Endometrial glandular changes in aged captive cheetahs (*Acinonyx jubatus*) in southern Africa

by

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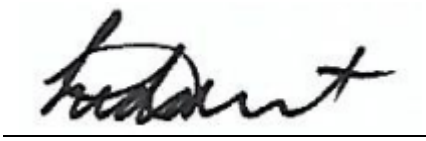
DECLARATION

I declare that this mini-dissertation hereby submitted to the University of Pretoria for the degree of MMedVet(Pathology) has not been previously submitted by me or anyone for a degree at this or any other University, that it is my own work in design and in execution, and that all material contained herein has been duly cited.



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26/04/2021

Date

ETHICS STATEMENT

I, Alida Avenant, have obtained the necessary research ethics approval (V074-18) for the research described in this dissertation, I declare that I have observed the ethical standards required in terms of the University of Pretoria's code of ethics for researchers and the policy guidelines for responsible research. (See Appendix 1 - Proof of ethical clearance)

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ABBREVIATIONS

ANOVA	Analysis of variance
CEH	Cystic endometrial hyperplasia
EGF	Epidermal growth factor
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
H&E	Haematoxylin and Eosin
IUCN	International Union for Conservation of Nature
IQR	Interquartile range
LH	Luteinising hormone
MGA	Melengesterol acetate
µm	Micrometer
mm	Millimeter
O	Referring to sample collected at ovariectomy
P	Referring to sample collected at post mortem
ROI	Region of interest
S	Referring to sample collected one year post-salpingectomy

Endo/myo ratio	The ratio of the average endometrial- to myometrial width per sample
Gland lumen area %	The fraction/percentage of the endometrial area occupied by gland lumens

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ABSTRACT

Endometrial glandular changes in aged captive cheetahs (Acinonyx *jubatus*) in southern Africa

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The cheetah, *Acinonyx jubatus*, is classified as “vulnerable” on the IUCN red list of threatened species. With declining population numbers in the wild, conservation efforts have focused on captive breeding and investigating the long-term effects of reproduction control strategies. Cheetahs in captivity survive longer than their wild counterparts and are purportedly more predisposed to conditions such as endometrial hyperplasia, cystic endometrial glandular changes, endometritis and pyometra. Uterine samples were collected from 21 captive aged cheetahs (mean and median age of 10 years) from three research conservation organisations in southern Africa. Endometrial changes from cheetahs sampled one year post-laparoscopic salpingectomy (n=7) were compared with samples collected at ovariectomy (n=7) or post mortem (n=7). Histological examination showed minimal endometrial pathology with only mild glandular ectasia (calculated as gland lumen area %) in most cases. This contrasts with previous studies in cheetahs, and other domestic and wild felids and canids where severe cystic and/or hyperplastic endometrial glandular changes were frequently reported. Increased age was positively correlated with increased gland lumen area (% and median) and endometrial area. There were no differences in endometrial changes between the post-salpingectomy and other samples. No notable endometrial changes were observed in the 10 cheetahs previously implanted with deslorelin as a contraceptive. It is possible that the observed mild endometrial changes are within the realm of physiological normality and not representative of overt pathology. This requires further investigation before new grading systems are developed to evaluate endometrial glandular pathology in cheetahs.

1. INTRODUCTION

The cheetah, *Acinonyx jubatus*, is classified as “vulnerable” on the International Union for Conservation of Nature (IUCN) red list of threatened species. The global adult cheetah population is estimated to be around 6700, of which 4190 are found in southern Africa (Durant et al., 2015). The global captive cheetah population is estimated to be 1600 (Jago, 2014) and numerous population control and breeding strategies have been developed for these populations, including artificial insemination, in vitro fertilisation, contraception with e.g. GnRH analogues or progestin-based contraceptives, separation of male and female animals and permanent surgical sterilisation (Munson et al., 2002; Cocchia et al., 2015; Hartman et al., 2015). Long term effects of these reproductive control strategies on the animals are of increasing interest as Namibian legislation now requires the permanent sterilisation of all captive large female carnivores, including cheetahs (Ministry of Environment and Tourism, 2012). However controversial, this legislation has led to scientific evaluation of the female reproductive tract (Schulman et al., 2015; Penfold, Soley & Hartman, 2019; Penfold et al., 2020) and evaluation of various methods of laparoscopic sterilisation (Hartman et al., 2015) in the southern African cheetah population.

Histopathology of the endometrium provides a tool to assess the effects of reproduction management strategies on the uterus and effects of these on subsequent fertility (Munson et al., 2002; Moresco, Munson & Gardner, 2009; Asa et al., 2013). The importance of histological evaluation of the endometrium is supported by the numerous grading systems that have been developed in diverse species (Dow, 1959, 1962; Kenney & Doig, 1986; De Bosschere et al., 2001; Munson et al., 2002; Marsden & Hacker, 2003; Snider, Sepoy & Holyoak, 2011; Sivridis & Giatromanolaki, 2013; Emons et al., 2015; Binder et al., 2019). Many of these systems focus on specific conditions of the endometrium, including cystic endometrial hyperplasia.

Several factors that may affect the presence and severity of endometrial pathology in the cheetah have been investigated in North American captive cheetah populations (Munson et al., 2002; Crosier et al., 2011), however, to date, no studies have been conducted on the southern African captive cheetah population. The potential long-term

effects of salpingectomy as a method of permanent sterilisation on the feline uterus have also never been evaluated. This study provided an opportunity to characterise endometrial changes in southern African captive cheetah populations, to investigate the effect of salpingectomy on the endometrium, and to identify potential factors associated with these changes.

2. LITERATURE REVIEW

2.1 The feline oestrous cycle

Cats are seasonally polyoestrus. The normal oestrous cycle in felids consists of four phases, namely pro-oestrus, oestrus, dioestrus and anoestrus. During pro-oestrus, oestrogen levels rise in accordance with the cyclical development of follicles on the ovaries. In oestrus, follicle development and oestrogen levels peak. Oestrogen functions to enhance the uterine responses to oestrogen and progesterone by increasing the number of receptors for both these hormones in the uterus (von Reitzenstein, Archbald & Newell, 2000; Agudelo, 2005; Pires et al., 2016). Oestrogen also causes dilation of the cervix which may increase the risks for ascending bacterial infections from normal vaginal flora (von Reitzenstein, Archbald & Newell, 2000; Agudelo, 2005; Root Kustritz, 2005).

Felids are generally considered to be induced ovulators (Agudelo, 2005; Root Kustritz, 2005; Pires et al., 2016) and coitus is required to stimulate events resulting in eventual ovulation. Neuronal stimulation of the medial basal hypothalamus induced by coitus results in the release of gonadotropin-releasing hormone (GnRH), which is transported to the adenohypophysis. This stimulates the release of luteinising hormone (LH) and follicle stimulating hormone (FSH) from the hypophysis, which in turn stimulates follicular development, oocyte maturation and oestrogen production in the ovaries. This cascade of events leads to eventual ovulation (Troedsson & Madill, 2004; Brown, 2006). However, spontaneous ovulation has been reported to occur in about 30-40% of queens as a result of tactile, visual or pheromonal stimulation, stress or even with increased weight (Lawler et al., 1993; Root Kustritz, 2005; Pires et al., 2016; Binder et al., 2019).

The cheetah has also been classified as an “induced ovulator” (Bertschinger et al., 1984; Wildt et al., 1993), but numerous reports of spontaneous ovulation exist, albeit at low incidence (Wildt et al., 1993; Brown et al., 1996; Wielebnowski et al., 2002; Terio et al., 2003). It is believed that the presence of male cheetahs in auditory and-/or olfactory range or other psychosocial stimuli (Brown et al., 1996; Wielebnowski et al., 2002; Terio et al., 2003) may be responsible.

Dioestrus follows ovulation, where the presence of corpora lutea lead to high levels of progesterone, which suppresses further release of LH from the hypophysis (negative feedback) (Troedsson & Madill, 2004). Progesterone is associated with hyperplasia of endometrial glands and the endometrial epithelium, reduced contractility of the myometrium, increased secretion by endometrial glands and closure of the cervix (von Reitzenstein, Archbald & Newell, 2000; Pires et al., 2016). Anoestrus occurs between waves of follicular development and follows oestrus when ovulation does not occur. Cheetahs cycle throughout the year, with cycles averaging 13 days (Brown et al., 1996; Terio et al., 2003).

2.2 Physiological cyclical changes to the uterus

The mammalian uterus undergoes several architectural changes during the oestrous cycle. These changes are similar in the dog, cat and horse (Barrau et al., 1975; Kenney, 1978; Pineda, 2003a,b). As oestrogen levels rise during pro-oestrus and oestrus the uterine wall thickness increases due to epithelial hyperplasia and hypertrophy, stromal oedema and hyperaemia in the endometrium (Barrau et al., 1975; Pineda, 2003a). Glandular crypts increase in size and mucous differentiation of glandular epithelium take place during this stage. The luminal epithelium is low to medium columnar, reaching maximum height during oestrus (Barrau et al., 1975; Kenney, 1978; Pineda, 2003b). This results in a high endometrium to myometrium (endo/myo) ratio.

After ovulation, under the influence of progesterone, there is marked hyperplasia and hypertrophy of the endometrial glandular epithelium, with increased branching and coiling of the glands and the luminal epithelium can be low to tall columnar (Barrau et al., 1975; Kenney, 1978; Pineda, 2003a,b). The endo/myo ratio decreases significantly during oestrus due to endometrial and myometrial hypertrophy, and then increases slightly during dioestrus (De Bosschere et al., 2001).

Late dioestrus and anoestrus are characterised by a decreased uterine wall thickness, with cuboidal luminal epithelium and straight glands lined by low columnar to cuboidal epithelium (Barrau et al., 1975; De Bosschere et al., 2001; Pineda, 2003b). This

reduction in epithelial cell size during anoestrus occurs more rapidly than that of the associated glandular lumens, and may lead to a cystic appearance of the glands (De Bosschere et al., 2001).

2.3 Endometrial pathology

2.3.1 Terminology

The definitions associated with histological changes in endometrial glandular lumen area are largely unclear and even differ among authors (Chatdarong et al., 2005). This complicates the choice of nomenclatures to apply in different situations. In general, according to the Saunders Comprehensive Veterinary Dictionary the term “cyst” refers to a semisolid material- or fluid-filled cavity lined by epithelium (Blood, Studdert & Gay, 2007). “Dilation” and “dilatation” is used interchangeably (Mahroo, Shalchi & Hammond, 2014) and refers to a tubular structure (e.g. an endometrial gland) expanding beyond it’s normal extent, while the term “ectasia” can be applied to any dilation, expansion or distention (Blood, Studdert & Gay, 2007). The terminology can create confusion when applied to varying endometrial gland lumen size or area and there are no clear histological characteristics that define thresholds, similarities or differences (if any) between these terms or specifies when changes are considered either physiological or pathological.

2.3.2 Pathogenesis

Apart from physiological hormonal changes to the endometrium, various pathological changes are also characterised histologically. Some pathological changes in the endometrium are thought to be under hormonal influence and the normal oestrous cycle may play a role in their development. Endometrial hyperplasia, with associated changes to the endometrial glands (cystic endometrial hyperplasia/CEH) is commonly reported in domestic and wild canids and felids (Feldman & Nelson, 2004a; Agudelo, 2005; Chatdarong et al., 2005; Schlafer & Gifford, 2008; Keskin et al., 2009; Moresco, Munson & Gardner, 2009). The risk of developing CEH in domestic and wild felids appears to increase with age (Perez et al., 1999; Munson et al., 2002; Agudelo, 2005; Crosier et al., 2011; Binder et al., 2019). Even though the pathogenesis, severity and significance of CEH remains incompletely understood in many species, including the

cheetah, both progesterone and oestrogen have been implicated to varying degrees (De Bosschere et al., 2001; Feldman & Nelson, 2004b; Agudelo, 2005; Binder et al., 2019).

