Ultrasonographic and laparoscopic evaluation of the reproductive tract in older captive female cheetahs (*Acinonyx jubatus*)

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ABSTRACT

The study uniquely described the clinical value of trans-abdominal ultrasonography for monitoring features characterising the oestrous cycle in female cheetahs (*Acinonyx jubatus*). The reproductive tracts of 21 female, nulliparous and relatively aged (median: 11 and interquartile range: 9.25 - 14 years) captive cheetahs resident on two sites in Namibia were assessed by trans-abdominal ultrasound. Subsequently, the ovarian findings on ultrasound were compared with direct visualisation whilst performing laparoscopic sterilization. A combination of these observations supported by concurrent sampling for vaginal cytology and serum progesterone concentrations (SPC) defined the oestrous status of individual animals. At one site, six cheetahs had been implanted with the GnRH agonist, deslorelin as a contraceptive at least once within the preceding 11 years. On ultrasound, 31 uterine horns and 35 ovaries with discernible structures on 28 (86%) were visualised in the 21 cheetahs. The uterine body was difficult to visualise due to its intrapelvic location. Eleven of 19 uteri (58%) visualised showed endometrial oedema suggestive of oestrogenisation. The uteri of four cheetahs (19%) showed evidence of mild cystic endometrial hyperplasia (CEH). Paraovarian (PO) cysts were seen on ultrasound (n = 21) and laparoscopy (n = 26) in 16 (76.2%) and 18 (85.7%) cheetahs respectively. Ovarian volumes obtained from ultrasonographically determined dimensions predicted cyclic activity. Laparoscopy showed that 19 ovaries had discernible follicular structures. In the study population, 10 (47.6%) were in pro-oestrus or oestrus; none in the luteal phase and 11 (52.4%) in anoestrus. Trans-abdominal ultrasound in combination with SPC and vaginal cytology was used with acceptable accuracy to assess cyclic ovarian activity in captive cheetahs. A considerable

proportion of this aged population showed ovarian activity and the prevalence of PO cysts was notable. A history of prior deslorelin treatment was not associated with either reproductive activity or uterine pathology

1. Introduction

The increased availability and technological advances in ultrasonography together with improvements in hormone analyses and reproductive control methodologies have facilitated current reproductive management of free-range or captive wild carnivore populations, including cheetahs (*Acinonyx jubatus*). Behavioural observations, clinical and laboratory methods are all reported for oestrous monitoring and defining the reproductive status of individual female cheetahs and lions [1-4]. These reports include hormonal analyses and the application of vaginal cytology in both free-ranging and captive populations and ultrasonography via trans-rectal and trans-abdominal approaches [5,6]. Descriptions of the normal reproductive tract, particularly with regard to imaging of the ovaries are limited [1-3,7-9]. The utilization of ovarian imaging to observe changes in ovarian function in domestic felids is partially limited by the small size of the ovarian structures but has been reported as an adjunct to oestrous monitoring in non-domestic felids [5,6,8,10-13]. Ultrasonography provides a non-invasive, practical and relatively uncomplicated means to rapidly assess reproductive status in both free-ranging and captive animals. Laparoscopy facilitates direct visualization of the ovaries and uterus thus enabling evaluation of the dynamic changes associated with follicular and luteal phases of the oestrous cycle as well as for the diagnosis (and staging) of pregnancy and pathologies of the reproductive tract of wild felids [5,6,13]. Additionally, uterine changes may also be indicative of the different stages of the reproductive cycle. Cheetahs in oestrus display a thicker uterine wall with oedematous, more anechoic bands compared to anoestrus cheetahs which have thinner more echogenic uterine walls [6]. Trans-abdominal diagnostic ultrasound is particularly useful for in-the-field application in defining reproductive status of both free-ranging and captive wild felid populations potentially providing a rapid alternative to laboratory-based methods for their reproductive management [5,6,14].

The reproductive cycles of many wild felids, including cheetahs have been comprehensively reviewed [3].The female cheetah, reproductively active throughout the year, shows relatively short cycles (7 - 21 d) characterised by oestrogen surges that distinguish oestrus (2 - 6 d) from interoestrous

periods. Four phases of the felid oestrous cycle: pro-oestrus, oestrus, dioestrus and anoestrus are distinguished by observation of ovarian structures. Pro-oestrus and oestrus are associated with sequential development of visible follicles in the ovaries preceding the dioestrus (or luteal phase) which is seen as $\geq$ one visible *corpus luteum* (CL), often with a visible transition in development from a *corpus haemorrhagicum* (CH) [3]. Anoestrus, the period of follicular quiescence is associated with visibly non-functional follicular and luteal sites. [1,3]. Variable periods of ovarian quiescence, particularly in captive groups are possibly due to socially-induced and stress-associated ovarian suppression [3,15].

The GnRH analogue deslorelin is administered via a subcutaneous implant to down-regulate reproductive activity as a safe and reliable contraceptive method in both male and female captive and free-ranging wild carnivores, including cheetahs [2,21]. Adverse side effects have not been reported after long-term administration in treated female lions and tigers, however similar data obtained from female cheetahs are currently limited [2,16,17].

Hormonal methods describing the oestrous cycle, dependant on either serum or faecal sample-derived steroids, demonstrate cyclical elevations of oestrogens differentiating anoestrus from oestrus. Currently both induced (post-mating) and more rarely, spontaneous ovulations preceding the luteal phase (with associated elevations in progestagens following an oestrogen surge) are recognized [1,3,7]. The pregnant luteal phase in the cheetah may be associated with a greater elevation in mean serum progesterone concentrations (SPC) [2,9].

Vaginal cytology detects the effects of oestrogenisation resulting in an increased proportion of cornified vaginal epithelial cells and a clear background on fixed and stained vaginal epithelium smears to describe pro-oestrus and oestrus and is well described in the dog and cat [14,18]. The use of vaginal cytology in non-domestic felids although not commonly performed is reported as a valuable adjunct in determining cyclical activity [1,2,5].

It has been variously reported that captive breeding programs are typified by poor reproductive performance ascribed anecdotally to a plethora of causes including captive stress, lack of genetic diversity, poor husbandry conditions and seasonal climatic changes [3,4]. Asymmetric reproductive aging has been demonstrated in cheetahs similar to other captive populations of non-carnivore species such as elephants and rhinoceros where older captive females demonstrate irregular oestrous cycles or may cease cyclic activity [6,19,20].

