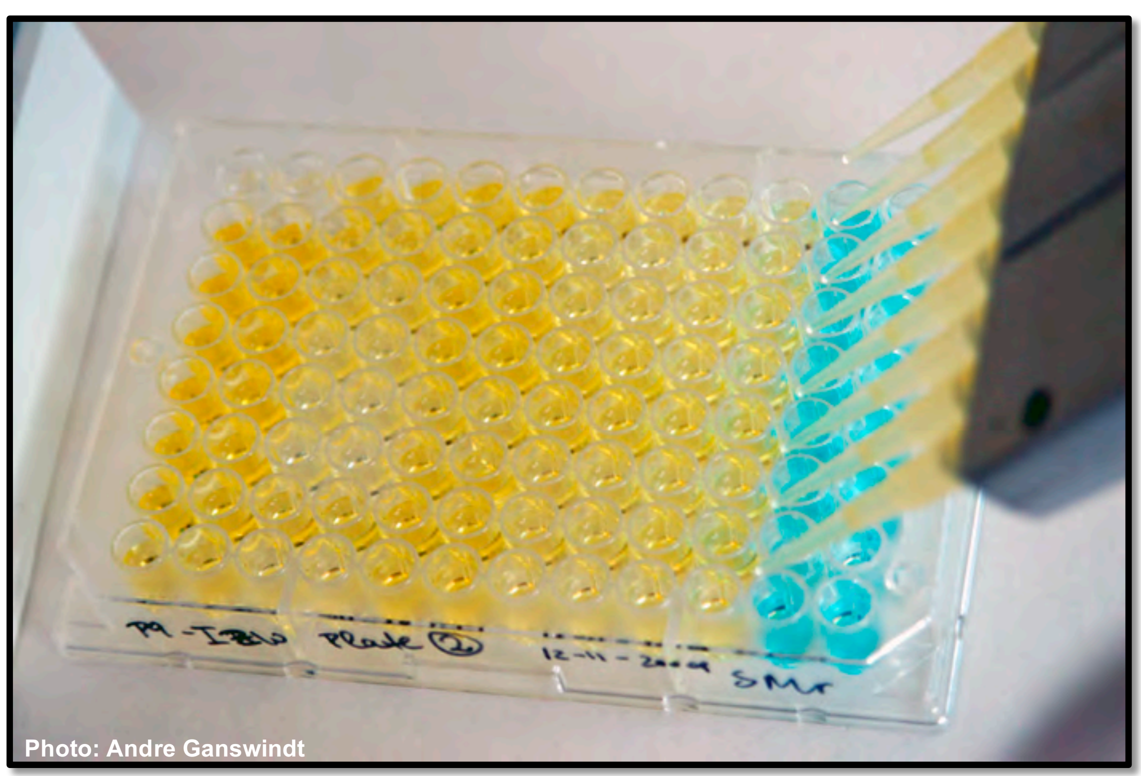


Photo: Andre Ganswindt

This chapter has been submitted to the journal "Hormones and Behavior" as a "Research Paper" on the 17th of November 2019, and is currently under review.

3.1 ABSTRACT

To develop successful *ex-situ* breeding programs for the conservation of threatened wildlife, information about reproductive endocrinology in the species of interest is essential. Faecal hormone metabolite patterns are widely used in this regard, but limited information exists on how these reflect blood hormone profiles. This study aimed to longitudinally monitor and compare serum progestagen (sP) and oestrogen (sE) concentrations, as well as faecal progestagen (fPM) and oestrogen (fEM) metabolite concentrations, with reproductive behaviour in African lionesses. Further, the study examined the endocrine effect of a 20 μg buserelin-acetate single dose to induce ovulation on days 4–6 of behavioural oestrus in this species. For 18 months, blood sampling (n=309) from five captive lionesses took place 1-7 times per week, during positive reinforcement training. In parallel, over a period of nine months, faecal samples (n=129) were searched for daily and collected when found. Daily behavioural monitoring enabled classification of reproductive stage. Competitive enzyme immunoassays, utilizing antibodies against 5β-pregnane-3β-ol-20-one-3HS:BSA and 17β-oestradiol-17-HS:BSA, were used for steroid quantification. Pattern of fPM concentrations matched sP levels (n=115; r=0.77; ρ=0.69; τ=0.53), while determined fEM and sE levels were not correlated (n=115; r=0.06; ρ=0.17; τ=0.09). Overall, pattern of faecal hormone metabolite concentrations matched reproductive behaviour more reliably. FPM and sP patterns enabled identification of luteal phases, and may serve to distinguish between pregnant and non-pregnant females. Detection of oestrus by measurement of fEM and sE was not accurate. Induced ovulation occurred on average 60.1 h (n=10, range: 24-96 h) after buserelin-acetate administration, with some lionesses showing oestrus signs up to 72 h after ovulation.

3.2 KEYWORDS

Blood; oestrogen; faeces; non-invasive hormone monitoring; ovarian cycle; ovulation induction; *Panthera leo*; progestagens; reproduction.

3.3 INTRODUCTION

While behaviour analysis laid the foundation of wildlife research long ago (Martin *et al.* 1993), endocrinology constitutes an essential tool for the study of reproductive physiology in threatened species, and the improvement of conservation breeding efforts (Kersey and Dehnhard, 2014). Over the last four decades, non-invasive hormone monitoring has emerged as the most commonly used approach in this regard (Kersey and Dehnhard, 2014). Quantification of faecal hormone metabolites has advanced the understanding of ovarian activity in a substantial number of wildlife species, including numerous small and large felid species (Graham *et al.* 1995; Moreira *et al.* 2001; van Dorsser *et al.* 2007; Brown, 2011; Putman *et al.* 2015). Most felids are induced ovulators (Schramm *et al.* 1994; Brown, 2011), and their ovarian cycle is generally divided into three phases (Andrews *et al.* 2019). The anoestrus phase is associated with reproductive inactivity; the follicular phase (which includes "oestrus" and "interoestrous interval") is characterized by follicular waves and fluctuating oestrogen concentrations; and the luteal phase (or dioestrus, which occurs exclusively after ovulation) is associated with elevated progesterone concentrations (Andrews *et al.* 2019). The "pregnant luteal phase" (PLP, pregnancy, or gestation) occurs after ovulation when conception takes place, but when fecundation does not occur, this phase is referred to as "non-pregnant luteal phase" (NPLP, pseudopregnancy, or pseudogestation).

African lionesses (*Panthera leo*) are polyoestrous, not affected by season or photoperiod, and seem to show the above-mentioned kind of reproductive cycle (Brown, 2011; Putman *et al.* 2015). Based on faecal endocrine results, African lionesses have an ovarian cycle length of between 8-30 days, with oestrus ranging between 2-9 days (Putman *et al.* 2015). Although regarded as induced ovulators, lionesses also present occasional spontaneous ovulations (Schramm *et al.* 1994).

Due to opportunistic feeding patterns and prolonged intestinal passage time, lions do not usually defecate more often than once every 24 hours (Smith *et al.*

2006). The use of faeces as a non-invasive monitoring tool for specific reproductive effects in this species can be thus disadvantageous. Firstly, because hormone metabolite excretion may be variable (not only between defecation events, but also between individuals) and secondly, because availability of faecal samples may be limited. Therefore, the ability to detect or monitor specific reproductive events utilizing faecal samples may be hampered (Touma and Palme, 2005; Wielebnowski and Watters, 2007). On the other hand, the use of minimally-invasive techniques enables collection of scheduled, regular blood samples, which by definition are the ideal matrix to monitor endocrine events in animals (Kersey and Dehnhard, 2014). However, performing routine blood collection in generally unhandled and potentially dangerous wild animals, such as lions, is extremely challenging (Graham *et al.* 1995; Kersey and Dehnhard, 2014; Callealta *et al.* 2019). Additionally, repeated anaesthetic procedures may increase the probability of deleterious side effects derived from hypoventilation and hypotension (Gilroy and DeYoung, 1986).

Information related to longitudinal blood hormone profiles of non-domestic felids and the extent to which faecal oestrogen and progesterone metabolites actually reflect circulating hormone levels is limited (Schmidt *et al.* 1979, 1993; Seal *et al.* 1985; Briggs *et al.* 1990; Moreland *et al.* 2002). However, precise longitudinal data related to reproductive endocrine characteristics in non-domestic felids would, for instance, help to improve the current protocols for ovulation induction in these species (Pelican *et al.* 2006). Ovulation induction is a prerequisite for the application of any assisted reproduction technique in felids and thus key to the development of *ex-situ* breeding and conservation programs (Swanson, 2006; Lermen *et al.* 2009).

With the final goal of achieving a better understanding of the reproductive physiology of the female African lion, and laying the foundation for any ART procedure by studying ovulation induction in this species, this study had three specific aims. The first aim was to compare longitudinal profiles of female serum reproductive hormone concentrations and faecal metabolites. The second, to compare the pattern of serum and faecal steroid concentrations with reproductive events such as overt oestrus and pregnancy. The third and final aim was to investigate endocrine and behavioural responses to a single dose of the exogenous GnRH analogue buserelin-acetate to induce ovulation in lionesses presenting natural oestrus.

3.4 MATERIALS AND METHODS

3.4.1 Study animals and housing

The subjects of this study were five female African lions held in captivity at a private conservation centre in South Africa. Individuals were identifiable based on unique exterior features and by microchips. Three adult lionesses (7-9 years) that had previously reproduced, cohabited for four months with an adult male (6 years) in an 800-1200 m^2 outdoor enclosure with natural substrate, trees, and a shelter. At the end of the four-month period and prior to the start of the hormonal ovulation induction experiments, the male was relocated to a similar adjacent enclosure. The two remaining juvenile females (2.5 years), nulliparous at the beginning of the study, were kept together in an adjacent enclosure under the same environmental conditions, within visual, auditory, and olfactory range of the other four animals, but without direct contact to a male. Animals were fed every 7-10 days and water was accessible *ad libitum*. Food items consisted mainly of complete cow and horse carcasses, supplemented with game meat and farmed chicken. Based on veterinary assessment, all lions were healthy and in good body condition. The five focal females were trained by positive reinforcement conditioning to voluntarily allow blood collection, and drug administration by hand-syringe (Callealta *et al.* 2019). This study was conducted under the approval of the Animal Ethics, Use and Care, and Research Committees (V052-17) of the University of Pretoria, South Africa.

3.4.2 Behavioural monitoring

We used the standardized ethogram for felids suggested by Stanton *et al.* (2015) to define targeted base state and event behaviours, with special attention to sexual and oestrous behaviours (Table 3.1). Thorough observations of all lionesses were conducted over a period of 12 months in an attempt to identify females in oestrus based on changes in/or expression of typical behaviours. Presuming the nocturnal and crepuscular activity pattern of lions (Hayward and Hayward, 2007), behavioural monitoring sessions took place around sunrise and dusk. Meteorological data such as temperature, wind direction and speed, humidity, and the probability of rain were noted at the beginning of each session. Scan sampling allowed us to record the state (*i.e.* “drinking”, “eating”, “exploring”, “lying”, “pacing”,

"patrolling", "sitting", "standing", "other", or "out of sight") of each focal female at five minute intervals during 30-120 minutes per day, five days per week. *Ad-libitum* sampling, on the other hand, facilitated continuous recording of all targeted behavioural events that occurred during each monitoring session (Martin *et al.* 1993). Two different observers videoed focal individuals from different angles during each session, and all behavioural data were subsequently logged in capture sheets and scored by one investigator. The distance between observers and animals ranged from 5 to 100 m, and binoculars (Sonoma™, 8 x 40 mm, Tasco®) were used when necessary. The behavioural monitoring conducted during this study caused no harm to the animals or disturbance to their natural behaviour.

Table 3.1: Definition of reproductive base "event" behaviours recorded during the study, slightly modified from Stanton *et al.* 2015. *For statistical analysis, these behaviours were included in a new category: "marking".

Behaviour	Definition
Allowing mount	Lioness stays and does not reject male (or female) when this tries to mount her.
Allogrooming	Lion licks the fur of another lion's head or body.
Anogenital grooming	Lion grooms (licks) its own genitals.
Body rubbing*	Lion rubs any part or entire length of body against (modifier).
Copulation	Male mounts female and intromission is achieved.
Flehmen	Lion makes a grimaced facial expression, where the mouth is open, upper lip is elevated, and tongue may protrude out of the mouth. Generally, follows sniffing of an object, scent, bodily excretion, or another lion.
Flirting run	Female feigns running away from a breeding partner (or another female). May include the female running short distance, then stopping to roll or look around before continuing to run as the partner approaches. Both lions may also walk around or circle each other.
Head rubbing*	Lion rubs any part of its head against (modifier).
Hind feet scraping*	Lion scrapes hind feet on the ground in a backwards direction, shuffling one foot after the other.

Table 3.1 (cont.): Definition of reproductive base "event" behaviours recorded during the study, slightly modified from Stanton *et al.* 2015. *For statistical analysis, these behaviours were included in a new category: "marking".

Behaviour	Definition
Lordosis	Female raises hindquarters while lowering forequarters to the ground, presenting genitals to male. Tail is often averted to one side. The position is sometimes accompanied by treading of the hind legs.
Mount	Male attempts intromission by straddling over the female with front and hind feet. This behaviour may as well be performed by a female instead of a male.
Purring	Female makes a quiet, continuous, soft sound, similar to the one domestic cats make.
Rejection	Lioness backs away from male (or female) when this tries to mount or approach her.
Rolling	While lying on the ground, lion rotates body from one side to another, repeatedly. During the roll, the back is rubbed against ground, the belly is exposed and all paws are in the air.
Urine spray*	While standing with tail raised vertically, lion releases a jet of urine backwards against a vertical surface or object.

3.4.3 Reproductive stage classification

In this study, we considered three phases of the ovarian cycle, as described for other felids (Andrews *et al.* 2019): anoestrus (juvenile or lactational), follicular phase (oestrus and interoestrus), and luteal phase (pregnancy or pseudopregnancy). Accordingly, the reproductive stages assessed by behavioural observations in this study were: 1) Juvenile anoestrus (prepubertal female lacking reproductive behaviour); 2) Oestrus (normally cycling female showing reproductive behavioural signs); 3) Interoestrus (normally cycling female between two oestrous events without ovulation; 4) Pregnancy (female conceives after ovulation and mating/artificial insemination, and lacks reproductive behaviour for a prolonged period of time); 5) Pseudopregnancy (female does not conceive after spontaneous or induced ovulation, with or without mating/artificial insemination, and lacks reproductive behaviour for a prolonged period of time); 6) Lactational anoestrus (female after parturition, nursing cubs and lacking reproductive behaviour).

The lionesses in this study were classified in behavioural oestrus when a high frequency of the reproductive events described in Table 3.1 was observed (Stanton *et al.* 2015). Oestrus was considered to start on the first day these signs were exhibited by a lioness after a resting period, and to end on the last day the signs were observed. For analysis purposes, each oestrus event was divided into two equally long parts (*i.e.* “first half” and “second half”). Behavioural interoestrus was identified by the absence of most reproductive events described in Table 3.1 in normally cycling females (after a period of intense exhibition of reproductive signs, with or without mating, and without ovulation). Behavioural pregnant and non-pregnant luteal phases were identified by the absence of most reproductive events described in Table 3.1, after a period of intense exhibition of reproductive signs, and ovulation. Pregnancy was defined to start on the first day the female ceased oestrous behaviour after mating/insemination, and end on the day of parturition. Pseudopregnancy was considered to start on the first day the female did not present typical oestrous behaviour, and end on the last day before the lioness began showing oestrous signs again after a non-conceptive ovulation. To enable practical comparison, pregnancy was divided into three distinct periods of 36 days each (*i.e.* “first part”, “second part”, and “third part”), while pseudopregnancy was divided into a period of 36 days (“first part”) and a second period of variable length (“second/last part”). Behavioural anoestrus was defined by the absence of reproductive signs in young, prepubertal females (juvenile anoestrus), and adult lactating females (lactational anoestrus). Lactational anoestrus was considered to start on the day of parturition, and lasted a maximum of three weeks. After this period, the cubs were relocated to a different enclosure and hand-reared further.

3.4.4 Faecal sample collection and steroid extraction

Over the course of nine months, daily faecal searches overlapped with behavioural observations. To facilitate individual faecal sample collection, animals received 500-750 grams of red meat mixed with a distinctive marker of corn, a canary seed mix, or green peas, every day during positive reinforcement conditioning training (Callealta *et al.* 2019). For each sample collected, three 20 g fractions of fresh faecal material were deposited into a double-layer Ziploc plastic

bag, using gloves, and immediately frozen and stored at -20°C to minimize microbial alteration of the steroid metabolites before analyses. In total, we collected 129 samples from four of the five focal females (32.25 ± 3.90 samples per female; range: 23-42 samples). The fifth lioness remained prepubertal during the nine-month faecal sampling period, and none of her faecal samples was assessed. After transportation to the Endocrine Research Laboratory of the University of Pretoria (South Africa), the frozen faecal samples were lyophilized, pulverized, and sieved through a wire-mesh filter to remove any remains of the marker and other undigested materials. Based on Webster *et al.* (2018), 0.050 – 0.055 g faecal powder was extracted using 3 ml of 80% ethanol in water. The suspension was vortexed for 15 minutes and, after centrifugation for 10 minutes at 1500 g, the supernatant was decanted into 1.5 ml Eppendorf microtubes, and stored at -20°C until analyses.

3.4.5 Blood sample collection and ovulation induction

Over a period of 18 months, blood sampling took place 1-7 times per week, during positive reinforcement conditioning training. Blood samples were generally collected daily during oestrus, every 48 hours during interoestrus, every 3-5 days during pseudopregnancy and lactational anoestrus, and every 5-7 days during pregnancy and juvenile anoestrus. Approximately 5 ml of whole blood was collected from the dorsal or lateral coccygeal veins using a 21G butterfly needle attached to a 10 ml syringe. Samples were immediately deposited in serum clot-activator tubes and then centrifuged for 15 minutes at 1 000 g. The resulting serum was placed in cryovials, and stored at -80°C until analyses. In total, we assessed 309 blood samples: 121 samples (24.20 ± 3.81 samples per female; range: 13-35 samples) that overlapped with the faecal sampling period, and additional 188 samples (37.60 ± 4.07 samples per female; range: 23-47 samples) collected during the ovulation induction experiments described by Callealta *et al.* (2019) for artificial insemination (AI). Briefly, a single intramuscular dose of the GnRH analogue buserelin-acetate (20 μg; 5 ml Receptal®, Intervet, South Africa) was administered by hand-syringe at the end of the lioness natural oestrus, on days 4 (n=3), 5 (n=5), or 6 (n=2), to induce ovulation prior to AI trials.

3.4.6 Steroid analyses

Faecal extracts and serum samples were measured for immunoreactive faecal oestrogen (fEM) and progestagen (fPM) metabolites, and serum oestrogen (sE) and progestagen (sP) concentrations using two different enzyme-immunoassays (EIA). We utilized antibodies against 17β-oestradiol-17-HS:BSA (oestrogen EIA) and 5β-pregnane-3α-ol-20-one-3HS:BSA (progestagen EIA) on microtiter plates coated with goat anti-rabbit IgG. Detailed assay characteristics, including full descriptions of the assay components and cross-reactivities have been provided for both the oestrogen EIA (Palme, 1993) and the progestagen EIA (Schwarzenberger *et al.* 1996). The two assays were biologically validated for faecal and serum reproductive steroid measurement in female lions by detection of matching hormonal patterns and observed reproductive events. Further validation was achieved by comparing fEM and sE concentrations of three lionesses on day 4 of oestrus and day 7 of the following interoestrous interval, as well as fPM and sP concentrations of the same lionesses on day 10 prior to parturition, and day 30 after parturition (Table 3.2). Percentage change was -73.4% and -63.7% for fEM and sE respectively, and
-92.9% and -88.3% for fPM and sP, respectively. Additionally, serial dilutions of the extracted faecal samples and serum aliquots resulted in displacement curves that were parallel to their respective standard curves. The sensitivities of the EIAs for the faecal steroid extracts were 0.48 ng/g dry weight (DW) for the oestrogen EIA, and 19.1 ng/g DW for the progestagen EIA. Intra-assay coefficients of variation (CV) of high- and low-quality controls were 5.40% and 6.04% for the oestrogen EIA, and 4.07% and 5.33 % for the progestagen EIA, respectively. Inter-assay CV of high- and low-quality controls were 8.34% and 9.27% (oestrogen EIA), and 12.06% and 14.69% (progestagen EIA), respectively. Sensitivities of the serum EIA analyses were 8.0 pg/ml for the oestrogen EIA and 320 pg/ml for the progestagen EIA. Intra-assay CV of high- and low-quality controls were 4.68% and 5.36% for the oestrogen EIA, and 4.07% and 5.33% for the progestagen EIA, respectively. Inter-assay CV of high- and low-quality controls were 15.53% and 15.92%, respectively, for the oestrogen EIA, and 14.90% in both cases for the progestagen EIA. Assay procedures were conducted following published protocols (Ganswindt *et al.* 2002) in the Endocrine Research Laboratory of the University of Pretoria, South Africa.

Table 3.2: Biological validation. Faecal oestrogen (fEM) and progestagen (fPM) metabolites concentrations (µg/g DW), serum oestrogen (sE) and progesterone (sP) concentrations (ng/ml), and percentage change of three different African lionesses on days 4 of oestrus and 7 of the following interoestrus, and days 10 and 30 before and after parturition, respectively.

		fEM			**sE**	
Day	F1	F3	F4	F1	F3	F4
4	2.107	1.272	1.025	0.622	0.880	0.832
7	0.695	0.429	0.134	0.196	0.276	0.382
%	-67.00	-66.25	-86.96	-68.42	-68.68	-54.04
		fPM			**sP**	
Day	F1	F3	F4	F1	F2	F4
4	114.0	158.3	214.3	572.4	251.5	877.7
7	11.8	3.1	19.1	30.6	55.6	67.4
%	-89.65	-98.02	-91.09	-94.65	-77.89	-92.32

3.4.7 Data analysis

Endocrine data were plotted against time and compared to data resulting from behavioural monitoring to correlate oestrogen peaks with behavioural oestrous events, and clusters of elevated progesterone concentration with behavioural luteal phases. We estimated total and individual baselines for fEM and fPM concentrations by iteratively excluding all values that exceeded the mean plus 2.0 and 1.75 times the standard deviation (SD), respectively, as previously described by Moreira *et al.* (2001). After all high values had been excluded, the resulting means were used as the respective baselines. FEM concentrations higher than 2.0 times the SD above the baseline were considered oestrous peaks. Clusters of elevated fPM concentrations (starting the day when at least two consecutive samples exceeded the baseline 1.75 times the SD, and ending the day when fPM concentration remained below the baseline for at least two consecutive samples) were considered luteal phases. Likewise, we calculated total and individual baselines for sE and sP concentrations by excluding all values exceeding the mean plus 1.5 SD and 1.75 SD, respectively. All statistical analyses were performed with the R version 3.4.4

(The R Foundation for Statistical Computing, Vienna, Austria), and using a significance level of $P<0.05$. Basic results are reported as mean ± standard error of the mean (SEM), unless stated otherwise. Descriptive statistics for concentrations of the two reproductive hormones and their metabolites in every phase of the ovarian cycle were calculated using the R RcmdrMisc package. Differences between groups of females and phases of the reproductive cycle were tested with Mann–Whitney (when two groups were considered) or Kruskal–Wallis tests (when more than two groups were considered), and we used the canonical correlation coefficient (η) to estimate effect sizes. When statistically significant differences were found in more than two groups, we applied Dunn's test of multiple comparison with Bonferroni correction, using the R dunn.test package as post-hoc. All variables were tested for normality using Shapiro-Wilk's normality test, and for equality of variances using Levene's and Fligner-Killeen's tests. Concentrations of the two circulating reproductive hormones were correlated with respective faecal metabolite concentrations using Pearson's r correlation coefficient, Kendall's τ and Spearman's ρ rank correlation coefficients, and the odds ratio (OR), using R stats and epiR packages. To do this, non-available endocrine data were obtained by cubic spline interpolation using nearby available observations (maximum two days apart). Interpolated data were used to compare daily faecal and serum concentrations only when one of both variables counted with available data for each specific day. To plot the steroid concentration trends along pregnancy and pseudopregnancy (Figures 3.2 and 3.6), we used the method of decomposition of a non-stationary time series proposed by Kitagawa (1984) using the R timsac package, and including all interpolated data.

3.5 RESULTS

3.5.1 Behavioural observations

The total number of hours dedicated to behavioural monitoring of the five focal females was 1197.18 (about 240 hours per female), from which 81.1% corresponded to observations of resting behaviour or inactivity (*i.e.* “lying”). Only 18.9% of time corresponded to non-resting or active states such as “standing” (6.16%), “pacing” along enclosure fences (3.53%), “patrolling” the enclosure

(3.48%), “sitting” (1.72%), “eating” (1.62%), “exploring” (1.0%), “drinking” (0.19%), or performing “other” activities (1.10%). Lionesses were found “out of sight” 0.10% of time. The two youngest lionesses did not show any oestrous signs during the initial four-month period or subsequent nine-month period respectively, and thus were considered to be in juvenile anoestrus until specific reproductive signs appeared. We confirmed behavioural oestrus in adult females with significantly high frequencies of the following specific reproductive behaviours: “allowing mount”, “anogenital grooming”, “copulation”, “flirting run”, “lordosis”, “mount”, “purring”, and “rolling” (Table 3.3). Absence of these reproductive behaviours during specific time intervals enabled detection of interoestrus (no signs for about two weeks) and luteal phases (pseudopregnancy: no signs for more than three weeks; pregnancy: no signs for more than 60 days).

Observed non-ovulatory cycles (follicular phases) lasted 16.15 ± 1.13 days (n=13; range: 8-23 days), with oestrus of 6.43 ± 0.47 days (n=14; range: 4-9 days), and interoestrous intervals of 9.69 ± 1.18 days (n=13; range: 3-19 days). In total, 16 luteal phases were observed during this study: seven PLP (three of which occurred after natural mating, and four after AI) and nine NPLP. Three of these non-pregnant luteal phases occurred after spontaneous ovulation (sNPLP), and the remaining six were observed after ovulation induction by GnRH administration (iNPLP). Based on the lack of reproductive behavioural signs after mating/AI, pregnancy lasted on average 108.7 days ± 0.56 days (n=6; range: 107-110 days). Based on the lack of reproductive behavioural signs after ovulation with or without mating/AI, spontaneous NPLP lasted 49.33 ± 0.88 days (n=3; range: 48-51 days), while iNPLP lasted 54.67 ± 1.09 days (n=6; range: 51-58 days). Induced NPLP were significantly longer than sNPLP (Mann-Whitney; U=0.5; W=38.5; n=6, 3; p=0.024; η=0.74). All females entered behavioural, lactational anoestrus after parturition.

Table 3.3: Frequency of occurrence of the recorded reproductive base event behaviours. Results are shown as "mean ± SEM (Kruskal-Wallis rank mean)". "n" indicates the number of cases of each specific cycle stage observed throughout the study. "*" shows behaviours that enabled oestrus detection.

