Animal Reproduction Science 213 (2020) 106260

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

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

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

[a] Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110, South Africa
[b] Mammal Research Institute, Department of Zoology and Entomology, Faculty of Natural and Agricultural Sciences, University of Pretoria, Private Bag X20, Hatfield, 0028, South Africa
[c] Centre for Veterinary Wildlife Studies, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110, South Africa

ARTICLE INFO

Keywords:
African lion
Estrus detection
Non-domestic felids
Ovarian cycle
Vaginal smear

ABSTRACT

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

1. Introduction

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

Most felids only have ovulations if mating occurs (induced ovulations) and during the period of the ovarian cycle there may be no ovulations (alternate periods of estrus and inter-estrus without ovulation), or ovulations (diestrus occurs after estrus and ovulation; Fig. 1) (Andrews et al., 2019). When there is conception after ovulation, a luteal phase ensues and is maintained beyond the typical period of luteal function if there is a signal from the developing fetal tissues indicative of pregnancy. If there is no pregnancy, the

* Corresponding author.
E-mail addresses: icallealta@gmail.com (I. Callealta), andre.ganswindt@up.ac.za (A. Ganswindt), imke.lueders@geolifes.com (I. Lueders).

https://doi.org/10.1016/j.anireprosci.2019.106260
Received 3 October 2019; Received in revised form 30 November 2019; Accepted 16 December 2019
Available online 17 December 2019
0378-4320/

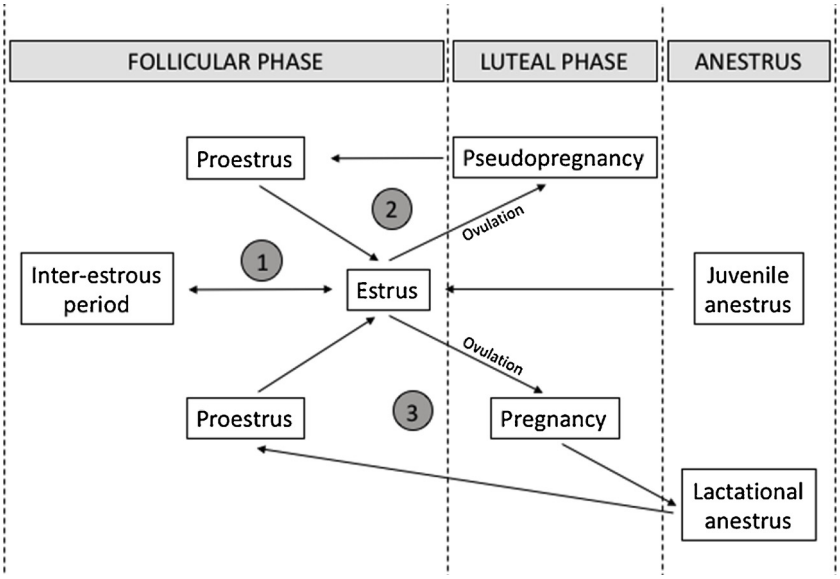

Fig. 1. Feline reproductive cycle: non-ovulatory cycle (1), cycle without associated ovulation (2), and cycle with associated ovulation (3).

period of diestrus ensues and the luteal tissues regress after a typical period of function (Andrews et al., 2019).

Variations in the concentration of circulating estrogen during the estrous cycle are associated with changes in the cells of the mucous membrane of the reproductive tract (Johnston et al., 2001). Thus, evaluation of the type and proportion of epithelial cells in serial vaginal smears serves as an indirect method to monitor fluctuations in estradiol concentrations, and therefore, progression of the reproductive cycle (von Heimendahl and England, 2010). In addition, vaginal cytology evaluations may potentially be used to predict the time for mating and artificial insemination in non-domestic felids, similar to what occurs in domestic dogs (Johnston et al., 2001). In the domestic dog, cytological changes of the vaginal epithelium have been precisely determined (*e.g.*, Schutte, 1967; Bell et al., 1973). There, however, are few reports on vaginal cytology evaluations of domestic cats (Mowrer et al., 1975; Herron, 1977; Shille et al., 1979; Mills et al., 1979; Cline et al., 1980) and even fewer with non-domestic felids (Liche and Wodzicki, 1939; Asa et al., 1992). The results from these previous studies indicated vaginal cytology observations could be used for detection of estrus in domestic cats, but was not useful to distinguish between inter-estrous periods and diestrus (Johnston et al., 2001; von Heimendahl and England, 2010). Additionally, in cheetahs (*Acinonyx jubatus*), there seemed to be a larger number of leukocytes in the vaginal cytology samples during inter-estrus than during gestation (Asa et al., 1992). In most studies performed in non-domestic cats, there was use only of opportunistic or infrequent vaginal samples, and therefore, there was a lack of a systematic approach with many of these previous assessments.