Progesterone plays an important role in increasing secretory activity of endometrial glands and suppression of local uterine immunity (von Reitzenstein, Archbald & Newell, 2000; Pires et al., 2016), especially after oestrogen priming (Asa et al., 2013), increasing the risk for developing uterine infections and pathology later in life. Excessive glandular secretion by proliferating epithelium may result in fluid accumulation in endometrial gland lumens, resulting in cystic changes (Kenney, 1978; Feldman & Nelson, 2004b).

Prolonged high levels of oestrogen may also be associated with hyperplastic changes (Schlafer & Foster, 2016). Proliferative and hyperplastic changes seen in older animals may be the result of oestrogen increasing the levels of the epidermal growth factor (EGF) receptors and subsequent EGF binding in the uterus. EGF also mimics the effects of oestrogen, leading to cellular proliferation in the endometrium (Mukku & Stancel, 1985; Ignar-Trowbridge et al., 1995). A study by Potter, et al. (1991) in 79 cats suggested that oestrogen stimulation may play a more important role in endometrial hyperplasia (than progesterone), because of the absence of corpora lutea in queens that showed histological signs of endometrial hyperplasia. Munson, et al. (2002) hypothesised that glandular cyst formation in zoo felids was the result of excessive epithelial hyperplasia in gland necks. The use of an exogenous progestin, melengestrol acetate (MGA) as a contraceptive has also been associated with increased cystic hyperplastic changes (Munson et al., 2002). These conflicting reports support the further investigation of the pathogenesis of cystic endometrial gland changes in felids.

Although cystic changes are mostly reported in association with endometrial hyperplasia (CEH), some authors distinguish between “cystic endometrial hyperplasia” and “endometrial cysts”. Munson et al. (2002) differentiated these pathologies based on evidence of glandular epithelial hyperplasia, together with multiple cystic glands in CEH, but did not describe characteristics associated with endometrial cysts. This suggests that cystic changes in the endometrium may not only

be associated with endometrial hyperplasia. In equines, cystic dilation of endometrial glands is thought to be secondary to lamellated periglandular fibrosis (Kenney, 1978; Hoffmann et al., 2009), where myofibroblastic differentiation is present in periglandular fibrous tissue that causes reduced outflow, with subsequent dilatation of glands (Hoffmann et al., 2009). This has, however, not been reported in canids or felids and further investigation into the development of endometrial cysts is indicated.

2.3.3 Histological grading systems:

Numerous endometrial grading systems have been developed in various species. These systems have been developed based on histomorphology alone, or in combination with reproductive performance.

2.3.3.1 Equines

In equines, the most extensively used classification system is based mainly on the degree of fibrosis and inflammation in the endometrium (Kenney & Doig, 1986; Snider, Sepoy & Holyoak, 2011). This classification correlates with the ability of the mare to carry a foal to term (Snider, Sepoy & Holyoak, 2011).

2.3.3.2 Domestic canids and felids

Grading systems in domestic dogs and cats were first published in 1959 and 1962, respectively, developed specifically for the cystic endometrial hyperplasia-pyometra complex. In dogs the classification was based on clinical, macroscopic and microscopic findings (Dow, 1959). Similarly in cats, severity of disease was based on reproductive status and macroscopic and histologic morphology of the endometrium (Dow, 1962). De Bosschere, *et al*. (2001) proposed a new histological classification in dogs, which categorised cystic endometrial hyperplasia and pyometra as two separate entities, which may occur independently. This classification evaluated the inflammatory reaction in the endometrium and myometrium, the percentage of the endometrium occupied by gland lumens, presence, number and size of endometrial cysts, the ratio of endometrial- to myometrial width and the presence and degree of fibroblast proliferation (De Bosschere et al., 2001).

2.3.3.3 Wild canids and felids

Munson *et al.* (2002) developed a grading system for proliferative and cystic changes of the endometrial glands in wild felids. This system has been applied in studies with cheetahs (Crosier et al., 2011) and wild canids (Moresco, Munson & Gardner, 2009). The overall cumulative increase in endometrial thickness and glandular proliferative- or cystic changes were evaluated (Munson et al., 2002). Endometrial changes were graded as follows (Munson et al., 2002; Crosier et al., 2011): Grade 0 lesions showed no hyperplastic changes. In grade 1 lesions, mild cystic or proliferative changes were observed in the glands, but without evidence of increased endometrial width. In grade 2 lesions, the endometrium was less than or equal to twice the height of the normal endometrium and the glandular changes were moderate. Grade 3 lesions showed severe glandular changes as well as a more than two times increase in endometrial width. When there was variation in lesion severity within a uterus, the most severe lesions were used for grading. Several other pathological changes in the endometrium were also reported in the studies by Munson, *et al*. (2002) and Crosier *et al.* (2011) in wild felids, including endometrial cysts, endometrial fibrosis, hydrometra, pyometra, adenomyosis, endometritis, endometrial atrophy and endometrial polyps (Munson et al., 2002; Crosier et al., 2011).

2.4 Effects of contraception

Contraceptives have been used to control reproduction in captive wildlife for over 40 years (Asa, 1997). Contraception implies the prevention of, or disruption of pregnancy, while maintaining fertility (Munson, 2006). Exogenous hormones are applied to disrupt normal cyclicity by interfering with the normal hypothalamic-pituitary-gonadal axis, or to prevent specific hormone production (Munson, 2006). The two main contraceptives that have been reported in wild canid and felid females are the progestin-based contraceptives (e.g. MGA, megesterol acetate and medroxyprogesterone actetate) and GnRH analogues (e.g. deslorelin or leuprolide acetate) (Munson, 2006; Asa et al., 2013).

Progestin-based contraceptives are typically administered as implants, depot injections or orally in wild felids. They function by stimulating growth of the endometrium, increasing glandular secretions and relaxing the uterine smooth muscles. This most likely results in diminished oocyte transport or changes the uterine

environment, reducing receptivity and leading to failure of implantation (Munson, 2006). Various negative effects on the endometrium have been reported in wild carnivores that may result in irreversible infertility, including the development of severe cystic or glandular endometrial hyperplasia, hydrometra and mineralisation (Munson et al., 2002; Munson, 2006; Moresco, Munson & Gardner, 2009; Asa et al., 2013). They also predispose to the development of endometritis, pyometra and endometrial polyp formation (Munson et al., 2002; Pineda, 2003a). The progestin-based contraceptive MGA, which has been commonly used in wild felids (Munson, 2006), does not appear to affect ovarian follicular development, indicating that endogenous oestrogens and progesterone are still produced in these species (Kazensky, Munson & Seal, 1998). This likely exacerbates the negative effects on the endometrium caused by the contraceptive and may even play a more direct role in CEH development in the contracepted animals.

The GnRH analogues interfere with the hypothalamic-pituitary-gonadal axis by cancelling the function of normal endogenous GnRH. The GnRH analogue formulations are administered via depot injection or subcutaneous implant. After administration there is an initial surge of follicle stimulating hormone (FSH) and luteinising hormone (LH) secretion, stimulating ovulation. This is followed by six to twelve months (Asa, Boutelle & Bauman, 2012) of ovarian inactivity due to the slow-release effect of the implant or injectable contraceptive (Munson, 2006; Asa et al., 2013). Because GnRH analogues do not directly affect the endometrium or abnormally increase exposure to progesterone, no negative effects are expected. No evidence of long term side effects were observed in cheetahs (Bertschinger et al., 2002; Schulman et al., 2015), lions (Bertschinger et al., 2002, 2008), tigers (Bertschinger et al., 2008), wild dogs (Bertschinger et al., 2002) or domestic cats (Munson et al., 2001; Toydemir, Kiliçarslan & Olgaç, 2012) treated with deslorelin. However, Asa et al. (2013) reported negative effects on the endometrium that were similar to progestin-based contraceptives in wild canids.

The association of other risk factors (e.g. age, parity, etc.) with specific pathologies that have been reported with contraceptive use have not been widely investigated in wildlife (Asa et al., 2013). Notably however, separation of the sexes as an alternative

to contraceptives, did not alter the risk of developing uterine pathology compared to either progestin-based- or GnRH analogue contraceptives (Asa et al., 2013).

2.5 Effects of captivity on the reproductive tract

Captive cheetahs survive longer than their wild counterparts, living to an average age of 12 years in the North American population (Crosier et al., 2011). In the wild, female cheetahs live an average of 6 years (Marker et al., 2003; Crosier et al., 2011). Wild cheetahs usually have their first litter at around two years of age and then every two years thereafter, until death (Kelly et al., 1998; Marker et al., 2003). In captivity first litters are produced at an average age of two to five years and these populations include many nulliparous female cheetahs over eight years of age (Crosier et al., 2011; Hartman et al., 2015; Schulman et al., 2015).

The influence of age, years barren and parity on the reproductive tract have been investigated in many wildlife species (Agnew, Munson & Ramsay, 2004; Hermes et al., 2006; Napier et al., 2009; Crosier et al., 2011; Asa et al., 2013). Long periods without breeding are associated with increased prevalence of uterine pathology in various species (Napier et al., 2009; Asa et al., 2013). Asa, et al. (2013) found that frequent breeding had a protective effect against conditions like endometrial hyperplasia and pyometra in wild canids. This may be subsequent to progesterone levels dropping rapidly just before parturition and endometrial remodelling occurring after parturition, which effectively returns the endometrium back to the normal pre-gravid state (Asa et al., 2013).

3. MOTIVATION FOR THE STUDY

Hartman et al. (2015) investigated laparoscopic ovariectomy and salpingectomy as surgical techniques for permanent sterilisation in cheetahs at two Namibian cheetah conservation centres. In order to investigate any histological effects on the uterus subsequent to salpingectomy, uterine tips were obtained one year post-salpingectomy. During the initial study similar samples were also collected from the cheetahs undergoing ovariectomies. The original aim of this study was to adapt published methods of grading endometrial pathology on the uteri obtained from these cheetahs, and other uterine samples collected from cheetahs post mortem. However, a preliminary examination showed that the wild felid grading system described by Munson *et al.* (2002) and Crosier *et al.* (2011) lacked sufficient detail to define this study's aims . These publications used the overall cumulative increase in endometrial thickness as one of its main criteria to differentiate different grades of cystic and/or proliferative endometrial hyperplasia. The "normal" values with which the pathological findings were compared to determine a "cumulative increase" in endometrial thickness was determined in species of similar body size and stage of the oestrous cycle, using samples that showed no hyperplastic changes (Munson et al., 2002; Moresco, Munson & Gardner, 2009). Even at a preliminary glance, samples in our study showed that uterine sizes were too variable to apply overall cumulative increase in endometrial thickness, even in animals of similar body size and oestrous cycle stage.

4. PROBLEM STATEMENT

- As a vulnerable species globally, there has been increased focus on, and research into reproductive management strategies in the cheetah. In this context uterine- and, more specifically, endometrial histology is critically important to assist in the understanding of reproductive management and its effects. However, there are currently no objective histologic criteria with which to evaluate cheetah uteri. Therefore, the development of a morphometric matrix for this purpose is the logical next step.
- There is also limited data available on the association of age and deslorelin treatment with uterine and endometrial histomorpholgy, particularly from southern African cheetahs.

5. STUDY OBJECTIVES

- To develop a morphometric matrix to characterise histological uterine changes observed in captive southern African cheetahs and use it to:
    a. evaluate the effect of laparoscopic salpingectomy on uteri after one year, compared to similar samples collected at the time of ovariectomy and post mortem
    b. explore associations between uterine morphology and age and deslorelin treatment

6. BENEFITS

- Develop a morphometric matrix to characterise histological uterine changes observed in cheetahs.
- Determine whether salpingectomy results in uterine changes in the cheetah one year after laparoscopy.
- To contribute to the understanding of the effect of age and deslorelin treatment on uterine histomorphology by contributing data from southern African cheetahs.