The primary objective of this study was to assess the clinical value of trans-abdominal ultrasound observations of the reproductive tracts of 21 nulliparous, aged captive cheetahs *via* comparison with direct laparoscopic visualisation at the time of sterilisation. These methods in

combination with data derived from concurrent measurement of SPC and vaginal cytology were used to define the reproductive status of the cheetahs.

2. Materials and Methods

2.1. Animals

All cheetahs in this study were obtained as orphaned wild cubs and maintained thereafter as non-releasable wild cheetahs by two independent cheetah conservation organizations (Sites A and B) in northern Namibia. The females were kept in small groups of 2 - 5 animals under free range conditions in large, fenced bushveld camps (ranging from 3 - 50 ha) separated by at least a fence-line or roadway from similar small groups of males. Their diet consisted mainly of donkey and horse meat on the bone with a commercial vitamin and mineral supplement (Predator supplement, V-Tech, Centurion, South Africa).

Current Namibian legislation requires surgical sterilization of all captive female carnivores [21]. The University of Pretoria was requested to initiate a surgical sterilization programme at the above two conservation sites to comply with this legislation. The project was approved by the University's Animal Use and Care Committee and Research Committee (protocol number V014-14) and conducted under the terms of permit 1919/2014 from the Namibian Ministry of Environment and Tourism.

Twenty-one sexually mature nulliparous female cheetahs were included in a laparoscopic surgical sterilization trial comparing ovariectomy to salpingectomy during July 2014 [22]. The animals' numbers, ages (median and interquartile range (IQR)), body-weights (median and IQR) at both sites and their combined data respectively were as follows:

a) site A: n = 11, 11 (IQR 9.25 - 12 years), 33.2 (IQR 31.6 - 33.4 kg);

b) site B: n = 10, 13 (IQR 10 - 14 years), 32.75 (IQR 30.0 - 36.0 kg); and

c) combined population: n = 21, 11 (9.25 - 14 years), 33.2 (IQR 31.2 - 35.0 kg).

Records showed that at site A, six cheetahs had received at least one 4.7 mg implant of the GnRH agonist deslorelin (Suprelorin, Virbac, Milperra, NSW, Australia) within the preceding 11 years (Table 1).

All cheetahs were examined under general anaesthesia during a nine-day observation period. Cheetahs were fasted 24 - 48 h preoperatively, but had free access to water. Cheetahs at site A were immobilised by remote injection using a dart gun with combinations of tiletamine and zolazapam (Zoletil, Virbac, Halfway House, South Africa) and medetomidine (Domitor, Pfizer Animal Health,

Sandton, South Africa). At site B the cheetahs were trained to enter a capture cage in which the above drugs were injected intramuscularly. Cheetahs were maintained on isoflurane (Isofor, Safeline Pharmaceuticals, Johannesburg, South Africa). Morphine sulphate (Fresenius Kabi, Port Elizabeth, South Africa) was given for pain control during surgery. After the procedure the anaesthesia was antagonised with atipamazole (Antisedan, Pfizer, Sandton, South Africa). For post-operative analgesia, 0.3 mg/kg meloxicam (Metacam®, 5 mg/kg, Boehringer Ingelheim, Randburg, South Africa) was administered subcutaneously prior to anaesthetic recovery. One cheetah at site A had a concomitant intra-abdominal granuloma with associated abdominal effusion secondary to an abdominally-located thorn. This granuloma was removed laparoscopically [23].

2.2. Ultrasonography

Each cheetah underwent a dorsally recumbent trans-abdominal ultrasound examination of her reproductive tract. A portable ultrasound machine was used with a 3 - 6 MHz convex array transducer for the general abdominal examination and a 5 - 10 MHz linear array transducer for evaluation of the reproductive tract (Mindray Model M7 Vet, Shenzhen Mindray Biomedical from Lomaen, Johannesburg, South Africa). The uterine body, horns, ovaries and surrounding structures were examined and their associated dimensions, echogenicity, wall-layering, luminal content and presence of cyclical activity recorded where visible. The uterine horns were categorised as being either cyclically active or inactive [6] and the average diameter of both horns and the presence of uterine oedema (associated with oestrogenisation) were compared retrospectively to the designated reproductive status of individual cheetahs to determine a difference in size between the cyclically active and inactive groups. Round, intra-ovarian visualized structures were classified as follicles if they were anechoic and had acoustic enhancement and as periovulatory structures which included late, luteinizing follicles or CH (slightly echogenic with minimal acoustic enhancement) and CL (slightly hyperechoic to the surrounding ovarian tissue with no acoustic enhancement) [10]. The location of the ovaries relative to the ipsilateral kidney and surrounding structures were recorded. The ovarian size and structure was compared to direct visualization during the ensuing laparoscopic procedure. Single or multiple, round, anechoic cystic structures in close proximity to the ovaries were defined as paraovarian (PO) cysts.

Table 1
Reproductive status categorisation and ultrasonographic and laparoscopic observed features of the ovaries and uteri of 21 female cheetahs examined at two sites in Namibia.