	Frequency of Occurrence (per hour)					
	Anoestrus (n=278)	Oestrus (n=132)	Interoestrus (n=146)	Pregnancy (n=222)	Pseudo-pregnancy (n=321)	Kruskal-Wallis test
Allowing mount*	0.01 ± 0.00 (528.6)	0.59 ± 0.10 **(672.2)**	0.06 ± 0.03 (543.2)	0.01 ± 0.01 (525.8)	0.06 ± 0.02 (538.2)	H = 150.81 $p < 0.001$ η = 0.37
Allogrooming	0.22 ± 0.04 (544.1)	0.29 ± 0.07 (553.8)	0.25 ± 0.05 (546.4)	0.25 ± 0.04 (576.7)	0.38 ± 0.07 (536.7)	H = 4.86 $p = 0.304$ η = 0.03
Anogenital grooming*	0.09 ± 0.02 (523.2)	0.37 ± 0.06 (629.3)	0.21 ± 0.05 (572.7)	0.10 ± 0.02 (537.9)	0.16 ± 0.03 (538.7)	H = 33.46 $p < 0.001$ η = 0.16
Copulation*	0 (541)	0.27 ± 0.07 (615.9)	0 (541)	0 (541)	0 (541)	H= 133.92 $p < 0.001$ η = 0.34
Flehmen	0.11 ± 0.03 (515.9)	0.21 ± 0.05 (555.4)	0.23 ± 0.05 (558.7)	0.38 ± 0.05 (625.4)	0.19 ± 0.04 (521.2)	H = 48.72 $p < 0.001$ η = 0.20
Flirting run*	0.03 ± 0.01 (518.4)	1.45 ± 0.16 (823.6)	0.04 ± 0.04 (509.0)	0.01 ± 0.00 (516.5)	0.00 ± 0.00 (506.7)	H = 494.17 $p < 0.001$ η = 0.67
Lordosis*	0.01 ± 0.01 (537.0)	0.45 ± 0.10 (651.6)	0 (535)	0.00 ± 0.00 (537.4)	0 (535)	H = 192.92 $p < 0.001$ η = 0.42
Mount*	0.04 ± 0.02 (542.6)	0.36 ± 0.08 (629.3)	0.11 ± 0.05 (551.5)	0.01 ± 0.01 (532.2)	0.04 ± 0.02 (535.4)	H = 74.87 $p < 0.001$ η = 0.25
Purring*	0.06 ± 0.04 (518.1)	1.95 ± 0.23 (828.0)	0.05 ± 0.04 (514.1)	0.00 ± 0.00 (508.8)	0.00 ± 0.00 (508.1)	H = 525.98 $p < 0.001$ η = 0.69
Marking	0.81 ± 0.07 (515.5)	1.28 ± 0.16 (573.7)	0.85 ± 0.14 (485.6)	1.47 ± 0.11 (640.5)	1.24 ± 0.11 (536.8)	H = 32.67 $p < 0.001$ η = 0.16
Rejection after mount*	0.01 ± 0.01 (530.5)	0.23 ± 0.06 (606.2)	0.04 ± 0.02 (545.2)	0.06 ± 0.01 (565.6)	0.02 ± 0.01 (535.2)	H = 52.59 $p < 0.001$ η = 0.21
Rolling*	0.43 ± 0.06 (547.1)	2.92 ± 0.25 (859.3)	0.37 ± 0.08 (511.2)	0.30 ± 0.04 (537.5)	0.15 ± 0.03 (451.6)	H = 258.47 $p < 0.001$ η = 0.48

3.5.2 Relation between faecal hormone metabolite and circulating steroid levels

Overall, no strong linear correlation or ordinal association was found between faecal oestrogen metabolite and circulating oestrogen concentrations for any of the three phases of the lionesses' ovarian cycle during this study (n=115; r=0.06, p=0.270; ρ=0.17, p=0.034; τ=0.09, p=0.066). However, there was a weak negative association between fEM and sE concentrations during behavioural oestrus (n=38; r=-0.27, p=0.048; ρ=-0.34, p=0.020; τ=-0.25, p=0.014). In contrast, a strong positive linear correlation was detected between faecal progestagen metabolite concentrations and circulating progesterone concentrations (n=115; r=0.77, p<0.001; ρ=0.69, p<0.001; τ=0.53, p<0.001) throughout all phases of the ovarian cycle. FPM and sP concentrations were positively associated during anoestrus (n=10; r=0.96, p<0.001; ρ=0.58, p=0.044; τ=0.51, p=0.023), the follicular phase (n=64; ρ=0.30, p=0.008; τ=0.21, p=0.008), and the luteal phase (n=41; r=0.68, p<0.001; ρ=0.74, p<0.001; τ=0.56, p<0.001). During anoestrus, all samples with fPM concentration either higher or lower than 1.75 SD above the baseline presented equally high or low results of sP, according to the respective baseline (n=10, OR=Inf; 95% CI: 1.71, Inf). During the follicular phase, all samples presented fPM concentrations lower than the baseline plus 1.75 SD, and so did 98.4% of related sP values (97.4% during oestrus, and 100% during interoestrus). During the luteal phase, 92% of the samples presenting fPM concentrations higher than the baseline plus 1.75 SD, were mirrored by high sP concentrations, according to the respective baseline (n=41; OR=10.71; 95% CI: 2.10, Inf).

3.5.3 Faecal hormone metabolite concentrations and behavioural observations

During anoestrus, both fEM and fPM concentrations remained low (up to 2-fold above baseline) (Table 3.4). Even though fPM concentrations were higher in lactating females than in juveniles, the difference was found non-statistically significant (Mann-Whitney; U=11; W=26; n=5, 7; p=0.172; η= 0.3). Overall, fEM concentrations during the follicular phase were elevated (up to 4-fold above baseline), and were significantly higher during oestrus than during interoestrus

(Mann-Whitney; U=127; W=677; n=24, 21; p=0.002; η=0.42). Likewise, fEM concentrations were significantly higher during the second half of oestrus compared to the first half (Mann-Whitney; U=28; W=106; n=12, 12; p=0.005; η=0.52). In contrast, fPM concentrations remained at baseline without significant differences throughout this phase of the ovarian cycle. In pregnant females, overall fEM concentrations remained low (up to 2-fold above baseline), while fPM concentrations were elevated (up to 20-fold above baseline). Both fEM and fPM concentrations seemed to increase in parallel throughout gestation (Table 3.4). Differences in fEM concentrations between the three periods of pregnancy were found non-significant, (Kruskal-Wallis; H=3.76; n=13 14 17; p=0.162; η=0,21), however fPM concentrations were significantly higher during the second (Dunn; Z=-2.59; p=0.029) and third (Dunn; Z=-3.09; p=0.006) parts of gestation (Kruskal-Wallis; H= 10.75; n=13 14 17; p=0.003; η=0.46) (Fig 3.1). On the other hand, fEM concentrations remained low (up to 2-fold above baseline) throughout pseudopregnancy, while fPM concentrations increased during the first part, and decreased significantly during the second part (Mann-Whitney; U=44; W=284; n=16, 12; p=0.008; η=0.46). Overall, fPM concentrations were significantly higher during pregnancy compared to pseudopregnancy (Mann-Whitney; U= 223; W=1999; n=44, 28; p<0.001; η=0.53) (Fig 3.2, Table 3.4). FPM concentrations did not differ significantly between sNPLP (28.95 ± 3.23 μg/g DW, n=20, range: 6.94-52.42 μg/g DW) and iNPLP (30.33 ± 11.35 μg/g DW, n=8, range: 12.47-107.24 μg/g DW) (Mann-Whitney; U=57; W=93; n=8, 20; p=0.129; η=0.22), but higher fPM concentrations were found during iNPLP.

Individual baseline determination enabled identification of 17 fEM peaks, and seven clusters of elevated fPM concentration. Observed behavioural oestruses were paired by fEM peaks in 64.29% (9/14) of cases. Eight of the nine paired behavioural oestruses were detected by elevated fEM concentrations occurring during the second half of oestrus. However, 47.06% of identified fEM peaks were not correlated with oestrous behaviour (Table 3.3), but with pregnant luteal phases (Fig 3.3). Clusters of elevated fPM concentrations matched behavioural luteal phases in 100% (7/7) of cases. Further, all three observed pregnancies were matched by periods of elevated fPM concentration (n=44; 80.02 ± 8.66 μg/g DW; range: 10.22-214.28 μg/g DW). Periods of comparatively lower, but still elevated

fPM concentration (n=28; 29.34 ± 3.85 μg/g DW; range: 6.94-107.24 μg/g DW) were correlated with observed pseudopregnancies in 75% (3/4 cases) (Fig 3.4). The remaining cluster of elevated fPM concentrations matched the only iNPLP observed.

3.5.4 Circulating hormone concentrations and behavioural observations

During anoestrus, overall sE and sP concentrations were low, up to 2-fold above the baseline (Table 3.4). Concentrations of both circulating steroids were significantly higher during lactation compared to juvenile anoestrus (Mann-Whitney; U=0; W=6; n=3, 5; p=0.018; η=0.79) (Fig 3.5). During the follicular phase, sE concentrations generally remained up to 2-fold above baseline, but were significantly higher during oestrus than interoestrus (Mann-Whitney; U= 1892.5; W=7512.5; n=95, 51; p=0.015; η=0.18). SE concentrations did not differ between the first and second half of oestrus (Mann-Whitney; U=1059; W=1961; n=40, 55; p=0.622; η=0.03). SP concentrations remained at baseline throughout the follicular phase, but were significantly higher during the second half of oestrus (Mann-Whitney; U=793; W=1613; n=40, 55; p=0.01; η=0.24). In pregnant females, sE concentrations increased throughout gestation and were significantly higher during the third part of pregnancy compared to the first and second parts (Mann-Whitney; U=340; W=1880; n=55, 21; p=0.003; η=0.32) (Fig 3.1). During the first part of pregnancy, sP concentrations increased significantly compared to oestrus (Mann-Whitney; U=40; W=4600; n=95, 31; p=0.000; η=0.72), and then reached a plateau until parturition (Fig 3.6). During the second and third parts of pregnancy, sP concentrations were significantly higher compared to the first part (Mann-Whitney; U=418; W=914; n=31, 45; p=0.001; η=0.34). During pseudopregnancy, both sE and sP concentrations mimicked respective faecal metabolite patterns. SE concentrations remained stable throughout pseudogestation (Table 3.4), and sP concentrations increased during the first part, and decreased significantly during the second part (Mann-Whitney; U=225; W=2529; n=51, 28; p=0.000; η=0.56). Overall sP concentrations were significantly higher during pregnancy than during pseudopregnancy (Mann-Whitney; U=1112; W=7818; n=76, 79; p=0; η=0.54), which was also reflected in fPM results (Table 3.4). We found no significant difference between sP concentrations during sNPLP (166.25 ± 29.72 ng/ml, n=20,

range: 27.70-434.90 ng/ml) and iNPLP (179.13 ± 22.88 ng/ml, n=59, range: 32.17-665.55 ng/ml), but higher sP concentrations were found during iNPLP.

Individual baseline determination enabled identification of 78 sE peaks, and 13 clusters of elevated sP concentrations. Observed behavioural oestruses were paired with sE peaks in 66.67% (20/30) of cases. However, 66.67% of identified sE peaks were not correlated with oestrous behaviour (Table 3.3), but occurred during luteal phases (confirmed by both, prolonged absence of oestrous signs and elevated sP levels) (Fig 3.3 and 3.4). Periods of elevated sP concentrations matched behavioural luteal phases in 100% (13/13) of cases, resembling faecal results. Clusters of elevated sP concentrations matched all five observed pregnancies (n=76; 388.78 ± 20.86 ng/ml; range: 47.56-877.71 ng/ml), while periods of comparatively lower, but still elevated sP concentrations (n=79; 175.87 ± 18.59 ng/ml; range: 27.70-665.55 ng/ml) matched detected pseudopregnancies in 50% (4/8) of cases. The four remaining clusters of elevated sP matched four of the six recorded iNPLPs (Fig 3.4).

3.5.5 Ovulation induction with buserelin-acetate

All females that received a single intramuscular dose of the exogenous GnRH analogue buserelin-acetate at the end of behavioural oestrus, entered either a PLP (n=4) after artificial insemination, or a NPLP (n=6). Thus, ovulation occurred in all cases (100%, n=10). Ovulation was additionally confirmed by a rise in sP concentrations in the days following injection. Females that received buserelin-acetate on day 4 of behavioural oestrus continued to show related signs for 72-96 hours post-administration. In the three lionesses that were exposed to this prescript, sP concentrations surpassed individual baseline concentrations 24-60 hours after injection. When GnRH was administered on day 5 of behavioural oestrus, no related signs were observed after 48-72 h post-administration. In the five lionesses exposed to this protocol, sP concentrations rose above individual baseline concentrations after 48-72 h post-administration. Females that received buserelin-acetate on day 6 ended behavioural oestrus on the same day of injection. However, in the two females that were exposed to this prescript, sP concentrations only exceeded the estimated baseline after 85 and 96 h post-injection, respectively.

Table 3.4: Faecal oestrogen (fEM) and progestagen (fPM) metabolites concentrations (μg/g DW) and serum oestrogen (sE) and progesterone (sP) concentrations (ng/ml) for the different phases of the African lioness' ovarian cycle (mean ± SEM). Minimum and maximum values are reported inside the parentheses. "n" indicates the number of faecal and blood samples collected during each stage of the reproductive cycle.

Reproductive Stage	n	fEM	fPM	n	sE	sP
ANOESTRUS	**12**	**0.34 ± 0.07 (0.12-0.97)**	**27.89 ± 9.54 (10.0-128.84)**	**8**	**0.79 ± 0.18 (0.23-1.62)**	**77.39 ± 17.89 (38.90-190.80)**
Juvenile	5	0.33 ± 0.10 (0.12-0.65)	14.84 ± 1.52 (11.97-19.97)	3	0.31 ± 0.05 (0.23-0.41)	41.13 ± 1.58 (38.90-44.20)
Lactation	7	0.34 ± 0.11 (0.12-0.97)	37.21 ± 15.82 (10.0-128.84)	5	1.08 ± 0.19 (0.59-1.62)	99.14 ± 24.08 (55.55-190.80)
FOLLICULAR PHASE	**45**	**0.74 ± 0.11 (0.04-3.89)**	**11.57 ± 0.65 (3.14-21.54)**	**146**	**0.54 ± 0.02 (0.15-1.18)**	**38.63± 1.21 (18.05-143.27)**
Oestrus	24	1.06 ± 0.18 (0.06-3.89)	10.67 ± 0.79 (3.14-17.65)	95	0.57 ± 0.02 (0.26-1.18)	38.63 ± 1.61 (18.05-143.27)
1st half	12	0.63 ± 0.17 (0.06-2.11)	10.02 ± 1.23 (3.14-17.65)	40	0.58 ± 0.03 (0.31-1.01)	34.04 ± 1.38 (18.05-53.86)
2nd half	12	1.48 ± 0.27 (0.07-3.89)	11.32 ± 1.02 (6.63-17.62)	55	0.56 ± 0.03 (0.26-1.18)	41.96 ± 2.50 (20.64-143.27)
Interoestrous interval	21	0.38 ± 0.06 (0.04-0.97)	12.60 ± 1.03 (4.10-21.54)	51	0.49 ± 0.03 (0.15-0.98)	38.62 ± 1.80 (18.42-92.31)
LUTEAL PHASE	**72**	**0.39 ± 0.04 (0.02-1.59)**	**60.31 ± 6.21 (6.94-214.28)**	**155**	**0.52 ± 0.02 (0.19-1.20)**	**280.27 ± 16.33 (27.70-877.71)**
Pregnancy	44	0.41 ± 0.06 (0.02-1.59)	80.02 ± 8.66 (10.22-214.28)	76	0.58 ± 0.03 (0.19-1.20)	388.78 ± 20.86 (47.56-877.71)
1st part	13	0.27 ± 0.08 (0.03-1.12)	39.74 ± 6.99 (10.22-90.03)	31	0.52 ± 0.03 (0.25-1.00)	307.28 ± 36.74 (47.56-724.56)
2nd part	14	0.37 ± 0.09 (0.02-0.93)	87.61 ± 14.29 (23.23-197.40)	24	0.53 ± 0.05 (0.19-1.15)	460.82 ± 25.69 (295.63-801.91)
3rd part	17	0.55 ± 0.11 (0.05-1.59)	104.57 ± 15.44 (26.37-214.28)	21	0.70 ± 0.06 (0.36-1.20)	426.77 ± 34.48 (199.62-877.71
Pseudopregnancy	28	0.37 ± 0.05 (0.08-1.02)	29.34 ± 3.85 (6.94-107.24)	79	0.47 ± 0.02 (0.23-1.02)	175.87 ± 18.59 (27.70-665.55)
1st part	16	0.37 ± 0.06 (0.08-0.89)	36.37 ± 5.68 (12.82-107.24)	51	0.45 ± 0.02 (0.23-0.98)	238.65 ± 24.42 (32.17-665.55)
2nd part	12	0.37 ± 0.08 (0.11-1.02)	19.97 ± 3.47 (6.94-50.27)	28	0.50 ± 0.03 (0.29-1-02)	61.52 ± 7.47 (27.70-172.00)

Figure 3.1: Faecal oestrogen (fEM) and progestagen (fPM) metabolite mean ± SEM concentrations of four African lionesses (left panel), and serum oestrogen (sE) and progesterone (sP) mean ± SEM concentrations of five African lionesses (right panel) throughout pregnancy (n=3 and n=5, respectively) and pseudopregnancy (n=4 and n=8, respectively).

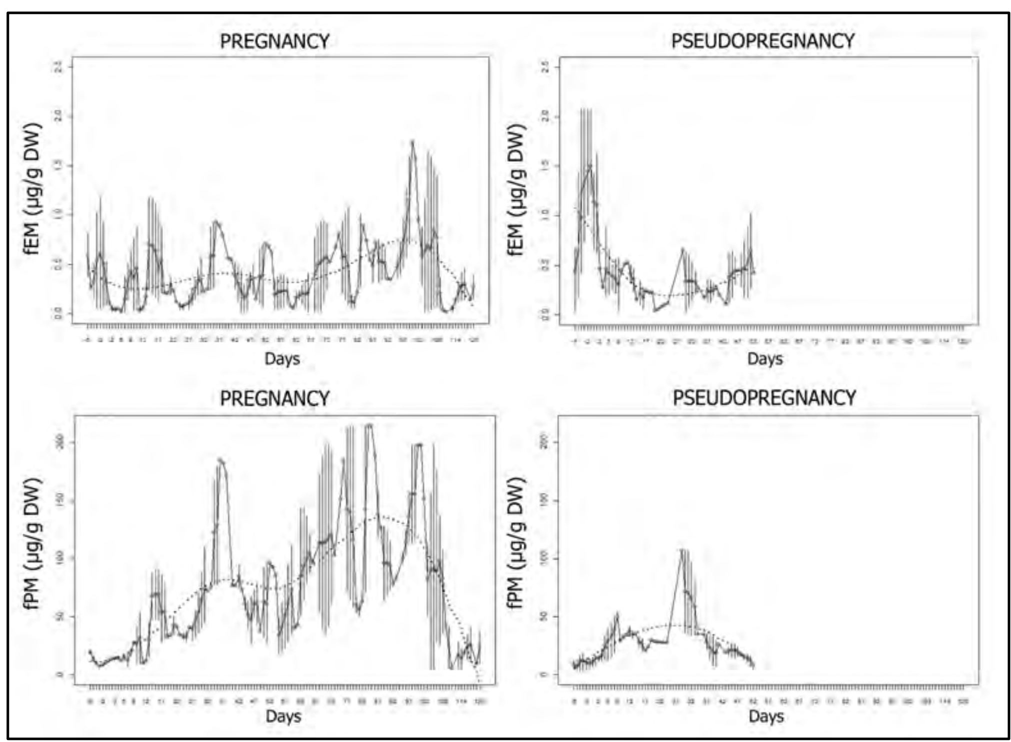


Figure 3.2: Mean (min-max) faecal oestrogen (fEM) and progestagen (fPM) metabolite concentrations of four African lionesses during pregnancy (n=3) and pseudopregnancy (n=4) plotted against time.

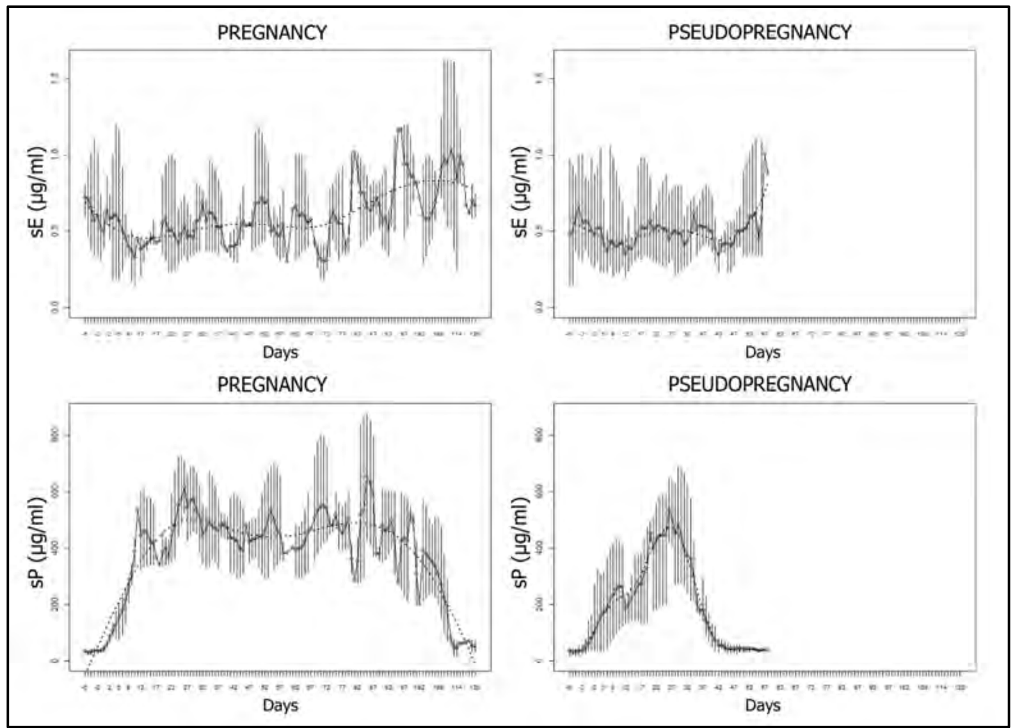


Figure 3.6: Mean (min-max) serum oestrogen (sE) and progesterone (sP) concentrations of five African lionesses during pregnancy (n=5) and pseudopregnancy (n=8) plotted against time.

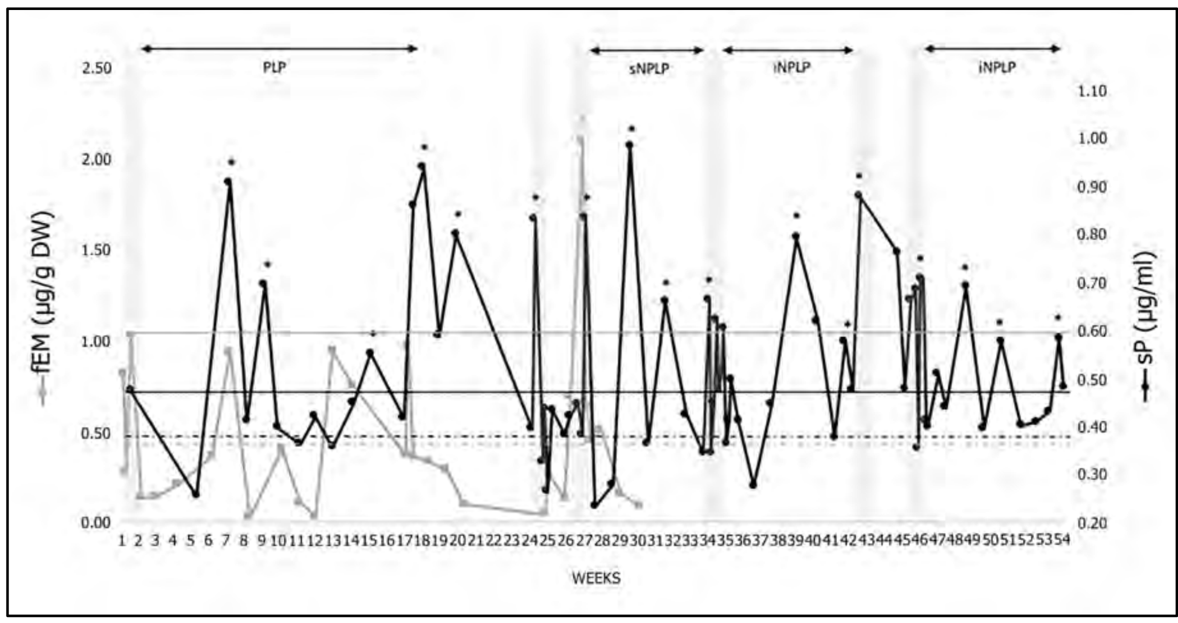


Figure 3.4: Faecal oestrogen metabolite (fEM, in grey) and serum oestrogen (sE, in black) concentrations of an adult lioness plotted against time. Periods of behavioural oestrus appear as shaded bars. Overall fEM and sE concentration baselines are represented by a straight line (grey for fEM, black for sE). Elevated levels of fEM (2.0SD above the baseline) and sE (1.5SD above the baseline) appear above the dotted grey and black lines, respectively. Behavioural luteal phases are indicated with arrows (pregnant luteal phase, PLP; induced non-pregnant luteal phase, iNPLP; spontaneous non-pregnant luteal phase, sNPLP).

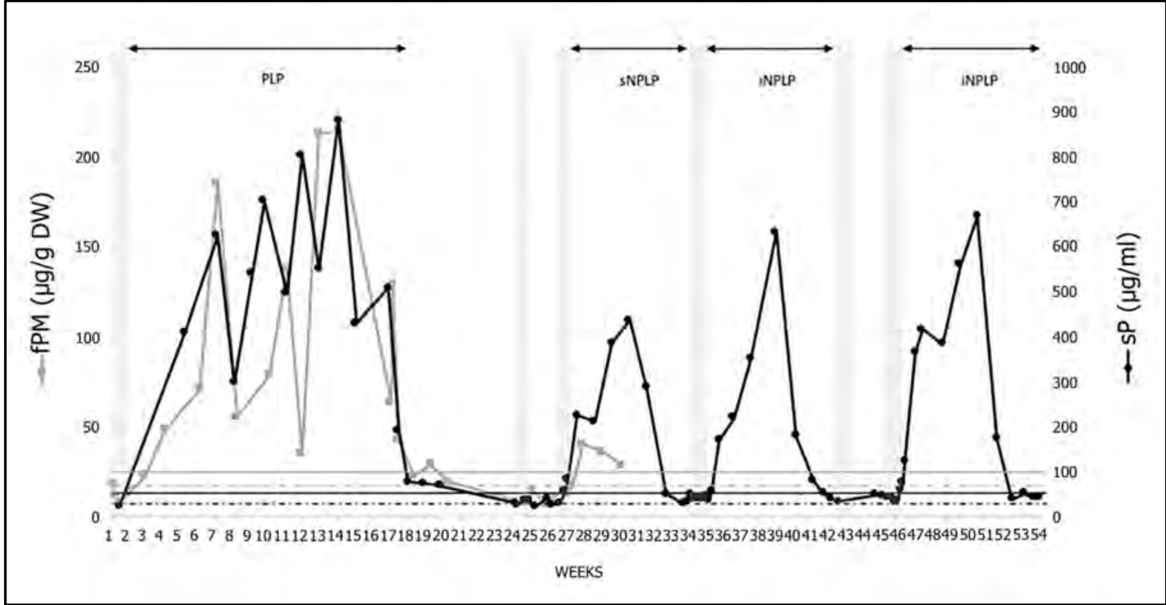


Figure 3.4: Faecal progestagen metabolite (fPM, in grey) and serum progesterone (sP, in black) concentrations of an adult lioness plotted against time. Periods of behavioural oestrus appear as shaded bars. Overall fPM and sP concentration baselines are represented by a straight line (grey for fPM, black for sP). Elevated levels of fPM and sP (1.75SD above the baseline) appear above the dotted grey and black lines, respectively. Behavioural luteal phases are indicated with arrows (pregnant luteal phase, PLP; induced non-pregnant luteal phase, iNPLP; spontaneous non-pregnant luteal phase, sNPLP).