7. MATERIALS AND METHODS

In this study a morphometric matrix was developed in order to quantify glandular changes observed in 21 nulliparous cheetah endometrial samples. Morphometric analysis was used to evaluate endometrial width and total endometrial area, as well as gland profile number, the area occupied by gland lumens and myometrial width in order to discover any potential relationship between these measurements, which could be used to grade endometrial glandular changes.

7.1 Study population and sample collection procedures

Uterine samples were collected from 21 nulliparous cheetahs (Table 1) originating from two different research conservation organisations in Namibia and one in South Africa during previous studies (Hartman et al., 2015; Penfold, Soley & Hartman, 2019). Hartman *et al.* (2015) studied two different laparoscopic sterilisation procedures in cheetahs, namely ovariectomy and salpingectomy. During their study, approximately 1 cm of the tip of the right uterine horn was collected at the time of bilateral ovariectomy (O) and the uterine tube was transected bilaterally in salpingectomy cases. One year after these laparoscopic procedures the right sided ovaries and uterine tips were removed from cheetahs that originally underwent salpingectomy (S). Seven uterine tip samples were selected from each of the O and S laparoscopy groups, eliminating samples with electrocautery artefacts as well as samples at the utero-tubal junction. An additional seven uteri were collected from non-sterilised cheetahs at post mortem (P) (Penfold, Soley & Hartman, 2019; Penfold et al., 2020) and samples approximately 1 cm from the uterine tip, distal to the utero-tubal junction, were selected. The post mortem uterine samples from the Penfold et al. (2020) study were utilised in this study, assessing only the samples from the uterine tip and not those obtained from other sites.

Contraceptive treatment history of all animals was recorded. The only contraceptive used was deslorelin implants (4.7 mg) (Suprelorin®, Virbac, Milperra, NSW, Australia).

Table 1. Individual animal data collected from the cheetah conservation organisations in Namibia (A and B) and South Africa (C)

Cheetah no.*	Origin (conservation centre)	Age (years)	Oestrous cycle stage [$]	Year of sample collection	Deslorelin treatment
O1	A	6	Anoestrus	2014	2010, 2012, 2013
O2	A	9	Anoestrus	2014	0
O3	A	11	Anoestrus	2014	2008, 2011, 2012
O4	A	14	Anoestrus	2014	0
O5	B	14	Pro-/oestrus	2014	0
O6	B	11	Pro-/oestrus	2014	0
O7	B	8	Pro-/oestrus	2014	0
S1	A	12	Anoestrus	2015	2012
S2	A	12	Anoestrus	2015	0
S3	A	11	Pro-/oestrus	2015	2003
S4	A	6	Anoestrus	2015	2012
S5	B	14	Anoestrus	2015	0
S6	B	10	Pro-/oestrus	2015	0
S7	B	10	Anoestrus	2015	0
P1	C	3	Unknown	2017	0
P2	A	10.5	Anoestrus	2012	2006
P3	A	10	Anoestrus	2012	2006, 2007
P4	A	7	Inter-/anoestrus	2012	0
P5	A	10	Anoestrus	2012	2007
P6	A	10	Anoestrus	2012	2006, 2007
P7	A	10.5	Anoestrus	2012	2006, 2007

*O = samples collected at ovariectomy

S = Samples collected one year post-salpingectomy

P = samples collected at post mortem

[$] The oestrous cycle stages at time of surgery for both ovariectomy and post-salpingectomy groups were determined using a combination of transabdominal ultrasonography and direct visualisation during laparoscopy supported by vaginal cytology and serum progesterone concentrations (Schulman et al., 2015). For cheetahs that presented for post-mortem, macroscopic measurements and histological evaluation of the ovaries determined cyclic activity and reproductive status (Penfold et al., 2020).

7.2 Sample processing

The selected formalin-fixed uterine tip samples were cut perpendicular to the long axis and cross sections placed in labelled tissue cassettes. Tissue cassettes were routinely processed, and haematoxylin and eosin (H&E) sections were prepared according to standard procedures used at the Section of Pathology, Department of Paraclinical Sciences, Faculty of Veterinary Sciences, University of Pretoria. Histopathology slides were randomly labelled with numbers in order to prevent reader bias.

7.3 Morphometric analysis

To quantify uterine and glandular changes observed, the H&E stained sections were photographed using an Olympus BX63 light microscope with the Olympus D72 camera, controlled by Olympus cellSens® Dimension imaging software (Olympus Life Sciences Solutions, Japan). Images were stitched together to obtain an image of the entire cross section of each section at 10x objective magnification. After the images were captured, Olympus cellSens imaging software was used to perform image measurements. A colour threshold range was selected to detect glandular lumens automatically within the selected endometrial area. The selected areas were checked manually, and areas not associated with glandular lumens were deleted. Any glandular lumens not detected by the imaging software, were manually selected to be included in the data obtained. The total endometrial area was automatically estimated by the software, and comprised the entire area occupied by the endometrium, including endometrial gland lumens, but excluding the uterine lumen (**Figure 1**A & D).

Endometrial glands are not always straight depending on the stage of the oestrous cycle and the angle at which the section was cut. Therefore, it was not possible to differentiate on cross-section between the cut profile of a single tortuous gland and multiple small glands or gland branches in the same area (Wang et al., 2007). It was impractical to quantify or identify individual glands and therefore gland profiles were counted (gland profile number), and the areas of each of these gland profile lumens were calculated by the software. The median area occupied by individual gland lumens (median gland lumen area) was determined manually for each case.

The sum of the lumen areas (total gland lumen area) was also calculated automatically (Figure 1B & D). This information was exported into an Excel spreadsheet for each case. The total endometrial area and the total gland lumen area were used to determine the fraction of the endometrial area occupied by gland lumens (gland lumen area %) (as reported by De Bosschere et al., 2001). The gland lumen area % was calculated in order to account for the large variation in endometrial area among the different samples.

Endometrial and myometrial width was also measured using Olympus cellSens software. Six to seven (depending on sample quality) radial measurements of the endometrium (from the luminal epithelium to the endometrium-myometrium junction) (Figure 1C) and myometrium (from the endometrium-myometrium junction to the serosal surface of the uterine wall) were taken for each case. The individual measurements were automatically recorded and exported to an Excel spreadsheet. The individual measurements were averaged and the endometrium-to-myometrium width ratio (endo/myo ratio) was calculated for each case.

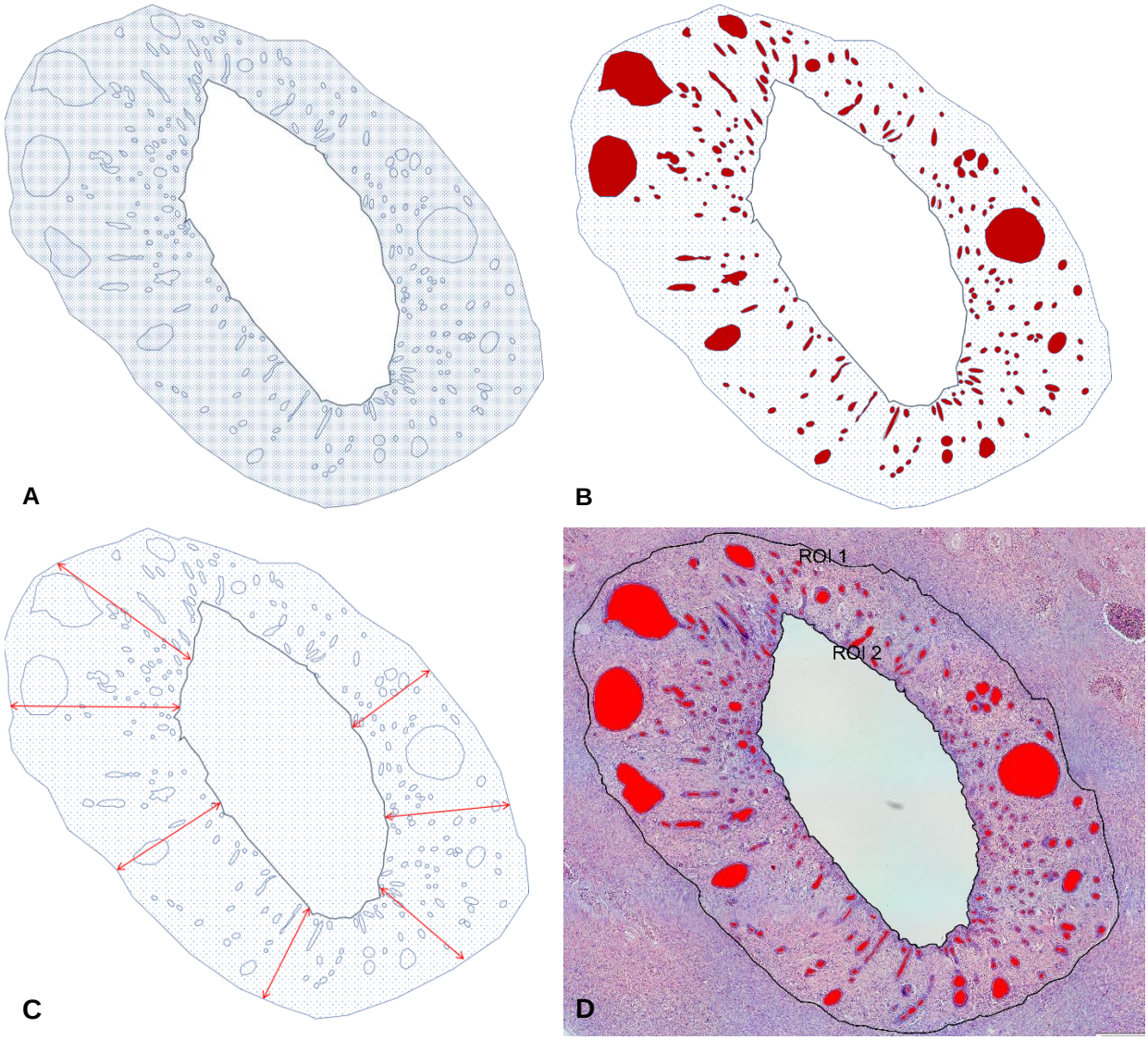

Figure 1. Diagrammatic representation of variables measured using Olympus cellSens® Dimension imaging software, based on cross sectional profile of sample S1: the total endometrial area (A), total gland lumen area (B) and measurements of endometrial width (C). Use of Olympus cellSens® Dimension imaging software on a stitched image of the endometrium of sample S1 to demonstrate the selected (red) gland lumen areas. Region of interest (ROI) 1 represents the endometrial area and ROI 2 represents the uterine lumen area (D).

7.4 Histopathology

Histological examination of all the samples were performed by the primary investigator in order to evaluate glandular and luminal epithelium, with specific attention to epithelial height (cuboidal, low columnar, high columnar) in both ectatic and normal glands. Glandular distribution and any additional changes in the glands and stroma were also noted.

7.5 Statistical analysis

The morphometric matrix was used to compare uterine morphology related to sampling group: ovariectomy (O), one year post-salpingectomy (S) and post mortem (P). The age, stage of the oestrous cycle and history of prior deslorelin contraceptive treatment of each individual cheetah were also correlated with the morphometric matrix. For each variable (age, total endometrial area, total gland lumen area, gland lumen area %, median gland lumen area, gland profile number, mean endometrial width, mean myometrial width and endo/myo ratio) a histogram was created to visualize the distribution of the variable. Correlations between the variables were assessed using Spearman's rank correlation. These were considered significant when $P<0.05$. Box plots were created to visually compare the distribution of each variable between animals in anoestrus and those in pro-oestrus/oestrus, between animals previously treated with deslorelin and those not, and between O, S and P uterine tip samples.

Differences in the medians of each variable between animals in anoestrus and those in pro-oestrus/oestrus, and between animals previously treated with deslorelin and those not treated, were assessed using a two-sample Wilcoxon rank-sum (Mann-Whitney) test. Differences between O, S and P samples were assessed using the Kruskal-Wallis one-way ANOVA (nonparametric version of ANOVA), with Dunn's multiple comparison test and the Bonferroni adjustment for multiple comparisons.