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

2. Materials and methods

2.1. Study animals

Five adult (3.5–9 years of age) female African lions located in a private conservation center in South Africa served as study subjects for this research. Three of these females were proven fertile, cohabiting with an adult male (6 years of age) in an 800–1200 m^2 outdoor enclosure which included natural substrate, trees, and a shelter. The remaining two females were nulliparous and were located together in another enclosure with similar conditions as those for the females housed with the male. All animals were within visual, auditory, and olfactory range of each other. All lions were healthy and in good body condition. The five lionesses were trained by positive reinforcement conditioning to voluntarily allow for collection of vaginal cytology samples using swabs (Callealta et al., in press). This study was conducted with the permission of the Animal Ethics, Use and Care, and Research Committees of the University of Pretoria, South Africa (V052-17).

2.2. Behavioral monitoring

For 12 months, the behavior of the five females was monitored twice a day (at sunrise and dusk) 5–7 days per week, in sessions of 15 − 60 min. A relative increase in the frequency of specific reproductive symptoms such as purring, flirting run, lordosis, allowing for mounting, copulation, and rolling, enabled detection of females in natural estrus (Stanton et al., 2015). Behavioral estrus usually

lasted about 6 days, while inter-estrous periods and diestrus were identified by absence of specific reproductive symptoms for fewer than 21 consecutive days and more than 21 consecutive days, respectively (Callealta et al., submitted-a). Lactational anestrus was observed after parturition and was sustained for a maximum of 3 weeks.

2.3. Collection of vaginal cytology samples, sample smear preparation, and sample interpretation

In parallel to behavioral monitoring, vaginal samples were collected during training every 1–3 days from females in estrus and during the inter-estrous interval, and every 3–7 days from females during the diestrous period (Callealta et al., in press).

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

The samples on every slide were assessed using a microscope at x40 and x200 magnification to quantify the amount of mucous and cellular debris and the extent to which cell clumping occurred. The quantity of mucus and/or cellular debris (*i.e.*, clearing) were rated from 0 to 6 and classified as "minimal quantity/no debris" (0–1), "small quantity/small debris abundance" (1–2.5), "moderate quantity/moderate debris abundance" (2.5–4.5), and "large quantity/large debris abundance" (4.5–6). Cellular distribution (*i.e.*, clumping) was rated from 0 (single epithelial cells) to 3 (large piles or clusters of epithelial cells). There was assessment of 200 epithelial cells at x1000 magnification and cells were classified into groups (*i.e.*, basal, parabasal, intermediate, superficial nucleated, and superficial enucleated) as described for other species (Johnston et al., 2001). To standardize these groups, the major axes of 30 cells of each type were measured in six vaginal cytology samples of three different females in estrus and diestrus, using an ACA Basler 1300-200 µc camera attached to a Nikon E50i microscope and the software Sperm Class Analyser SCA 6.3 with Morphology Module (Microptic SL, Barcelona, Spain). The relative number of epithelial cells was classified as "small" (0 %–20 %), "moderate" (20 %–50 %), and "large" (50 %–100 %). In addition, the number of polymorphonuclear neutrophils (PMN) observed per 100 epithelial cells, as well as the quantity of microbial presence (including genus Simonsiella) were recorded. The number of PMN was classified as "minimal or absent" (0–2/100 epithelial cells), "small" (2–20/100 epithelial cells), "moderate" (20–100/100 epithelial cells), "large" (100–400/100 epithelial cells), and "very large" ($>$ 400/100 epithelial cells). The number of bacteria was classified as "minimal" ($<$ 10 microorganisms per field), "small/few" (10–25 microorganisms per field), "moderate" (25–50 microorganisms per field), and "large" ($>$ 50 microorganisms per field). In total, there was evaluation of 541 vaginal smears (108.2 ± 13.9 samples per female; range: 56–133), collected within the 12-month observation period.

2.4. Data analysis

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

3. Results

3.1. Vaginal epithelial cell types

Basal epithelial cells of female African lions were small (15.6 ± 0.48 µm diameter), rounded, and stained pink to pale blue (Fig. 2a). These cells had a large nucleus and small cytoplasm, and were not frequently detected in the vaginal smear at any stage of the reproductive cycle. Parabasal epithelial cells were similar to basal cells in shape and color of staining, but had a larger diameter (22.3 ± 0.67 µm; Fig. 2b). Intermediate epithelial cells had large nuclei and stained pink to pale blue; however, there was considerable variation in shape and size (36.0 ± 1.12 µm diameter; Fig. 2c). Superficial epithelial cells were large (54.4 ± 0.82 µm diameter), angulated, and generally stained dark blue. The nuclei of these cells were generally small, dark, and pyknotic; however, could also be stained to a minimal extent or staining could be completely absent due to degeneration and apoptosis (Fig. 3).