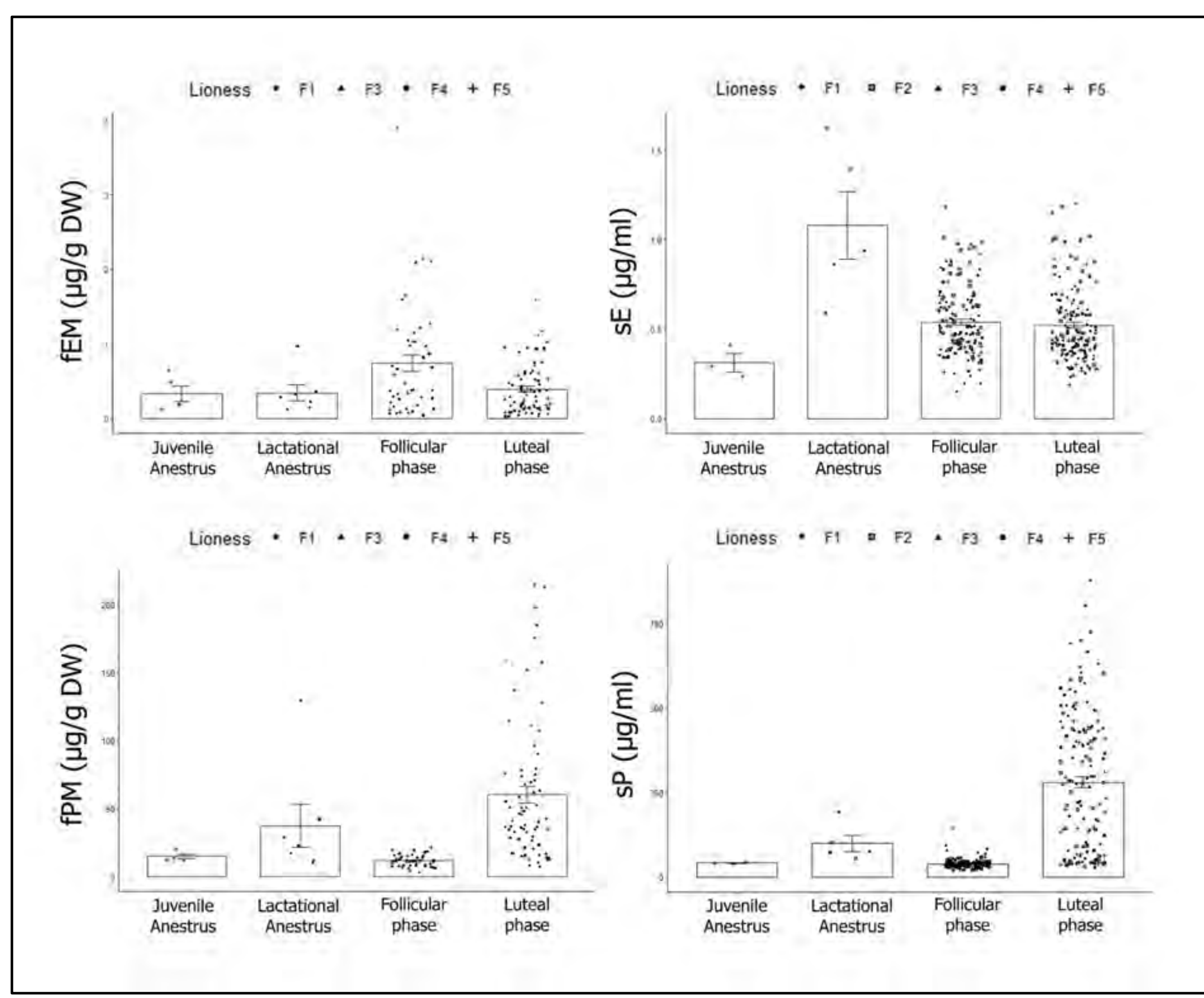


Figure 3.5: Faecal oestrogen (fEM) and progesterone (fPM) metabolite concentrations (left panel) and serum oestrogen (sE) and progesterone (sP) concentrations (right panel) during the anoestrus, follicular phase and luteal phase of five African lionesses. Bar plots show mean ± SEM with jittered symbols representing individual measurements.

3.6 DISCUSSION

To our knowledge, this is the first time both serum oestrogen and progesterone concentrations and their immunoreactive faecal metabolite counterparts were longitudinally monitored and compared to behavioural reproductive traits in a non-domestic felid. Overall, serum oestrogen (sE) and faecal oestrogen metabolite (fEM) concentrations of female African lions were not correlated, while serum progesterone (sP) regularly matched faecal progestagen metabolite (fPM) concentrations. This study showed that measurement of oestrogen concentration alone is not a valuable tool for oestrus detection in lionesses, regardless of the matrix. In contrast, determining progesterone concentrations in either of the two tested matrixes may serve to distinguish between follicular and luteal phases, as well as pregnancy and pseudopregnancy in the African lioness. Further, we confirmed that ovulation in this species occurred 24-96 hours after i.m. administration of buserelin-acetate at the end of natural behavioural oestrus.

Overall serum oestrogen and progestagen cyclic patterns seemed to reflect those previously described for other felids, including lions (Brown *et al*. 2011). Generally, sE concentrations increased during oestrous events and rapidly decreased during interoestrus, while sP remained at baseline throughout the ovarian cycle, unless ovulation occurred. In our study, sE peaks did not match behavioural oestrus in all cases. This is in contrast to a previous study on African lionesses, during which weekly serum samples from three females over a period of six months enabled detection of nine oestrogen peaks, all of them correlated with behavioural oestrus signs (Schmidt *et al*. 1979). A previous study on Siberian tigers (*Panthera tigris altaica*) showed that serum oestrogen concentration in two samples collected 30 minutes apart could differ up to 4-fold (Seal *et al*. 1985). Thus, in our study, the collection of one single serum sample per day may have limited the detection of sE peaks during behavioural oestrus. Additionally, in most species, the maximum concentration of oestrogen is reached about 24 hours before the LH peak, and 48-72 hours before ovulation (Ptaszynska, 2009). In our study, the focal females were not mated, nor was GnRH administered during every observed oestrus event. Thus, it is possible that the concentration of circulating oestrogen during oestrus in these cases did not reach sufficiently high concentrations (able to induce an LH surge and ovulation). This could explain the difficulty of detecting oestrogen peaks

during some of the behavioural oestrus events observed in our study, and would match Schmidt *et al*. (1979) study in lionesses, in which seven of the nine detected oestrogen peaks were followed by a luteal phase, according to endocrine results and later histopathology. In our study, serum oestrogen concentrations in pregnant lionesses seemed to increase during the last month of gestation, peak right before parturition, and decrease during the first week of lactation. This pattern differs from that observed in domestic cats, where oestrogen levels generally peak around the last week of pregnancy, and rapidly decrease before parturition (Johnston *et al.* 2001; England, 2010). It is known that the foeto-placental unit produces oestrogen throughout gestation in most species (Hadley and Levine, 1993), and oestrogen surges during pregnancy have been observed in other felid species such as the cheetah (*Acinonyx jubatus*, Brown *et al.* 1996) and the Pallas' cat (*Otocolobus manul*, Brown *et al*. 2002), thus the different sE pattern observed in pregnant lionesses could be purely species-specific. Our serum progesterone results during pregnancy, on the other hand, were in line with those previously reported by Schmidt *et al*. (1979), confirming sP in pregnant lionesses increases and reaches a peak at the end of the first part of pregnancy, which then remains elevated until parturition.

Given our results, faecal oestrogen and progestagen metabolite patterns throughout the lioness' ovarian cycle resembled those observed in other non-seasonal felids, with high fEM concentration waves differentiating oestrus from interoestrus, and overall low fPM concentrations throughout the follicular phase (Brown, 2006). However, a previous study in African lions reported a high frequency of silent oestrus (up to 73% fEM peaks unrelated to oestrus behavioural signs; Putman *et al.* 2015), which we did not observe. Silent oestrus is common in lions and other felid species (Schmidt *et al*. 1979, 1993; Wildt *et al*. 1982; van Dorsser *et al*. 2007; Axnér *et al*. 2008), and seems to appear more frequently in lionesses housed with males than in females housed alone (Putman *et al*. 2015). The absence of silent oestrus in our study may thus be biased by the distribution of the targeted animals, which enabled visual and olfactory contact with an adult breeding male, but also by the sample size of the study group, and/or the length of the study itself. During pregnancy, the patterns of both faecal reproductive steroid metabolites (fEM and fPM) were similar to those described for other non-domestic felids, such as the Pallas' cat (Brown, 2002), the Arabian leopard (*Panthera pardus nimr*, van Dorsser

et al. 2007), and also the African lion (Putman *et al*. 2015). In these previous studies, fEM remained low, but seemed to increase after mid gestation, while fPM quickly increased after ovulation and remained elevated until parturition.

Our results show that overall sE levels did not seem to be related to fEM patterns, while sP concentrations matched fPM levels quite consistently. This is in contrast to similar structured studies conducted on other species such as black rhinoceros (*Diceros bicornis,* Berkeley *et al*. 1997), dairy goats (*Capra aegagrus hircus,* Capezzuto *et al*. 2008), and red wolves (*Canis rufus,* Walker *et al*. 2002), that found both oestrogen and progestagen concentrations to be correlated in serum and faeces. The absence of correlation between sE and fEM in our study could mainly be attributed to the high fluctuation of circulating oestrogen concentrations in contrast to the cumulative effect of the faecal oestrogen metabolites. As in other large-bodied species, circulating steroids in lions seem to be metabolized and stored in the intestine for about 24-48 hours before final excretion of the corresponding metabolites, due to erratic feeding patterns, slow digestion, and gastro-intestinal transit (Hodges *et al*. 2010, Kumar *et al*. 2013). This would explain why, unlike the serum counterpart, fEM concentrations of lionesses in the current study were remarkably higher during the second half of oestrus, compared to the first half, and also seemed to progressively increase throughout the three parts of pregnancy. In addition, unlike the focal species in the aforementioned studies, lions are induced ovulators and, as hypothesized above, it is possible that circulating oestrogen concentrations did not reach remarkably high levels at any time during oestrus in this species. Thus, with a single serum sample per day, we may have missed, for instance, a high value of sE at the end of oestrus, while a faecal sample collected the same day would have shown an elevated fEM concentration due to presumed accumulation of oestrogen metabolites within faeces along the entire oestrous event. We observed a distinct difference between sE and fEM concentrations during lactational anoestrus. However, the number of samples collected from females in this reproductive stage was limited (twelve in total; five serum and seven faecal samples). Most serum samples were collected during the first week after parturition, while faecal samples belonged to the second and third weeks of lactation. Therefore, it is possible that sE values corresponded to elevated residual levels still present after parturition, while faecal metabolites reflected the actual “classic”

endocrine profile of a lactational anoestrus. This difference between the time that both types of samples were collected may explain the observed hormonal discrepancy. Nevertheless, these results should be interpreted carefully, considering the low total sample size during anoestrus. A larger sample pool would help to clarify this point, and further research is thus recommended in the future. On the other hand, even though sP and fPM concentrations were strongly correlated throughout the ovarian cycle of the African lionesses under study, we observed that fPM progressively increased until the end of pregnancy, instead of reaching a plateau as detected by sP results. This, again, may be due to the suspected cumulative signal of hormone metabolite concentrations detected within a faecal sample.

In our case, even though blood sampling was more frequent than faecal sampling, the results obtained from faecal material seemed to be more reliable according to behavioural observations. Overall, both matrixes enabled oestrus detection by oestrogen concentration determination with about 60% accuracy. However, neither matrix was considered ideal to reliably detect oestrus in lionesses by endocrine analyses alone, due to a high number of false positives. Our specificity results were remarkably lower than the 100% reported for lions by Schmidt *et al.* (1979) with weekly serum samples, and the 95% reported for Arabian leopards by van Dorsser *et al.* (2007) with daily faecal samples. These differences may be due to the high number of ovulatory cycles observed in the first study, and the higher sampling frequency in the second study compared to ours. In this regard, the accuracy of oestrus detection reported by van Dorsser *et al*. (2007), together with the consistent length of the different reproductive stages determined here (in contrast to other studies where non-ovulatory cycles were reported to last between 1-20 weeks, and non-pregnant luteal phases between 2-10 weeks; Andrews *et al.* 2019), support the importance of frequent sampling to enable an accurate monitoring of the ovarian cycle in lionesses. Both matrixes enabled detection of luteal phases by progestagen concentration determination with 100% accuracy. Despite the wide range of progesterone concentration results observed in this study, sP and fPM levels were significantly higher during luteal phase than during follicular phase, as well as during pregnancy compared to pseudopregnancy. The latter was especially evident when comparing intra-individual results. These observations are

in agreement with previous studies in domestic cats (Verhage *et al*. 1976), Arabian leopards (van Dorsser *et al*. 2007), and lions (Putman *et al.* 2015). Our results suggest that a high concentration of progesterone (according to preferably individual baseline concentrations) at least 36 days after the end of behavioural oestrus may serve to diagnose pregnancy in African lions.

On the other hand, we found a significant difference between the duration of spontaneous and induced non-pregnant luteal phases. This observation is in contrast to the results of Putman *et al.* (2015), who found no significant differences between the length of spontaneous and induced-by-mating pregnant luteal phases. In our case, pseudopregnancies occurring after buserelin-acetate administration were significantly longer than spontaneous pseudopregnancies. The difference between these two studies could be due to the effect of buserelin-acetate, the different methods utilized for length estimation, or a bias due to the reduced size of our study population. In this regard, our results indicate a consistent rise of sP concentrations above the baseline following buserelin-acetate administration which served to confirm ovulation during the AI trials performed by Callealta *et al.* (2019). Here, most ovulations occurred within 24-72 hours after GnRH administration. This is in line with studies conducted in different species, where most ovulations took place 24-60 hours after an LH surge (England, 2010; Miki *et al.* 2016). While, in domestic cats, the ovulatory LH surge seems to occur within minutes of mating (Shille *et al.* 1983), in cattle, the LH surge was reported to appear within six hours after buserelin injection (Picard-Hagen *et al.* 2015). GnRH stimulates both FSH and LH secretion (Conn and Crowley, 1991). Thus, it seems buserelin-acetate in lionesses induced rupture of already mature follicles when injected on day 4, and growth and rupture of premature follicles when injected on days 5 and 6. As described in some other species (England, 2010), this study confirmed that lionesses may show behavioural oestrus signs for 24 to 72 hours after ovulation. In contrast to previous studies where GnRH failed to induce growth of fully functional *corpora lutea* in Rhesus monkeys (*Macaca mulatta*; Zelinski-Wooten *et al*. 1991), buserelin-acetate administration in lionesses led to both successful pregnancies, and induced pseudopregnancies longer and with higher maximum concentrations of progesterone than sNPLP.

In conclusion, this study compared longitudinal reproductive faecal hormone metabolites and circulating steroids of female African lions for the first time. Our results showed that progesterone patterns were similar in both matrixes, while punctual oestrogen concentrations could not be correlated. Overall, we do not recommend the use of oestrogen concentration determination alone for oestrus detection, regardless of the matrix. In contrast, one sample of either matrix collected about five weeks after mating/AI could be used for pregnancy diagnosis. Furthermore, buserelin-acetate proved to be a valuable tool to induce ovulation in African lions, and its use may help to advance and improve assisted reproduction techniques for this and other threatened large felids. In addition, blood collected from captive felids trained by positive reinforcement conditioning may serve, in the future, to investigate the yet unknown patterns of peptide reproductive hormones such as FSH or LH in these species.

3.7 DECLARATIONS OF INTEREST

None.

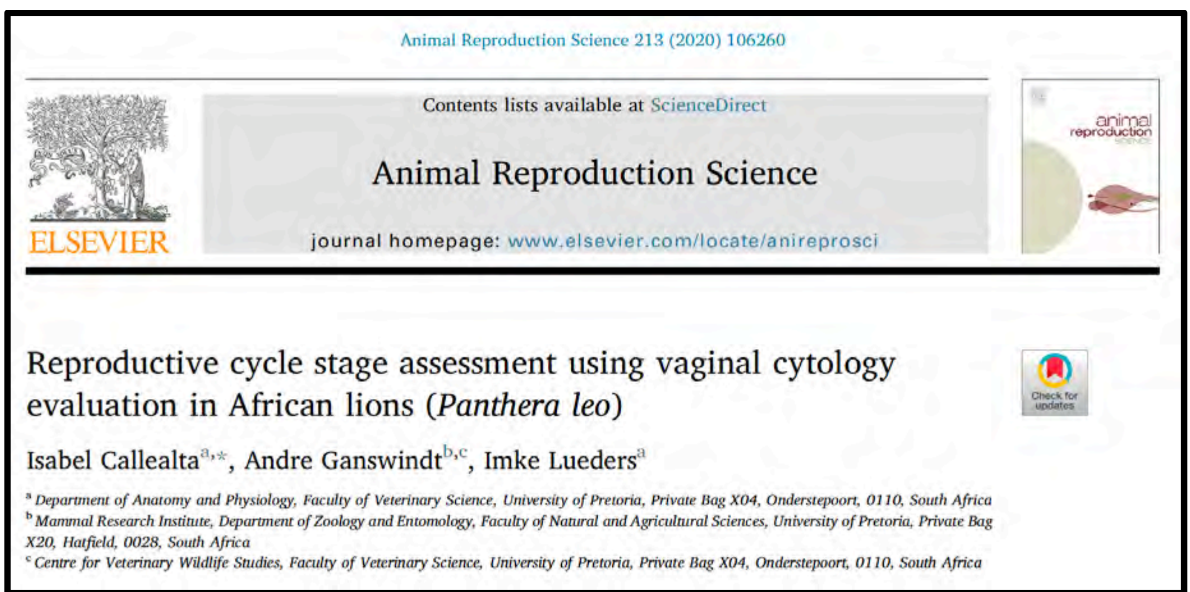
3.8 ACKNOWLEDGEMENTS

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, Willie Jr Jacobs, and the staff, volunteers, and students involved in the training of the lionesses; Nicole Hagenah-Shrader, Stefanie Ganswindt, and Abongile Ndzungu of the Endocrine Research Lab (University of Pretoria, RSA), as well as the “Poop Group”, for their help, patience, and support. 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.

4. CHAPTER FOUR:

REPRODUCTIVE CYCLE STAGE ASSESSMENT USING VAGINAL CYTOLOGY EVALUATION IN AFRICAN LIONS (*PANTHERA LEO*)

Photo: Isabel Callealta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Reproductive cycle stage assessment using vaginal cytology evaluation in African lions (*Panthera leo*)

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4.1 ABSTRACT

Vaginal cytology evaluation is an economic, non-invasive technique for indirect monitoring of fluctuations in oestrogen concentrations, and thus progression of the oestrous cycle. This technique is widely used in domestic dogs for determining timing of artificial insemination. There, however, are only a few reports on the vaginal cytology of non-domestic felids, including lions. This study was conducted, therefore, to describe the vaginal epithelial changes throughout the reproductive cycle of African lions, and to investigate the efficacy of vaginal cytology assessments for predicting reproductive stages. During a 12-month period, reproductive behavioural data and vaginal swabs were collected daily from five lionesses. In total, 541 vaginal smears were evaluated for the proportion of mucosal epithelial cells, neutrophils, bacterial cells, and amount of mucous, cellular debris. One single swab with a large proportion of superficial cells, absence of neutrophils, large number of bacteria, without cellular debris was sufficient for detecting lionesses in oestrus. Likewise, one cytology sample with a large proportion of parabasal and intermediate cells, few neutrophils, few bacteria, and large amount of mucous, cellular debris enabled detection of females in advanced dioestrus or gestation. To distinguish lionesses in early dioestrus from those in an interoestrous period, at least two consecutive swabs were necessary for satisfactory classification. Overall, evaluation of vaginal cytology samples was an effective

technique for differentiation among different stages of the reproductive cycle, confirmation of oestrus, and pregnancy diagnosis in lionesses. This technique, therefore, has the potential for application in classifying different stages of the reproductive cycle in other feline species.

4.2 KEYWORDS

African lion; oestrus detection; non-domestic felids; ovarian cycle; vaginal smear.

4.3 INTRODUCTION

The International Union for the Conservation of Nature (IUCN) considers most non-domestic felids threatened with extinction, including lions (IUCN, 2019). Basic information about the reproductive physiology of these species, therefore, is needed to develop successful conservation and *ex situ* breeding programs (Swanson, 2006).

Most felids only have ovulations if mating occurs (induced ovulations) and during the period of the ovarian cycle there may be no ovulations (alternate periods of oestrus and interoestrus without ovulation), or ovulations (dioestrus occurs after oestrus and ovulation; Fig 4.1) (Andrews *et al.* 2019). When there is conception after ovulation, a luteal phase ensues and is maintained beyond the typical period of luteal function if there is a signal from the developing foetal tissues indicative of pregnancy. If there is no pregnancy, the period of dioestrus ensues and the luteal tissues regress after a typical period of function (Andrews *et al.* 2019).

Variations in the concentration of circulating oestrogen during the oestrous cycle are associated with changes in the cells of the mucous membrane of the reproductive tract (Johnston *et al.* 2001). Thus, evaluation of the type and proportion of epithelial cells in serial vaginal smears serves as an indirect method to monitor fluctuations in oestradiol concentrations, and therefore, progression of the reproductive cycle (von Heimendahl and England, 2010). In addition, vaginal cytology evaluations may potentially be used to predict the time for mating and artificial insemination in non-domestic felids, similar to what occurs in domestic dogs (Johnston *et al.* 2001). In the domestic dog, cytological changes of the vaginal

epithelium have been precisely determined (*e.g.* Schutte, 1967; Bell *et al.* 1973). There, however, are few reports on vaginal cytology evaluations of domestic cats (Mowrer *et al.* 1975; Herron, 1977; Shille *et al.* 1979; Mills *et al.* 1979; Cline *et al.* 1980) and even fewer with non-domestic felids (Liche and Wodzicki, 1939; Asa *et al.* 1992). The results from these previous studies indicated vaginal cytology observations could be used for detection of oestrus in domestic cats, but was not useful to distinguish between interoestrous periods and dioestrus (Johnston *et al.* 2001; von Heimendahl and England, 2010). Additionally, in cheetahs (*Acinonyx jubatus*), there seemed to be a larger number of leukocytes in the vaginal cytology samples during interoestrus than during gestation (Asa *et al.* 1992). In most studies performed in non-domestic cats, there was use only of opportunistic or infrequent vaginal samples, and therefore, there was a lack of a systematic approach with many of these previous assessments.

The aims of the present study, therefore, were: 1) to describe the type and proportion of cells as well as related changes observed in the vaginal cytology samples of African lions throughout the period of the reproductive cycle, and 2) to investigate the use of this technique to predict the reproductive stage of the lioness.

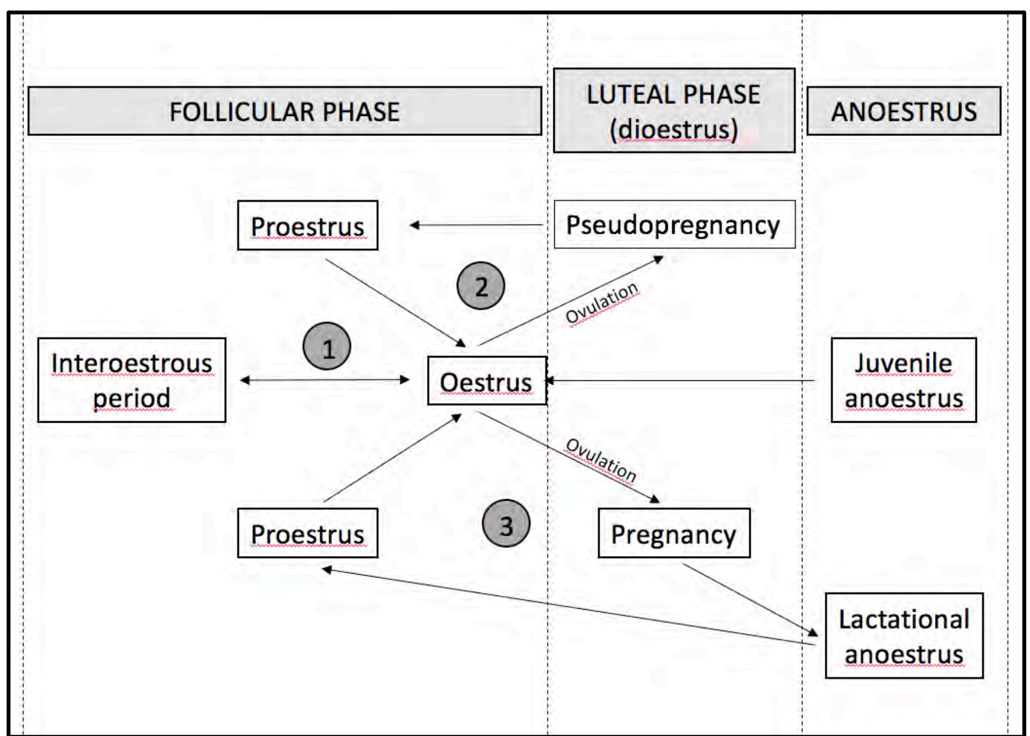

Figure 4.3: Feline reproductive cycle. Non-ovulatory cycle (1), cycle without associated ovulation (2), and cycle with associated ovulation (3).

4.4 MATERIALS AND METHODS

4.4.1 Study animals

Five adult (3.5-9 years of age) female African lions located in a private conservation center in South Africa served as study subjects for this research. Three of these females were proven fertile, cohabiting with an adult male (6 years of age) in an 800 to 1200 m^2 outdoor enclosure which included natural substrate, trees, and a shelter. The remaining two females were nulliparous and were located together in another enclosure with similar conditions as those for the females housed with the male. All animals were within visual, auditory, and olfactory range of each other. All lions were healthy and in good body condition. The five lionesses were trained by positive reinforcement conditioning to voluntarily allow for collection of vaginal cytology samples using swabs (Callealta *et al.* 2019).

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).

4.4.2 Behavioural monitoring

For 12 months, the behaviour of the five females was monitored twice a day (at sunrise and dusk) 5 to 7 days per week, in sessions of 15 to 60 minutes. A relative increase in the frequency of specific reproductive symptoms such as purring, flirting run, lordosis, allowing for mounting, copulation, and rolling, enabled detection of females in natural oestrus (Stanton *et al.* 2015). Behavioural oestrus usually lasted about 6 days, while interoestrous periods and dioestrus were identified by absence of specific reproductive symptoms for fewer than 21 consecutive days and more than 21 consecutive days, respectively (Callealta *et al.* submitted). Lactational anoestrus was observed after parturition and was sustained for a maximum of 3 weeks.

4.4.3 Collection of vaginal cytology samples, sample smear preparation, and sample interpretation

In parallel to behavioural monitoring, vaginal samples were collected during training every 1 to 3 days from females in oestrus and during the interoestrous interval, and every 3 to 7 days from females during the dioestrous period (Callealta *et al.* 2019).