Site & animal ID & age (years)	SPC (nmol/L)	Vaginal cytology oestrogenised (Y/N)	Reproductive status	Uterine horns US visible Left horn (Y/N)	Uterine horns US visible Right horn (Y/N)	Uterine horns US oedema (Y/N)	Left ovary follicle N° US	Left ovary follicle N° Lap	Left ovary PO cyst N° US	Left ovary PO cyst N° Lap	Right ovary follicle N° US	Right ovary follicle N° Lap	Right ovary PO cyst N° US	Right ovary PO cyst N° Lap
A1 (6) [α]	0.222	N	anes	Y	N	N	-	0	0	0	-	0	0	0
A2 (12) [α]	0.590	N	anes	Y	Y	Y	3	0	1	1	2	2	0	0
A3 (12)	0.852	N	anes	Y	N	Y	3	0	0	0	-	0	0	1
A4 (9)	2.185	N	anes	Y	N	N	2	0	1	1	0	1	0	1
A5 (9)	0.413	N	anes	N	N	-	0	0	0	0	0	0	0	3
A6 (11) [α]	1.463	N	anes	Y	Y	Y	-	0	0	0	-	0	0	7
A7 (11) [α]	1.176	N	anes	Y	Y	N	ms	mul	4	4	ms	mul	2	2
A8 (11) [α]	1.263	Y	pro/est	Y	Y	Y	1	1	2	2	3	2	0	0
A9 (7) [α]	0.801	N	anes	Y	N	N	0	0	0	2	0	0	1	1
A10 (12)	2.445	N	anes	N	Y	N	ms	0	3	3	1	0	5	5
A11 (14)	2.462	Y	pro/est	N	N	-	-	1	0	0	2	1	2	2
B12 (14)	7.058	Y	pro/est	Y	Y	Y	ms	1	1	0	ms	0	0	0
B13 (14)	4.093	Y	pro/est	Y	Y	Y	6	0	10	mul	2	0	0	0
B14 (14)	2.760	undefined	anes	Y	Y	Y	2	mul	1	2	3	0	1	1
B15 (10)	2.784	Y	pro/est	Y	Y	N	4	1	1	1	1	1	0	0
B16 (11)	1.031	Y	pro/est	Y	Y	Y	1	1	0	0	5	0	2	2
B17 (8)	1.526	Y	pro/est	Y	Y	Y	3	2	0	0	1	0	1	1
B18 (8)	1.359	N	anes	N	Y	N	-	0	0	1	0	0	5	5
B19 (14)	7.352	Y	pro/est	Y	Y	Y	ms	mul	1	1	2	2	3	mul
B20 (12)	6.411	N	pro/est	Y	Y	Y	4	4	3	3	0	0	1	1
B21 (14)	1.849	Y	pro/est	Y	N	N	2	2	0	0	5	5	0	0

Abbreviations: A, site A; B, site B; [α], prior deslorelin treatment; SPC, serum progesterone concentration; Y, yes; N, no; anes, anoestrus; pro/est, pro-oestrus or oestrus; US, ultrasound;
Lap, laparoscopy; ms, multiple small follicles(< 2mm diameter); mul, multiple cysts or follicles; PO, para-ovarian; -, ovary not seen

2.3. *Laparoscopy*

Immediately following the ultrasonographic examination, each cheetah underwent a single incision laparoscopic surgery (SILS) starting in dorsal recumbency. A 5 mm 0° endoscope (Hopkins Optik, Karl Storz GMBH & Co. KG, Tuttlingen, Germany) was inserted for visual inspection of the abdominal cavity. The cheetahs were tilted 45° to the left followed by 45° to the right, and the right and left ovaries and associated structures, respectively were identified. Digital images were captured using a H3 camera head and HD camera Image 1 HUB with a Xenon 300 nova light source, AIDA data recording device and 26 inch screen (Karl Storz GMBH & Co. KG, Tuttlingen, Germany).

Thereafter, all cheetahs underwent bilateral salpingectomy (n = 10) or ovariectomy (n = 11). The authors retrospectively examined the laparoscopic images obtained and described both the observed ovarian structures, follicles, periovulatory or luteal structure (either CH or CL) and PO cysts and compared them to the ultrasonographic images obtained.

2.4. *Reproductive status*

2.4.1. *Vaginal cytology*

Vaginal smears were obtained from each cheetah immediately prior to ultrasonography after passage of a sterilized, clear, perspex tube speculum of 12 mm diameter *via* the vulva and vestibulum. Thereafter, a sterile cotton-tipped swab attached to a stainless steel wire was passed through the tube until making contact with the cranial vaginal mucous membrane, rotated several times, retrieved and rolled gently on a glass microscope slide that was air-dried, and stained with Diff-Quik® (Kyroquick, Kyron Laboratories, Benrose, South Africa) before permanent mounting using Entellan® (Merck, Halfway House, South Africa) and a glass cover slip. The slide was examined microscopically to describe the presence and percentage of superficial epithelial cells expressed as the Superficial Cell Index (SCI) and the clarity of the background as an indicator of the presence or absence of oestrogenisation [1,2].

2.4.2. Hormone assays

Serum samples were collected for SPC determination as an indication of periovulatory or luteal status by radioimmune assay (RIA) [1, 2]. The samples were stored at -40 °C until transfer to the Immunocontraception Laboratory, Faculty of Veterinary Science, University of Pretoria followed by

thawing for assay using progesterone RIA kits (Progesterone Coat-a-Count, Diagnostic Products) as previously described [17].

2.4.3. Ovarian structures

The ovaries were observed *in situ* by a combination of ultrasonography and laparoscopy. The ultrasonographic ovarian dimensions (length, width and height) were measured for each observed ovary and volumes calculated using the method of Pavlik *et al* as an indicator of cyclic ovarian activity [24]. The calculated ovarian volumes from the individual cheetahs in which both ovaries were visualised (n = 16) were compared with their reproductive status as assessed from the SPC and vaginal cytology data. The ultrasonographic characteristics and dimensions of all observed ovarian structures (follicles, CH and CL) and adnexal PO cysts, were recorded. Ovarian activity on laparoscopic visualisation was defined by the observation of prominent structures. Clear, vesicular-appearing structures were recorded as follicles. The periovulatory or luteal phases were defined by observation of one or more prominent, somewhat irregular yellowish structures with evidence of increased vascularization [13,25].

2.4.4. Categorisation of cyclic activity

Based on the combination of vaginal cytology, SPC measurements and the observed ultrasonographic and laparoscopic ovarian structures, the cheetahs were categorised as being cyclically active (pro-oestrus or oestrus and dioestrus) or inactive (anoestrus) [10].

2.4.5. Deslorelin treatment

At Site A, six cheetahs received $\geq$ one (range: 1 - 3) treatments between 2003 to 2013 with 4.7 mg deslorelin implants (Suprelorin®, Virbac, Milperra, NSW, Australia) with the most recent administration in Cheetah No. 1 approximately 12 months prior to examination. The effects of this treatment on uterine pathology and reproductive activity were compared with the non-treated study populations from both sites (n = 15).

2.5. Statistical analysis

Data was tested for normality using the Kolmogorov-Smirnov test. Non-parametric data was reported as median and interquartile range (IQR) according to Tukey's Hinges. The Wilcoxon single rank test was used to compare contralateral ovarian volume. The Mann-Whitney U test was used to compare total uterine volume and uterine horn diameter in cyclically active and inactive cheetahs.