3.2. Cytological cellular characteristics of the lioness reproductive cycle

One single vaginal smear was sufficient to identify when a lionesses was in estrus (Fig. 4a, b).This stage of the reproductive cycle

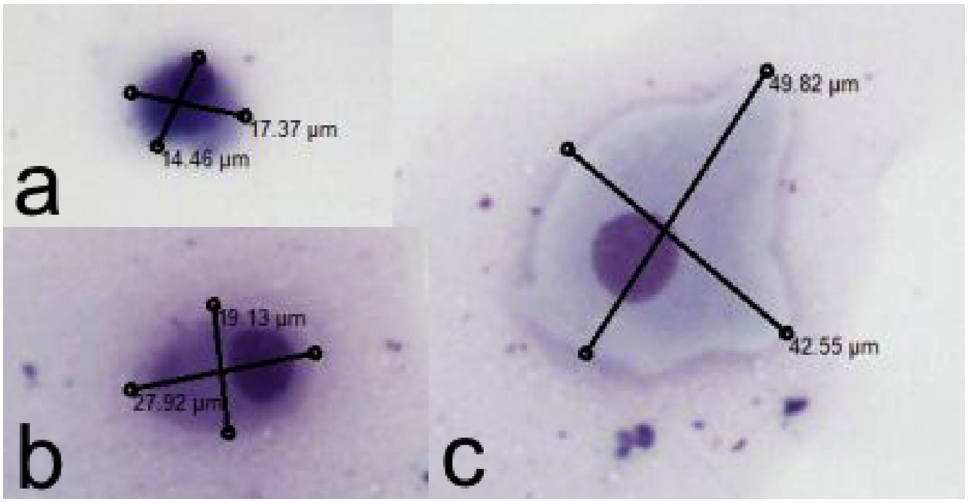

Fig. 2. Microscopic images of vaginal basal (a), parabasal (b), and intermediate (c) epithelial cells of African lions, stained with modified Wright-Giemsa.

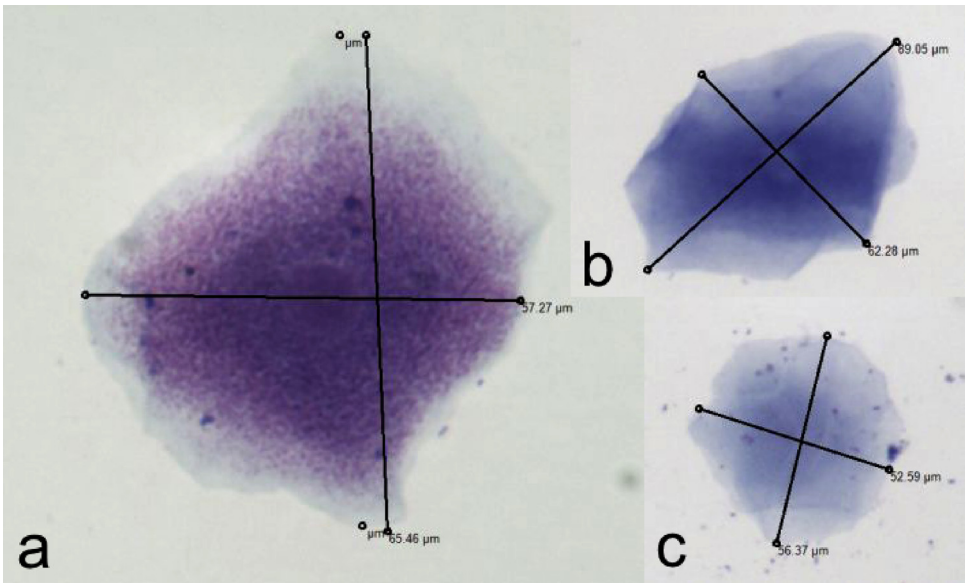

Fig. 3. Microscopic images of vaginal superficial epithelial cells in different amounts of degeneration, stained with modified Wright-Giemsa: nucleated (a), nucleated with partially pyknotic nucleus (b), and enucleated (c).

was precisely characterized when there was presence of a large proportion of superficial epithelial cells (n = 155; mean = 99.79 %; 95 % CI [99.69, 100]), absence of PMN (n = 155; mean = 0.36 PMN; 95 % CI [0, 0.59]), moderate-to-large number of bacteria (n = 139; median = moderate; 95 % CI [moderate, large]), and minimal quantity of mucus/cellular debris (n = 155; median = minimal; 95 % CI [minimal, minimal]) (Table 1). *Simonsiella* spp were detected in 52.9 % of vaginal smears collected during estrus and were most prevalent from the third day of estrous behavior (72.6 %), compared with the first (16.7 %) and second (36.7 %) days of the estrous period.