Before vaginal swabbing, the vulvar labia were externally examined and the appearance classified as "covered" (vulva was obscured by hair) or "exposed" (obvious hairless vulvar labia). The presence/absence of vaginal discharge was also recorded. With the lioness positioned in sternal recumbency and after separating the labia with a gloved hand, a dry cotton-tipped swab was carefully introduced about 4 cm dorsally into the vagina to avoid the urethral orifice. The swab was gently rotated against the vaginal walls and rolled twice onto a clean glass microscope slide (Johnston *et al.* 2001). The prepared slide was then air-dried at room temperature (26 °C) and the sample on the slide was fixed and stained using the modified Wright-Giemsa method (Rapidiff Fixative®, Clinical Sciences Diagnostics CC, South Africa).

The samples on every slide were assessed using a microscope at x40 and x200 magnification to quantify the amount of mucous and cellular debris and the extent to which cell clumping occurred. The quantity of mucus and/or cellular debris (*i.e.* clearing) were rated from 0 to 6 and classified as "minimal quantity/no debris" (0-1), "small quantity/small debris abundance" (1-2.5), "moderate quantity/moderate debris abundance" (2.5-4.5), and "large quantity/large debris abundance" (4.5-6). Cellular distribution (*i.e.* clumping) was rated from 0 (single epithelial cells) to 3 (large piles or clusters of epithelial cells). There was assessment of 200 epithelial cells at x1000 magnification and cells were classified into groups (*i.e.* basal, parabasal, intermediate, superficial nucleated, and superficial enucleated) as described for other species (Johnston *et al.* 2001). To standardize these groups, the major axes of 30 cells of each type were measured in six vaginal cytology samples of three different females in oestrus and dioestrus, using an ACA Basler 1300-200 µc camera attached to a Nikon E50i microscope and the software Sperm Class Analyser SCA 6.3 with Morphology Module (Microptic SL, Barcelona, Spain). The

relative number of epithelial cells was classified as “small” (0%-20%), “moderate” (20%-50%), and “large” (50%-100%). In addition, the number of polymorphonuclear neutrophils (PMN) observed per 100 epithelial cells, as well as the quantity of microbial presence (including genus Simonsiella) were recorded. The number of PMN was classified as “minimal or absent” (0-2/100 epithelial cells), “small” (2-20/100 epithelial cells), “moderate” (20-100/100 epithelial cells), “large” (100-400/100 epithelial cells), and “very large” (>400/100 epithelial cells). The number of bacteria was classified as “minimal” (<10 microorganisms per field), “small/few” (10 to 25 microorganisms per field), “moderate” (25 to 50 microorganisms per field), and “large” (>50 microorganisms per field). In total, there was evaluation of 541 vaginal smears (108.2 $\pm$ 13.9 samples per female; range: 56-133), collected within the 12-month observation period.

4.4.4 Data analysis

Statistical analyses were conducted using the R version 3.6.1 (The R Foundation for Statistical Computing, Vienna, Austria). Basic results appear as untransformed mean ± standard error of the mean (SEM), unless otherwise indicated. All data sets were tested for normality using the Shapiro-Wilk’s normality test (R stats package), and for equality of variances using Levene's and Fligner-Killeen’s tests. In general, confidence intervals (CI) were calculated for the mean (metric variables) and the median (ordinal variables) using normal approximation. For small samples (n<30), the CI were estimated using non-parametric bootstrapping. Differences in epithelial cell groups, number of PMN, number of bacteria, and extent of background debris between every two stages of the reproductive cycle were tested using the Mann–Whitney test. Corresponding canonical correlation coefficients (η) were calculated to estimate the effect sizes of groups. Homogeneity between proportions of epithelial cell groups during interoestrus was investigated using the Friedman rank sum test and the pairwise *post-hoc* Nemenyi test for multiple comparisons, using the R stats and PMCMR packages. Significances were determined at the P <0.05 α level.

4.5 RESULTS

4.5.1 Vaginal epithelial cell types

Basal epithelial cells of female African lions were small (15.6 ± 0.48 µm diameter), rounded, and stained pink to pale blue (Fig 4.2a). These cells had a large nucleus and small cytoplasm, and were not frequently detected in the vaginal smear at any stage of the reproductive cycle. Parabasal epithelial cells were similar to basal cells in shape and colour of staining, but had a larger diameter (22.3 ± 0.67 µm; Fig 4.2b). Intermediate epithelial cells had large nuclei and stained pink to pale blue; however, there was considerable variation in shape and size (36.0 ± 1.12 µm diameter; Fig 4.2c).

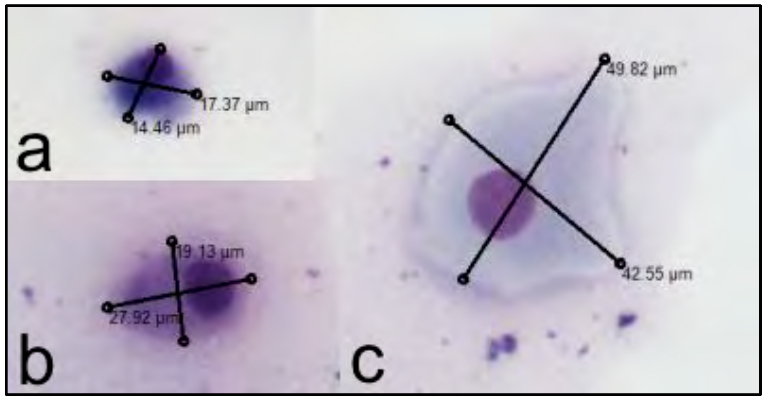

Figure 4.2: Microscopic images of vaginal basal (a), parabasal (b), and intermediate (c) epithelial cells of African lions, stained with modified Wright-Giemsa.

Superficial epithelial cells were large (54.4 ± 0.82 µm diameter), angulated, and generally stained dark blue. The nuclei of these cells were generally small, dark, and pyknotic; however, could also be stained to a minimal extent or staining could be completely absent due to degeneration and apoptosis (Fig 4.3).

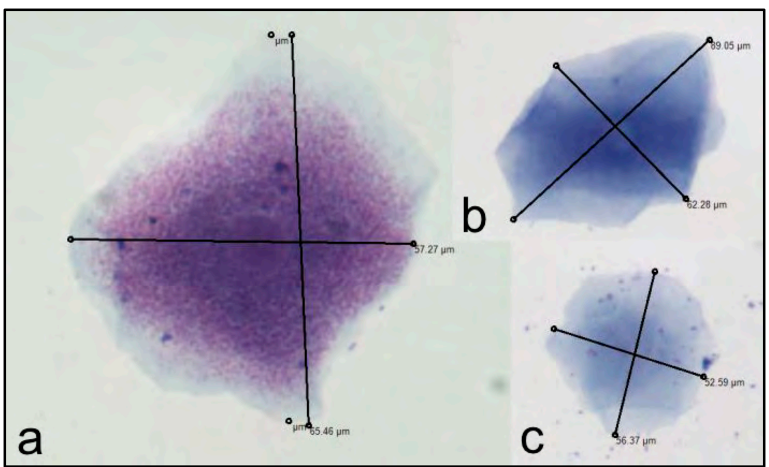

Figure 4.4: Microscopic images of vaginal superficial epithelial cells in different amounts of degeneration, stained with modified Wright-Giemsa: nucleated (a), nucleated with partially pycnotic nucleous (b), and enucleated (c).

4.5.2 Cytological cellular characteristics of the lioness reproductive cycle

One single vaginal smear was sufficient to identify when a lionesses was in oestrus (Fig 4.4). This stage of the reproductive cycle was precisely characterized when there was presence of a large proportion of superficial epithelial cells (n = 155; mean = 99.79%; 95% CI [99.69, 100]), absence of PMN (n = 155; mean = 0.36 PMN; 95% CI [0, 0.59]), moderate-to-large number of bacteria (n = 139; median = moderate; 95% CI [moderate, large]), and minimal quantity of mucus/cellular debris (n = 155; median = minimal; 95% CI [minimal, minimal]) (Table 4.1). *Simonsiella* spp were detected in 52.9% of vaginal smears collected during estrus and were most prevalent from the third day of oestrous behaviour (72.6%), compared with the first (16.7%) and second (36.7%) days of the oestrous period.

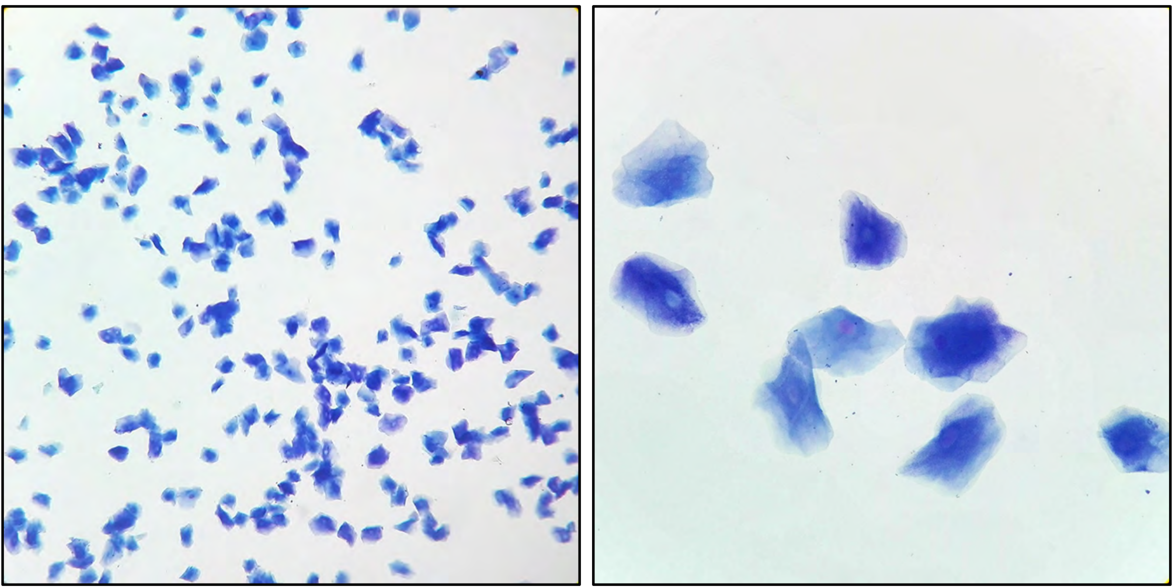

Figure 4.4. Microscopic images of stained (modified Wright-Giemsa) vaginal smears of a lioness in oestrus. x40 (left) and x200 (right).

Likewise, the assessment of one single cytology sample enabled detection of females in advanced dioestrus (Fig 4.5). In this stage, there was a large proportion of parabasal and intermediate cells (n = 222; mean = 79.76%; 95% CI [78.14,100]), small number of PMN (n = 222; mean = 9.96 PMN; 95% CI [0, 13.9]), few bacteria (n = 36; median = few; 95% CI [minimal, few]), and a moderate quantity of mucus/cellular debris (n = 222; median = moderate; 95% CI [moderate, moderate; Table 4.1).

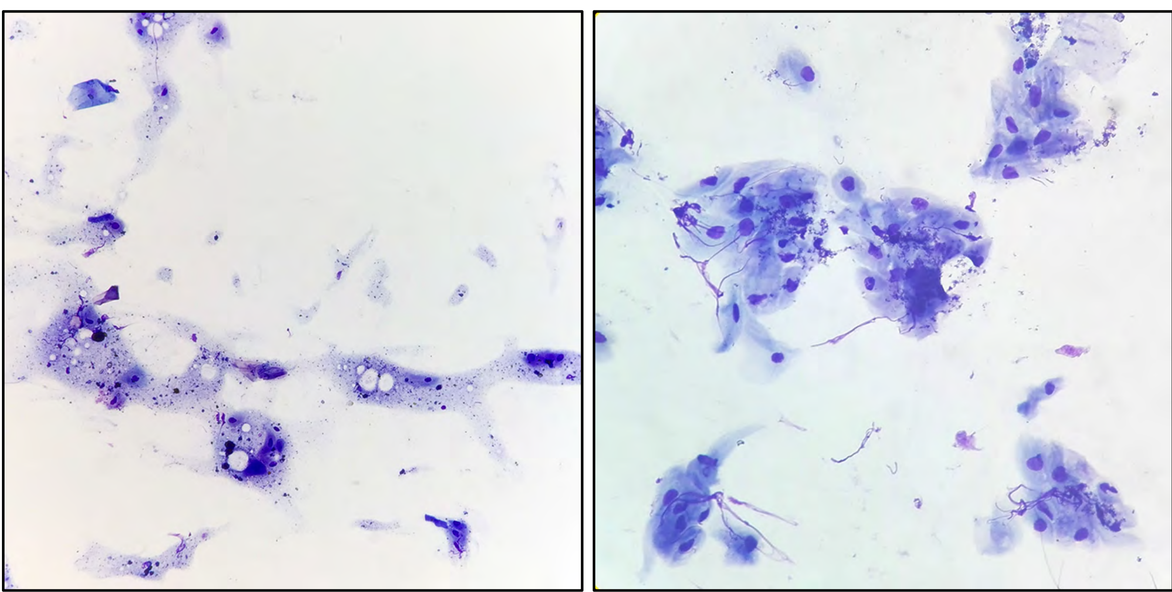

Figure 4.5: Microscopic images of stained (modified Wright-Giemsa) vaginal smears of a lioness in dioestrus. x40 (left) and x200 (right).

To distinguish females in early dioestrus from those in interoestrous period of the reproductive cycle, at least two to three consecutive vaginal cytology samples needed to be assessed. This transitional stage was considered to be "post-oestrus" (Fig 4.6). During the post-oestrous period, there was a large proportion of generally aggregated superficial cells (n = 63; mean = 81.93%; 95% CI [76.52, 100]), moderate-to-large number of PMN (n = 63; mean = 114.5 PMN; 95% CI [56.71,

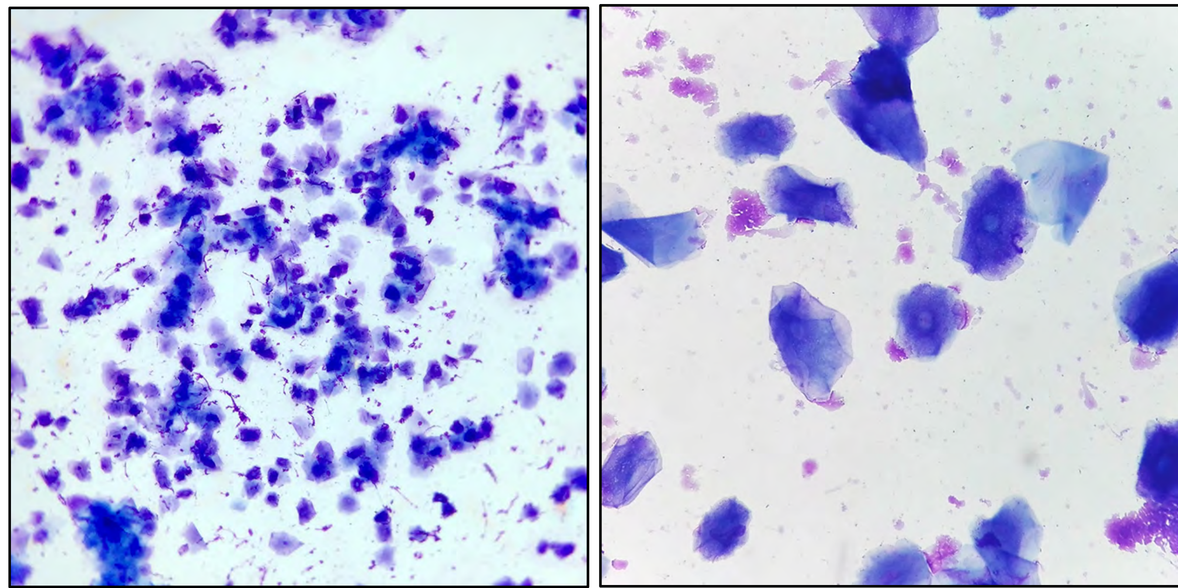

Figure 4.6: Microscopic images of stained (modified Wright-Giemsa) vaginal smears of a lioness in post-oestrus. x40 (left) and x200 (right).

172.29]), moderate number of bacteria (n = 24; median = moderate; 95% CI [moderate, large]) including *Simonsiella* spp (n = 63; prop = 20.63%; 95% CI [10.36, 30.91]), and moderate abundance of mucous and cellular debris, mainly due to a moderate-to-large amount of cellular debris (n = 63; median = moderate; 95% CI [moderate, large]) (Table 4.1).

Interoestrus was generally characterized by similar moderated proportions of parabasal, intermediate, and superficial cells (Friedman Test: χ^2 (2) = 1; n = 24; P = 0.664), moderate number of PMN (n = 24; mean = 30.62 PMN; 95% CI [14.21, 79.25]), small number of bacteria (n = 7; median = few; 95% CI [minimal, few]), and moderate abundance of mucosal and cellular debris, mainly due to a moderate content of cellular debris (n = 24; median = moderate; 95% CI [small, moderate]) (Fig 4.7, Table 4.1). There were similar cytology images during the interoestrus period of the reproductive cycle and lactational anoestrus.

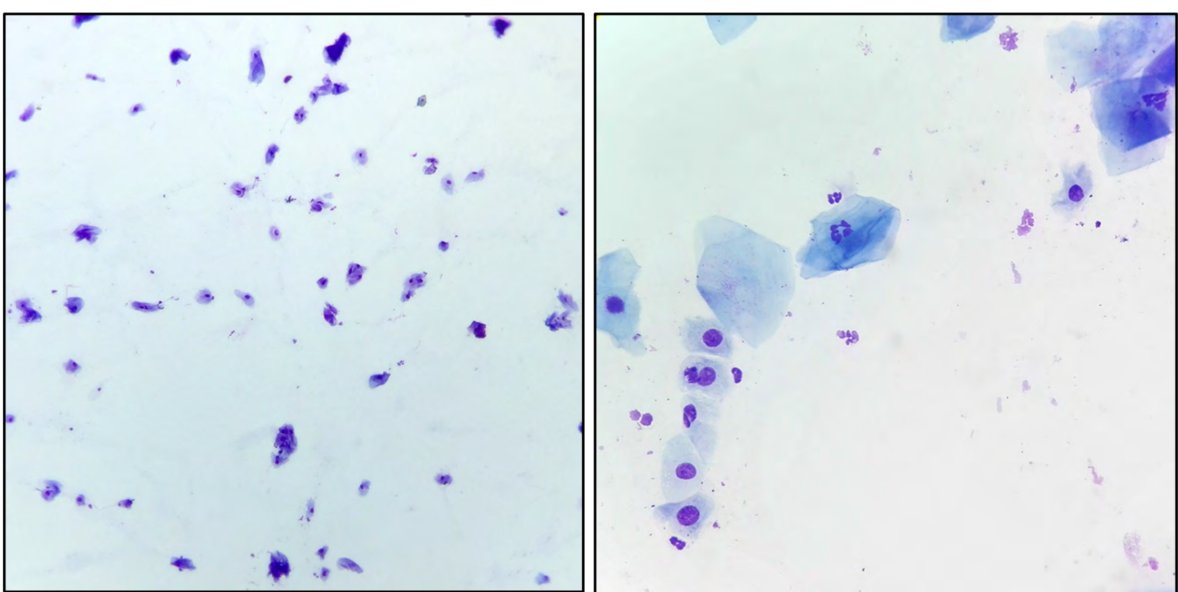

Figure 4.7: Microscopic images of stained (modified Wright-Giemsa) vaginal smears of a lioness in interoestrus. x40 (left) and x200 (right).

Results from vaginal cytology evaluations during the initial days after parturition indicated there was a similar proportion of epithelial cells as that during dioestrus (Table 4.1). During the post-partum period, however, there were a large number of red and white blood cells (n = 6; mean = 439.42 PMN; 95% CI [76.6, 1448.2]), and a few *Simonsiella* spp (n = 6; prop = 16.67%; 95% CI [0, 33.33]) (Fig 4.8).

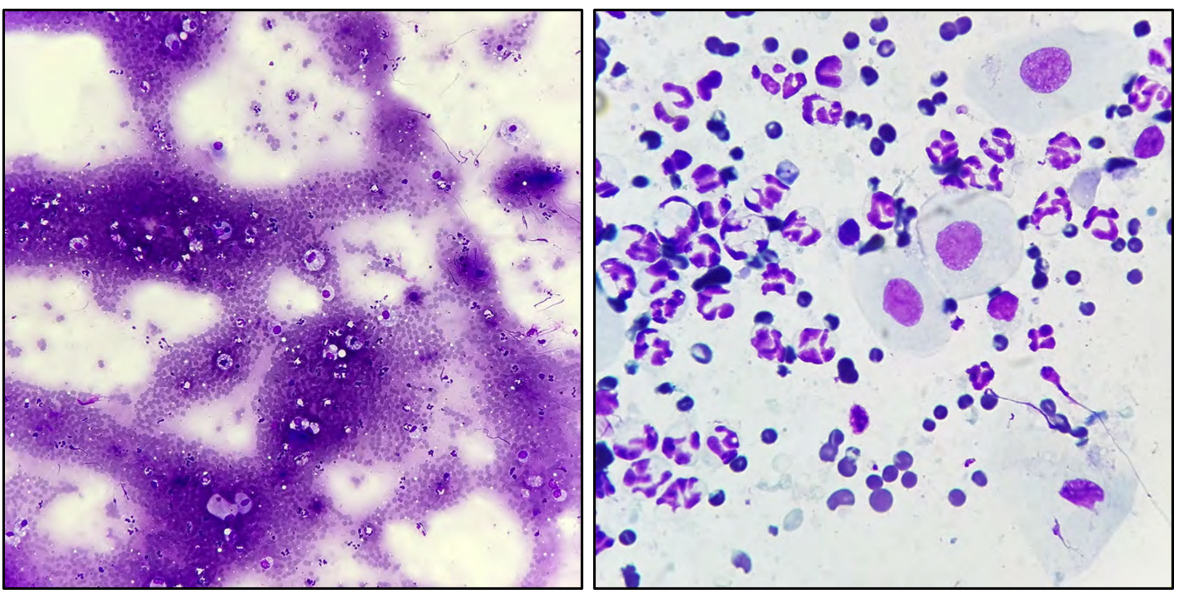

Figure 4.8: Microscopic images of stained (modified Wright-Giemsa) vaginal smears of a lioness in post-partum. x40 (left) and x1000 (right).

During the period when there were no specific reproductive symptoms, frequent vaginal swabbing allowed for detection of the proestrous period as a result of cytological assessments (Fig 4.9). This stage of the reproductive cycle was characterized with a large proportion of superficial cells (n = 71; mean = 56.58%; 95% CI [51.38, 100]), a very large number of PMN with the numbers gradually decreasing until the time of oestrus (n = 70; mean = 648.73 PMN; 95% CI [477.1, 3502.5]), few bacteria (n = 18; median = few; 95% CI [minimal, few]), and amounts of mucous and cellular debris ranging from a large to minimal quantities (n = 71; median = moderate; 95% CI [moderate, moderate]) (Table 4.1).

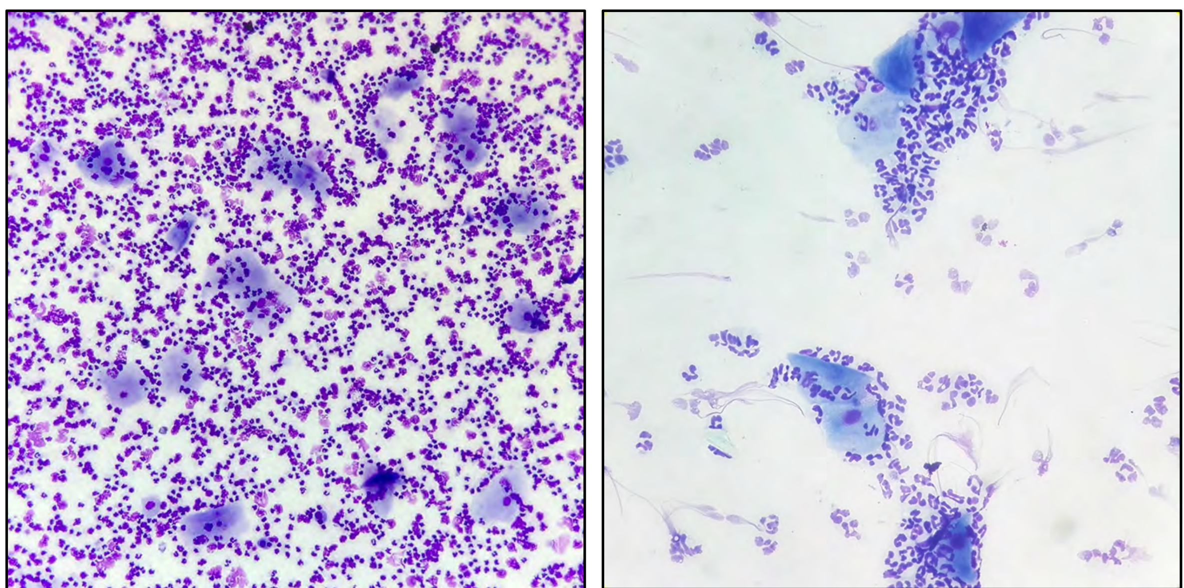

Figure 4.9: Microscopic images of stained (modified Wright-Giemsa) vaginal smears of a lioness in proestrus. x40 (left) and x200 (right).

The stage of proestrus, as ascertained from cytological assessment, was evident at the end of pseudopregnancy, but the cytology evaluation during proestrus was similar to that during the post-oestrous and interoestrous periods when there was assessment of a single slide (Fig 4.10). Nevertheless, the proportion of basal, parabasal, and intermediate epithelial cells was markedly less in post-oestrus than during both the proestrous (Mann-Whitney; U=923; W=6106; n= 71, 63; P=0.000; η=0.51) and interoestrous periods (Mann-Whitney; U = 207.5; W = 2223.5; n = 63, 24; P = 0.000; η = 0.56). Additionally, the number of PMN was markedly larger during the proestrus as compared with the post-oestrous period (Mann-Whitney; U = 1052; W = 5843; n = 70, 63; P = 0.000; η = 0.45) and interoestrous period (Mann-Whitney; U = 267.5; W = 3897.5; n = 70, 24; P = 0.000; η = 0.51).

Figure 4.10: Proportion of epithelial cells and polymorphonuclear neutrophils (PMN) observed in the different stages of the female African lion when there was not an associated ovulation (Non-ovulatory; A) and when there was an associated ovulation (Ovulatory; B) during the reproductive cycle.

Table 4.1: Means and the standard errors (SEM) or medians of the vaginal cytology findings throughout the reproductive cycle of five female African lions, with number of slides analysed for each stage of the cycle (n), and percentiles 10th and 90th inside the parentheses.