8. RESULTS

All the samples in this study originated from 21 nulliparous cheetahs of which 16 animals were nine years of age or older (post-prime). Only five of the animals showed evidence of follicular development at the time of sampling, while the remaining cheetahs were in anoestrus. Ten of the animals had been treated one to three times during their lifetime by implantation with deslorelin. Uterine tip samples were collected either at the time of ovariectomy (O) or one year after salpingectomy (S) or at post mortem (P).

8.1 Morphometric analysis

The frequency of the range of observations for each of the measured morphometric variables is illustrated in Figure 2. The mean endometrial width ranged from 0.5 mm to 1.4 mm (Figure 2A) and the total endometrial area varied between 1.2 mm^2 and 10.1 mm^2 (Figure 2B). Total gland lumen area varied between 0.0009 mm^2 and 0.5 mm^2 in 95% (20/21) of the animals (Figure 2C); and the fraction of the endometrium comprising glandular lumens (gland lumen area%) in most of the cases (19/21) was between 0.4 - 4.8% (Figure 2D). The median gland lumen area per case were between 22-261 μm^2 (0.00002-0.0003 mm^2) in 19/21 of the cheetahs (Figure 2E). Gland profile numbers varied between 121-904 per case (Figure 2F). The mean myometrial width ranged from 0.6 mm to 1.5 mm in 19/21 of the cases (Figure 2G). The endometrium-to-myometrium width ratio (endo/myo ratio) varied between 0.4 and 1.5 (Figure 2H).

Individual measurements from six cheetahs were outliers. The total gland lumen area in cheetah S2 was considerably higher than the other cheetahs (1.4 mm^2, outlier Figure 2C). Cheetah S1 (Figure 1D) and S2 (Figure 6C) had the highest gland lumen area % measurements (8.9% and 21.4% respectively, outliers Figure 2D). Both cheetahs were 12 years of age and in anoestrus. Cheetah S1 had a history of being treated with deslorelin once (three years prior to sampling), while S2 was never treated with deslorelin. Cheetah O4 had the highest mean endometrial width (1.41 mm, outlier Figure 2A), number of endometrial gland profiles (904, outlier Figure 2F) and endo/myo ratio (1.5, outlier Figure 2H). The second-highest endometrial width (1.36 mm) value was observed in cheetah O2. These cheetahs were 14 and 9 years old respectively, in anoestrus and with no history of deslorelin treatment. The highest

mean myometrial width (2.1 mm, outlier Figure 2G) and second highest median gland lumen area (484 µm2/0.0005 mm^2, outlier Figure 2E) were observed in cheetah S5, a 14-year old animal in anoestrus, with no history of deslorelin treatment. Cheetah O6, an 11-year old animal in pro-/oestrus with no history of previous deslorelin treatments had the highest median gland lumen area (517 µm2/0.0005 mm2, outlier Figure 2E).

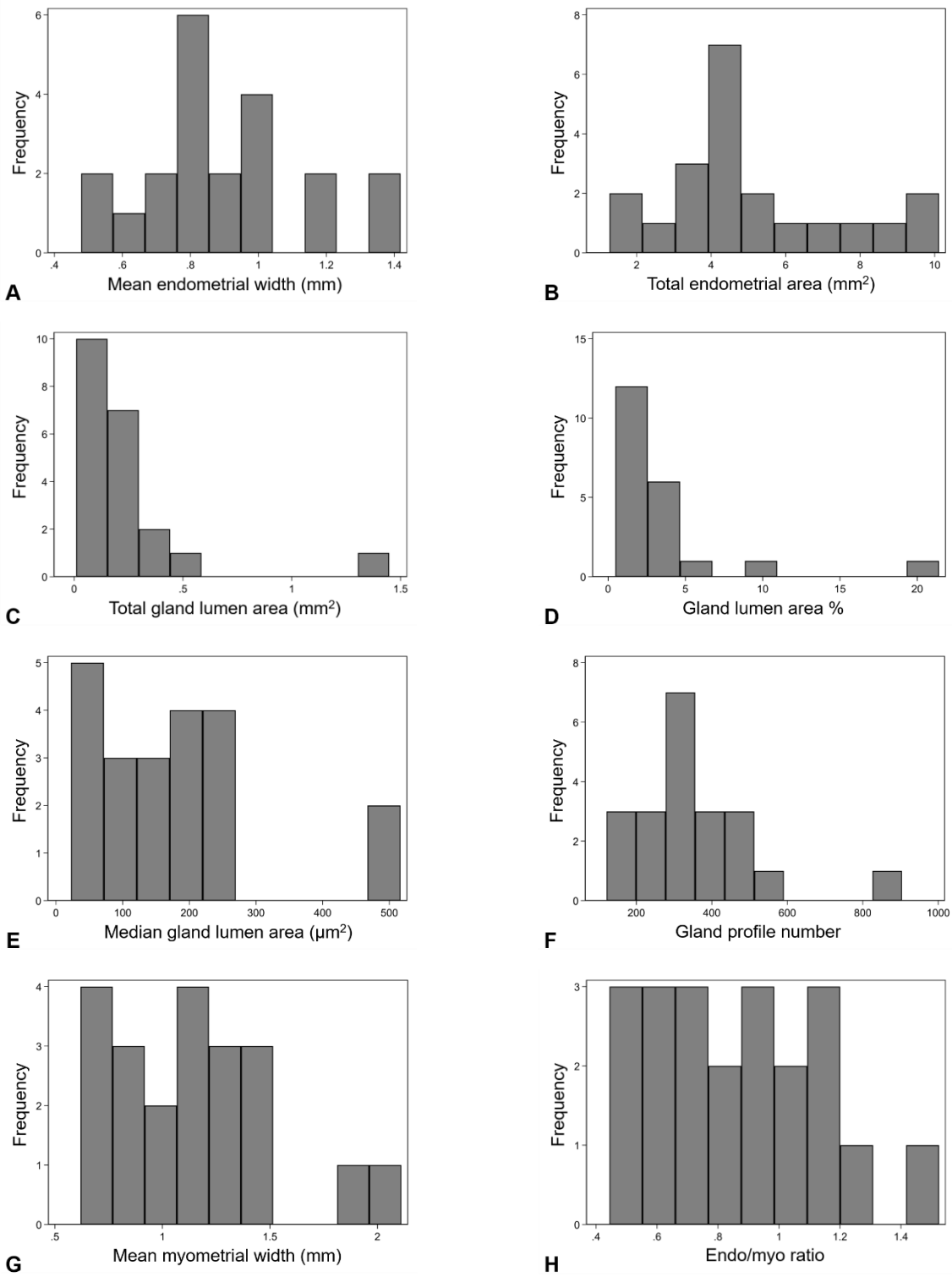

Figure 2. Frequency distribution of range of observations for mean endometrial width (A), total endometrial area (B), total gland lumen area (C), gland lumen area % (D), median gland lumen area (E), gland profile number (F), mean myometrial width (G) and the endo/myo ratio (H).

8.1.1 Morphometric matrix data

Table 2 illustrates the correlations between the variables. Correlations were considered significant when $P<0.05$. Endometrial area was positively correlated with mean endometrial width, gland lumen area %, median gland lumen area and mean myometrial width. Gland lumen area % was also positively correlated with median gland lumen area and mean myometrial width. Mean endometrial width and mean myometrial width were also both correlated with median gland lumen area, as well as with each other. Positive correlations were also observed between endo/myo width ratio and gland profile number. Median gland lumen area and mean myometrial width were negatively correlated with the endo/myo ratio.

Table 2. Spearman's rank correlation coefficient (rho) and significance level of each correlation between variables

	Age	Deslorelin treatment	Mean endometrial width	Total endometrial area	Gland lumen area %	Median gland lumen area	Gland profile number	Mean myometrial width	Endo/myo ratio
Age	1.0000								
Deslorelin treatment	-0.0875	1.0000							
	0.7061								
Mean endometrial width	0.2112	-0.6298	1.0000						
	0.3580	**0.0022**							
Total endometrial area	0.4434	-0.5196	0.8727	1.0000					
	0.0441	**0.0158**	**<0.0001**						
Gland lumen area %	0.5799	-0.2047	0.3390	0.5299	1.0000				
	0.0059	0.3734	0.1328	**0.0135**					
Median gland lumen area	0.5412	-0.4409	0.5494	0.6013	0.6351	1.0000			
	0.0113	**0.0454**	**0.0099**	**0.0039**	**0.0020**				
Gland profile number	-0.1263	-0.1260	0.3124	0.3917	-0.0188	-0.2930	1.0000		
	0.5853	0.5863	0.1679	0.0791	0.9354	0.1975			
Mean myometrial width	0.6193	-0.3149	0.4935	0.6000	0.5974	0.9104	-0.2403	1.0000	
	0.0028	0.1644	**0.0230**	**0.0040**	**0.0042**	**<0.0001**	0.2940		
Endo/myo ratio	-0.4566	-0.0945	0.2338	0.0688	-0.2805	-0.5000	0.5463	-0.6935	1.0000
	0.0375	0.6838	0.3078	0.7669	0.2181	**0.0210**	**0.0104**	**0.0005**	

Key:
Rho
Significance level (**P<0.05**)

8.1.2 Effect of age

The animals were mostly in the post-prime age category (≥ nine years of age) (Crosier et al., 2011). Cheetah ages ranged from three to 14 years, but cheetahs in the O and S groups were of a similar mean age (10.4 and 10.7 years, respectively) while the P group was slightly younger (mean of 8.7 years) (Figure 3A).

The median age between all three groups was similar: 10 years for the P group and 11 years for the O and S groups (Figure 3B). In general, older animals were more likely to have a higher endometrial area, gland lumen area % and median gland lumen area (Table 2).

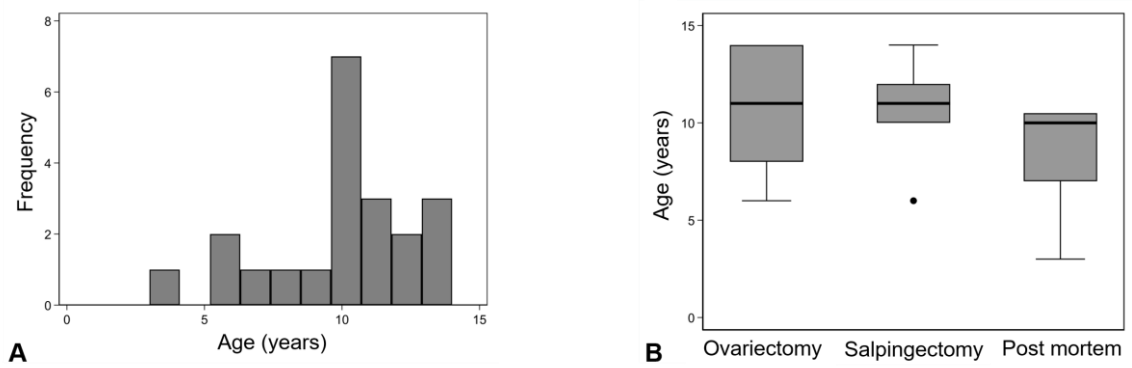

Figure 3. Histogram of the age distribution of all cheetahs used in this study (A). Box plot illustrating the age distribution of cheetahs where samples were collected at O, S and P procedures (B). Median age (dark horisontal line) was similar between the different procedure groups.

8.1.3 Effect of sampling procedure

The distribution of measures for the different procedures by which uterine samples were collected are shown in Table 3. Endometrial area (P=0.021) and mean myometrial width (P=0.015) were greater in samples collected by ovariectomy than in those collected at post-mortem, but neither differed significantly from the S group (Figure 4, highlighted in Table 3). There was also a tendency for the median gland lumen area to be greater in samples collected at ovariectomy than those collected at post mortem (p=0.047), but no significant differences were observed between the O and S, or the S and P groups. No significant associations were observed between the procedure by which uterine samples were collected and age, gland lumen area %, gland profile number, mean endometrial width or endo/myo ratio.