Likewise, the assessment of one single cytology sample enabled detection of females in advanced diestrus (Fig. 4c, d). In this stage, there was a large proportion of parabasal and intermediate cells (n = 222; mean = 79.76 %; 95 % CI [78.14,100]), small number of PMN (n = 222; mean = 9.96 PMN; 95 % CI [0, 13.9]), few bacteria (n = 36; median = few; 95 % CI [minimal, few]), and a moderate quantity of mucus/cellular debris (n = 222; median = moderate; 95 % CI [moderate, moderate; Table 1).

To distinguish females in early diestrus from those in inter-estrous period of the reproductive cycle, at least two to three consecutive vaginal cytology samples needed to be assessed. This transitional stage was considered to be “post-estrus” (Fig. 4e, f). During the post-estrous period, there was a large proportion of generally aggregated superficial cells (n = 63; mean = 81.93 %; 95 % CI [76.52, 100]), moderate-to-large number of PMN (n = 63; mean = 114.5 PMN; 95 % CI [56.71, 172.29]), moderate number of bacteria (n = 24; median = moderate; 95 % CI [moderate, large]) including *Simonsiella* spp (n = 63; prop = 20.63 %; 95 % CI

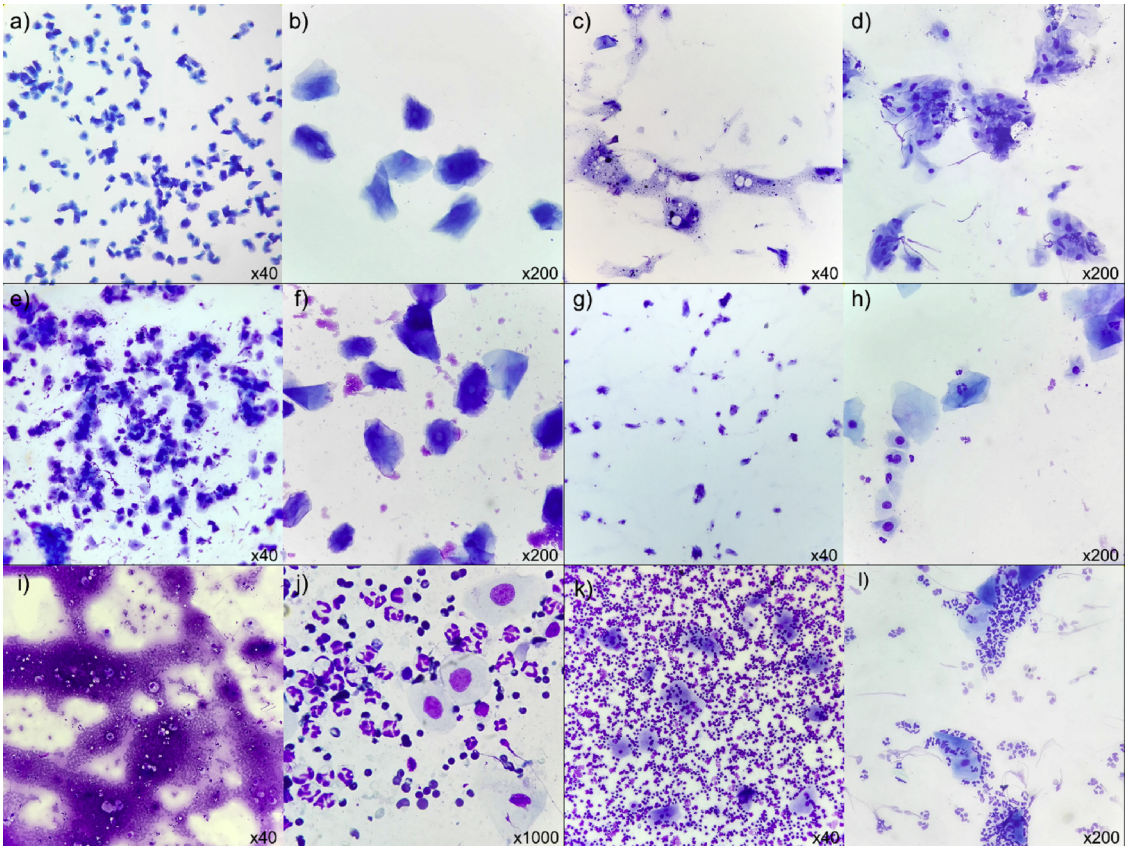

Fig. 4. Microscopic images of vaginal smears of African lionesses in estrus (a, b), diestrus (c, d), post-estrus (e, f), inter-estrus (g, h), post-partum (i, j), and proestrus (k, l) stained with modified Wright-Giemsa.