		Proestrus (n=71)	**Oestrus** (n=155)	**Post-oestrus** (n=63)	**Interoestrus** (n=24)	**Dioestrus** (n=222)	**Post-partum** (n=6)
Epithelial cells (%)	Basal	2.74 ± 0.52 (0-9.00)	0.01 ± 0.00 (0-0.00)	0.25 ± 0.10 (0-0.50)	1.33 ± 0.30 (0-3.35)	5.20 ± 0.46 (0-15.50)	4.33 ± 2.55 (1-9.75)
	Parabasal	22.99 ± 1.85 (5.50-43.00)	0.09 ± 0.03 (0.00-0.00)	7.97 ± 1.38 (0.00-25.40)	27.33 ± 4.12 (9.05-48.20)	53.61 ± 1.10 (31.55-76.95)	56.25 ± 4.14 (46.75-62.50)
	Intermediate	17.69 ± 1.62 (2.50-36.5)	0.12 ± 0.04 (0.00-0.0)	9.86 ± 2.13 (0.00-35.7)	24.54 ± 2.46 (9.15-40.5)	26.15 ± 0.73 (12.55-40.5)	30.25 ± 1.57 (26.00-33.5)
	Sup. Nucleated	34.31 ± 1.88 (12.00-56.50)	62.26 ± 0.99 (47.40-76.50)	56.18 ± 2.54 (33.30-76.00)	30.58 ± 3.76 (4.25-56.45)	7.82 ± 0.45 (1.00-18.00)	5.75 ± 1.06 (3.50-8.25)
	Sup. Enucleated	22.27 ± 1.86 (4.50-47.00)	37.53 ± 1.00 (23.00-52.60)	25.75 ± 1.74 (8.10-47.40)	16.21 ± 2.55 (1.00-32.75)	7.22 ± 0.55 (0.00-20.85)	3.42 ± 1.83 (1.25-7.50)
PMN (*per* 100 epithelial cells)		648.73 ± 102.94 (2.50-2115.00)	0.36 ± 0.14 (0.00-0.50)	114.50 ± 28.91 (0.00-326.50)	30.63 ± 13.65 (0.00-43.50)	9.96 ± 2.38 (0.00-12.45)	439.42 ± 329.15 (12.25-1167.50)

Table 4.1 (cont.): Means and the standard errors (SEM) or medians of the vaginal cytology findings throughout the reproductive cycle of five female African lions, with number of slides analysed for each stage of the cycle (n), and percentiles 10th and 90th inside the parentheses.

		Proestrus (n=71)	**Oestrus** (n=155)	**Post-oestrus** (n=63)	**Interoestrus** (n=24)	**Dioestrus** (n=222)	**Post-partum** (n=6)
Bacteria	Load	Small (Small-Mod.)	Moderate (Small-Large)	Moderate (Small-Mod.)	Small (Small-Mod.)	Small (Min.-Mod.)	Minimal (Min.-Min.)
	Simonsiella spp (%)	2.82 ± 1.98 (0-0)	52.90 ± 4.02 (0-100)	20.63 ± 5.14 (0-100)	4.17 ± 4.17 (0-0)	0.90 ± 0.64 (0-0)	16.67 ± 16.67 (0-50)
Clearing	Mucus	Minimal (Min.-Mod.)	Minimal (Min.-Min.)	Minimal (Min.-Small)	Minimal (Min.-Small)	Small-Mod. (Small-Large)	Moderate (Small-Mod.)
	Debris	Moderate (Small-Large)	Small (Min.-Mod.)	Moderate (Small-Large)	Small-Mod. (Small-Mod.)	Small-Mod. (Small-Mod.)	Small-Mod. (Small-Mod.)
	Clusters	S.cells-S.cluster (S.cluster-Med.)	Single cells (S.cells-S.cells)	Single cells (S.cells-Med.)	Small clusters (S.cells-Med.)	S.cells-S.cluster (S.cells-Med.)	Medium cluster (S.cluster-Med.)
	Piles	Small piles (S.cells-Med.)	Small piles (S.cells-Med.)	Medium piles (S.cells-Large)	Small piles (S.cells-Small)	Single cells (S.cells-S.cells)	Single cells (S.cells-S.cells)

4.5.3 Vulvar morphology

There were macroscopic changes in both the vulvar lips and the vaginal vestibulum, in association with the reproductive stage of the lioness (Fig 4.11). Overall, most females in behavioural oestrus had oedematous, exposed vulvas (81.6%, n = 103), a clear vaginal discharge (66.3%, n = 95), and prominent vaginal mucous membrane folds, with a rough surface when there were palpation assessments (79.1%, n = 129). During behavioural dioestrus, most vulva tissues were covered with hair (88.3%, n = 240), secretions were generally not observed (12.7%, n = 236; from which 53.3% actually corresponded to cytological proestrus), and the mucous membranes of the vestibulum were soft when palpated. During the behavioural interoestrus period, the lionesses generally had exposed vulva tissues (60.9%, n = 69), as well as a thick yellow vaginal discharge (43.1%, n = 65), and non-prominent vaginal folds, that were soft when evaluated by palpation (71.2%, n = 69). There was a moderately dark haemorrhagic vaginal discharge commonly observed until 9 days after parturition.

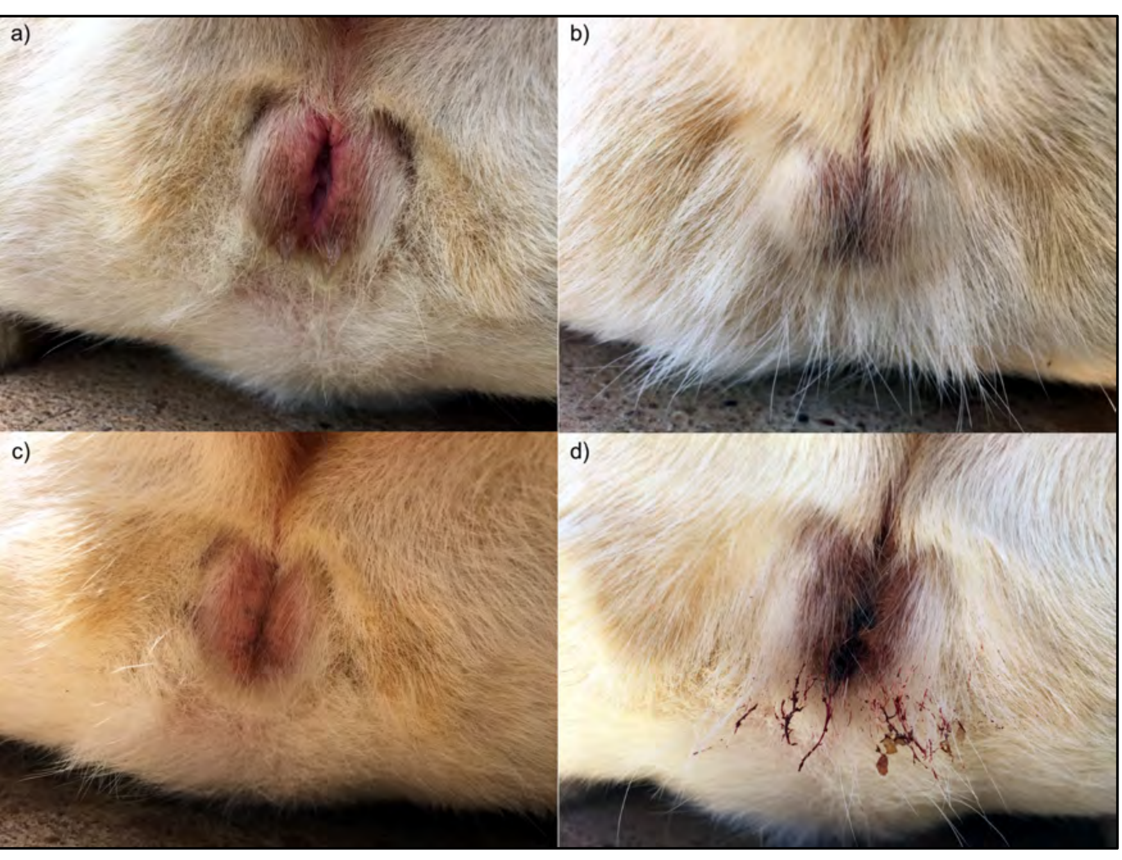

Figure 4.11: Vulvar morphology of one adult African lioness in sternal recumbency during oestrus (a), dioestrus (b), interoestrous interval (c), and post-partum (d).

4.6 DISCUSSION

In general, vaginal cytology evaluations are rarely used for endocrine and physiological assessments in felids, normally serving to confirm ovarian follicular functions by identification of either the physiological state of "proestrus/oestrus" during the reproductive cycle (when a large proportion of superficial cells is observed) or "interoestrus/dioestrus" (when there is a predominant proportion of intermediate cells, and few parabasal and superficial cells) (von Heimendahl and England, 2010). In the present study, there was a detailed assessment of the changes observed in the African lioness' vaginal epithelium throughout the different stages of the reproductive cycle as classified by animal behaviour. To the best of our knowledge, this is the first study in which there has been a report of the association between transitional reproductive stages such as "proestrus" and "post-oestrus" and vaginal cytological cellular populations for any non-domestic felid. In addition, there is reporting of concurrent macroscopic vulvar changes and physiological vaginal discharge patterns throughout the reproductive cycle.

Vaginal epithelial cells of the lioness resembled those previously described for domestic species in both shape and size (*i.e.* parabasal cells: 10-20 μm of diameter; intermediate cells: 20-30 μm; superficial cells: 30-75 μm; von Heimendahl and England, 2010). Likewise, vaginal cytology samples during oestrus and dioestrus in the present study had characteristics similar to those previously described for cats (Mills *et al.* 1979). The proportion of total superficial cells observed during behavioural oestrus in lionesses (>90%) is apparently greater than that of domestic cats (40%-60%; Shille *et al.* 1979) and cheetahs (>60%; Asa *et al.* 1992). This inconsistency in findings may result from inter-species differences; however, in these previous studies oestrus was defined with evaluation of endocrine correlates, which did not always match behavioural symptoms that are characteristic during the reproductive cycle. In domestic cats, maximum epithelial cell cornification coincides with peak concentrations of circulating oestradiol, which generally induces maximum manifestation of symptoms of behavioural oestrus (Mills *et al.* 1979). In the present study, oestrus detection methodology (*i.e.* identification of overt symptoms of behavioural oestrus exclusively) could also explain why there was a larger proportion of cornified cells during the oestrous period.

The presence of *Simonsiella* spp associated to superficial epithelial cells was most commonly indicative of an on-going oestrus that had started at least 2 to 3 days prior to the time of detection of this microorganism. *Simonsiella* spp originate in the oral cavity and are thought to colonize the vaginal epithelium during oestrus, due to a combination of increased anogenital grooming and absence of local white cells (Valle *et al.* 2006; Callealta *et al.* 2018). This hypothesis would explain why these bacteria were found during the period immediately after oestrus and during the post-partum period (although to a lesser extent) in the present study. Even though evidence from domestic cats indicates there can be ovulation induction with use of a vaginal swab (Porter *et al.* 1957; von Heimendahl and England, 2010; Malandain *et al.* 2011), there are results from studies with lions that indicate that induction of ovulations does not occur when there is use of this technique for assessing vaginal cellular populations (Callealta *et.al.* 2019). Even with regular collection of vaginal samples, only 19% of the oestrous cycles observed in this study (4 of 21) resulted in spontaneous ovulation and pseudopregnancy. If the mechanical stimulation derived from frequent vaginal swabbing were enough of a tactile stimulus to induce ovulation alone, the number of spontaneous ovulations observed would have been greater than the rates previously reported for lions (20%-26%) by Schramm *et al.* (1994) and Putman *et al.* (2015).

Furthermore, in previous research, polymorphonuclear neutrophils were usually not observed at any stage of the oestrous cycle in the vaginal cytology samples of lions (Schmidt *et al.* 1983), tigers (*Panthera tigris*; Seal *et al.* 1985), and pumas (*Felis concolor*; Bonney *et al.* 1981), but were occasionally detected in domestic cats during interoestrus and dioestrus (Mills *et al.* 1979), and immediately after oestrus cessation in cheetahs (Asa *et al.* 1992). In the present study, PMN were detected throughout the reproductive cycle of the lioness, consistently varying in number depending on the stage of the reproductive cycle. As observed in most domestic species, neutrophils were largely absent during the oestrous period, in relatively larger numbers after the oestrous period, and were occasionally detected during dioestrus. It appears that circulating neutrophils may enter the vaginal lumen across the epithelial layer lining of the vagina when there are relatively lesser oestrogen concentrations, which generally occurs after oestrus and sometimes during pregnancy (Asa *et al.* 1992). In lionesses, however, there was a relatively

larger number of PMN in the vaginal cytology samples approximately 7 to 10 days before oestrus when there were both oestrous symptoms expressed without an associated ovulation and when there were these symptoms in association with the timing of an ovulation. This finding complicated using the vaginal cytology evaluations for reproductive stage diagnosis when one single slide was collected around oestrus. For example, when samples were collected during late proestrus, using only the vaginal cytology assessment technique for evaluation of reproductive status, the vaginal cellular populations were similar to those during the early post-oestrous period. Furthermore, when samples were collected during early the proestrous period, using only the vaginal cytology technique for evaluation of reproductive status, the vaginal cellular populations were similar to those when there were conditions such as vaginitis or pyometra. The absence of this increase in number of leukocytes from 45 to 55 days after natural mating or artificial insemination is considered to be an indicator of pregnancy in lions. The increase in numbers of PMN during proestrus is thought to be the result of a local inflammation caused by luteolysis and regression of corpora lutea. Further research is needed to confirm this hypothesis and determine the pattern of change in PMN numbers when there is expression of oestrus without there being occurrence of an associated ovulation.

In most cases, there are specific characteristics of the vulva tissues that are indicative of females in oestrus. A small amount of vulvar oedema was also observed in females evaluated in the present study around oestrus, interoestrus, and even dioestrus. It, therefore, is only recommend that there be use vulvar assessments to determine the reproductive stage of female African lions when other techniques are used in combination with these vulva assessments such as vaginal palpation and/or cytology evaluations. Interestingly, in the present study the increased cornification of the vaginal mucosa during oestrus was not only associated with an increased proportion of superficial epithelial cells; this epithelial cell cornification was also associated with a rough vaginal surface when there was palpation of these tissues during oestrus.

4.7 CONCLUSIONS

In summary, frequent vaginal swabbing and immediate interpretation of cytological results enabled precise detection of specific reproductive stages in trained female African lions. It is suggested that vaginal cytology evaluation may be a practical, economical technique to closely monitor the reproductive cycle, confirm oestrus, and diagnose pregnancy in captive lionesses, in combination with behavioural observations. In this setting, it may be especially useful when anaesthesia is not possible, specialized equipment (such as an ultrasonic device) is not available, and/or endocrine analyses cannot be performed. Furthermore, it is suggested that the implementation of this technique into the routine management of captive feline populations may help to improve *ex-situ* breeding efforts for threatened felid species.

4.8 DECLARATIONS OF INTEREST

None.

4.9 ACKNOWLEDGEMENTS

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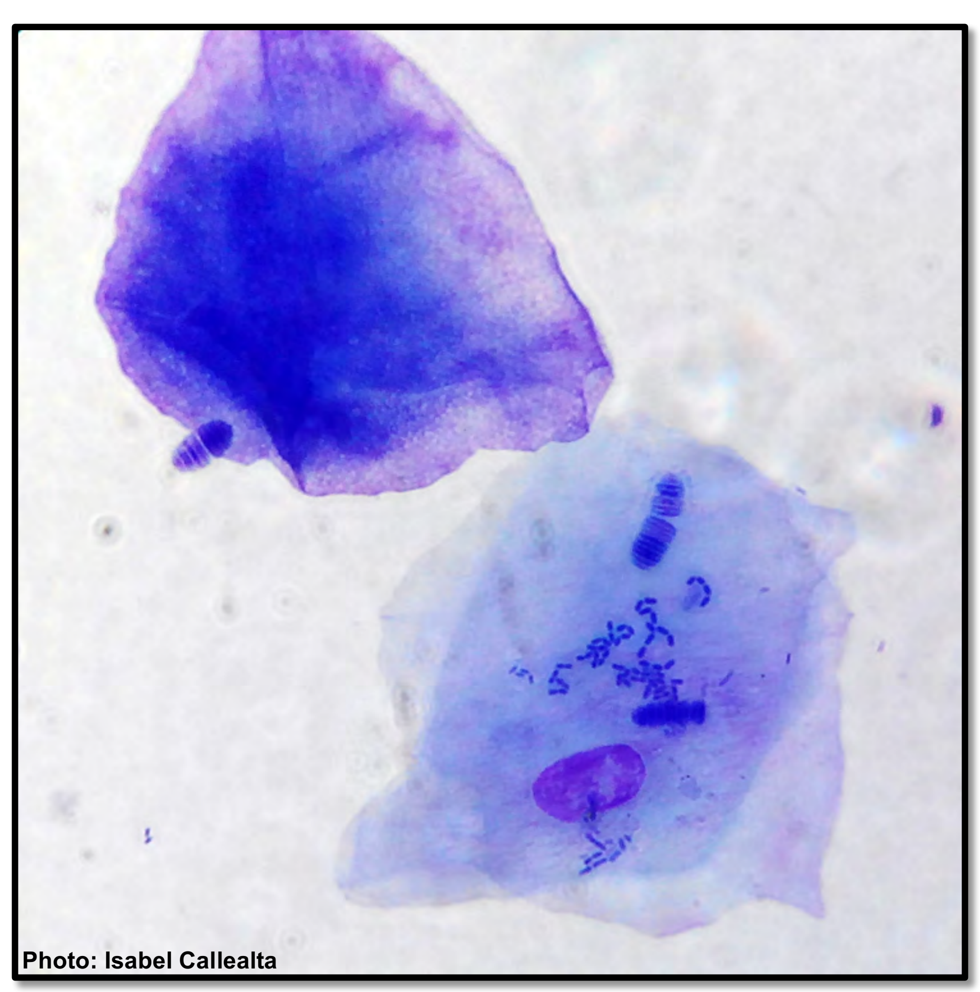
5. CHAPTER FIVE:

DETECTION OF *SIMONSIELLA SPP* IN THE VAGINA OF LIONS AND LEOPARD IN OESTRUS



Reproduction in Domestic Animals

SHORT COMMUNICATION

Detection of *Simonsiella* spp. in the Vagina of Lions and Leopard in Oestrus

Isabel Callealta, Andre Ganswindt, Sara Goncalves, Abraham Mathew, Imke Lueders

First published: 01 August 2018 | https://doi.org/10.1111/rda.13298 | Cited by: 1

5.1 ABSTRACT

Reports of the vaginal flora of wild cats such as lions or leopards are scarce. The microorganisms most commonly found in the vagina of clinically healthy cats are aerobic bacteria such as coagulase-negative *Staphylococcus*, *Streptococcus canis*, and *E. coli*. *Simonsiella* spp are large Gram-negative bacteria belonging to the Neisseriaceae family, typically found in the oral cavity and upper respiratory tract of many species. To date, there are no reports of the detection of *Simonsiella* spp in the vaginal flora of any felid. For a period of six months, daily behaviour monitoring was performed on six captive lionesses at a South African conservation centre, in parallel with the collection of vaginal swabs and interpretation of the resultant vaginal cytologies every other day. Oestrus was identified by typical female reproductive behaviours, as well as by enlarged and separated vulvar lips, and a predominant proportion of superficial cornified cells, clearing of the background, and high bacterial presence in the vaginal smear. *Simonsiella* spp were identified by their characteristic morphology in 58% (60 of 103) of the vaginal samples collected during oestrus. They were also found in oral swabs of 3 out of 3 lions tested. Additionally, *Simonsiella* spp were opportunistically found in a vaginal smear from a zoo housed female Sri Lankan leopard in oestrus, during a routine reproduction assessment. The finding of *Simonsiella* spp may be more common than previously suspected, transitory, and without detectable clinical relevance. A connection between occurrence of these bacteria and oestrus was apparent.

5.2 KEYWORDS

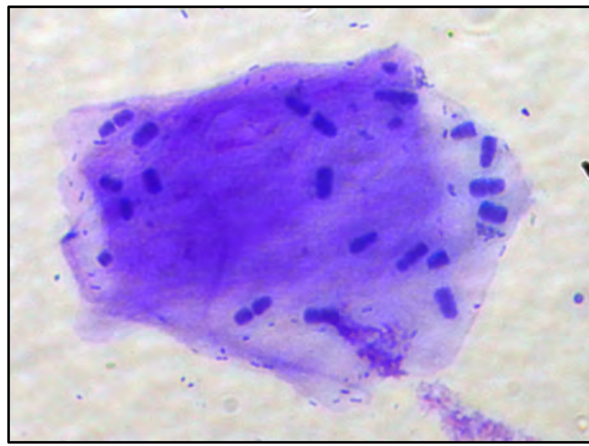
African lion; oestrus; *Simonsiella* spp; Sri Lankan leopard; vaginal cytology; vaginal flora.

5.3 INTRODUCTION

In the domestic cat, the vaginal bacterial flora is mainly constituted by aerobic bacteria of the genus Acinetobacter, Actinomyces, Corynebacterium, Escherichia, Haemophilus, Klebsiella, Lactobacillus, Pasteurella, Staphylococcus, and Streptococcus (Clemetson and Ward, 1990; Holst *et al.* 2003). Anaerobic bacteria such as Bacteroides and Peptococcus have also been isolated in a lesser extent (Clemetson and Ward, 1990; Holst *et al.* 2003). The most commonly found organisms in the vagina of clinically healthy domestic cats are coagulase-negative *Staphylococcus*, *Streptococcus canis*, and *E. coli*. (Clemetson and Ward, 1990; Holst *et al.* 2003). These bacteria originate from the skin and the bowel, and their presence, even in high concentration, is not considered to be an indication of reproductive disorder (Clemetson and Ward, 1990; Holst *et al.* 2003, Johnston *et al.* 2001). While mating does not seem to have a direct effect, the stage of the oestrous cycle does influence the bacterial populations present on the vagina of the cat (Holst *et al.* 2003). To date, however, information about the vaginal flora of non-domestic felids, such as African lions or leopards remains scarce.

Simonsiella spp are Gram-negative bacteria belonging to the Neisseriaceae family (Bruckner and Fahey, 1969). These bacteria are short (0.5-1.3 µm) and wide (1.9-6.4 µm), and usually stay together forming characteristic single-series groups of 8-12 cells that make them easily recognizable under the microscope (Fig 5.1) (Hedlund and Staley, 2002). They may be found attached to epithelial cells of the oral cavity and upper respiratory tract of many species (Bruckner and Fahey, 1969; Hedlund and Staley, 2002; Nyby *et al.* 1977; Kuhn *et al.* 1978). To the authors' knowledge, *Simonsiella* spp have only been opportunistically found once in the vagina of a bitch in oestrus (Valle *et al.* 2006).

In this short communication, we report the detection of *Simonsiella* spp in the vagina of six African lionesses and one female Sri Lankan leopard.



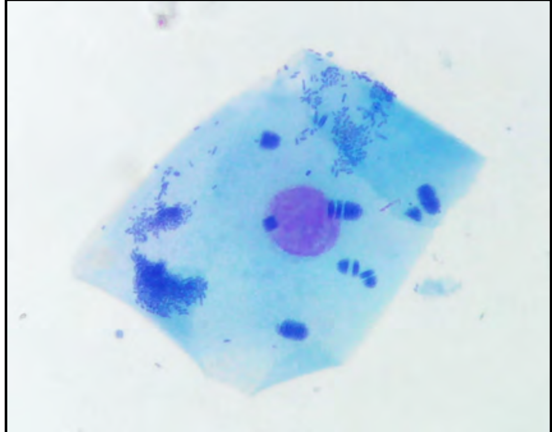


Figure 5.1: Vaginal cytology of two lionesses in oestrus. *Simonsiella* spp can be observed attached to a superficial epithelial cell, forming typical clusters. Multiple bacteria of smaller size can be observed as well. Diff-Quik stain. x1000.

5.4 MATERIALS AND METHODS

All lionesses, housed at a wildlife conservation centre in South Africa, were trained by means of positive reinforcement conditioning to voluntarily allow collection of vaginal swabs as part of a research project concerning wild felid reproductive physiology, approved by the Animal Ethics Committee of the University of Pretoria.

Vaginal samples were collected every other day, at least three times per week, for a period of six months in 6 animals. In parallel, behaviour monitoring was performed two hours a day, five days per week, throughout the same period of time, to record and describe any sexual activity and oestrous signs present on these females. In total, 22 oestrous cycles and 3 pregnancies were recorded, and 400 vaginal swabs were collected from the lionesses throughout the research period. Smears were prepared from the vaginal swabs, and these were stained with the Diff-Quik method (*i.e.* modified Wright-Giemsa) and evaluated according to the technique previously described by Johnston *et al.* (Johnston *et al.* 2001).

Oestrus was identified by the presence of specific behavioural signs such as: purring, flirting, lordosis, rolling, and increased anogenital grooming, as well as by enlarged and separated vulvar lips. The presence of a predominant proportion of

superficial cornified cells, the clearing of the background, and a moderate to high number of bacteria associated to the epithelial cells in the vaginal smear (Fig 5.2) was additionally considered as an indicator of oestrus as previously described for other carnivore species (Johnston *et al.* 2001).

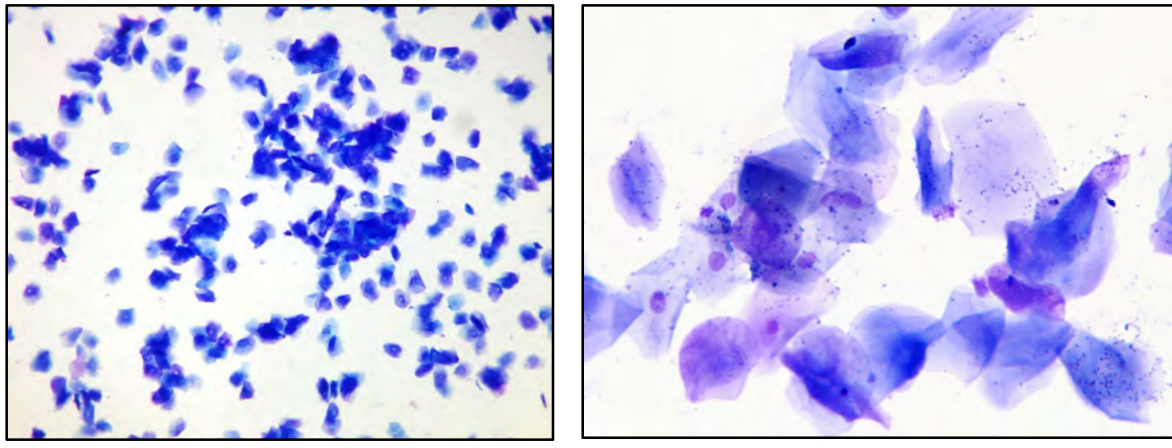


Figure 5.2: Vaginal cytology of one lioness in oestrus. A predominant proportion of superficial cornified cells, clearing of the background, and a high number of bacteria may be observed. Diff-Quik stain. x40 (left) and x200. (right)

5.5 RESULTS

Out of the 400 vaginal cytologies, 103 slides were classified as oestrus smears by means of the above described criteria. *Simonsiella* spp were identified by their characteristic morphology (*i.e.* large, clustered microorganisms associated to the exfoliated cells) in 60 of the oestrus smears (58%) belonging to all six females under study. Vaginal swabs from a female in oestrus presenting the presumed bacteria were sent to the Laboratory of Bacteriology of the Faculty of Veterinary Sciences of the University of Pretoria (South Africa) to confirm the identity of the suspected microorganism by means of bacterial culture and Gram staining. Although, *Simonsiella* spp were not isolated in the culture, they were clearly identified in the Gram stain as well as microscopically by the criteria described earlier (Hedlund and Staley, 2002). A heavy growth of *Streptococcus canis* and *E. Coli* was isolated in this sample; nevertheless, this growth was not associated with disease. No microorganisms belonging to genus Simonsiella were detected in the vaginal cytologies prepared from samples collected during interoestrous interval or dioestrus (*i.e.* predominant proportion of parabasal and intermediate cells, with or

without neutrophils) (Fig 5.3). Exceptionally, *Simonsiella* spp were found in the vaginal cytology of one female two days after parturition.