Table 3. The distribution of measures for the different procedures by which uterine samples were collected

Variable	Ovariectomy			Post-salpingectomy			Post mortem		
	Median	IQR	Range	Median	IQR	Range	Median	IQR	Range
Age (years)	11	8 - 14	6 - 14	11	10 - 12	6 - 14	10	7 - 10.5	3 - 10.5
Mean endometrial width (mm)	1.0	0.9 - 1.4	0.5 - 1.4	0.8	0.8 - 1.0	0.7 - 1.1	0.8	0.7 - 0.9	0.5 - 1.0
Total endometrial area (mm^2) [a]	**7.9**	**4.6 - 9.9**	**2.0 - 10.1**	4.8	4.5 - 6.1	2.9 - 6.8	**3.9**	**3.2 - 4.6**	**1.3 - 4.6**
Gland lumen area % (mm^2)	2.4	2.1 - 4.2	0.5 - 4.3	3.8	2.0 - 8.9	1.4 - 21.4	1.5	1.0 - 2.3	0.8 - 4.8
Median gland lumen area (μm^2)	231.1	130.5 - 261.0	22.4 - 516.8	175.1	78.3 - 245.3	59.2 - 484.8	88.1	48.2 - 146.8	44.7 - 193.9
Gland profile number	349	288 - 574	172 - 904	297	260 - 372	244 - 509	292	182 - 360	121 - 483
Mean myometrial width (mm)[b]	**1.4**	**0.9 - 1.5**	**0.7 - 1.8**	1.1	0.9 - 1.3	0.9 - 2.1	**0.8**	**0.7 - 1.1**	**0.6 - 1.2**
Endo/myo ratio	0.7	0.6 - 1.1	0.5 - 1.5	0.8	0.6 - 0.9	0.4 - 0.9	1.1	0.7 - 1.1	0.5 - 1.2

[a] significantly greater (p=0.021) in O than P samples

[b] significantly greater (p=0.015) in O than P samples

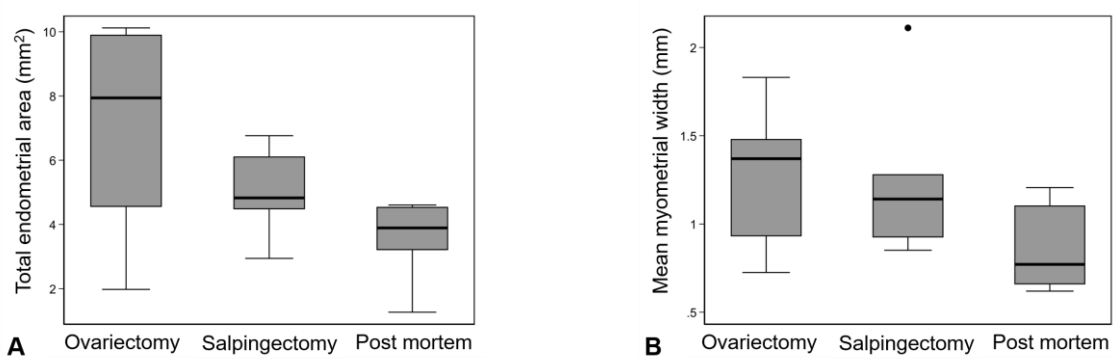

Figure 4. Box plots of the distribution of endometrial area (A) and myometrial width (B) in the different procedure groups (O,S or P).

8.1.4 Effect of reproductive cycle stage

The distribution of measurements at different stages of the reproductive cycle is shown in Table 4. The majority of the cheetahs (15/21; 71.4%) were in anoestrus at the time of sampling. Five (23.8%) cheetahs (O5, O6, O7, S3 and S6) were determined to be in pro-oestrus or oestrus and the reproductive status of one cheetah (P1) was unknown (Table 1). No significant associations were observed between the stage of

the oestrous cycle and age, gland lumen area %, median gland lumen area, gland profile number, mean endometrial width or endo/myo ratio. However, there was a tendency for endometrial area (p=0.089) and mean myometrial width (p=0.061) to be greater in animals in pro-oestrus/oestrus compared to those in anoestrous.

Table 4. The distribution of measures at different stages of the reproductive cycle

Variable	Oestrus/pro-oestrus			Anoestrus			p-value
	Median	IQR	Range	Median	IQR	Range	
Age (years)	11	10 - 11	8 - 14	10	9 - 12	6 - 14	0.566
Mean endometrial width (mm)	1.0	0.96 - 1.03	0.8 - 1.2	0.8	0.7 - 0.9	0.5 - 1.4	0.150
Total endometrial area (mm^2)	4.8	4.7 - 7.9	4.7 - 9.9	4.5	3.2 - 6.1	1.3 - 10.1	0.089
Gland lumen area % (mm^2)	4.0	3.8 - 4.2	2.2 - 4.3	2.1	1.4 - 3.8	0.5 - 21.4	0.127
Median gland lumen area (μm^2)	205.4	175.1 - 261.0	78.3 - 516.8	126.8	51.5 - 231.1	22.4 - 484.8	0.150
Gland profile number	372	349 - 501	172 - 509	292	266 - 360	121 - 904	0.315
Mean myometrial width (mm)	1.4	1.1 - 1.5	1.1 - 1.8	0.9	0.7 - 1.3	0.6 - 2.1	0.061
Endo/myo ratio	0.7	0.7 - 0.9	0.5 - 0.9	0.9	0.6 - 1.1	0.4 - 1.5	0.359

8.1.5 Effect of contraceptive treatment

The distribution of measures for history of contraceptive treatment is shown in Table 5. Ten (47.6%) cheetahs (O1, O3, S1, S3, S4, P2, P3, P5, P6 and P7) received at least one (range one to three) deslorelin treatments (Table 1). Animals that were treated with deslorelin and those that were never treated with contraceptives were of similar age (median ages of 10.3 and 10 years, respectively). Endometrial area, median gland lumen area and mean endometrial width were smaller in animals with a history of deslorelin treatment compared to animals that did not receive any contraceptive treatment (Figure 5). No significant associations were observed between a history of deslorelin treatment and age, gland lumen area %, gland profile number, mean myometrial width, or endo/myo ratio.

Table 5. The distribution of measures for history of contraceptive treatment

Variable	Deslorelin treatment			No treatment			p-value
	Median	IQR	Range	Median	IQR	Range	
Age (years)	10.3	10 - 11	6 - 12	10	8 - 14	3 - 14	0.696
Mean endometrial width (mm)	**0.8**	**0.7 - 0.8**	**0.5 - 0.9**	**1.00**	**0.9 - 1.2**	**0.7 - 1.4**	**0.005**
Total endometrial area (mm^2)	**4.4**	**3.5 - 4.6**	**1.3 - 5.7**	**6.1**	**4.6 - 9.0**	**2.9 - 10.1**	**0.020**
Gland lumen area % (mm^2)	2.1	1.0 - 3.8	0.5 - 8.9	3.7	1.5 - 4.2	1.0 - 21.4	0.360
Median gland lumen area (μm^2)	**113.0**	**48.2 - 188.8**	**22.4 - 231.1**	**205.4**	**88.1 - 261.0**	**59.2 - 516.8**	**0.049**
Gland profile number	292.5	287 - 360	121 - 483	349	244 - 509	172 - 904	0.573
Mean myometrial width (mm)	1.0	0.7 - 1.2	0.6 - 1.4	1.3	0.9 - 1.5	0.7 - 2.1	0.159
Endo/myo ratio	0.7	0.6 - 1.1	0.5 - 1.2	0.9	0.7 - 1.1	0.4 - 1.5	0.673

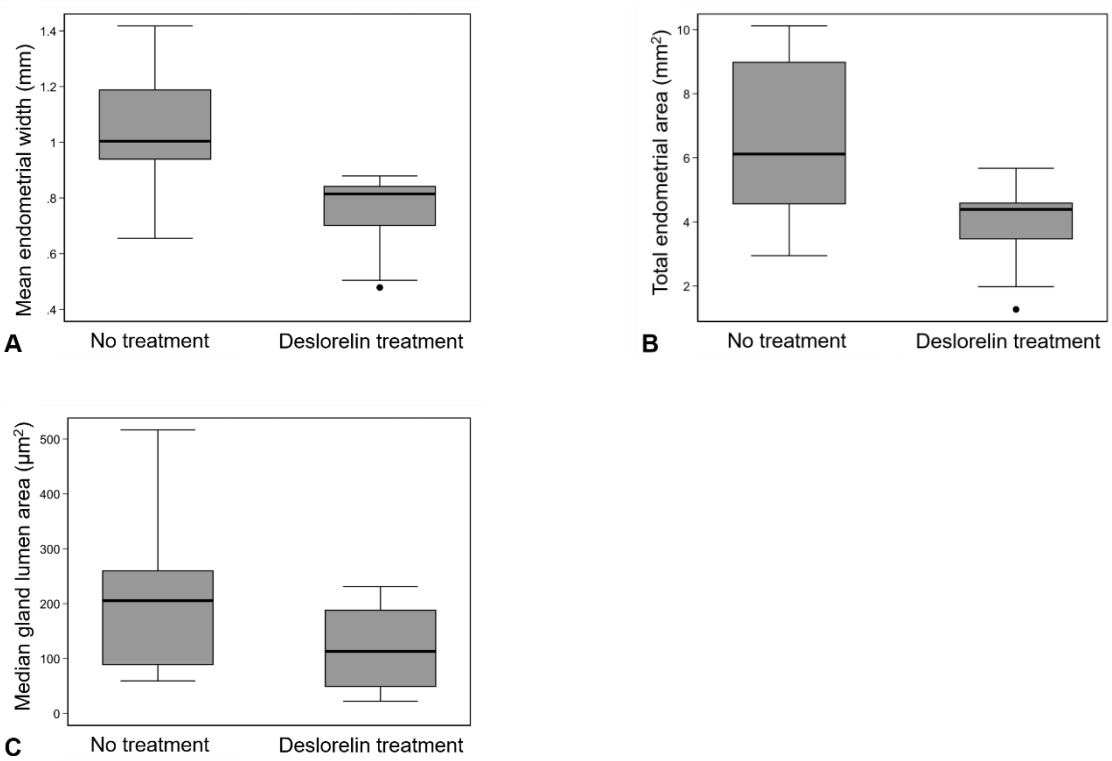

Figure 5. Box plots of the distribution of mean endometrial width (A), total endometrial area (B) and median gland lumen area (C) in animals with a history of deslorelin treatment compared to animals with no history of contraceptive treatment.

8.2 Histopathology

Preliminary views of uterine samples from the nulliparous, mainly anoestrus cheetahs used in this study showed minimal endometrial pathology, with no epithelial changes and no large glandular cysts encroaching on the uterine lumen. Therefore, it was decided to use the term “ectasia” to define any increase in glandular lumen area, whether pathological or physiological in nature.

Histological examination highlighted additional endometrial parameters to the morphometric matrix. Six of 21 (29%) of the samples demonstrated negligible to no increase in gland lumen area (e.g. Figure 6A). Eleven (52%) of the samples had single or randomly scattered, multifocal mildly ectatic glands, predominantly in the deep endometrium (e.g. Figure 6B). Only four (19%) of the samples revealed one or multiple prominent ectatic glands (e.g. Figure 6C) located mainly in the deep endometrium with no evidence of encroachment on the uterine lumen.

Glandular and luminal epithelial cells were largely low columnar, with occasional glands with cuboidal epithelium. Epithelial cell characteristics did not vary between glands with different lumen areas. Mild, lymphoplasmacytic inflammatory cell infiltrates were observed in the stroma of the superficial endometrium in 38% (8/21) of the samples from animals in anoestrus (6/8; O2, O4, S1, S5, P2 and P4) and pro-/oestrus (2/8; O7 and S3). Two of these samples also revealed scattered neutrophils in the superficial endometrial stroma (O4 and S5), both from animals in anoestrus. Moderate, acute endometritis was present in one uterus from an animal in pro-/oestrus (O5). One uterus from an animal in anoestrus (O3) revealed moderate haemorrhage in the superficial endometrial stroma.