[10.36, 30.91]), and moderate abundance of mucous and cellular debris, mainly due to a moderate-to-large amount of cellular debris (n = 63; median = moderate; 95 % CI [moderate, large]) (Table 1).

Inter-estrus was generally characterized by similar moderated proportions of parabasal, intermediate, and superficial cells (Friedman Test: χ^2 (2) = 1; n = 24; P = 0.664), moderate number of PMN (n = 24; mean = 30.62 PMN; 95 % CI [14.21, 79.25]), small number of bacteria (n = 7; median = few; 95 % CI [minimal, few]), and moderate abundance of mucosal and cellular debris, mainly due to a moderate content of cellular debris (n = 24; median = moderate; 95 % CI [small, moderate]) (Fig. 4g, h, Table 1). There were similar cytology images during the inter-estrus period of the reproductive cycle and lactational anestrus.

Results from vaginal cytology evaluations during the initial days after parturition indicated there was a similar proportion of epithelial cells as that during diestrus (Table 1). During the post-partum period, however, there were a large number of red and white blood cells (n = 6; mean = 439.42 PMN; 95 % CI [76.6, 1448.2]), and a few *Simonsiella* spp (n = 6; prop = 16.67 %; 95 % CI [0, 33.33]) (Fig. 4i, j).

During the period when there were no specific reproductive symptoms, frequent vaginal swabbing allowed for detection of the proestrous period as a result of cytological assessments (Fig. 4k, l). This stage of the reproductive cycle was characterized with a large proportion of superficial cells (n = 71; mean = 56.58 %; 95 % CI [51.38, 100]), a very large number of PMN with the numbers gradually decreasing until the time of estrus (n = 70; mean = 648.73 PMN; 95 % CI [477.1, 3502.5]), few bacteria (n = 18; median = few; 95 % CI [minimal, few]), and amounts of mucous and cellular debris ranging from a large to minimal quantities (n = 71; median = moderate; 95 % CI [moderate, moderate]) (Table 1).

The stage of proestrus, as ascertained from cytological assessment, was evident at the end of pseudopregnancy, but the cytology evaluation during proestrus was similar to that during the post-estrous and inter-estrous periods when there was assessment of a single slide (Fig. 5). Nevertheless, the proportion of basal, parabasal, and intermediate epithelial cells was markedly less in post-estrus than during both the proestrous (Mann-Whitney; U = 923; W = 6106; n = 71, 63; P = 0.000; η = 0.51) and inter-estrous periods (Mann-Whitney; U = 207.5; W = 2223.5; n = 63, 24; P = 0.000; η = 0.56). Additionally, the number of PMN was markedly larger during the proestrus as compared with the post-estrous period (Mann-Whitney; U = 1052; W = 5843; n = 70, 63; P = 0.000; η = 0.45) and inter-estrous period (Mann-Whitney; U = 267.5; W = 3897.5; n = 70, 24; P = 0.000; η = 0.51).

Table 1
Means and the standard errors (SEM) or medians of the vaginal cytology findings throughout the reproductive cycle of five female African lions, with number of slides analyzed for each stage of the cycle (*n*), and percentiles 10th and 90th inside the parentheses.