The mean number of follicles $\geq$ 2 mm diameter detected in each cheetah by ultrasound or laparoscopic examination were compared between these modalities using a paired samples T-test. Proportions of cyclically-active and inactive animals that showed uterine oedema was analysed using a 2-sided Fishers exact test. Similarly a 2-sided Fishers exact test was used to determine the ratios of deslorelin-treated animals that showed uterine pathology and additionally, their reproductive activity. Sensitivity and specificity data were obtained using empirical ROC curves and a cut-off value for 100% specificity was determined. A linear regression model with Bonferroni correction was used to determine the function between ovarian volume and uterine horn diameter. All analyses were performed with SPSS v.17 (IBM, New York, USA) statistical software package. Statistical significance was defined as $P<0.05$ in all cases with the exception of the regression model ($P<0.025$).

3. Results

3.1. Ultrasonography

The uterine body dorsal to the bladder was difficult to detect as the caudally converging uterine horns entered the pelvic canal at the pubic rim before joining and could not be seen further caudally due to pubic bone acoustic shadowing. The cervix and vagina were thus also not observed. At least one ovary or uterine horn was seen in all but two cheetahs (Table 1). Thirty one of a possible 42 uterine horns (76.2%) were seen either by imaging transversely cranial to the pubis and detecting the horns dorsal or adjacent to the bladder, or by following the ovaries caudally. Eleven cheetahs had oedematous uterine walls, usually with visible wall-layering and thus likely to be in either pro-oestrus or oestrus (Fig. 1A), whereas eight had non-oedematous uterine walls, likely to be associated with anoestrus (Fig. 2A) (Table 1). In cheetahs categorised as cyclically active (i.e. pro-oestrus or oestrus); uterine horns were seen in 17 of 20 (85%) and 14 of 22 horns (64%) were seen in those categorised as inactive (i.e. anoestrus) (Table 1). There was no statistical difference in uterine horn diameter between cyclically active (4.7 IQR 4.4 - 5.2 mm; n = 9) and inactive individuals (4.2
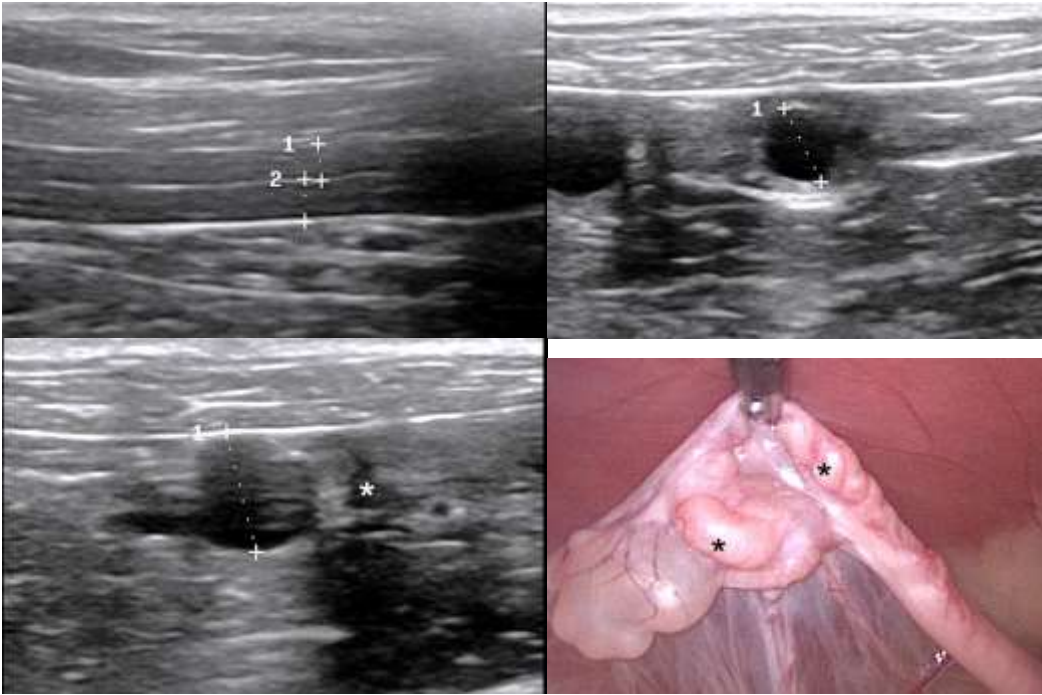
Fig. 1. Cheetah 8, an 11-year-old, in pro-oestrus or oestrus. Ultrasonographic (A-C) and laparoscopic images (D): (A) oedematous right uterine horn with wall layering. Walls were 2.6 and 3.0 mm thick (between cursors) giving a total wall diameter of 5.6 mm (B) left ovary with 6.2 mm diameter anechoic follicle (between cursors) demonstrating acoustic enhancement. Anechoic structure to left of follicle was a paraovarian cyst (C) paraovarian cyst cranial to the left ovary 8.7 mm in diameter (between cursors). Anechoic structure (*) is a section of left fallopian tube with hydrosalpinx. (D) left ovary with large paraovarian cyst cranial to the ovary and hydrosalpinx seen prominently to the right of the forceps and superimposed on the ovary (*). Cranial is to the left.

Table 2
Ovarian cut-off volume for cyclic reproductive activity.

Ovarian volume	Cyclic	Non-Cyclic	Total
<1 200 mm^3	3	7	10
>1 200 mm^3	6	0	6
Total	9	7	16