Five (23.8%) of the sections (O6, S6, P3, P4 and P5) demonstrated glandular crowding in the deep endometrium, often associated with mild ectasia of the gland(s) involved (e.g. Figure 6D). Two affected animals were in pro-/oestrous (S6 and O6) and three in anoestrous (P3, P4 and P5).

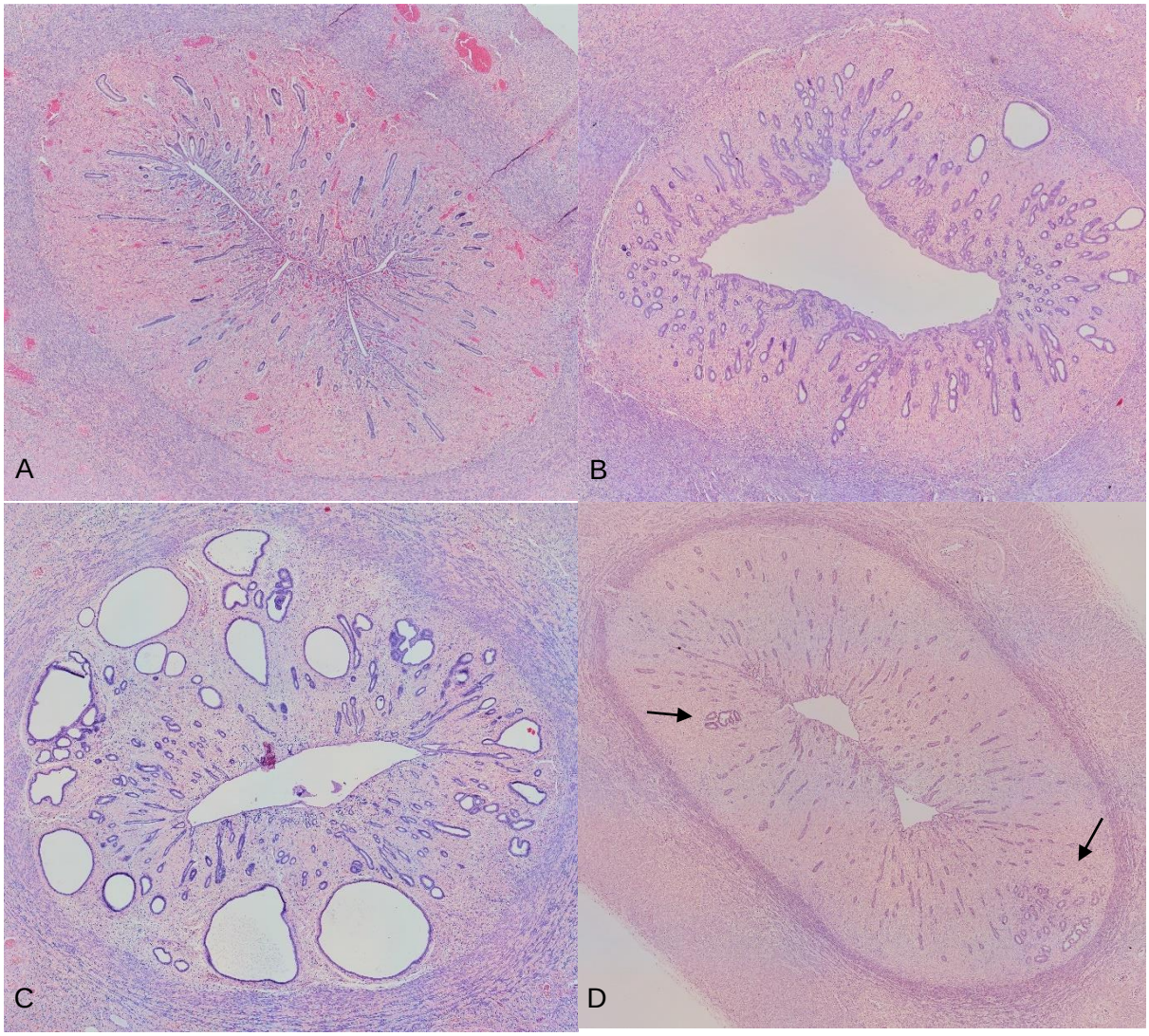

Figure 6. Examples of the variation in endometrial glandular changes observed. Glandular ectasia varied from negligible - cheetah P1 (A), randomly scattered ectatic glands - cheetah S3 (B) to multiple prominent ectatic glands - cheetah S2 (with highest total gland lumen area and gland lumen area %) (C). Glandular crowding in the deep endometrium with mild ectasia of associated glands - cheetah P3 (D)

9. DISCUSSION

The wild felid grading system used by Munson et al., (2001) and Crosier et al. (2011) could not be applied in this study. These previous studies specifically graded the severity of hyperplastic changes in the endometrium. However, no hyperplastic changes were observed in the current study. Samples from the predominantly aged cheetahs in this study also did not show any signs of severe additional pathology, like cysts encroaching on the uterine lumen, endometrial fibrosis, adenomyosis, hydrometra, pyometra, endometrial polyps, or endometrial atrophy, which were observed in 22% and 56% of prime (six to eight years old) and post-prime (≥ nine years old) North American cheetahs, respectively by Crosier et al., (2011).

The overall cumulative increase in endometrial width was also an important criterion in the wild felid endometrial hyperplasia grading system (Munson et al., 2002; Crosier et al., 2011). Endometrial width from animals of similar body size and oestrous cycle stage, with no hyperplastic changes, were used as a baseline to determine the cumulative increase in endometrial width in these studies (Munson et al., 2002; Moresco, Munson & Gardner, 2009). Endometrial width typically increases when endometrial glands become hyperplastic and/or cystic (physiological and pathological) (Barrau et al., 1975; De Bosschere et al., 2001; Munson et al., 2002). In the present study, however, there were large variations in measured mean endometrial widths and total endometrial areas, even in animals of similar body size and oestrous cycle stage, even though no severe pathology was observed. The endometrial width varied too much to establish a baseline to calculate a cumulative increase in endometrial width, which made it difficult to practically apply this measurement in this study. To overcome this obstacle, gland lumen area % was calculated.

Gland lumen area % was generally low, with only two of the samples (cheetah S1 and S2) with a gland lumen area % higher than 5% (8.88% and 21.41% respectively). Even in the two samples with the most outspoken increases in gland lumen area, the affected portions were confined to the mid- to deep endometrium. This contrasted with severe cystic lesions frequently observed in dogs and also reported in cheetahs, where cystic glands were superficial and encroached on the uterine lumen (Munson

et al., 2002; Schlafer & Gifford, 2008; Crosier et al., 2011). Approximately 89% of samples evaluated during the Crosier, et al. (2011) study originated from North American zoos with an associated focus on conservation, reproduction and husbandry. (Wildt et al., 1993; Swanson, 2006; Crosier et al., 2011). The remaining 11% of samples in the same study originated from cheetahs from Namibia (free-ranging or at a conservation organisation), but no further distinctions were made regarding the severity of lesions between samples originating in North America versus Namibia. Thirty-two percent of the cheetah samples evaluated by Crosier, et al. (2011) showed moderate to severe hyperplastic and/or cystic changes. This contrasted with cheetahs in southern Africa, where severe cystic endometrial changes are rarely observed in cheetahs (E.P. Mitchell, personal communication, 23/04/2021). This interesting discrepancy between the severity in endometrial pathology observed in the current study and the North American population remains to be investigated.

The total gland lumen area and gland lumen area % were relatively constant among the different samples evaluated indicating that absolute or relative increases in gland lumen area are not the source of the observed variation in endometrial width. This suggests that the variability in endometrial width and endometrial area are most likely the result of tangential cuts during sample processing. Therefore, using endometrial width as a measure in any pathological grading system should only be considered when it is possible to ensure a 90° cross-sectional cut of the uterus.

Interestingly, significant ($p<0.05$) positive correlations were observed in this study between endometrial area, mean endometrial width, median gland lumen area and gland lumen area %. These correlations indicate that endometrial area and endometrial width are related to gland lumen area. Increased endometrial area is probably due to increased gland lumen area, which in turn, appears to be due to increased gland size rather than number. The implication is that increasing endometrial width and/or area can predict, or be predicted by increasing gland lumen area and that endometrial width measurements (as used by Munson et al. (2002) and Crosier et al. (2011)) may be a proxy for gland lumen area. However, considering that the methods described by Munson et al. (2002) and Crosier et al. (2011) are insufficiently specific, practical application of these measurements and establishing baselines still require further investigation.

Endometrial width, endometrial area and gland lumen area (% and mean) were not correlated with gland profile number. Animals in oestrus are anticipated to show slightly coiled endometrial glands, increasing in tortuosity and branching after ovulation (Dawson & Kosters, 1944; Pineda, 2003a,b; Wang et al., 2007), resulting in increased gland profile numbers when viewed in cross-section. However, in this study the cheetah with the highest gland profile number (O4) was in anoestrus, indicating that gland profile number alone cannot be used to predict an animal's reproductive stage. The small number of samples evaluated in this study may mask potential true relationships between these variables. Interestingly, cheetah O4 also had the highest endometrial width, even though gland profile number was not positively correlated with endometrial width. This suggests that the gland profile number is not the reason for the high endometrial width. Again, tangential cuts may have played a role in increasing the number of gland profiles

No consistent relationships were observed between endometrial width, myometrial width, endo/myo ratio and other variables. The cheetah with the highest endometrial width (O4) also had the highest endo/myo ratio. This was likely as a result of the high endometrial width and an average myometrial width, increasing the ratio between these two variables. Cheetah O2 had the second highest endometrial width. Both cheetahs were in anoestrus at the time of sampling, which is typically associated with endometrial quiescence and reduced endometrial width. In these cases, the paradoxical high mean endometrial width may be artefactual due to tangential cut.

Cheetahs with the highest myometrial width were S5, an animal in anoestrus, and O5, in pro-/oestrus. All of the uterine samples collected post mortem had endometrial- and myometrial widths at the bottom half of the range of measurements. These findings indicate that the time between death/sampling and fixation may have an effect on endometrial and myometrial width. Dawson & Kosters (1944) suggested that differences in the degree of myometrial contraction in different phases of fixation may account for variation in uterine lumen appearance. The variation in muscular contraction of the myometrium in samples fixed some time after death and samples placed directly into formalin after collection during surgical procedures may also play a role in the endometrial and myometrial widths in samples evaluated histologically.

The nulliparous, captive cheetah population from this study consisted of predominantly prime and post-prime cheetahs. Age was positively correlated with endometrial area, gland lumen area % and median gland lumen area. This finding may explain the considerably higher gland lumen area % observed in cheetahs S1 and S2 (both in the post-prime category). S2 also had a much higher total gland lumen area than the other cheetahs. Increased pathology of the endometrial glands have been reported with increasing age in domestic and wild canids and felids (Dow, 1959; Perez et al., 1999; Munson et al., 2002; Agudelo, 2005; Crosier et al., 2011; Pires et al., 2016; Binder et al., 2019). Crosier et al. (2011) reported severe endometrial hyperplasia and/or cysts only in post-prime cheetahs and additional severe endometrial pathologies were highly age-associated. (Crosier et al., 2011). Nulliparity is also reported to increase the risk of uterine pathology in many wildlife species, especially in captivity (Agnew, Munson & Ramsay, 2004; Hermes et al., 2006; Crosier et al., 2011; Asa et al., 2013). The nulliparous status of all cheetahs in the current study likely contributed to the changes observed, however nulliparity does not seem to be associated with severe endometrial pathology.

A uterine tip sample of only one young cheetah (three years old) was used in this study, collected post mortem (cheetah P1). This may have skewed some of the data for the P group. For example, the average age of the P group, without cheetah P1 would have been 9.7 years (vs. 8.7 years including P1).