		Proestrus (*n* = 71)	Estrus (*n* = 155)	Post-estrus (*n* = 63)	Inter-estrus (*n* = 24)	Diestrus (*n* = 222)	Post-partum (*n* = 6)
Epithelial cells (%)	Basal	2.74 ± 0.52 (0–9.00)	0.01 ± 0.00 (0-0.00)	0.25 ± 0.10 (0-0.50)	1.33 ± 0.30 (0–3.35)	5.20 ± 0.46 (0–15.50)	4.33 ± 2.55 (1–9.75)
	Parabasal	22.99 ± 1.85 (5.50–43.00)	0.09 ± 0.03 (0.00-0.00)	7.97 ± 1.38 (0.00–25.40)	27.33 ± 4.12 (9.05–48.20)	53.61 ± 1.10 (31.55–76.95)	56.25 ± 4.14 (46.75–62.50)
	Intermediate	17.69 ± 1.62 (2.50–36.5)	0.12 ± 0.04 (0.00-0.0)	9.86 ± 2.13 (0.00–35.7)	24.54 ± 2.46 (9.15–40.5)	26.15 ± 0.73 (12.55–40.5)	30.25 ± 1.57 (26.00–33.5)
	Sup. Nucleated	34.31 ± 1.88 (12.00–56.50)	62.26 ± 0.99 (47.40–76.50)	56.18 ± 2.54 (33.30–76.00)	30.58 ± 3.76 (4.25–56.45)	7.82 ± 0.45 (1.00–18.00)	5.75 ± 1.06 (3.50–8.25)
	Sup. Enucleated	22.27 ± 1.86 (4.50–47.00)	37.53 ± 1.00 (23.00–52.60)	25.75 ± 1.74 (8.10–47.40)	16.21 ± 2.55 (1.00–32.75)	7.22 ± 0.55 (0.00–20.85)	3.42 ± 1.83 (1.25–7.50)
PMN[a]		648.73 ± 102.94 (2.50–2115.00)	0.36 ± 0.14 (0.00-0.50)	114.50 ± 28.91 (0.00–326.50)	30.63 ± 13.65 (0.00–43.50)	9.96 ± 2.38 (0.00–12.45)	439.42 ± 329.15 (12.25–1167.50)
Bacteria	Load	Small (Small-Mod.)	Moderate (Small-Large)	Moderate (Small-Mod.)	Small (Small-Mod.)	Small (Minimal-Mod.)	Minimal (Min.-Min.)
	Simonsiella spp (%)	2.82 ± 1.98 (0-0)	52.90 ± 4.02 (0–100)	20.63 ± 5.14 (0–100)	4.17 ± 4.17 (0-0)	0.90 ± 0.64 (0-0)	16.67 ± 16.67 (0–50)
Clearing	Mucus	Minimal (Min.-Mod.)	Minimal (Min.-Min.)	Minimal (Min.-Small)	Minimal (Min.-Small)	Small-Moderate (Small-Large)	Moderate (Small-Mod.)
	Debris	Moderate (Small-Large)	Small (Minimal-Mod.)	Moderate (Small-Large)	Small-Mod.(Small-Mod.)	Small-Mod.(Small-Mod.)	Small-Mod.(Small-Mod.)
	Clusters	S.cells-S.cluster (S.cluster-Med.)	Single cells (S.cells-S.cells)	Single cells (S.cells-Med.)	Small clusters (S.cells-Med.)	S.cells-S.cluster (S.cells-Med.)	Medium cluster (S.cluster-Med.)
	Piles	Small piles (S.cells-Med.)	Small piles (S.cells-Med.)	Medium piles (S.cells-Large)	Small piles (S.cells-Small)	Single cells (S.cells-S.cells)	Single cells (S.cells-S.cells)

[a] Polymorphonuclear neutrophils observed per 100 epithelial cells).