Sensitivity 67% Specificity 100%

IQR 3.7-5.2 mm; n = 9) (P = 0.340; U = 29) or between cyclic reproductive activity and uterine oedema (P = 0.250) (Table 2). Ultrasonography identified five cheetahs (23.8%) with features consistent with mild uterine pathology. Mild cystic endometrial hyperplasia (CEH) with one to three ultrasonographically visible intra-mural cysts (largest = 5.9 mm diameter) was present in four cheetahs (19.0%) ages 11, 12, 14 (2x) years (Fig. 3A). A small accumulation of intra-luminal uterine fluid, believed to be a
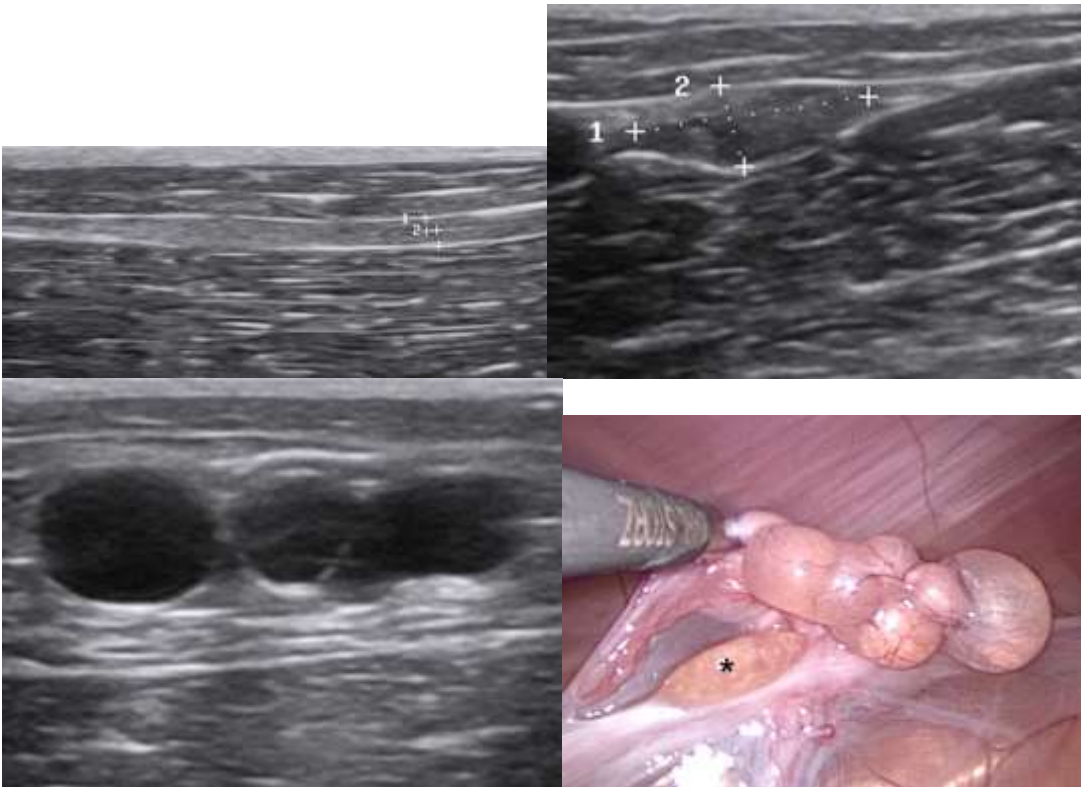
Fig. 2. Cheetah 18, an 8-year-old, in anoestrus. Ultrasonographic (A-C) and laparoscopic images (D): (A) homogenous mildly hyperechoic right uterine horn with no wall layering. Walls were 0.9 and 1.1 mm thick (between cursors) giving a total wall diameter of 2 mm (B) inactive right ovary 16.7 mm long and 5.9 mm wide (between cursors) (C) multiloculated paraovarian cyst cranial to the right ovary (D) right ovary with large paraovarian cyst cranial to the ovary (*) Normal fallopian tube just below forceps.

mucometra, was seen in one 12 year-old cheetah. Hydrosalpinx, which mimicked small ovarian or PO cystic structures, was present in four cheetahs (19%) ages 8, 16, 19, 20 years (Fig. 1C, 1D), two of which additionally showed CEH.

Thirty five of a possible 42 ovaries (83.3%) were seen in 19 cheetahs with none in two and unilaterally in three cheetahs (Table 1). On the left side, colonic gas and on the right side, small intestines, respectively occasionally interfered with attempts to image the ovaries. The left ovary was generally easier to find than the right where small intestines often hampered ovarian identification. The ovary was usually located immediately caudal to the region where the caudal hyperechoic edge of the caudal perirenal fat joined the ventral hyperechoic surface edge of the iliopsoas muscle fascia at an angle of approximately 60 degrees as described in lions [5]. In some cheetahs the ovaries were more readily found by tracing the uterine horns cranially.
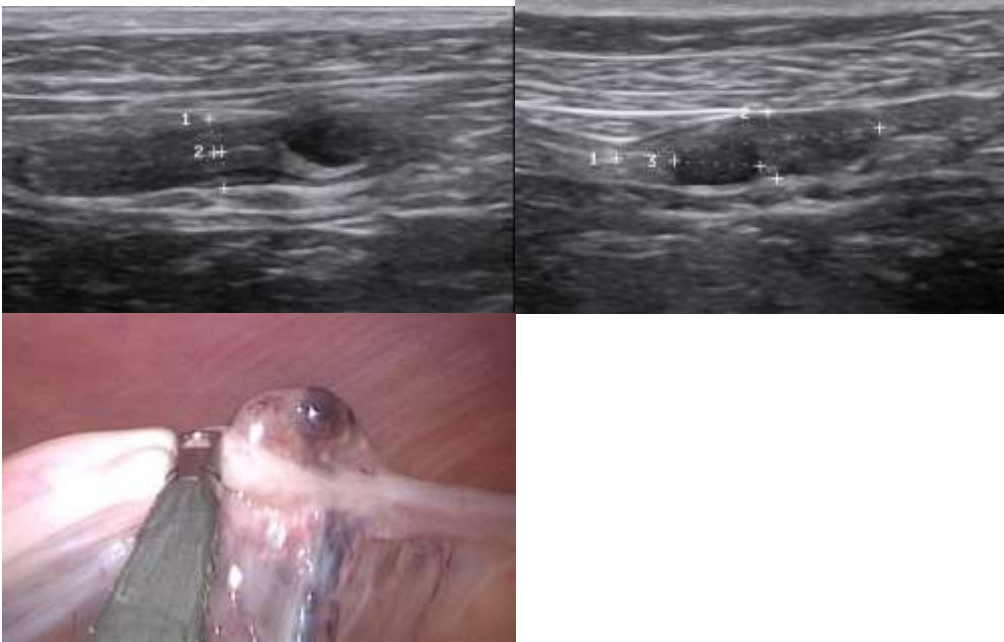
Fig. 3. Cheetah 21, a 14-year-old, in pro-oestrus or oestrus. Ultrasonographic (A-B) and laparoscopic images (C): (A) oedematous left uterine horn with wall layering. Walls were 2.5 and 2.7 mm thick giving a total wall diameter of 5.2 mm Note the anechoic mural structure to the right of the cursors with acoustic enhancement which was a large endometrial cyst 5.9 mm in diameter. This uterus had 2 additional smaller cysts (B) left ovary with 6.3 mm diameter (between cursors no. 3) anechoic to hypoechoic peri-ovulatory structure with very mild acoustic enhancement. (C) left ovary showing a vascularised purple-coloured periovulatory follicle superficially. Cranial is to the right.