This study did not show a difference in the glandular changes in the endometrium at one year after salpingectomy, compared to animals in which ovariectomies were performed. However, further investigation is required to confirm this with a larger sample size. The Namibian Government controversially requires sterilisation of all female wild carnivores in captivity (Ministry of Environment and Tourism, 2012), but conservation of this threatened species remains a priority in most other countries worldwide. Therefore, opportunities for further studies on different sterilisation procedures in cheetahs to obtain more samples may be limited.

The stage of the reproductive cycle at the time of sampling was not significantly associated with any of the variables evaluated. The tendency for endometrial area to

be greater in pro-oestrus/oestrus is a normal physiological finding, due to stromal oedema, hyperaemia, as well as endometrial glandular hyperplasia and hypertrophy (Dawson & Kosters, 1944; Barrau et al., 1975; Pineda, 2003a). Myometrial contractility decreases under the influence of progesterone (von Reitzenstein, Archbald & Newell, 2000; Pires et al., 2016), which may result in the observed tendency for increased myometrial width during oestrus. However, with only five of the animals in our study in pro-oestrus/oestrus at the time of sampling (cheetahs O5, O6, O7, S3 and S6), definitive statements cannot be made based on these results and further investigation is required to determine whether the stage of the reproductive cycle plays a role in endometrial glandular and other uterine changes in the cheetah.

The GnRH-analogue deslorelin was used in ten out of the 21 cheetahs included in this study. The lack of significant pathology associated with deslorelin treatment in this study is consistent with findings in previous studies in cheetahs (Bertschinger et al., 2002; Schulman et al., 2015). The only effects of deslorelin observed in this study were a decrease in median gland lumen area, endometrial area and mean endometrial width. The majority of the treated cheetahs (9/10) were in anoestrus at the time of sampling, a period during which the endometrial wall is normally thinner without the cyclical hormonal influences of oestrogen and progesterone (Barrau et al., 1975; De Bosschere et al., 2001; Pineda, 2003b; Chatdarong et al., 2005). Deslorelin treatment may arguably have exacerbated the observed uterine effect in the cheetahs in anoestrous, due to its induction of ovarian inactivity (Munson, 2006).
In this study the luminal and glandular epithelium was predominantly low columnar, with no consistent pattern of flattening of epithelium in ectatic glands that may have resulted from excessive glandular secretions (Pineda, 2016). Without evidence of epithelial attenuation or hyperplasia associated with ectatic glands in this study, it could be argued that the increases in glandular lumen area in these cases are largely the result of physiological processes and not pathological in nature.

An interesting observation in this study that has not been previously reported was the crowding of glands/gland profiles in the deep endometrium in five of the 21 samples (cheetahs O6, S6, P3, P4 and P5). Similar nested or crowded glands have been observed in endometrial biopsies from horses and humans. In horses “glandular nesting” is typically the result of focal clustering of a few branches of endometrial

glands due to periglandular fibrosis (Evans et al., 1998; Snider, Sepoy & Holyoak, 2011). This study did not reveal any evidence of periglandular fibrosis, reducing the likelihood of this pathogenesis in cheetahs. Focal non-pathological clustering of glands or gland branches, not associated with fibrosis, can also occur in horses due to stromal oedema during proestrus (Kenney, 1978). Oedema was not observed in the cheetahs in this study. In humans glandular “crowding” is seen in complex endometrial hyperplasia. Focal branching or clustering of glands are seen, with compression of surrounding stroma, but no fibrosis is present. This condition in humans is thought to result from persistent oestrogen stimulation, but has also been reported when no cyclic abnormalities were present. (Sivridis & Giatromanolaki, 2013). The majority of the affected samples in this study originated from animals in anoestrus (P3, P4, P5) and therefore oestrogenic stimulation and/or oedema is unlikely to be the cause in these cases. This lesion and its significance remain to be investigated in cheetahs.

The main limitation of this study was the small sample size. As the cheetah is classified as a vulnerable species on the IUCN red list of threatened species and samples are not routinely collected from cheetahs in zoological or conservation collections in southern Africa, samples collected during previous reproductive management studies were used. Due to the small sample size, the study had limited power and was unable to account for potential confounders such as age, deslorelin treatment and cyclicity. This may explain some of the perceived differences between the results obtained in the current and other studies. There were also only relatively mild changes observed in the available uterine samples, which limited comparisons between these samples, and with other studies.

Another limitation was that only uterine tip samples were available for examination. It is possible that the uterine tip is not representative of the changes present throughout the uterus. However, a study conducted by Penfold et al. (2020) in a small number of cheetahs (n=7) found that the tip of the uterine horn is a reliable sample for endometrial glandular changes throughout the uterus.

In this study no severe pathology, like that described by Munson et al. (2002) and Crosier et al. (2011) as being “severe hyperplastic- and/or cystic changes” was observed. Without any great degree of variation in the observed glandular

abnormalities, it is difficult to judge the severity and significance of the observed changes. Therefore, further studies are indicated in animals with a wider range of age, parity reproductive cycle stage, geographical distribution and history of contraceptive treatment in order to more definitively describe the difference between normal physiological and pathological changes in the cheetah endometrium.

10. CONCLUSION

This study evaluated endometrial glandular changes at the uterine tip collected at the time of ovariectomy, one year post-salpingectomy, or at post mortem in 21 captive southern African cheetahs. In addition to characterising endometrial glandular changes, we investigated the associations between uterine morphology and age and deslorelin treatment. The animals were nulliparous and predominantly in the post-prime age group (≥ nine years old). Changes in gland lumen area were absent to minimal in most of the samples in this study and very little additional pathology was observed. There were no significant differences between samples collected one year post-salpingectomy and those collected at ovariectomy, or post mortem. As expected, based on the current understanding of cheetah reproductive physiology, we found that 1) older animals had larger gland lumen areas and endometrial areas (median and %); 2) animals in pro-oestrus and oestrus were inclined to have larger endometrial areas; and 3) deslorelin-treated cheetahs had thinner endometria and smaller gland lumens.

This study emphasised the importance of practical methods to evaluate and describe the range of endometrial glandular pathology and the normal physiological endometrial changes in cheetahs of a wide range of ages, parity, reproductive cycle stages, and variable history of contraceptive treatment. The normal physiological variations and pathology in the cheetah endometrium must be more completely defined before undertaking similar investigations that indicate the selection of specific parameters/variables for inclusion in a grading system for endometrial glandular pathology.

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12. APPENDICES

12.1 Appendix 1 - Proof of ethical clearance

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Histological classification of endometrial pathology in the cheetah (*Acinonyx jubatus*)**
PROJECT NUMBER	**V074-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. A Avenant**

STUDENT NUMBER (where applicable)	**U_28010842**
DISSERTATION/THESIS SUBMITTED FOR	**MMedVet**

ANIMAL SPECIES/SAMPLES	**Cheetah (*Acinonyx jubatus*)**
NUMBER OF ANIMALS	**28 previously collected samples (V014-14; V089-17)**
Approval period to use animals for research/testing purposes	**August 2018 – August 2019**
SUPERVISOR	**Prof. MJ Hartman**

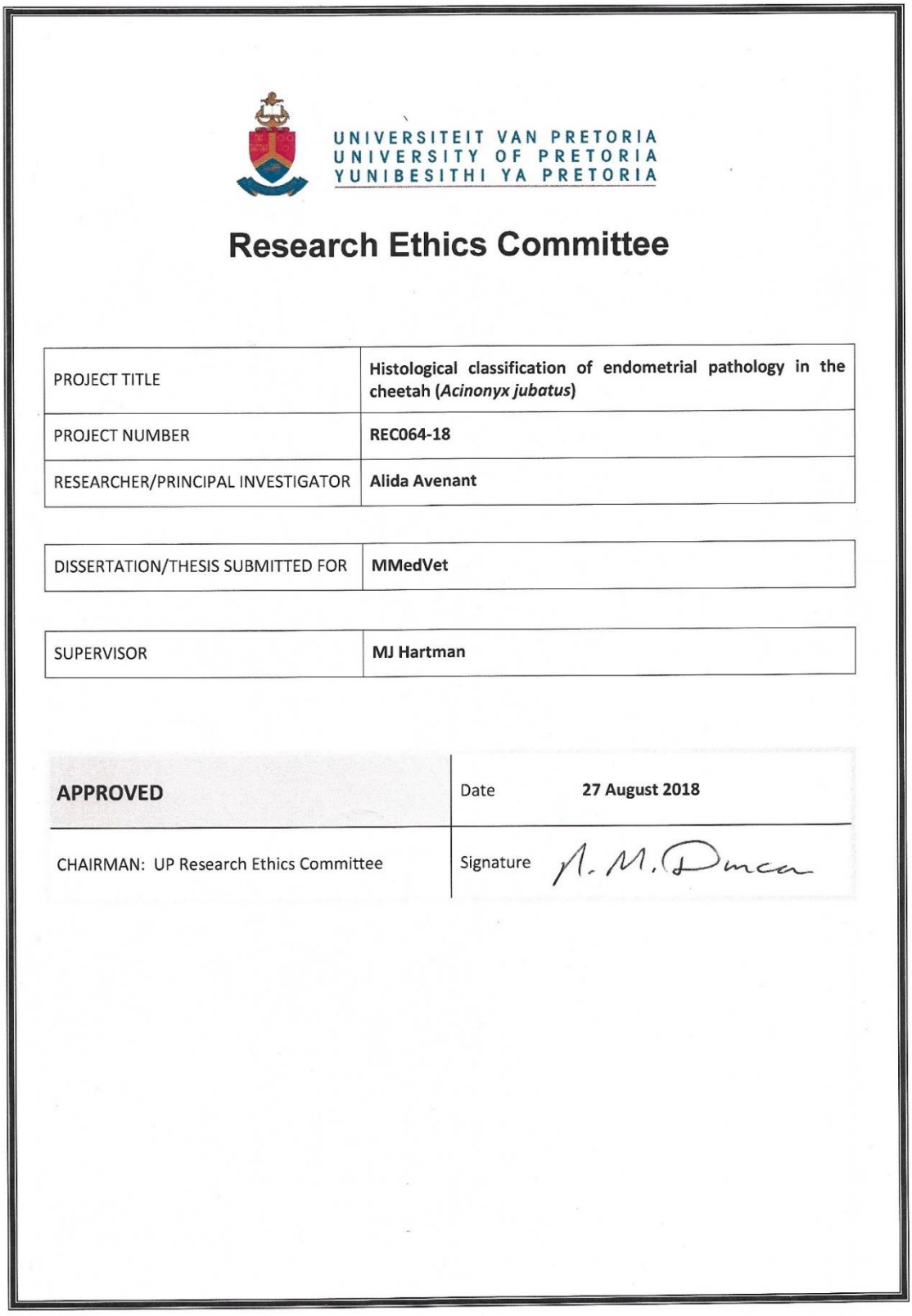
KINDLY NOTE:

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	28 August 2018
CHAIRMAN: UP Animal Ethics Committee	Signature	

S4285-15

Figure 7. Original Animal Ethics Committee approval certificate



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YUNIBESITHI YA PRETORIA

Research Ethics Committee

PROJECT TITLE	**Histological classification of endometrial pathology in the cheetah (*Acinonyx jubatus*)**
PROJECT NUMBER	**REC064-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Alida Avenant**

DISSERTATION/THESIS SUBMITTED FOR	**MMedVet**

SUPERVISOR	**MJ Hartman**

APPROVED	Date **27 August 2018**
CHAIRMAN: UP Research Ethics Committee	Signature M. M. Duncan