In addition, oral swabs were taken and cytologies performed from three females during anaesthetic procedures for routine health checks. *Simonsiella* spp were again identified by their characteristic morphology in all of these samples.

Noteworthy, *Simonsiella* spp were also found opportunistically in a vaginal cytology from a captive female Sri Lankan leopard in oestrus, at a zoological institution in Singapore, during a routine reproduction assessment.

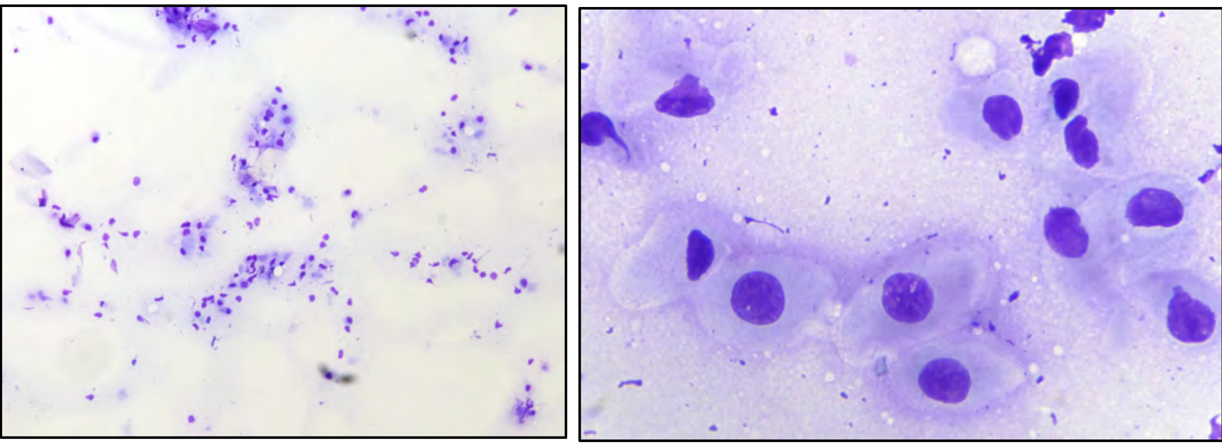


Figure 5.3: Vaginal cytology of one lioness in interoestrous interval. A predominant proportion of parabasal cells and some neutrophils may be observed. Diff-Quik stain. x40 (left) and x400 (right).

5.6 DISCUSSION

To date, there is no reports of the detection of *Simonsiella* spp in the vaginal flora of any felid, despite the large number of bacteria already identified to be normal in healthy cats (Clemetson and Ward, 1990; Holst *et al.* 2003).

The number of neutrophils present on the surface of the vaginal mucous membrane during oestrus is low, compared to any other stage of the oestrous cycle (Johnston *et al.* 2001). This may create a favourable environment, and facilitate the growth of the resident bacteria and a transient colonization of the vaginal surface by other microorganisms such as *Simonsiella* spp. In this case, *Simonsiella* spp may have originated in the mouth, and migrated to the vagina as a result of increased ano-genital grooming. This behaviour was recorded in all females under study during oestrus stage and immediately postpartum.

The detection of *Simonsiella* spp in the vagina of six lionesses in oestrus, in addition to the casual finding of this microorganism in one Sri Lankan female leopard in oestrus may confirm this finding to be more common than previously suspected, transitory, and with no clinical relevance. As described previously in the bitch (Valle *et al.* 2006), the occurrence of *Simonsiella* spp may be an additional cytology result helping to identify oestrus by vaginal smears.

5.7 DECLARATIONS OF INTEREST

None.

5.8 ACKNOWLEDGEMENTS

The authors thank Ukutula Conservation Center for the support during the development and performing of the current research project, especially all the staff involved in the training of the lionesses, the Laboratory of Bacteriology of the Faculty of Veterinary Sciences of the University of Pretoria, and the veterinary team of Wildlife Reserves Singapore, as well as the South African National Research Foundation for the financial support provided.

6. CHAPTER SIX:

NON-SURGICAL ARTIFICIAL INSEMINATION USING A GnRH ANALOGUE FOR OVULATION INDUCTION DURING NATURAL OESTRUS IN AFRICAN LIONS (*PANTHERA LEO*)

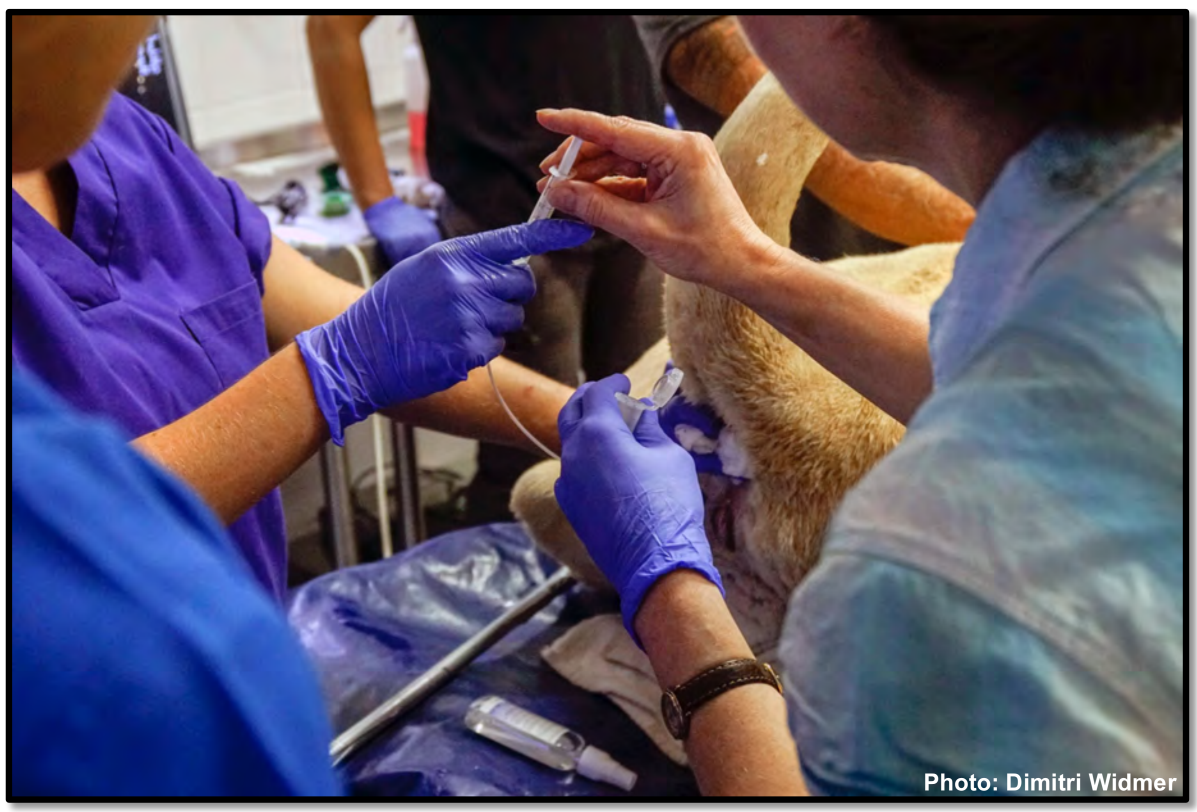


Photo: Dimitri Widmer

Theriogenology
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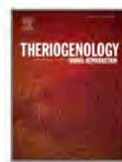

Non-surgical artificial insemination using a GnRH analogue for ovulation induction during natural oestrus in African lions (*Panthera leo*)

Isabel Callealta [a], Andre Ganswindt [b], Martin Malan [c], Imke Lueders [b]

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6.1 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 electroejaculation, 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®, Kruuse, South Africa) with a metal stylet. A single intramuscular dose of exogenous GnRH (20 μg buserelin-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%.

6.2 KEYWORDS

Artificial insemination; African lion; ovulation induction; GnRH; fresh sperm; natural oestrus.

6.3 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 (IUCN, 2019). 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 (Wildt *et al.* 1987; Lacy, 1997). *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 (Swanson, 2006; Lermen *et al.* 2009). Unfortunately, overall ART success rate in non-domestic felids remains <25% to date (Andrews *et al.* 2019). For the last decades, successful AI trials in non-domestic cats have involved the African lion (*Panthera leo*, Bowen *et al.* 1982; Goeritz *et al.* 2012), Persian leopard (*Panthera pardus saxicolor,* Dresser *et al.* 1982), cheetah (*Acinonyx jubatus*, Howard *et al.* 1992), Siberian tiger (*Panthera tigris altaica*, Donoghue *et al.* 1996; Silva *et al.* 2000), puma (*Puma concolor*, Barone *et al.* 1994), ocelot (*Leopardus pardalis*, Swanson *et al.* 1996), snow leopard (*Panthera uncia*, Roth *et al.* 1996), clouded leopard (*Neofelis nebulosa*, Howard *et al.* 1996; Tipkantha *et al.* 2007), and Asiatic Golden cat (*Catopuma temminckii,* Lueders *et al.* 2014). 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 (Pelican *et al.* 2006). 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 (Pukazhenthi and Wildt, 2004; Pelican *et al.* 2006).

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 (Bauer *et al.* 2016). In South Africa, however, there is an increasing number of lions living in private and national reserves that breed successfully (Bauer *et al.* 2016). 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 (Swanson, 2006). Lion females are polyoestrous, and not affected by season or photoperiod (Brown, 2001). The ovarian cycle of these felids lasts about 2-3 weeks, with an oestrus duration of 2-9 days (Putman *et al.* 2015). As other cats, they are induced ovulators, although may ovulate spontaneously (Schramm *et al.* 1994). The gestation length is around 110 days (Putman *et al.* 2015), and pseudopregnancy may appear after non-conceptive ovulation, ranging from 35 to 54 days in duration (Brown, 2011; Putman *et al.* 2015).

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.

6.4 MATERIALS AND METHODS

6.4.1 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 collection of vaginal swabs, and drug administration by hand-syringe (Callealta *et al.* 2019). 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 6.1.

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).

6.4.2 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.

Table 6.1: 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 Rese 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 dynamics	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

6.4.3 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 (Stanton *et al.* 2015). Behavioural oestrus was confirmed by the presence of a

predominant proportion of superficial cornified epithelial cells associated to a moderate-to-high number of bacteria and clearing of the background in the vaginal cytology (Callealta *et al.* 2018) (Fig 6.1). 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.

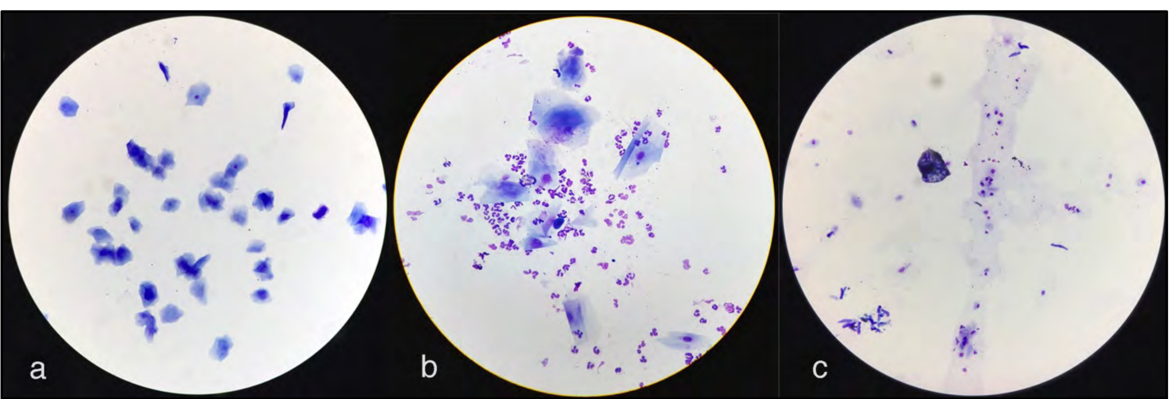

Figure 6.1: Microscopic images of Diff-Quik® stained vaginal smears of lionesses presenting different stages of the cycle. Vaginal cytologies were typically classified as a) oestrus smear: predominant proportion of superficial cornified epithelial cells associated to a moderate-to-high number of bacteria, 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).

6.4.4 Ovulation induction

Once oestrus was detected, the focal female received a single intramuscular dose of the GnRH analogue buserelin-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 (Lueders *et al.* 2014). 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.

6.4.5 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.* (2012), by inserting a sterile commercial 2.6 x 500 mm dog urinary catheter (Buster®, Kruuse, 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 electroejaculation (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 and Oko (1989).

6.4.6 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 6.2a) in an attempt to mimic the posture she would acquire during natural mating (Fig 6.2b). Also, this position is believed to help stabilize the cervix, which may facilitate the passage of the AI catheter through it (Lueders *et al.* 2014). After cleaning the perineal region, a commercial 2.0 x 500 mm dog urinary catheter (Buster®, Kruuse, 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.

Figure 6.2: 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.

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 6.2c), with the objective of mimicking the rolling behaviour shown by these animals right after natural mating (Fig 6.2d). Finally, the female was returned to the enclosure for recovery, and the anaesthesia was reversed as described above.

6.4.7 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.* 2015) 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.

6.5 RESULTS

6.5.1 Oestrus monitoring and AI timing

Oestrus lasted on average 6.84 ± 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.* (2015) (4.4 ± 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 6.1). In all remaining trials, the vaginal samples collected at the time of AI provided the classic oestrus image described in 6.4.3.

6.5.2 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 ± 11.5 hours after the injection (n=8; range: 36-120 hours). The females that received the GnRH analogue

on Day 4 (*protocol 3*) terminated oestrus 105 ± 9 hours after the injection (n=4; range: 84-120 hours). The median time interval between buserelin injection and first day of dioestrus for females treated on Day 4 (108 hours) and Day 5 (72 hours) differed by 36 hours (Table 6.2). 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 6.1). 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).

Table 6.2: Buserelin-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	Hours from GnRH to end of dioestrus	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

6.5.3 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 6.3). 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 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.

6.5.4 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 6.3, Table 6.4). 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 6.2).

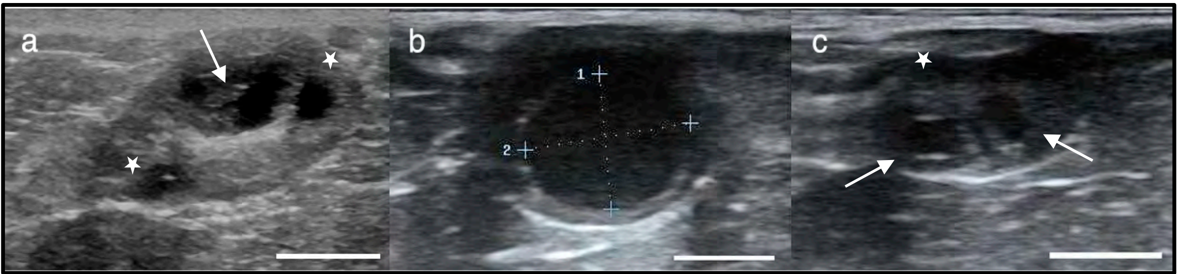

Figure 6.3: Ultrasonographic images of lion ovaries: a) ovarian follicles observed on Day 6 of oestrus, prior to GnRH administration and artificial insemination (typically observed with *protocol 1*). Arrow indicates a regressing dominant follicle (with hyperechoic content); stars show two subordinate 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.

6.5.5 Pregnancy rate

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 6.2).

Table 6.3. 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

Table 6.3 (cont.): 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
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
		EE	Grey	Watery	0.5	61.5	40	41	55	
10, 13	M6	UC	White	Thick milky	0.3*	2530	80	76	18	Yes**
		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	
		EE	Grey	Watery	5.0	30.5	85	37	60	
12	M1	EE	White	Thick milky	0.3 [1st]	956	80	83	54	No
		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	

Table 6.4: 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**	**no. of follicles**		**Diameter of largest follicle (mm)**		**no. 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

6.6 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.

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

Felids present exceptionally variable ovarian cycles (Andrews *et al.* 2019). Even within species, reports about cycle duration vary distinctively, depending on the number of animals and cycles observed, as well as the methodology used (Andrews *et al.* 2019). 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.* (2015) (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 buserelin-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 Putman *et al.* 2015; Schramm *et al.* 1994). We, therefore, support buserelin-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 (Swanson *et al.* 2001) and clouded leopards (Pelican *et al.* 2001). 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 6.5.4 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 (England, 2010; Miki *et al.* 2016). 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 (Putman *et al.* 2015). However, further investigation would be required to confirm whether this difference was related to the use of buserelin-acetate as an ovulation inductor, the distinct methods applied for length determination, or only a bias due to our small sample size.

6.6.2 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 (Dresser *et al.* 1982; Silva *et al.* 2000; Lueders *et al.* 2014). Only two prior studies described the achievement of successful non-surgical AI in African lions (Bowen *et al. 1982*; Goeritz *et al.* 2012). 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.* (2012) 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 (Lueders *et al.* 2012; Barbosa *et al.* 2019). Despite the total low volume used, and the moderate occurrence of teratoid sperm (typically observed in many felid species, Andrews *et al.* 2019), 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 (Tanaka *et al.* 2000; Tsutsui *et al.* 2000-a; Tsutsui *et al.* 2004) and wild felids (Wildt *et al.* 1986; Howard *et al.* 1997).

GnRH stimulates both FSH and LH secretion (Conn and Crowley, 1991). Thus, buserelin-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 6.2), and we concluded that Day 6, without previous ovulation induction was too late for

insemination. Most probably, buserelin-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 (Howard and Wildt, 2009) 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 6.1 and 6.2). Mechanic manipulation of the cervix through the rectal wall, as described for domestic (Zambelli and Cunto, 2005) and small wild cats (Lueders *et al.* 2014), 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) (Putman *et al.* 2015). 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) (AZA, 2012). In this study, the final pregnancy success rate achieved by non-surgical AI in lionesses presenting natural oestrus, after administration of buserelin-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 (Swanson, 2006), and the 28.6% achieved with intrauterine laparoscopic insemination in other large cats (Howard *et al.* 1992).

6.7 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, buserelin-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.

6.8 DECLARATIONS OF INTEREST

None.

6.9 ACKNOWLEDGEMENTS

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7. CHAPTER SEVEN:

FINDINGS AND RECOMMENDATIONS

This research project was carried out with the ultimate purpose of proving the feasibility of implementing basic assisted reproductive techniques (ARTs) into African lion breeding practices. For this, a better understanding of the fundamental aspects of the female African lion reproduction needed to be achieved in the first place. With this purpose, six African lionesses were trained by positive reinforcement conditioning (PRC) to voluntarily allow frequent collection of blood samples and vaginal swabs that were subsequently analysed. In parallel, the ovarian cycles of these females were non-invasively monitored by behavioural observation and measurement of faecal steroid metabolite concentrations. All gathered information served to determine the optimum timing for artificial insemination (AI) in this species, and to evaluate the effect of the exogenous synthetic GnRH analogue, buserelin-acetate, to induce ovulation in lionesses showing natural oestrus. The basic ARTs developed in the course of this research may be implemented as part of *ex-situ* conservation breeding programs for the African lion, and also potentially serve as a foundation for application in other large non-domestic felids.

The PRC training of captive African lionesses described in Chapter 2 resulted in a minimally-invasive, suitable, repeatable, and cost effective method for the collection of biological samples, such as vaginal swabs and blood. Over the course of this study, it enabled collection of about 750 vaginal swabs and 650 blood samples from the six focal females. Although, there are similar reports of single cases involving non-domestic felids trained by PRC (*e.g.* Bergman and Janssen, 2005; Broder *et al.* 2008; Lin and Wang, 2018), this was the first time, to the author's knowledge, that the training in parallel of such a number of large felids was documented in the scientific literature (Callealta *et al.* 2019). The results of this study support that PRC may reduce the psychological stress component associated with traditional sampling, which usually requires physical and chemical restraint techniques (Gilroy and DeYoung, 1986; Reinhardt, 2003). PRC sampling may also avoid physiological effects associated with anaesthesia, as observed on determined blood parameters in previous studies (Gilroy and DeYoung, 1986; Lambeth *et al.* 2006). Based on the positive responses displayed by all trained lionesses (*e.g.* voluntary approach to the training enclosure, quick learning of new routines), the

results of this study indicate that PRC training may be a feasible approach for both physiological studies and some veterinary procedures. In line with this observation, the scientific literature shows that PRC training is becoming an increasingly common practice in zoos as part of husbandry routines (Phillips *et al.* 1998; Savastano *et al.* 2003; Broder *et al.* 2008; Drews *et al.* 2011; Miller and King, 2013; Ward and Melfi, 2013; Magden, 2017). In addition, PRC training appeared to be stimulating for the lionesses under study (*e.g.* a few months after the training implementation, most females voluntarily participated in the training sessions even when there was food present in the main enclosure). PRC could be thus potentially considered a form of behavioural enrichment for African lions in captive settings, as previously suggested for other cats (Broder *et al.* 2008), birds (Miller and King, 2013), livestock (Hemsworth, 2003), primates (Reinhardt, 2003; Magden, 2017), and reptiles (Hellmuth *et al.* 2012). In summary, the implementation of PRC training as part of the handling routine of African lions (and other non-domestic felids) in captive settings is therefore highly recommended. Not only does it open new avenues of physiological research, veterinary diagnostic, and treatment options, but it may also help to progressively shape behaviours and management routines that initially warranted negative reinforcement or chemical immobilization.

Longitudinal patterns of female reproductive circulating steroids (serum oestrogen, sE, and progestagen, sP) and their faecal metabolite counterparts (faecal oestrogen metabolites, fEM, and faecal progestagen metabolites, fPM) were described and compared to behavioural reproductive traits in African lions, in Chapter 3 of this study. Previous studies in lions had already investigated alterations in female reproductive steroids in both serum (Schmidt *et al.* 1979) and faeces (Putman *et al.* 2015). The specialty of this study however was the possibility of assessing frequent blood samples (1-7 samples per week and female) collected by PRC for a long period of time (18 months). In addition, longitudinal patterns of serum oestrogen and progestagen concentrations were compared with corresponding faecal metabolite counterparts for the first time in a non-domestic felid. The results of this study showed that patterns of progestagen concentrations in female African lions were comparable when determined in either serum or faeces, whereas respective patterns of serum oestrogen concentrations and respective faecal

metabolites were not related. This was in contrast to findings of previous studies conducted on black rhinoceros (*Diceros bicornis,* Berkeley *et al*. 1997), dairy goats (*Capra aegagrus hircus,* Capezzuto *et al*. 2008), and red wolves (*Canis rufus,* Walker *et al*. 2002), where progestagen as well as oestrogen concentrations were found to be correlated in both matrixes. However, previous studies in non-domestic felids (Seal *et al*. 1985) and large-bodied species (Hodges *et al*. 2010, Kumar *et al*. 2013) confirmed the findings of this research. These studies speculated on the erratic fluctuation in sE concentrations and the cumulative effect, and thus less episodic fluctuation in faecal oestrogen metabolite concentrations, which may explain the lack of correlation between sE and fEM concentrations observed in this study. Additionally, and in contrast to previous observations in domestic cats (Johnston *et al*. 2001; England, 2010), oestrogen concentrations monitored in the pregnant lionesses seemed to increase during the last month of gestation, peak right before parturition, and decrease during the first week of lactation. Although oestrogen surges during pregnancy have been observed in other felids such as cheetahs (*Acinonyx jubatus*, Brown *et al*. 1996) and Pallas' cats (*Otocolobus manul*, Brown *et al*. 2002), elevations in oestrogen concentrations around and post-parturition had not been described before in other felids, and could be thus specific to African lions. Overall, the exclusive determination of oestrogen concentration for oestrus detection is not recommended, regardless of the matrix used, as sE levels showed to be rather fluctuant and fEM levels seemed to be predominantly elevated during the second half of oestrus, which makes it difficult to identify early oestrus stages. In agreement with the results obtained in lions by Putman *et al*. (2015), this study showed that one sample of either matrix may serve however to distinguish between follicular (baseline progestagen concentrations) and luteal phases (elevated progestagen concentrations), as well as between pregnant (highly elevated progestagen concentrations) and pseudopregnant lionesses (lower, but still elevated progestagen concentrations), especially if the samples are collected around week 5 after mating or AI. Overall, sP concentrations in this study were 120% higher in pregnant lionesses compared to pseudopregnant females, while fPM concentrations were 160% higher in gestating females compared to pseudogestating lionesses. However, revealed steroid values and subsequent classifications of ranges (*e.g.* baseline, low hormone concentrations, and highly

elevated hormone concentrations) were based on determined assay-specific absolute values, and will need to be adjusted if other immunoassays were to be applied.

In this research, we were able to identify specific reproductive cycle stages in trained female African lions by frequent vaginal swabbing and immediate interpretation of cytological results. Overall, and as described in Chapter 4, one single swab showing a high proportion of superficial cells, absence of neutrophils, large number of bacteria, and a clean background was enough to identify lionesses in oestrus. Likewise, one cytology showing a high proportion of parabasal and intermediate cells, occasional neutrophils, mild number of bacteria, and a dirty background enabled detection of females in advanced dioestrus or gestation. The cytological characteristics depicted for these two stages in lionesses were similar to those previously described for domestic cats (Mills *et al.* 1979) and cheetahs (*Acinonyx jubatus*; Asa *et al.* 1992). However, to distinguish lionesses in early dioestrus from lionesses in interoestrus, and females in pro- and post-oestrus, at least two consecutive swabs were necessary. The two latter stages (proestrus and post-oestrus) had not been previously described in the scientific literature from a cytological point of view for any non-domestic felid. Prior studies in pumas (*Felis concolor*; Bonney *et al.* 1981), lions (Schmidt *et al.* 1983), tigers (*Panthera tigris*; Seal *et al.* 1985), and cheetahs (Asa *et al.* 1992) assessed mainly weekly vaginal samples from anaesthetized animals. In this study, the possibility of collecting frequent vaginal swabs (1-7 samples per week and female) by PRC sampling enabled detailed description of all changes observed in the African lioness' vaginal epithelium during the ovarian cycle. Furthermore, the detection of *Simonsiella* spp in around 53% of evaluated oestrus vaginal smears, in addition to the casual finding of this micro-organism in one Sri Lankan female leopard in oestrus (Chapter 5), suggests that this finding may be more common than previously suspected (Valle *et al.* 2006). The results of this study suggest that the transient occurrence of *Simonsiella* spp in the vaginal smears of female African lions may be indicative of an on-going oestrus that had started at least 2-3 days prior. This research supports that vaginal cytology may serve as a practical, inexpensive tool to closely monitor the ovarian cycle, confirm oestrus, and diagnose pregnancy in captive lionesses,

providing appropriate conditioning training. The use of this technique is especially recommended in combination with behavioural observations, in captive settings where PRC training is possible, anaesthesia is not an option, specialized equipment (such as an ultrasound device) is not available or affordable, and/or endocrine analyses cannot be performed.