Structures classified as follicles were seen on 28 ovaries (Fig. 1B) and those structures classified as periovulatory follicles were seen on six ovaries (Fig. 3B). The largest follicular structure observed was 6.2 mm. The mean number of follicles detected per cheetah which included visualisation of both ovaries was 2.7 ± 2.6. Seven ovaries lacked discernible cyclic activity (Fig. 2B).

Single or clusters of multiple PO cysts (n = 21) (Table 1) in 16 (76.2%) cheetahs were observed on ultrasound, these were detected cranial to the ovaries with the exception of two cheetahs with unilateral PO cysts caudal to the ovary (Fig. 1C, 2C, 4). Ten sites had ≥ one cyst and one site was associated with >10 cysts. The PO cysts varied in diameter up to a maximum of 18.9 mm (Fig. 4). These relatively large, anechoic cystic structures facilitated ultrasonographic detection of the ipsilateral ovaries.
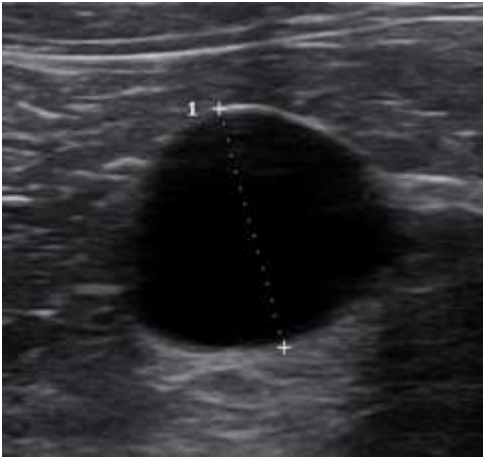
Fig. 4. Cheetah 20, a 12-year-old, in pro-oestrus or oestrus. Ultrasonographic image of the largest of 3 paraovarian cysts (diameter 18.9 mm) cranial to the left ovary.

3.2. Laparoscopy

The laparoscopic ovarian data are given in Table 1 and images presented in Figs. 1D, 2D and 3C. Comparing ultrasound with laparoscopy showed laparoscopy visualised all ovaries including the seven not seen on ultrasound in five cheetahs. Comparing the structures on the ovaries visualised with both ultrasonography and laparoscopy showed that 17 ovaries with follicles visible on ultrasound had corresponding structures on laparoscopy; 11 ovaries with follicles visible on ultrasound lacked corresponding structures on laparoscopy; two ovaries with follicles visible on laparoscopy showed none on ultrasound; the mean number of follicles detected per cheetah including both ovaries was 2.3 ± 3.1; and PO cysts were identified in two additional cheetahs on laparoscopy.

Nine of the 10 cheetahs categorised with cyclic ovarian activity had visible signs of cyclic ovarian activity apparent on both ultrasonography and laparoscopy, with follicles or periovulatory structures seen on 10 ovaries and these modalities agreed 100% on the identification of the type of activity (Table 1). There was no difference between the two modalities in detecting ovarian follicles ($P = 0.611$; $t = 0.517$).

3.3. Ovarian volumes

Sixteen cheetahs had bilaterally-observed ovaries; there was no difference between left (513 IQR 408 - 782 mm^3) and right (521 IQR 376 - 644 mm^3) ovarian volumes ($P = 0.642$), however total ovarian volumes for those categorised as cyclically active, i.e. pro-oestrus or oestrus (n = 9) were significantly greater (1322 IQR 1024 - 1363 mm^3) than those in anoestrus (n = 7) (906 IQR 387 - 964

mm^3) (P = 0.005; U = 6). Total ovarian volumes > 1 200 mm^3 had a specificity of 100%, a sensitivity of 67% and a 1.0 positive predictive value for cyclic ovarian activity (Table 2). The area under the ROC curve was 0.905 (95% confidence interval 0.75 – 1.00) which indicates a high diagnostic accuracy. There was a weak relationship between total ovarian volume and uterine horn diameter (R^2 = 0.306; P = 0.040).

3.4. Reproductive status

The reproductive status assignation of the study population's cheetahs showed 10 (47.6%) consistent with pro-oestrus or oestrus (follicular or periovulatory phase) and 11 (52.4%) with anoestrus (Table 1). None demonstrated a post-ovulatory luteal phase as evidenced by SPC.

3.5. Deslorelin treatment

One treated cheetah (No. 8), with a last treatment date approximately 11 years prior to the study showed obvious reproductive activity (Table 1). Neither the ratios of cheetahs that showed either reproductive activity (P = 0.149) (Table 3) or uterine pathology, as detected by ultrasound (P = 0.262) (Table 4) differed subsequent to treatment with this chemical contraceptive implant.

Table 3
Ratios for cyclic reproductive activity and uterine horn oedema

		Uterine horn oedema		
		Yes	No	Total
Cyclic	count	7	2	9
Non-cyclic	count	4	6	10
Total	count	11	8	19

Table 4
Ratios for deslorelin contraception and uterine pathology

			Uterine pathology		
			Pathology	No pathology	Total
Contracepted or not	Contracepted	count	0	6	6
	Not contracepted	count	5	10	15
Total		count	5	16	21

4. Discussion

The study populations' median age placed them within a cohort ideal for evaluating the effects of aging reportedly associated with markedly decreased reproductive competency where older cheetahs were classified as 9 to 15 years of age [13]. Ultrasonographic evaluation of the reproductive tract, uniquely enhanced by the concurrent direct laparoscopic visualisation of structures, provided a valuable insight into the reproductive status of cheetahs. Laparoscopically the absence of an enveloping ovarian bursa obscuring the felid ovary (as found in *Canidae)* enabled complete visualization of the ovary, its associated structures and adnexa. Laparoscopic visualization of the cheetah ovary had previously only been reported for assessing the effects of exogenous gonadotropin administration and oocyte retrieval [13].