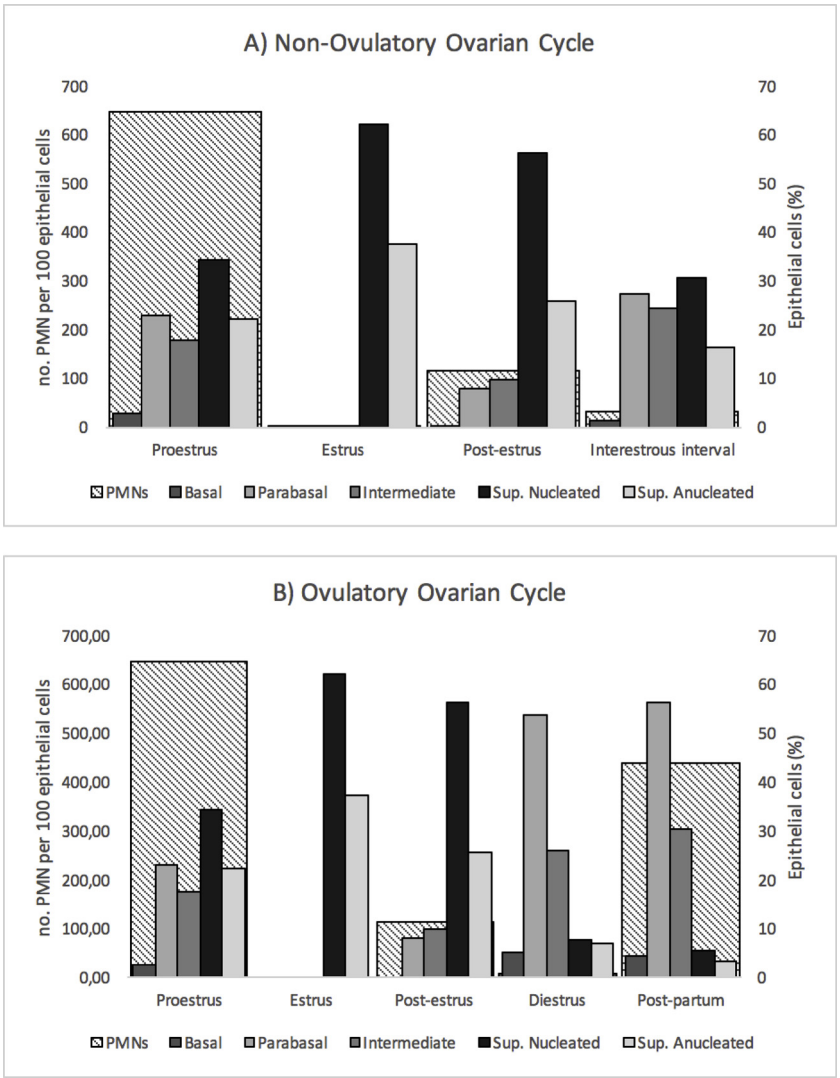

Fig. 5. Proportion of epithelial cells and polymorphonuclear neutrophils (PMN) observed in the different stages of the female African lion when there was not an associated ovulation (Non-ovulatory; A) and when there was an associated ovulation (Ovulatory; B) during the reproductive cycle.

3.3. Vulvar morphology

There were macroscopic changes in both the vulvar lips and the vaginal vestibulum, in association with the reproductive stage of the lioness (Fig. 6). Overall, most females in behavioral estrus had edematous, exposed vulvas (81.6 %, n = 103), a clear vaginal discharge (66.3 %, n = 95), and prominent vaginal mucous membrane folds, with a rough surface when there were palpation assessments (79.1 %, n = 129). During behavioral diestrus, most vulva tissues were covered with hair (88.3 %, n = 240), secretions were generally not observed (12.7 %, n = 236; from which 53.3 % actually corresponded to cytological proestrus), and the mucous membranes of the vestibulum were soft when palpated. During the behavioral inter-estrus period, the lionesses generally had exposed vulva tissues (60.9 %, n = 69), as well as a thick yellow vaginal discharge (43.1 %, n = 65), and non-prominent vaginal folds, that were soft when evaluated by palpation (71.2 %, n = 69). There was a moderately dark hemorrhagic vaginal discharge commonly observed until 9 days after parturition.

4. Discussion

In general, vaginal cytology evaluations are rarely used for endocrine and physiological assessments in felids, normally serving to confirm ovarian follicular functions by identification of either the physiological state of “proestrus/estrus” during the reproductive cycle (when a large proportion of superficial cells is observed) or “inter-estrus/diestrus” (when there is a predominant proportion of intermediate cells, and few parabasal and superficial cells) (von Heimendahl and England, 2010). In the present study, there was a

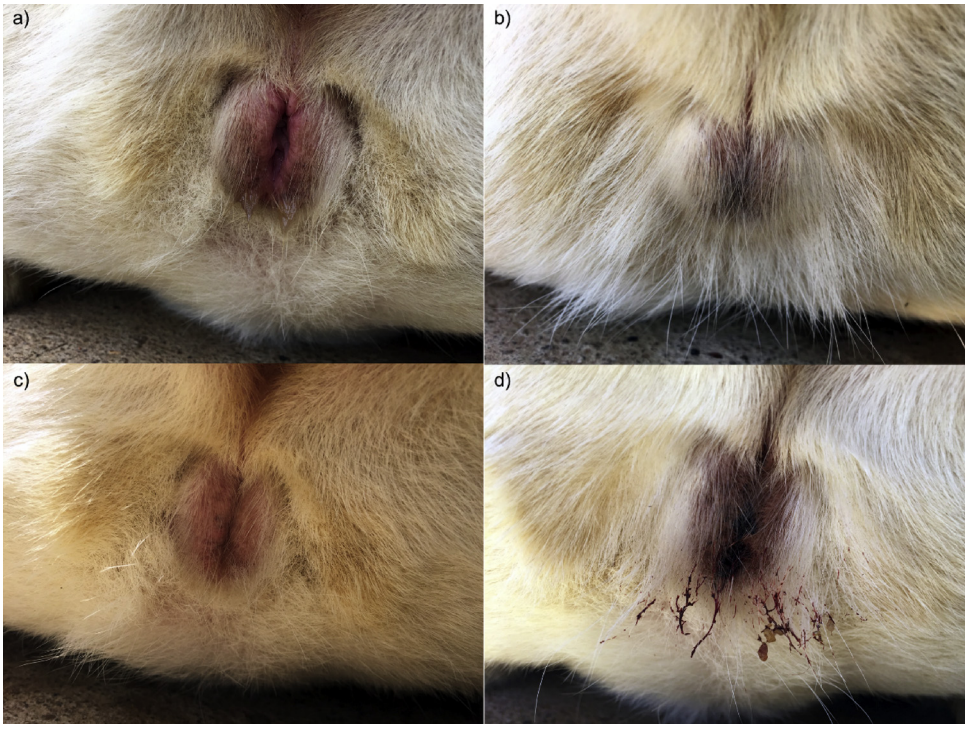

Fig. 6. Vulvar morphology of one adult African lioness in sternal recumbency during estrus (a), diestrus (b), inter-estrous interval (c), and post-partum (d).