Figure 8. Original Research Ethics Committee approval certificate

12.2 Appendix 2 - Histological images of the endometrial samples not shown in the main text

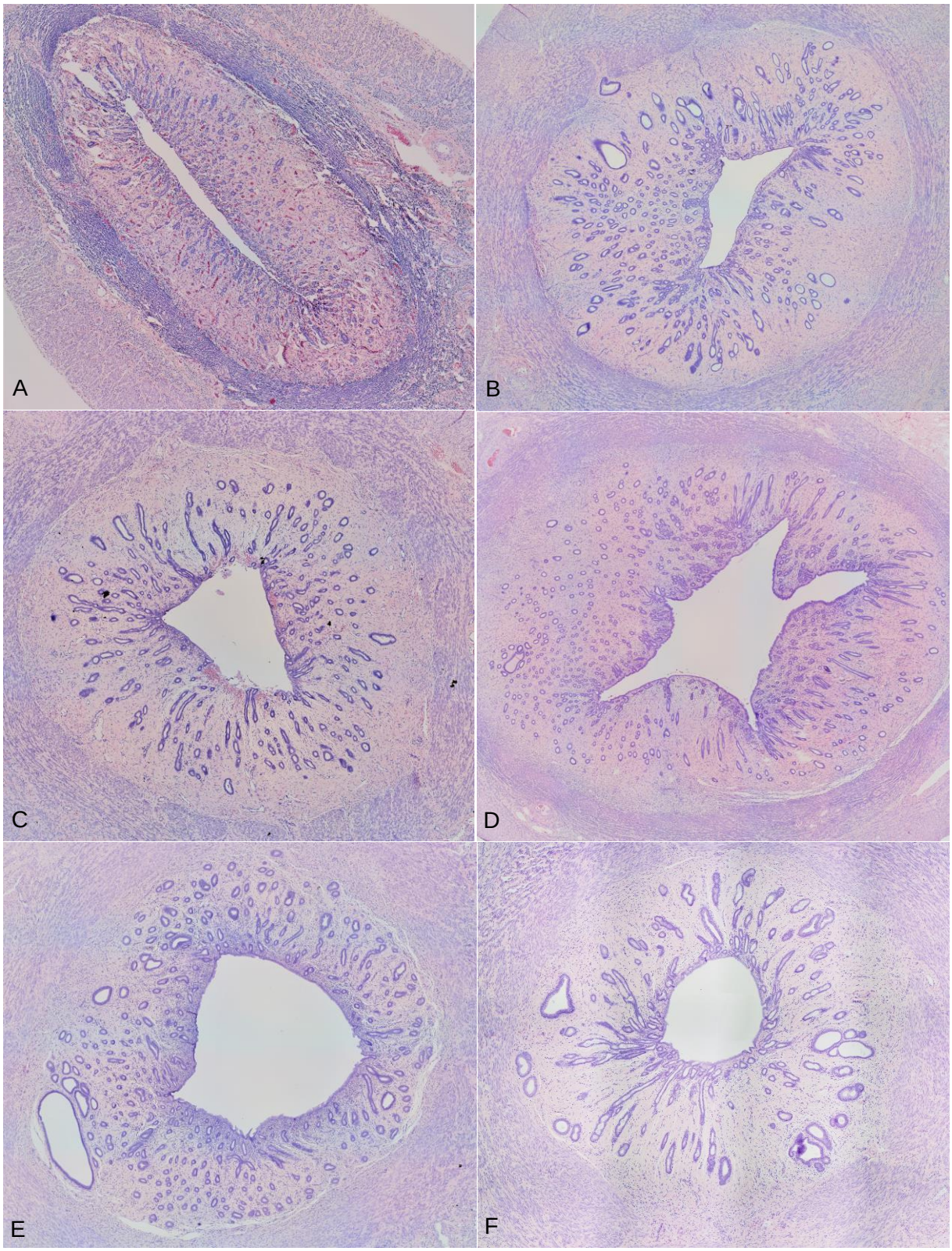

Figure 9. Stitched histological images of the cheetah endometrial samples not shown in the main text. Cheetah O1 (A), O2, with the second highest endometrial width (B), O3 (C), O4, with the highest mean endometrial width, gland profile number and endo/myo ratio (D), O5 (E) and O6, with glandular crowding (F).

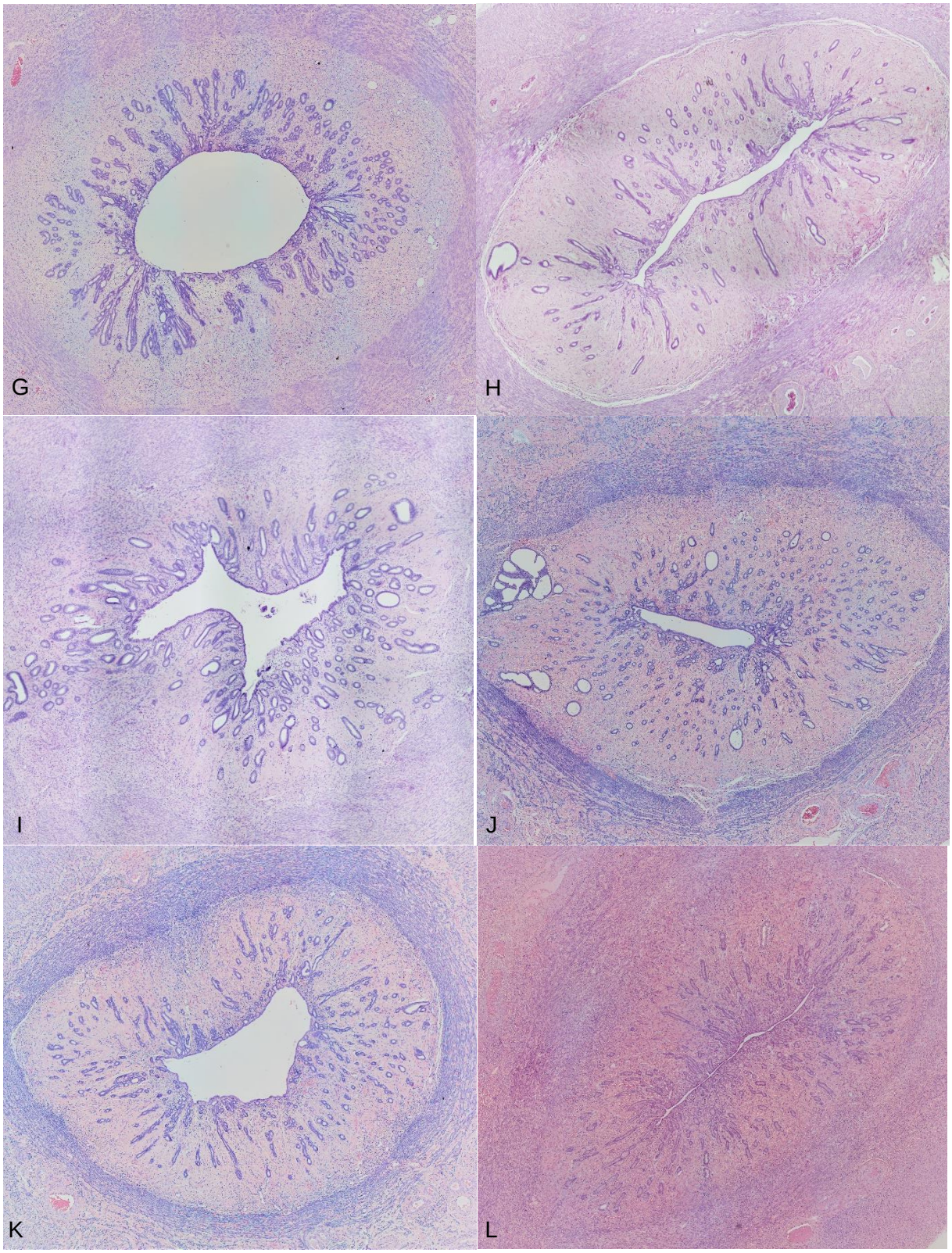

Figure 9 (continued). Stitched histological images of the cheetah endometrial samples not shown in the main text. Cheetah O7 (G), S4 (H), S5, with the highest myometrial width and second highest median gland lumen area (I), S6, with glandular crowding (J), S7 (K) and P2 (L).

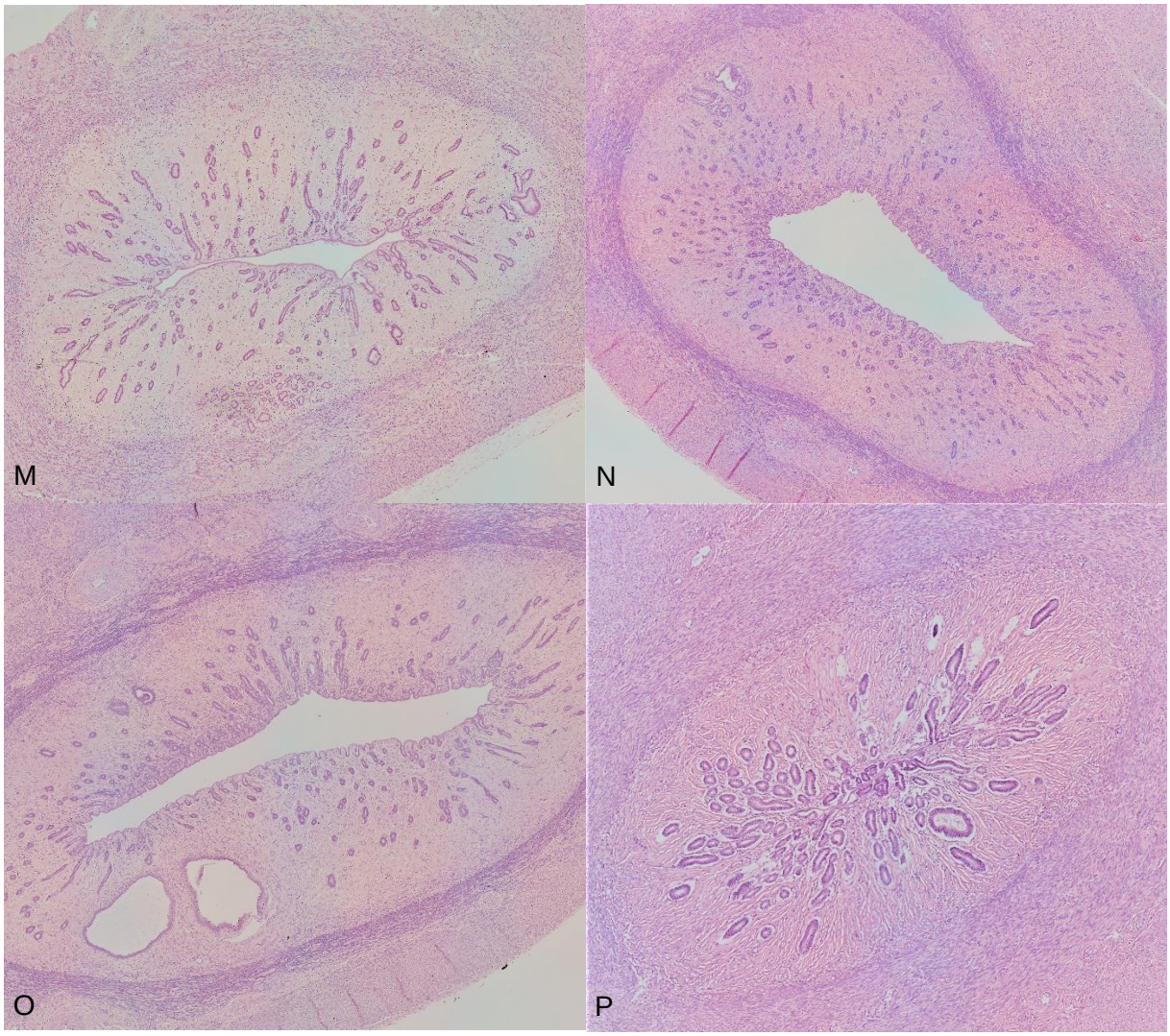

Figure 9 (continued). Stitched histological images of the cheetah endometrial samples not shown in the main text. Cheetah P4, with glandular crowding (M), P5, with glandular crowding (N), P6 (O) and P7 (P).