Ovaries lacking structures associated with normal cyclic activity proved relatively difficult to visualize on ultrasonography, however most had follicles and additionally many had associated adnexal PO cysts facilitating their localization. Following ovarian localization, the adjacent ipsilateral uterine horn was generally observed facilitating the process of following the uterine horns further caudally. The relatively low body weight of the cheetah together with numerous active ovaries facilitated identification and accurate ultrasonographic data collection when compared to larger wild felids. Comparing the cheetahs to a similar ultrasonograhic study in sexually mature lions, 76.2 *versus* 58% (uterine horns) and 83.3 *versus* 46% (ovaries) in cheetahs and lions respectively were recorded [5]. The greater detection rate in cheetah compared to lions is most likely due to cheetahs being smaller, weighing less and having less intra-abdominal fat. These factors allow higher frequency transducers to be used with associated improved resolution. In this study the presence of PO cystic structures primarily, and follicles secondarily, were the most important factors enhancing ovarian visibility and abdominal fat did not impede visualization of reproductive structures. The trans-

abdominal route utilises widely-available equipment and facilitated optimal imaging of the ovaries and adnexal structures in cheetahs in the field, a primary aim of this current study. Despite a paucity of reports in cheetahs, trans-rectal ultrasonography may provide enhanced visualisation of the uterus in particular [6] although its requirement for a specialised transducer and potential risks of this invasive approach may outweigh its potential benefits when applied in the field. To optimize visibility of the described structures it is advisable to use appropriate equipment and experienced ultrasonographers.

Although cyclically active and inactive animals were readily distinguished, the precise differentiation of follicular (including pro-oestrus, oestrus, periovulatory) and post-ovulatory (luteal) status was less straightforward. Certainty of detection of ovulation *via* definition of a post-ovulatory CL and hence precise determination of oestrous status by ultrasonography alone may be controversial as was supported by the current study [10,12]. The predictive value of ovarian volumes for determining cyclic activity in cheetahs was supported. The absence of any luteal phase in the current study supported the reported rarity of spontaneous ovulation in captive cheetahs when compared with lions, despite the relative close proximity of male cheetahs [1,3,5 7]. Ultrasonography of uterine horn diameter and uterine wall oedema were not significant predictors of cyclic activity. The uterine horns in cheetahs showing cyclic activity (85%) were however more readily visualised than horns in inactive cheetahs (64%).

Previous studies reporting effects of GnRH agonists including deslorelin in various domestic and non-domestic felids were limited by indirect methods such as laboratory-based serological and faecal steroid metabolite assays for evaluating cyclic ovarian activity when compared with direct ultrasonographic evaluation of ovarian and uterine appearance [2,13,14]. Inconsistent responses to GnRH agonists may make ultrasonography an ideal modality for monitoring any indication for additional administration of these agents in captive wild felids under field conditions [14]. The current study supported the previously reported absence of any obvious side effects despite repeated deslorelin administration [2].

This study supported a previous report describing ovarian activity in older, nulliparous female cheetahs where age had no apparent influence on ovarian volumes [13]. Relatively few cheetahs showed ultrasonographic evidence of either CEH or mucometra, correlating with a previous-report in a similar age cohort where these findings did not differ amongst three age categories [13]. Previous captive cheetah uterine histological studies have found increasing age associated with increasing incidences of uterine degenerative changes with variable effects of parity [13,26].

The relatively high prevalence of adnexal PO cystic structures (76.2%) in this study population in either a cranial location on the suspensory ligament (87.5%) or a caudal location between distal uterine horn and ovary (12.5%), associated with the reproductive tract was notable. Paraovarian (also

paratubal) cysts are benign intraperitoneal cysts arising from the broad ligament and are mesonephric duct remnants [27-29]. Paratubal cysts additionally include paramesonephric remnants, the small hydatid cysts of Morgagni attached to the fimbriae of the fallopian tube [27,28]. Their significance in cheetahs is undefined and reportedly they do not appear to influence fertility [6].

The current study's ultrasonographic identification of numerous incidents of adnexal cystic structures including hydrosalpinx must be considered in their differentiation from normal ovarian structures in similar populations. Paraovarian cysts have been described in domestic animal species including sheep and wild felids including cheetahs, lions and tigers, with parauterine (PU) cysts being reported in the lion [5,6,8,30]. Ultrasonography is the primary and preferred imaging modality for anechoic cystic structures visualised in women and these include follicles, functional follicular cysts, early CL's, PO cysts, and the convoluted appearing hydrosalpinx [31]. In our study there was no difference in the number of follicles observed between ultrasound and laparoscopic examination. Adnexal cysts are usually separated from the ovary but if closely adjacent may be mistaken for follicles. In women they occur in middle-age, tend to be benign but may predispose to ovarian torsion [31]. Concomitant laparoscopy was ideal for verification of these and other ultrasonographic findings. A limitation of this study was that of laparoscopy in clearly observing smaller, intra-ovarian follicles < 2mm in diameter. This may contribute to discrepancies but concurrent application of other clinical and laboratory modalities will not appreciably bias the ability to categorise cyclic activity.

Sterilization using various laparoscopic techniques are described in tigers and lions and similarly proved an appropriate surgical technique for captive cheetahs, being both rapid and with low complication rates that facilitated the postoperative care of these wild felids [22,30,32].

In conclusion, trans-abdominal diagnostic ultrasound in captive cheetahs proved accurate in determination of reproductive activity that was enhanced when combined with SPC and vaginal cytology. Ultrasonography showed a relatively high rate of apparently normal ovarian activity and limited evidence of functionally-significant pathology of the tubular tract in this aged population. These procedures offer potential benefits in assessment of individual reproductive function, including subfertility, and assisted reproduction techniques in similar populations of cheetahs.

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References

[1]   Schmidt AM, Nadal LA, Schmidt MJ, Beamer NB. Serum concentrations of estradiol and progesterone during normal oestrous cycle and early pregnancy in the lion (*Panthero leo*). J Reprod Fertil 1979;57:267-72.

[2]   Bertschinger HJ, de Barros Vaz Guimaraes MA, Trigg TE, Human, A. The use of deslorelin implants for the long-term contraception of lionesses and tigers. Wildlife Res 2008;35:525-30.

[3]   Brown JL. Female reproductive cycles of wild female felids. Anim Repro Sc 2010;3-4:155-62.

[4]   Bertschinger HJ, Meltzer DGA, van Dyk A.   Captive breeding of cheetahs in South Africa - 30 years of data from the de Wildt cheetah and wildlife centre. Reprod Dom Anim 2008;43 (Suppl. 2), 66-73.