The methods used in this research to daily monitor the ovarian cycle of the lionesses and determine the different reproductive stages (*i.e.* behavioural observations and vaginal cytology evaluation) proved to be valid, according to the similarities found between our results (Chapters 3 and 4) and those of previous studies in lions (Putman *et al.* 2015), domestic cats (Mills *et al.* 1979; Shille *et al.* 1979), and cheetahs (Asa *et al.* 1992). However, the exclusive use of one of these techniques alone for reproductive monitoring may lead to erroneous and confusing results. Based on the general findings of this research, and supported by Silva *et al.* (2017), to accurately monitor the reproductive cycle in lionesses, detect oestrus, help timing for AI, and distinguish between proestrus, interoestrous interval, pregnancy and pseudopregnancy, it is rather recommended to use a combination of different monitoring techniques, such as behavioural observation, vaginal cytology, hormone measurement, and ultrasonography.

As described in Chapters 3 and 6, the results of this research also showed that buserelin-acetate was a suitable drug to induce ovulation in African lions. All focal females entered either a pregnant luteal phase or a non-pregnant luteal phase after receiving one single dose of the exogenous synthetic GnRH analogue at the end of the oestrous period. These results dissent previous studies where GnRH agonists such as buserelin-acetate and leuprolide acetate were not recommended to induce follicular maturation and ovulation in felids, due to the unreliable effects in some species, such as domestic cats (Swanson *et al.* 2001) and clouded leopards (*Neofelis nebulosa*; Pelican *et al.* 2001). However, the results of this study were in agreement with more recent publications on other non-domestic feline species, such as the Asiatic golden cat (*Catopuma temminckii,* Lueders *et al.* 2014), and the Persian leopard (*Panthera pardus saxicolor*; Lueders *et al.* 2015). According to the endocrine results of this study, buserelin-acetate seemed to induce ovulation within

24-72 hours after administration. This is in line with previous studies where most ovulations in different species took place 24-60 hours after an LH surge (England, 2010; Miki *et al.* 2016). Some lionesses continued to show signs of behavioural oestrus for 24 to 72 hours after ovulation (up to 120 hours from administration of the GnRH analogue), which was in agreement with previous observations in at least another species (the domestic dog; England, 2010). Overall, one single 20 μg intramuscular dose of buserelin-acetate is recommended on day 5 from oestrus onset, about 24 hours before AI. Likewise, close monitoring of the reproductive cycle of the targeted female is highly endorsed to refine the ideal timing for ovulation induction and AI. As opposed to early studies in domestic cats (Howard *et al.* 1992), the anaesthetic protocol utilized during this study (*i.e.* medetomidine, ketamine, midazolam) did not interfere with ovulation. As described in Chapter 6, in numerous occasions, pre-ovulatory ovarian follicles (ranging from 9.5 to 13 mm of diameter) were present upon ultrasound examination (around 30-48 hours after buserelin-acetate administration). In these cases, sP concentrations rose above the baseline within days of injection in any case, indicating that successful ovulations occurred despite full anaesthetic immobilization of the focal lionesses. Additionally, this study showed that the pseudopregnancies induced by buserelin-acetate administration were around 10 days longer than the spontaneous pseudopregnancies (according to both, the results of this study and those reported by Putman *et al.* 2015), which should be taken into consideration when implementing the use of this drug for ovulation induction within breeding programs.

Numerous factors influence the outcome of AI (Thongphakdee *et al.* 2018). In addition to the choice of the right candidate and an effective ovulation induction protocol (Pelican *et al.* 2006), the time of insemination, the sperm concentration and preservation method (fresh versus frozen), as well as the site of semen deposition (intravaginal, intrauterine or oviductal insemination) are key factors to succeed (Tsutsui *et al.* 2000a; Tsutsui *et al.* 2000b; Chatdarong *et al.* 2007). This research confirmed that non-surgical AI with fresh semen can be performed in African lions both prior to and after ovulation, although semen deposition shortly after ovulation seemed to positively affect the success rate of the procedure (Chapter 6). Likewise, successful AI was achieved using both intravaginal and transcervical (intrauterine)

insemination, although an 8- to 10-fold higher dose was needed when sperm was deposited into the vagina, which was in accordance with previous studies in both domestic (Tanaka *et al.* 2000; Tsutsui *et al.* 2000a; Tsutsui *et al.* 2004) and non-domestic felids (Wildt *et al.* 1986; Howard *et al.* 1997). Laparoscopic intrauterine approach has enabled successful AI in domestic cats, leopards, cheetah, tigers, pumas, ocelots, and tigrinas, utilizing considerably lower sperm doses (>10^6 motile sperm per trial) (Swanson, 2012). In the last years, laparoscopic oviductal AI has become popular in wild felids, due to the promising pregnancy rates (>70%) achieved in domestic cats (Swanson, 2019). This approach has been successfully applied, for example, in Brazilian ocelots (*Leopardus pardalis mitis*) and Amur tigers (*Panthera tigris altaica*) (Lambo *et al.* 2014). However, the pregnancy rates achieved by laparoscopic AI are still low for most feline species, especially when cryopreserved sperm is used (Swanson, 2019). In addition, laparoscopic procedures, despite being minimally-invasive, may lead to complications and require postoperative care, which may be extremely challenging in generally unhandled and potentially dangerous wild animals, such as lions (Graham *et al.* 1995; Kersey and Dehnhard, 2014; Callealta *et al.* 2019). The non-surgical AI approach proposed in this study proved to be a valid minimally-invasive method for African lions that avoided surgical complications and specific postoperative attention. The achievement of four successful artificial inseminations, and the resulting birth of the first African lion cubs ever conceived by this procedure (Fig 7.1) crowned this research project. Two prior studies reported successful non-surgical AI in African lions (Bowen *et al. 1982*; Goeritz *et al.* 2012). However, although conception was confirmed in the related reports, all embryos obtained were retrieved and used for other purposes. Thus, live birth was not achieved in these studies. The final pregnancy success rate of non-surgical AI with fresh semen in lionesses presenting natural oestrus, after administration of buserelin-acetate to induce ovulation reported in Chapter 6 of this study, was 33.3%. While still low, this rate is higher than the last overall success rate reported for AI in small and large wild felids, using both laparoscopic insemination and non-surgical techniques (around 10%; Donoghue *et al.* 1996; Swanson, 2006; Howard and Wildt, 2009).

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 (Holt and Pickard, 1999). The AI protocols developed for lions during this study may serve as a foundation to establish, improve, and implement advanced ARTs already under investigation in this species, such as semen cryopreservation (Patil *et al.* 1998; Malo *et al.* 2003; Stander-Breedt *et al. 2004;* Luther *et al.* 2017), AI with frozen sperm (Lueders *et al.* unpublished results), *in vitro* embryo production (Armstrong *et al.* 2003; Damiani *et al.* 2003; Fernandez-Gonzalez *et al.* 2015), and embryo retrieval (Goeritz *et al.* 2012).

In summary, the information originated from this research allowed an in-depth description of the reproductive cycle of the African lioness, and helped to develop AI protocols for this species. All gathered information could be used as a baseline to adapt both established methods and newly created protocols, for other threatened felid species all around the world. This study presents a new research approach to felid reproduction physiology. Further studies on circulating peptide hormones, such as FSH, inhibin, and LH are still needed to fully understand regulative endocrine mechanisms in felids. However, the acquired ability to collect frequent blood samples from trained animals unfolds this possibility. Implementation of vaginal swabbing into the routine management of captive feline populations may help to improve *ex-situ* breeding efforts for threatened felid species. Furthermore, buserelin-acetate proved to be a valid, readily available exogenous hormone to induce ovulation in African lions, and its use may help to advance and improve assisted reproduction techniques for this and other non-domestic cats. Additionally, the results of this research suggest that the non-surgical approach for AI shown here for lions has potential to be applied to other endangered large felid species.

Altogether, the here-described approaches can provide new opportunities to improve breeding of captive and free ranging lion populations, and thereby assist global conservation efforts for this species, as well as for other threatened felids.

Photo: Isabel Callealta

Figure 7.1: two of the seven lion cubs conceived after artificial insemination during this research.

List of References

Andrews, C., Thomas, D., Yapura, J., Potter, M. (2019). Reproductive biology of the 38 extant felid species: a review. – *Mammal Review*, 49(1): 16-30.

Armstrong, D., Crichton, E., Dankof, S., Schwalbach, L., Gardner, D., Loskutoff, N. (2003). Ovarian stimulation, laparoscopic oocyte retrieval, ivf and blastocyst production using sequential media in the African lion (*Panthera leo*). – *Reproduction, Fertility and Development*, 16(2): 221-221.

Asa, C., Junge, R., Bircher, J., Noble, G., Sarri, K., Plotka, E. (1992). Assessing reproductive cycles and pregnancy in cheetahs (*Acinonyx jubatus*) by vaginal cytology. – *Zoo Biology*, 11(3): 139-151.

Axnér, E., Gustavsson, T., Ström Holst, B. (2008). Estradiol measurement after GnRH-stimulation as a method to diagnose the presence of ovaries in the female domestic cat. – *Theriogenology*, 70: 186–191.

AZA Lion Species Survival Plan. Lion Care Manual. (2012). Association of Zoos and Aquariums, Silver Spring, MD. Pp. 143.

Banks, D., Paape, S., Stabenfeldt, G. (1983). Prolactin in the cat: I. Pseudopregnancy, pregnancy and lactation. – *Biology of Reproduction*, 28: 923–932.

Barbosa, T., Angrimani, D., Rui, B., Losano, J., Bicudo, L., Blank M., *et al*. (2019). Functional sperm assessments of African Lion (*Panthera leo;* Mammalia; Carnivora; Felidae) in field conditions. – *Journal of Threated Taxa*, 11(1): 13114–13119.

Barone, M., Wildt, D., Byers, A., Roelke, M., Glass, C., Howard, J. (1994). Gonadotrophin dose and timing of anaesthesia for laparoscopic artificial insemination in the puma (*Felis concolor*). – *Reproduction*, 101(1): 103-108.

Barth, A., Oko, R. (1989). Abnormal morphology of bovine spermatozoa. Iowa State University Press. Pp. 285.

Bauer, H., Packer, C., Funston, P., Henschel P., Nowell, K. (2016). Panthera leo. *The IUCN Red List of Threatened Species 2016*: e.T15951A107265605. Downloaded on 08 March 2017.

Bauer, H., Nowell, K., Sillero-Zubiri, C., Macdonald, D. (2018). Lions in the modern arena of CITES. – *Conservation Letters*, 11(5): e12444.

Bell, E., Bailey, J., Christie, D. (1973). Studies on vaginal cytology during the canine oestrous cycle. – *Research in Veterinary Science*, 14: 173-179.

Bergman, L., Janssen, D. (2005). Treatment of generalized anxiety in a cheetah (*Acinonyx jubatus*). In: Current Issues and Research in Veterinary Behavioral Medicine. Ed: Mills, D., Levine, E., Landsberg, G. – *Purdue University Press: Indiana, USA*. Pp. 6.

Berkeley, E., Kirkpatrick, J., Schaffer, N., Bryant, W., Threlfall, W. (1997). Serum and fecal steroid analysis of ovulation, pregnancy, and parturition in the black rhinoceros (*Diceros bicornis*) – *Zoo Biology: Published in affiliation with AZA*, 16(2): 121-132.

Bertola, L., van Hooft, W., Vrieling, K., de Weerd, D., York, D., Bauer, H., *et al.* (2011). Genetic diversity, evolutionary history, and implications for conservation of the lion (*Panthera leo*) in West and Central Africa. – *Journal of Biogeography*, 38: 1356–1367.

Bjorklund, M. (2003). The risk of inbreeding due to habitat loss in the lion (*Panthera leo*). – *Conservation Genetics*, 4: 515–523.

Bloomsmith, M., Stone, A., Laule, G. (1998). Positive reinforcement training to enhance voluntary movement of group-housed chimpanzees within their enclosures. – *Zoo Biology,* 7: 333–341.

Bonney, R., Moore, H., Jones, D. (1981). Plasma concentrations of oestradiol-17β and progesterone, and laparoscopic observations of the ovary in the puma (*Felis concolor*) during oestrus, pseudopregnancy and pregnancy. – *Reproduction*, 63: 523-531.

Bowen, M., Platz, C., Brown, C., Kraemer, D. (1982). Successful artificial insemination and embryo collection in the African lion (*Panthera leo*). – *Proceedings American Association of Zoo Veterinarians*, 1: 57-59.

Briggs, M., Fithian, C., Starkey, P., Richards, R., Schramm, R., Reeves, J. (1990). Endocrine profiles in estrus, pregnant and pseudopregnant African lions (*Panthera leo*) throughout the year. – *Proceedings of the American Association of Zoo Veterinarians*, 279-281.

Bristol-Gould, S., Woodruff, T. (2006). Folliculogenesis in the domestic cat (*Felix catus)*. – *Theriogenology*, 66: 5-13.

Broder, J., MacFadden, A., Cosens, L., Rosenstein, D., Harrison, T. (2008). Use of positive reinforcement conditioning to monitor pregnancy in an unanesthetized snow leopard (*Uncia uncia*) via transabdominal ultrasound. – *Zoo biology,* 27(1): 78-85.

Brown, J., Wildt, D., Wielebnowski, N., Goodrowe, K., Graham, L., Wells, S., Howard, J. (1996). Reproductive activity in captive female cheetahs (*Acinonyx jubatus*) assessed by faecal steroids. – *Journal of Reproduction and Fertility,* 106: 337–346.

Brown, J., Graham, L., Wu, J., Collins, D., Swanson, W. (2002). Reproductive endocrine responses to photoperiod and exogenous gonadotropins in the Pallas' cat (*Otocolobus manul*). – *Zoo Biology*, 21: 347–64.

Brown, J. (2006). Comparative endocrinology of domestic and nondomestic felids. – *Theriogenology,* 66: 25-36.

Brown, J. (2011). Female reproductive cycles of wild female felids. – *Animal Reproduction Science*, 124: 155–162.

Bruckner, R., Fahey, S. (1969). A giant bacterial form (*Simonsiella*) seen in oral exfoliative cytology preparations. – *Oral Surgery, Oral Medicine, and Oral Pathology,* 28: 197-201.

Brockway, B., Hassler, C., Hicks, N. (1993). Minimizing stress during physiological monitoring. In: Refinement and reduction in animal testing. Ed: Niemi, S., Willson, J. – *Bethesda, MD: Scientists Center for Animal Welfare.* Pp. 56-69.

Byers, O., Lees, C., Wilcken, J., Schwitzer, C. (2013). The One Plan Approach: The philosophy and implementation of CBSG's approach to integrated species conservation planning. – *WAZA magazine*, 14: 2-5.

Callealta, I., Ganswindt, A., Gonçalves, S., Mathew, A., Lueders, I. (2018). Detection of *Simonsiella* spp. in the vagina of lions and leopard in oestrus. – *Reproduction of Domestic Animals*, 53(6): 1605-1608.

Callealta, I., Ganswindt, A., Malan, M., Lueders, I. (2019). Non-surgical artificial insemination using a GnRH analogue for ovulation induction during natural oestrus in African lions (Panthera leo). – *Theriogenology*, 139: 28-35.

Callealta, I., Ganswindt, A., Lueders, I. (2020). Reproductive cycle stage assessment using vaginal cytology evaluation in African lions (*Panthera leo*). – *Animal Reproduction Science*, 213, 106260.

Callealta, I., Lueders, I., Luther, I., Ganswindt, A. (2019). Positive reinforcement conditioning as a tool for frequent minimally-invasive blood and vaginal swab sampling in African lions (*Panthera leo*). – *Journal of Applied Animal Welfare Science*, 1-12.

Callealta, I., Lueders, I., Callealta, J., Ganswindt, A. (submitted). Blood and fecal reproductive steroid concentration patterns during the ovarian cycle of African lions (*Panthera leo*), and response to ovulation induction with a GnRH analogue. – *Hormones and Behavior*.

Capezzuto, A., Chelini, M., Felippe, E., Oliveira, C. (2008). Correlation between serum and fecal concentrations of reproductive steroids throughout gestation in goats. – *Animal Reproduction Science*, 103(1-2): 78-86.

Chatdarong, K., Axnér, E., Manee-In, E., Thuwanut, P., Linde-Forsberg, C. (2007). Pregnancy in the domestic cat after vaginal or transcervical insemination with fresh and frozen semen. – *Theriogenology*, 68(9): 1326-1333.

Clemetson, L., Ward, A. (1990). Bacterial flora of the vagina and uterus of healthy cats. – *Journal of the American Veterinary Medical Association*, 196(6): 902-906.

Cline, E., Jennings, L., Sojka, N. (1980). Analysis of the feline vaginal epithelial cycle. – *Feline Practice*, 10: 47-49.

Conn, P., Crowley, W. (1991). Gonadotropin-releasing hormone and its analogues. – *The New England Journal of Medicine*, 324(2): 93-103.

Crichton, E., Bedows, E., Miller-Lindholm, A., Baldwin, D., Armstrong, D., Graham, L., *et al*. (2003). Efficacy of porcine gonadotropins for repeated stimulation of ovarian activity for oocyte retrieval and in vitro embryo production and cryopreservation in Siberian tigers (*Panthera tigris altaica*). – *Biology of Reproduction*, 68: 105–113.

Damiani, P., Gomez, M., Cole, A., Pope, E., Aguilar, R., Hammond, B., *et al.* (2003). The production of intracytoplasmic sperm injection lion (*Panthera leo*) embyros using spermatozoa collected by percutaneous epididymal sperm aspiration from vasectomized males. – *Reproduction, Fertility and Development*, 16(2): 223-224.

DeMatteo, K., Porton, I., Kleiman, D., Asa, C. (2006). The effect of the male bush dog (*Speothos venaticus*) on the female reproductive cycle. – *Journal of Mammalogy*, 87(4): 723-732.

Donoghue, A., Byers, A., Johnston, L., Armstrong, D., Wildt, D. (1996). Timing of ovulation after gonadotrophin induction and its importance to successful intrauterine insemination in the tiger (*Panthera tigris*). – *Journal of Reproduction and Fertility,* 107: 53–58.

Dooley, M., Pineda, M., Hopper, J., Hsu, W. (1991). Retrograde flow of spermatozoa into the urinary bladder of cats during electroejaculation, collection of semen with an artificial vagina, and mating. – *American Journal of Veterinary Research*, 52: 687–691.

Dresser, B., Kramer, L., Reece, K., Russell, P. (1982). Induction of ovulation and successful artificial insemination in a Persian leopard (*Panthera pardus saxicolor*). – *Zoo Biology*, 1: 55–57.

Drews, B., Harmann, L., Beehler, L., Bell, B., Drews, R., Hildebrandt, T. (2011). Ultrasonographic monitoring of fetal development in unrestrained bonobos (*Pan paniscus*) at the Milwaukee County Zoo. – *Zoo biology*, 30(3): 241-253.

Durrant, B., Ann Olson, M., Amodeo, D., Anderson, A., Russ, K., Campos-Morales, R., *et al.* (2003). Vaginal cytology and vulvar swelling as indicators of impending estrus and ovulation in the giant panda (*Ailuropoda melanoleuca*). – *Zoo Biology: Published in affiliation with the American Zoo and Aquarium Association*, 22(4): 313-321.

Els, J. (2016). Determining the economic significance of the lion industry in the private wildlife tourism sector. Master´s Dissertation, North-West University, Potchefstroom Campus, South Africa. Pp. 149.

England, G. (2010). The reproductive cycle. In: BSAVA manual of canine and feline reproduction and neonatology. 2nd ed. Ed: England, G., Heimendahl, A. – *British Small Animal Veterinary Association*. Pp. 1-12.

Fernandez-Gonzalez, L., Hribal, R., Stagegaard, J., Zahmel, J., Jewgenow, K. (2015). Production of lion (*Panthera leo*) blastocysts after in vitro maturation of oocytes and intracytoplasmic sperm injection. – *Theriogenology*, 83(6): 995-999.

Ganswindt, A., Heistermann, M., Borragan, S., Hodges, J. (2002). Assessment of testicular endocrine function in captive African elephants by measurement of urinary and fecal androgens. – *Zoo Biology: Published in affiliation with AZA*, 21(1): 27-36.

Ganswindt, A., Brown, J., Freeman, E., Kouba, A., Penfold, L., Santymire, R., Vick, M., Wielebnowski, N., Willis, E., Milnes, M. (2012). International Society for Wildlife Endocrinology: the future of endocrine measures for reproductive science, animal welfare, and conservation biology. – *Biology Letters*, 8: 695-697.

Gillis, T., Janes, A., Kaufman, M. (2012). Positive reinforcement training in squirrel monkeys using clicker training. – *American journal of primatology,* 74(8): 712-720.

Gilroy, B., DeYoung, D. (1986). Anesthetic management and surgical technique. – *Veterinary Clinics of North America: Small Animal Practice,* 16: 483–494.

Goeritz, F., Painer, J., Jewgenow, K., Hermes, R., Rasmussen, K., Dehnhard, M., Hildebrandt, T. (2012). Embryo retrieval after hormonal treatment to control ovarian function and non-surgical artificial insemination in African lions (*Panthera leo*). – *Reproduction of Domestic Animals*, 47: 156-160.

Gonçalves, S. (2019). Correlation of Vaginal Cytology and Reproductive Tract Ultrasonography in Wild Felids for Oestrus Cycle Stage Determination. MSc Dissertation. Universidade de Trás-os-Montes e Alto Douro. Portugal. Pp. 70.

Graham, L., Goodrowe, K., Raeside, J., Liptrap, R. (1995). Non-invasive monitoring of ovarian function in several felid species by measurement of fecal estradiol-17β and progestins. – *Zoo Biology*, 14: 223-237.

Hadley, M., Levine, J. (1993). Endocrinology of pregnancy, parturition, and lactation, in: Endocrinology, 505-529.

Hamilton-Smith, C. (1842). Mammalia. An introduction to the Mammalia. In: The Naturalist's Library. Vol. 15. Ed: Jardine W. Chatto and Windus, London.

Hay, M., Goodrowe, K. (1993). Comparative cryopreservation and capacitation of spermatozoa from epididymis and *vasa deferentia* of the domestic cat. – *Journal of Reproduction and Fertility*, Suppl. 47: 297–305.

Hayward, M., Hayward, G. (2007). Activity patterns of reintroduced lion Panthera leo and spotted hyaena *Crocuta crocuta* in the Addo Elephant National Park, South Africa. – *African Journal of Ecology*, 45(2): 135-141.

Hayward, M., Obrien, J. Kerley, G. (2007). Carrying capacity of large African predators: Predictions and tests. – *Biological Conservation*, 139: 219–229.

Hedlund, B. Staley, J. (2002). Phylogeny of the genus Simonsiella and other members of the Neisseriaceae. – *International Journal of Systematic and Evolutionary Microbiology*, 52: 1377-1382.

Hellmuth, H., Augustine, L., Watkins, B., Hope, K. (2012). Using operant conditioning and desensitization to facilitate veterinary care with captive reptiles. – *Veterinary Clinics: Exotic Animal Practice,* 15(3): 425-443.

Hemsworth, P. (2003). Human-animal interactions in livestock production. – *Applied Animal Behavior Science,* 81: 185–98.

Herrick, J., Campbell, M., Levens, G., Moore, T., Benson, K., D'Agostino, J., *et al.* (2010). In vitro fertilization and sperm cryopreservation in the black-footed cat (*Felis nigripes*) and sand cat (*Felis margarita*). – *Biology of Reproduction*, 82: 552–562.

Herron, M. (1977). Feline vaginal cytologic examination. – *Feline Practice*, 7: 36-39.

Hodges, K., Brown, J., Heistermann, M. (2010). Endocrine monitoring of reproduction and stress. In: Wild mammals in captivity: principles and techniques for zoo management. Eds: Kleiman, D., Thompson, K., Kirk Baer, C. – *The University of Chicago Press*. *Chicago*. Pp. 447-468

Holst, B., Bergtröm, A., Lagerstedt, A., Karlstam, E, Englund, L., Baverud, V. (2003). Characterization of the bacterial population of the genital tract of adult cats. – *American journal of veterinary research*, 64(8): 963-968.

Holt, W., Pickard, A. (1999). Role of reproductive technologies and genetic resource banks in animal conservation. – *Reviews of Reproduction*; 4: 143-150.

Howard, J., Bush, M., Wildt, D. (1986). Semen collection, analysis and cryopreservation in nondomestic mammals. In: Current Therapy in Theriogenology. Ed: Morrow, D. – *W.B. Saunders Company, Philadelphia, PA, USA*. Pp. 1047-1053.

Howard, J., Barone, M., Donoghue, A., Wildt, D. (1992). The effect of preovulatory anaesthesia on ovulation in laparoscopically inseminated domestic cats. – *Journal of Reproduction and Fertililty*, 96: 175–186.

Howard, J., Donoghue, A., Barone, M., Goodrowe, K., Blumer, E., Snodgrass, K., *et al.* (1992). Successful induction of ovarian activity and laparoscopic intrauterine artificial insemination in the cheetah (*Acinonyx jubatus*). – *Journal of Zoo and Wildlife Medicine*, 288-300.

Howard. J., Byers, A., Brown, J., Barrett, S., Evans, M., Schwartz, R., Wildt, D. (1996). Successful ovulation induction and laparoscopic intrauterine artificial insemination in the clouded leopard (*Neofelis nebulosa*). – *Zoo Biology*, 15(1): 55-69.

Howard, J., Roth, T., Byers, A., Swanson, W., Wildt, D. (1997). Sensitivity to exogenous gonadotropins for ovulation induction and laparoscopic artificial insemination in the cheetah and clouded leopard. – *Biology of Reproduction*, 56: 1059–1068.

Howard, J., Wildt, D. (2009). Approaches and efficacy of artificial insemination in felids and mustelids. – *Theriogenology*, 71(1): 130-148.

Jewgenow, K., Braun, B., Dehnhard, M., Zahmel, J., Goeritz, F. (2017). Research on reproduction is essential for captive breeding of endangered carnivore species. – *Reproduction in Domestic Animals*, *52*: 18-23.

Johnston, S., Kustritz, M., Olson, P. (2001). Vaginal cytology. In: Canine and Feline Theriogenology. – *Philadelphia, W.B. Saunders*. Pp. 32-40.

Kersey, D,. Dehnhard, M. (2014). The use of noninvasive and minimally invasive methods in endocrinology for threatened mammalian species conservation. – *General and Comparative Endocrinology*, 203: 296-306.

Kirberger, R., Schulman, M., Hartman, M. (2011). Ultrasonographic and laparoscopic evaluation of the reproductive tract of the captive female African lion (Panthera leo). – *Theriogenology*, 76(5): 810-818.

Kitagawa, G. 1984. A smoothness priors-state space modelling of time series with trend and seasonality. – *Journal of the American Statistical Association*, 79(386): 378-389.

Kitchener A., Breitenmoser-Würsten C., Eizirik E., Gentry A., Werdelin L., Wilting A. *et al.* (2017). A revised taxonomy of the Felidae. The final report of the Cat Classification Task Force of the IUCN/ SSC Cat Specialist Group. – *Cat News Special Issue*, 11. Pp. 80.

Kuhn, D., Gregory, D., Buchanan Junior, G., Nyby, M., Daly, K. (1978). Isolation, characterization, and numerical taxonomy of *Simonsiella* strains from the oral cavities of cats, dogs, sheep, and humans. – *Archives of Microbiology*, 118: 235-241.

Kumar, A., Mehrotra, S., Dangi, S., Singh, G., Singh, L., Mahla, A.S., *et al.* (2013). Faecal steroid metabolites assay as a non-invasive monitoring of reproductive status in animals. – *Veterinary World*, 6(1): 59-63.

Lacy, R. (1997). Importance of genetic variation to the viability of mammalian populations. – *Journal of Mammalogy*, 78: 320–335.