[5]   Kirberger RM, Schulman ML, Hartman MJ. Ultrasonographic and laporoscopic evaluation of the reproductive tract of the female African lion (*Panthero leo)*. Theriogenology 2011;76:810-18.

[6]   Wachter B, Thalwitzer S, Hofer H,Lonzer J, Hildebrandt TB. Reproductive history and absence of predators are important determinants of reproductive fitness: the cheetah controversy revisited. Conserv Letters 2011;4:47-54.

[7]   Brown JL. Comparative endocrinology of domestic and non-domestic felids. Theriogenology 2006;6:25-36.

[8]   Goodrowe, KL. Feline reproduction and artificial breeding technologies. J Repro Sci 1992;28:389-97.

[9]   Brown JL, Bush M, Packer C, Pusey AE, Montfort SL, O'Brien JL, et al. Hormonal characteristics of free ranging female lions (*Panthera leo*) of the Serengeti plains and Ngorongoro crater J Reprod Fert 1993;87:107-14.

[10]   Davidson AP, Baker TW. Reproductive ultrasound of the bitch and queen. Top Comp An Med 2009;24:55-63.

[11]   Peter AT, Jakovljevic S. Real-time ultrasonography of the small animal reproductive organs. Comp Contin Educ Pract Vet 1992;14:739-44.

[12]   Root, CR and Spaulding, KA. Diagnostic imaging in companion animal theriogenology. Seminars in Vet Med and Surg (Small Animal) 1994;9:7-27.

 [13]   Crosier AE, Comizzoli P, Baker T, Davidson A, Munson L, Howard JG, et al.. Increasing age influences uterine integrity, but not ovarian function or oocyte quality, in the cheetah (*Acinonyx jubatus*). Biol Reprod 2011;85:243-53.

[14]   Ackermann CL, Volpato R, Destro FC, Trevisol E, Sousa NR, Guaitolini CRF, et al. Ovarian activity reversibility after the use of deslorelin acetate as a short-term contraceptive in domestic queens. Theriogenology 2012;78:817-22.

[15]   Jurke HM, Czekala NM, Lindburg DG, Millard SE. Fecal corticoid metabolite measurement in the cheetah (*Acinonyx jubatus*). Zoo Biol 1997;16:133-47.

[16]   Bertschinger HJ, Jago M, Nothling JO, Human A. Repeated use of the GnRH analogue deslorelin to down-regulate reproduction in male cheetahs (*Acinonyx jubatus*) Theriogenology 2006;66:1762-1767

[17]   Bertschinger HJ, Asa CS, Calle PP, Long JA, Bauman K, DeMatteo K, et al. Control of reproduction and sex related behaviour in exotic wild carnivores with the GnRH analogue deslorelin: preliminary observations. J Reprod Fertil Suppl 2001;57**:**275-83.

[18]   Johnston SD, Root-Kustritz MV, Olson PNS. In: Kersey R, editor. Canine and feline theriogenology. W. B. Saunders Company; 2001, p. 1-15.

[19]   Hildebrandt TB, Hermes R, Jewgenow K, Goritz F. Ultrasonography of the   urogenital tract in elephants (*Loxodonta africana and Elephas maximus):* an important tool for assessing female reproductive function. Zoo Biol 2000;19:321-32.

[20]   Hermes R, Hildebrandt TB, Goritz F. Reproductive problems directly attributable to long-term captivity – asymmetric reproductive aging. Anim Reprod Sci 2004;82-83:49-60.

[21]   Republic of Namibia: National conservation action plan for cheetah in Namibia. http://www.met.gov.na/SiteDirectory/Documents/Final%20draft_Cheetah%20NAP_Namibia_Sep%202013.pdf. Accessed 14 July 2014.

[22]   Hartman MJ, Monet E, Kirberger RM, Schmidt-Kuntzel A, Schulman ML, Stander JA, et al. Single-Incision laparoscopic sterilization of the cheetah (*Acinonyx jubatus*). Vet Surg DOI: 10.1111/vsu.12341.

[23]   Hartman MJ, Kirberger R, Tordiffe A, Boy S, Schoeman J. Laparoscopic removal of a large abdominal foreign body granuloma using single incision laparoscopic surgery (SILS) and extraction bag in a cheetah (*Acinonyx jubatus*) Vet Record Case Reports 2015 doi:10.1136/vetreccr-2014-000162

 [24]   Pavlik EJ, DePriest PD, Galcheetah HH, Ueland FR, Reedy MB, Kryscio RJ, et al. Ovarian volume related to age. Gyn Oncol 2000;77:410-412.

[25]   Nothling JO, de Cramer KGM, Gerber D, Kammer VR. Luteal and follicular count in bitches: Assessment by means of magnetic resonance imaging. Theriogenology 2006;66:1343-54.

[26]   Munson L, Gardner IA, Mason RJ, Chassy LM, Seal US. Endometrial hyperplasia and mineralization in zoo felids treated with melengesterol acetate contraceptives. Vet Pathol 2002; 39: 419-27.

[27]   Muolokwu E, Sanchez J, Bercaw JL, Sangi-Haghpeykar H, Banszek T, Brandt ML, et al. The incidence and surgical management of paratubal cysts in a pediatric   and adolescent population. J Ped Surg 2011;46:2161-63.

[28]   Palmieri C, Schiavi E, Saldaa LD. Congenital and acquired pathology of ovary and tubular genital organs in ewes: A review. Theriogenology 2011;75;393-410.

[29] Schlafer DH, Gifford AT. Cystic endometrial hyperplasia, pseudo-placentation, endometrial hyperplasia, and other cystic conditions of the canine and feline uterus. Theriogenology 2008; 70: 349-58.

[30] Emerson JA, Case JB, Brock AP, Vigani A, Graham DR, Isaza R. Single-incision, multicannulated, laparoscopic ovariectomy in two tigers (*Panthera tigris*). Vet Quarterly 2013;33:108-11.

[31] Ackerman S, Irshad A, Lewis M, Anis M. Ovarian cystic lesions. A current approach to diagnosis and management. Radiol Clin N Am 2013;51:1067-85.

[32] Hartman MJ, Monet E, Kirberger RM, Venter LJ, Bester L, Schulman ML, et al. Laparoscopic sterilization of the African lioness (*Panthera leo*). Vet Surg 2013;5:559-64.