Lagendijk, D., Gusset, M. (2008). Human-carnivore coexistence on communal land bordering the greater Kruger area, South Africa. – *Environmental Management*, 42: 971–976.

Lambeth, S., Hau, J., Perlman, J., Martino, M., Schapiro, S. (2006). Positive reinforcement training affects hematologic and serum chemistry values in captive chimpanzees (*Pan troglodytes*). – *American Journal of Primatology: Official Journal of the American Society of Primatologists,* 68(3): 245-256.

Lambo, C., Bateman, H., Swanson, W. (2014). Application of laparoscopic oviductal artificial insemination for conservation management of Brazilian ocelots and Amur tigers. – *Reproduction, Fertility and Development*, *26*(1): 116-116.

Laule, G., Bloomsmith, M., Schapiro, S. (2003). The use of positive reinforcement training techniques to enhance the care, management, and welfare of primates in the laboratory. – *Journal of applied animal welfare science,* 6(3): 163-173.

Lermen, D., Blomeke, B., Browne, R., Clarke, A., Dyce, P, Fixemer, T. *et al*. (2009). Cryobanking of viable biomaterials: implementation of new strategies for conservation purposes. – *Molecular Ecology*, 18: 1030–1033.

Liche, H., Wodzicki, K. (1939). Vaginal smears and the oestrous cycle of the cat and lioness. – *Nature*, 144: 245-246.

Lin, Y., Wang, L. (2018). Animal training and acupuncture in a Bengal tiger (*Panthera tigris tigris*) with hind limb paraparesis. – *Journal of Zoo and Wildlife Medicine,* 49(2): 493-496.

Lindsey, P. A., Alexander, R., Mills, M., Romañach, S., Woodroffe, R. (2007). Wildlife viewing preferences of visitors to protected areas in South Africa: implications for the role of ecotourism in conservation. – *Journal of Ecotourism*, 6(1): 19-33.

Linkie, M., Wibisono, H., Martyr, D., Sunarto, S. (2008). *Panthera tigris ssp. sumatrae*. *The IUCN Red List of Threatened Species 2008*: e.T15966A5334836. Downloaded on 21 March 2017.

Linnaeus, C. (1758). Systema Naturae per regna tria naturae, secundum classis, ordines, genera, species cumcharacteribus, differentiis, synonymis, locis (10th ed.), Vol. 1. Laurentii Salvii, Holmiae.

Lueders, I., Luther, I., Scheepers, G., Van der Horst, G. (2012). Improved semen collection method for wild felids: urethral catheterization yields high sperm quality in African lions (Panthera leo). – *Theriogenology*, 78(3): 696-701.

Lueders, I., Ludwig, C., Schroeder, M., Mueller, K., Zahmel, J., Dehnhard, M. (2014). Successful nonsurgical artificial insemination and hormonal monitoring in an Asiatic golden cat (Catopuma temmincki). – *Journal of Zoo and Wildlife Medicine*, 45(2): 372-379.

Lueders I, Ludwig C. Weber H. (2015). Nonsurgical artificial insemination in felids: Asiatic golden cat (*Catopuma temmincki*) and Persian leopard (*Panthera pardus saxicolor*). – *International Conference on Diseases of Zoo and Wild Anim*als, *Barcelona, Spain, June 2015, extended abstract, p.59.*

Luther, I., Jakop, U., Lueders, I., Tordiffe, A., Franz, C., Schiller, J., *et al*. (2017). Semen cryopreservation and radical reduction capacity of seminal fluid in captive African lion (Panthera leo). – *Theriogenology*, 89: 295-304.

Lutz, H., Soldner, R. (2011). Chapter 1: Basic physics of ultrasound. In: Manual of diagnostic ultrasound. Geneva, Switzerland: World Health Organization.

Magden, E. (2017). Positive reinforcement training and health care. In: Handbook of primate behavioral management. Ed: Schapiro, S. – *CRC press.* Pp. 201-215.

Maia, K., Peixoto, G., Campos, L., Bezerra J., Ricarte, A., Moreira, N., *et al*. (2014). Estrus cycle monitoring of captive collared peccaries (*Pecari tajacu*) in semiarid conditions. – *Pesquisa Veterinária Brasileira*, 34: 1115-1120.

Malandain, E., Rault, D., Froment, E., Baudon, S., Desquilbet, L., Begon, D., Chastant-Maillard, S. (2011). Follicular growth monitoring in the female cat during estrus. – *Theriogenology*, 76(7): 1337-1346.

Malo, A., Martinez-Pastor, F., Olivier, F., Spies, T., Roldan, E., Bartels, P. (2003). Effect of refrigeration and cryopreservation on the quality of lion epididymal spermatozoa. – *Reproduction, Fertility and Development*, 16(2): 225-226.

Martin, P., Bateson, P., Bateson, P. (1993). Measuring behaviour: an introductory guide. – *Cambridge University Press*.

Mead, R., Neirinckx, S., Czekala, N. (1990). Reproductive cycle of the steppe polecat (*Mustela eversmanni*). – *Reproduction*, 88(1): 353-360.

Melfi, V. (2013). Is training zoo animals enriching? – *Applied Animal Behaviour Science,* 147(3-4): 299-305.

Mellen, J., Shepherdson, D. (1997). Environmental enrichment for felids: an integrated approach. – *International Zoo Yearbook,* 35(1): 191-197.

Miki, W., Oniyama, H., Takeda, N., Kimura, Y., Haneda, S., Matsui, M., *et al*. (2016). Effects of a single use of the GnRH analog buserelin on the induction of ovulation and endocrine profiles in heavy draft mares. – *Journal of Equine Science*, 27(4): 149-156.

Miller, R., King, C. (2013). Husbandry training, using positive reinforcement techniques, for Marabou stork (*Leptoptilos crumeniferus*) at Edinburgh Zoo. – *International Zoo Yearbook*, 47(1): 171-180.

Mills, J., Valli, V., Lumsden, J. (1979). Cyclical changes of vaginal cytology in the cat. – *The Canadian Veterinary Journal*, 20(4): 95.

Moreira, N., Monteiro-Filho, E., Moraes, W., Swanson, W., Graham, L., Pasquali, O.L., *et al.* (2001). Reproductive steroid hormones and ovarian activity in felids of the Leopardus genus. – *Zoo Biology*, 20(2): 103-116.

Moreland, R., Brown, J., Wildt, D., Howard, J. (2002). Basic reproductive biology of the fishing cat (*Prionailurus viverrinus*). – Biology of Reproduction, 66: 328-328.

Mowrer, R., Conti, P., Rossow, C. (1975). Vaginal cytology: An approach to improvement of cat breeding. – *Veterinary Medicine, Small Animal Clinician: VM, SAC*, 70(6): 691-696.

Munson, L., Terio, K., Kock, R., Mlengeya, T., Roelke, M., Dubovi, E., *et al.* (2008). Climate extremes promote fatal co-infections during canine distemper epidemics in African lions. – *PLoSONE*, 3: 25-45.

Nyby, M., Gregory, D., Kuhn, D., Pangborn, J. (1977). Incidence of *Simonsiella* in the oral cavity of dogs. – *Journal of Clinical Microbiology*, 6: 87-88.

Packer, C., Kosmala, M., Cooley, H., Brink, H., Pintea, L., Garshelis, D., *et al.* (2009). Sport hunting, predator control, and conservation of large carnivores. – *PLoS One*, 4: 59-41.

Painer, J., Goeritz, F., Dehnhard, M., Hildebrandt, T., Naidenko, S., Sánchez, I., *et al.* (2014). Hormone-induced luteolysis on physiologically persisting corpora lutea in Eurasian and Iberian lynx (*Lynx lynx* and *Lynx pardinus*). – *Theriogenology*, 82(4): 557-562.

Palme, R. (1993). Biotin-streptavidin enzyme immunoassay for the determination of oestrogens and androgens in boar faeces. – *Advances of steroid analysis*, 111-117.

Patil, S., Jayaprakash, D., Shivaji, S. (1998). Cryopreservation of semen of tigers and lions: computerized analysis of the motility parameters of the spermatozoa. – *Current Science*, 930-935.

Pelican, K., Wildt, D., Howard, J. (2001). Short-term inhibition of ovarian activity using the GnRH agonist, leuprolide acetate, in the clouded leopard. – *Theriogenology*, 55: 394.

Pelican, K., Wildt, D., Howard, X. (2003). Ovarian control and assisted reproduction in felids. In: *'Proceedings of American Association of Zoo Veterinarians, 4-10 October 2003, Minneapolis, Minnesota'*, 127-131.

Pelican, K., Wildt, D., Pukazhenthi, B., Howard, J. (2006). Ovarian control for assisted reproduction in the domestic cat and wild felids. – *Theriogenology*, 66: 37–44.

Phillippi-Falkenstein, K., Clarke, M. (1992). Procedure for training corral-living Rhesus monkeys for fecal and blood-sample collection. – *Laboratory Animal Science,* 42: 83–85.

Phillips, M., Grandin, T., Graffam, W., Irlbeck, N. A., Cambre, R. C. (1998). Crate conditioning of bongo (*Tragelaphus eurycerus*) for veterinary procedures at the Denver zoological gardens. – *Zoo Biology,* 17: 25–32.

Picard-Hagen, N., Lhermie, G., Florentin, S., Merle, D., Frein, P., Gayrard, V. (2015). Effect of gonadorelin, lecirelin, and buserelin on LH surge, ovulation, and progesterone in cattle. – *Theriogenology*. 84(2): 177-183.

Pistey, W., Wright, J. (1961). The immobilization of captive wild animals with succinylcholine II. – *Canadian journal of comparative medicine and veterinary science,* 25(3): 59.

Platz, C., Seager, S. (1978). Semen collection by electroejaculation in the domestic cat. – *Journal of the American Veterinary Medical Association*, 173: 1353–1355.

Pope, C., Gomez, M., Dresser, B. (2006). In vitro embryo production and embryo transfer in domestic and non-domestic cats. – *Theriogenology*, 66(6-7): 1518-1524.

Porter, R., Cavanaugh, E., Critchlow, B., Sawyer, C. (1957). Localized changes in electrical activity of the hypothalamus in estrous cats following vaginal stimulation. – *American Journal of Physiology*, 189(1): 145-151.

Proulx, G., Cattet, M., Powell, R. (2012). Humane and efficient capture and handling methods for carnivores. In: Carnivore Ecology and Conservation: A Handbook of Techniques. Ed: Boitani, L., Powell, R. – *Oxford University Press: London, UK.* Pp. 70-129.

Ptaszynska, M. (2009). Compendium of animal reproduction. Intervet/Schering-Plough Animal Health. Pp. 398.

Pukazhenthi, B., Wildt, D. (2004). Which reproductive technologies are most relevant to studying, managing and conserving wildlife? – *Reproduction, Fertility and Development*, 16: 33-46.

Putman, S., Brown, J., Franklin, A., Schneider, E., Boisseau, N., Asa, C., Pukazhenthi, B. (2015). Characterization of ovarian steroid patterns in female lions (*Panthera leo*) and effects of contraception on reproductive function. – *PLoS One*, 10: 140-373.

Reinhardt, V. (2003). Working with rather than against macaques during blood collection. – *Journal of Applied Animal Welfare Science,* 6(3): 189-197.

Roth, T., Armstrong, D., Barrie, M., Garell, D., Wildt, D. (1996). Seasonal effects on ovarian responsiveness to exogenous gonadotropins and successful artificial insemination in the snow leopard (*Panthera uncia*). – *Theriogenology*, 1(45): 246.

Roth, T., Swanson, W. (2018). From petri dishes to politics – a multipronged approach is essential for saving endangered species. – *Nature communications*, 9(1): 2588.

Savastano, G., Hanson, A., McCann, C. (2003). The development of an operant conditioning training program for New World primates at the Bronx Zoo. – *Journal of Applied Animal Welfare Science,* 6(3): 247-261.

Schaller, G. (1972). The Serengeti lion. Chicago: Academic Press.

Schramm, R., Briggs, M., Reeves, J. (1994). Spontaneous and induced ovulation in the lion (*Panthera leo*). – *Zoo Biology*, 13: 301–307.

Schmidt, A., Nadal, L., Schmidt, M., Beamer, N. (1979). Serum concentrations of oestradiol and progesterone during the normal oestrous cycle and early pregnancy in the lion (*Panthera leo*). – *Reproduction*, 57(2): 267-272.

Schmidt, P., Chakraborty, P., Wildt, D. (1983). Ovarian activity, circulating hormones and sexual behavior in the cat. II. Relationships during pregnancy, parturition, lactation and the postpartum estrus. – *Biology of Reproduction*, 28: 657-671.

Schmidt, A., Hess, D., Schmidt, M., Lewis, C. (1993). Serum concentrations of oestradiol and progesterone and frequency of sexual behaviour during the normal oestrous cycle in the snow leopard (*Panthera uncia*). – *Reproduction*, 98(1): 91-95.

Schramm, R., Briggs, M., Reeves, J. (1994). Spontaneous and induced ovulation in the lion (*Panthera leo*). – *Zoo Biology*, 13: 301–307.

Schulman, M., Kirberger, R., Tordiffe, A., Marker, L., Schmidt-Küntzel, A., Hartman, M. (2015). Ultrasonographic and laparoscopic evaluation of the reproductive tract in older captive female cheetahs (*Acinonyx jubatus*). – *Theriogenology*, 84(9): 1611-1619.

Schutte, A. (1967). Canine vaginal cytology. – *Journal of Small Animal Practice*, 8: 301-317.

Schwarzenberger, F., Tomášová, K., Holečková, D., Matern, B., Möstl, E. (1996). Measurement of fecal steroids in the black rhinoceros (*Diceros bicornis*) using group-specific enzyme immunoassays for 20-oxo-pregnanes. – *Zoo Biology*, 15(2): 159-171.

Seal, U., Plotka, E., Smith, J., Wright, F., Reindl, N., Taylor, R., *et al*. (1985). Immunoreactive luteinizing hormone, estradiol, progesterone, testosterone, and androstenedione levels during the breeding season and anestrus in Siberian tigers. – *Biology of Reproduction*, 32: 361-368.

Shille V., Lundström, K., Stabenfeldt, G. (1979). Follicular function in the domestic cat as determined by estradiol-17β concentrations in plasma: relation to estrous behavior and cornification of exfoliated vaginal epithelium. – *Biology of Reproduction*, 21(4): 953-963.

Shille, V., Munro, C., Farmer, S., Papkoff, H., Stabenfeldt, G. (1983). Ovarian and endocrine responses in the cat after coitus. – *Journal of Reproduction and Fertility*, 69: 29–39.

Silva, A., Moreira, N., Pereira, A., Peixoto, G., Maia, K., Campos, L., Borges, A. (2017). Estrus Cycle Monitoring in Wild Mammals: Challenges and Perspectives. – *Theriogenology*, 21-45.

Silva, J., Leitao, R., Lapao, N., da Cunha, M., da Cunha, T., da Silva, J., *et al.* (2000). Birth of Siberian tiger (*Panthera tigris altaica*) cubs after transvaginal artificial insemination. – *Journal of Zoo and Wildlife Medicine*, 31(4): 566-570.

Smith, Y., De Waal, H., Kok, O. (2006). Aspects of carcass digestibility by African lions (*Panthera leo* Linnaeus, 1758) under captive conditions. – *Pakistan Journal of Biological Science*, 9: 2149-2152.

Smuts, G., Hanks, J., Whyte, I. (1978). Reproduction and social organization of lions from the Kruger National Park. – *Carnivore*, 1: 17–28.

Smuts, G., Robinson, G., Whyte, I. (1980). Comparative growth of wild male and female lions (*Panthera leo*). – *Journal of Zoology*, 190: 365–373.

Stander-Breedt, H., Schwalbach, L., Geyling, J., Loskutoff, N. (2004). Effect of different cryodiluents and thawing methods on the post-thaw motility of African lion (*Panthera leo*) spermatozoa. – *South African Journal of Animal Science*, 34(2): 74-76.

Stanton, L., Sullivan, M., Fazio, J. (2015). A standardized ethogram for the Felidae: a tool for behavioral researchers. – *Applied Animal Behaviour Science*, 173: 3-16.

Stein, A., Athreya, V., Gerngross, P., Balme, G., Henschel, P., Karanth, U. *et al.* (2016). *Panthera pardus*. *The IUCN Red List of Threatened Species 2016*: e.T15954A102421779. Downloaded on 21 March 2017.

Stewart, D., Stabenfeldt, G. (1985). Relaxin activity in the pregnant cat. – *Biology of Reproduction*, 32: 848–854.

Steyn, T. (1951). The breeding of lions in captivity. – *Fauna and Flora*, 2: 37–55.

Swanson, W., Howard, J., Roth, T., Brown, J., Alvarado, T., Burton, M., *et al*. (1996). Responsiveness of ovaries to exogenous gonadotrophins and laparoscopic artificial insemination with frozen–thawed spermatozoa in ocelots (*Felis pardalis*). – *Reproduction*, 106(1): 87-94.

Swanson, W., Horohov, D., Godke, R. (1995). Production of exogenous gonadotrophin-neutralizing immunoglobulins in cats after repeated eCG-hCG treatment and relevance for assisted reproduction in felids. – *Reproduction*, 105(1): 35-41.

Swanson, W., Bond, J., Steinetz, B., McRae, M. (2001). Fetal and neonatal development of domestic cats produced from in vitro fertilization and laparoscopic oviductal embryo transfer versus natural mating. – *Theriogenology*, 55: 371.

Swanson, W. (2003). Research in nondomestic species: experiences in reproductive physiology research for conservation of endangered felids. – *ILAR journal*, *44*(4): 307-316.

Swanson, W. (2006). Application of assisted reproduction for population management in felids: the potential and reality for conservation of small cats. – *Theriogenology*, 66: 49–58.

Swanson, W., Stoops, M., Magarey, G., Herrick, J. (2007). Sperm cryopreservation in endangered felids: developing linkage of in situ-ex situ populations. – *Society of Reproduction and Fertility*. Suppl, 65: 417-432.

Swanson, W. (2012). Laparoscopic oviductal embryo transfer and artificial insemination in felids–challenges, strategies and successes. – *Reproduction in Domestic Animals*, 47: 136-140.

Swanson, W. (2019). Practical application of laparoscopic oviductal artificial insemination for the propagation of domestic cats and wild felids. – *Reproduction, Fertility and Development*, 31(1): 27-39.

Tanaka, A., Takagi, Y., Nakagawa, K., Fujimoto, Y., Hori, T., Tsutsui, T. (2000). Artificial intravaginal insemination using fresh semen in cats. – *Journal of Veterinary Medicine and Science*, 62: 1163–1167.

Terio, K., Marker, L., Munson, L. (2004). Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx Jubatus*) based on adrenal morphology and function. – *Journal of Wildlife Diseases*, 40 (2): 259-266.

Tipkantha, W., Thuwanut, P., Maikeaw, U., Thongphakdee, A., Yapila, S., Kamolnorranath, S., *et al*. (2017). Successful laparoscopic oviductal artificial insemination in the clouded leopard (*Neofelis nebulosa*) in Thailand. – *Journal of Zoo and Wildlife Medicine*, 48(3): 804-812.

Thongphakdee, A., Tipkantha, W., Punkong, C., Chatdarong, K. (2018). Monitoring and controlling ovarian activity in wild felids. *Theriogenology*, 109: 14-21.

Touma, C., Palme, R. (2005). Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. – *Annals of the New York Academy Science*, 1046 (1): 54-74.

Tsutsui, T., Stabenfeldt, G. (1993) Biology of ovarian cycles, pregnancy and pseudopregnancy in the domestic cat. – *Journal of Reproduction and Fertility*, Suppl. 47: 29–35.

Tsutsui, T., Tanaka, A., Takagi, Y., Nakagawa, K., Fujimoto, Y., Murai, M., *et al*. (2000a). Unilateral intrauterine horn insemination of fresh semen in cats. – *Journal of Zoo and Wildlife Medicine*, 62: 1241–1245.

Tsutsui, T., Tanaka, A., Takagi, Y., Nakagawa, K., Fujimoto, Y., Murai, M., *et al*. (2000b). Unilateral intrauterine horn insemination of frozen semen in cats. – *Journal of Veterinary Medical Science*, 62:1247-1251.

Tsutsui, T., Nagakubo, K., Hori, T. (2004). Relationship between the sperm count and the fertilization rate of ova ovulated from the contralateral ovary in intrauterine horn insemination in cats. – *Journal of Veterinary Medical Scienc*e, 66: 1143–1145.

The IUCN red list of threatened species. URL: https://www.iucnredlist.org. Last accessed: April 2019.

Valdespino, C., Asa, C., Bauman, J. (2002). Estrous cycles, copulation, and pregnancy in the fennec fox (*Vulpes zerda*). – *Journal of Mammalogy*, 83(1): 99-109.

Valle, G., Toledo, J., De Figueiredo, C. (2006). Detection of *Simonsiella* species in the vagina of a bitch in heat. – *Veterinary Record*, 159:156-157.

van der Merwe, P., Saayman, M., Els, J., Saayman, A. (2017). The economic significance of lion breeding operations in the South African Wildlife Industry. – *International Journal of Biodiversity and Conservation*, 9(11): 314-322.

van Dorsser, F., Green, D., Holt, W., Pickard, A. (2007). Ovarian activity in Arabian leopards (*Panthera pardus nimr*): sexual behaviour and faecal steroid monitoring during the follicular cycle, mating and pregnancy. – *Reproduction, Fertility and Development,* 19(7): 822-830.

Verhage, H., Beamer, N., Brenner, R. (1976). Plasma levels of estradiol and progesterone in the cat during polyestrus, pregnancy and pseudopregnancy. – *Biology of Reproduction*, 14(5): 579-585.

von Heimendahl, A., England, G. (2010). Determining breeding status. In: BSAVA manual of canine and feline reproduction and neonatology. 2nd ed. Ed: England, G., Heimendahl, A. – *British Small Animal Veterinary Association*. Pp. 44-50.

Walker, S., Waddell, W., Goodrowe, K. (2002). Reproductive endocrine patterns in captive female and male red wolves (*Canis rufus*) assessed by fecal and serum hormone analysis. – *Zoo Biology*, 21(4): 321-335.

Ward, S., Melfi, V. (2013). The implications of husbandry training on zoo animal response rates. – *Applied Animal Behaviour Science*, 147(1-2): 179-185.

Webster, A., Burroughs, R., Laver, P., Ganswindt, A. (2018). Non-invasive assessment of adrenocortical activity as a measure of stress in leopards *Panthera pardus*. – *Journal of African Zoology*, 53(2): 53-60.

Weinhardt, D., Rodden, M. (1995). Management of reproduction. In: Husbandry Manual for the Maned Wolf (*Chrysocyon brachyurus*). Eds: Fletchall, N., Rodden, M., Taylor, S. Washington. Pp. 29-31.

Wielebnowski, N., Watters, J. (2007). Applying fecal endocrine monitoring to conservation and behavior studies of wild mammals: important considerations and preliminary tests. – *Israel Journal of Ecology and Evolution*, 53(3-4): 439-460.

Westlund, K. (2014). Training is enrichment—and beyond. – *Applied Animal Behaviour Science,* 152: 1-6.

Wildt, D., Platz, C., Seager, S., Bush, M. (1982). Induction of ovarian activity in the cheetah (*Acinonyx jubatus*). – *Biology of Reproduction*, 24: 217–222.

Wildt, D., Schiewe, M., Schmidt, P., Goodrowe, K., Howard, J., Phillips, L., *et al*. (1986). Developing animal model systems for embryo technologies in rare and endangered wildlife. – *Theriogenology,* 25: 33–51.

Wildt, D., Bush, M., Goodrowe, K., Packer, C., Pusey, A., Brown, J., *et al.* (1987). Reproductive and genetic consequences of founding isolated lion populations. – *Nature*, 329: 328–331.

Wildt, D., Brown, J., Swanson, W. (1998). Reproduction in cats. In: Encyclopedia of Reproduction. 1st ed. Ed: Knobil, E., Neill, J. – *Academic Press, New York*. Pp. 497–510.

Zambelli, D., Cunto, M. (2005). Transcervical artificial insemination in the cat. – *Theriogenology*, 64: 698–705.

Zambelli, D., Cunto, M. (2005). Vaginal and cervical modifications during the estrus cycle in the domestic cat. – *Theriogenology*, 64(3): 679-684.

Zambelli, D., Prati, F., Cunto, M., Iacono, E., Merlo, B. (2008). Quality and in vitro fertilizing ability of cryopreserved cat spermatozoa obtained by urethral catheterization after medetomidine administration. – *Theriogenology*, 69: 485–490.

Zambelli, S., Bini, C., Cunto, M. (2015). Endoscopic transcervical catheterization in the domestic cat. – *Reproduction in Domestic Animals*, 50: 13-16.

Zelinski-Wooten, M., Lanzendorf, S., Wolf, D., Chandrasekher, Y., Stouffer, R. (1991). Titrating luteinizing hormone surge requirements for ovulatory changes in primate follicles. I. Oocyte maturation and corpus luteum function. – *The Journal of Clinical Endocrinology and Metabolism*, 73(3): 577-583.

ANNEXURES

ANNEXURE A – ANIMAL ETHICS COMMITTEE APPROVAL

Animal Ethics Committee

PROJECT TITLE	**Implementing Assisted Reproduction Techniques into Large Felids Conservation: Study of the female African lion *(Panthera leo)* reproductive physiology, and development of artificial insemination protocols**
PROJECT NUMBER	**V052-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Ms. IC Rodriguez**

STUDENT NUMBER (where applicable)	**U_17403074**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Lions *(Panthera leo)***	
NUMBER OF SAMPLES	**22**	
Approval period to use animals for research/testing purposes		**June 2017- June 2018**
SUPERVISOR	**Prof. A Ganswindt**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 24 July 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

ANNEXURE B – ANIMAL ETHICS COMMITTEE APPROVAL (Cont.)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 1

PROJECT TITLE	**Implementing Assisted Reproduction Techniques into Large Felids Conservation: Study of the female African lion *(Panthera leo)* reproductive physiology, and development of artificial insemination protocols**
PROJECT NUMBER	**V052-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Ms. IC Rodriguez**

STUDENT NUMBER (where applicable)	**U_17403074**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Lions *(Panthera leo)***	
NUMBER OF SAMPLES	**22**	
Approval period to use animals for research/testing purposes		**January 2018 – January 2019**
SUPERVISOR	**Prof. A Ganswindt**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 16 December 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

ANNEXURE C – PUBLICATIONS ARISEN FROM THIS PHD THESIS

Callealta, I., Ganswindt, A., Gonçalves, S., Mathew, A., Lueders, I. (2018). Detection of *Simonsiella* spp. in the vagina of lions and leopard in oestrus. – *Reproduction of Domestic Animals*, 53(6): 1605-1608. (Attached)

Callealta, I., Ganswindt, A., Malan, M., Lueders, I. (2019). Non-surgical artificial insemination using a GnRH analogue for ovulation induction during natural oestrus in African lions (Panthera leo). – *Theriogenology*, 139: 28-35. (Attached)

Callealta, I., Ganswindt, A., Lueders, I. (2020). Reproductive cycle stage assessment using vaginal cytology evaluation in African lions (*Panthera leo*). – *Animal Reproduction Science*, 213, 106260. (Attached)

Callealta, I., Lueders, I., Luther, I., Ganswindt, A. (2019). Positive reinforcement conditioning as a tool for frequent minimally-invasive blood and vaginal swab sampling in African lions (*Panthera leo*). – *Journal of Applied Animal Welfare Science*, 1-12. (Attached)