detailed assessment of the changes observed in the African lioness' vaginal epithelium throughout the different stages of the reproductive cycle as classified by animal behavior. To the best of our knowledge, this is the first study in which there has been a report of the association between transitional reproductive stages such as "proestrus" and "post-estrus" and vaginal cytological cellular populations for any non-domestic felid. In addition, there is reporting of concurrent macroscopic vulvar changes and physiological vaginal discharge patterns throughout the reproductive cycle.

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

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

Furthermore, in previous research, polymorphonuclear neutrophils were usually not observed at any stage of the estrous cycle in the vaginal cytology samples of lions (Schmidt et al., 1983), tigers (*Panthera tigris*; Seal et al., 1985), and pumas (*Felis concolor*; Bonney et al., 1981), but were occasionally detected in domestic cats during inter-estrus and diestrus (Mills et al., 1979), and

immediately after estrus cessation in cheetahs (Asa et al., 1992). In the present study, PMN were detected throughout the reproductive cycle of the lioness, consistently varying in number depending on the stage of the reproductive cycle. As observed in most domestic species, neutrophils were largely absent during the estrous period, in relatively larger numbers after the estrous period, and were occasionally detected during diestrus. It appears that circulating neutrophils may enter the vaginal lumen across the epithelial layer lining of the vagina when there are relatively lesser estrogen concentrations, which generally occurs after estrus and sometimes during pregnancy (Asa et al., 1992). In lionesses, however, there was a relatively larger number of PMN in the vaginal cytology samples approximately 7–10 days before estrus when there were both estrous symptoms expressed without an associated ovulation and when there were these symptoms in association with the timing of an ovulation. This finding complicated using the vaginal cytology evaluations for reproductive stage diagnosis when one single slide was collected around estrus. For example, when samples were collected during late proestrus, using only the vaginal cytology assessment technique for evaluation of reproductive status, the vaginal cellular populations were similar to those during the early post-estrous period. Furthermore, when samples were collected during early the proestrous period, using only the vaginal cytology technique for evaluation of reproductive status, the vaginal cellular populations were similar to those when there were conditions such as vaginitis or pyometra. The absence of this increase in number of leukocytes from 45 to 55 days after natural mating or artificial insemination is considered to be an indicator of pregnancy in lions. The increase in numbers of PMN during proestrus is thought to be the result of a local inflammation caused by luteolysis and regression of corpora lutea. Further research is needed to confirm this hypothesis and determine the pattern of change in PMN numbers when there is expression of estrus without there being occurrence of an associated ovulation.

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

5. Conclusions

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

Authors contributions

IC, AG and IL designed the study. IC collected and analyzed the data. IC prepared a draft of the manuscript. AG and IL finalized the manuscript. All authors approved the final version of the manuscript.

Declaration of Competing Interest

None.

Acknowledgement

The authors thank Mr. & Mrs. Jacobs of the Ukutula Conservation Center (UCC, Brits, RSA), for hosting this project and making their animals available; Pierre Grobler and Willi Jr Jacobs, for their assistance during animal training and sample collection; Professor Gerhard van der Horst and Sara Gonçalves for their time and patience behind the microscope, and Professor Javier Callealta for sharing his statistical knowledge. This research was supported by the South African National Research Foundation (grant numbers SFH150721128779, UID108947); the South African Veterinary Foundation; and the Zebra Foundation for Veterinary Zoological Education.

References

Andrews, C.J., Thomas, D.G., Yapura, J., Potter, M.A., 2019. Reproductive biology of the 38 extant felid species: a review. Mamm. Rev. 49 (1), 16–30. https://doi.org/10.1111/mam.12145.

Asa, C.S., Junge, R.E., Bircher, J.S., Noble, G.A., Sarri, K.J., Plotka, E.D., 1992. Assessing reproductive cycles and pregnancy in cheetahs (*Acinonyx jubatus*) by vaginal cytology. Zoo Biol. 11 (3), 139–151. https://doi.org/10.1002/zoo.1430110303.

Bell, E.T., Bailey, J.B., Christie, D.W., 1973. Studies on vaginal cytology during the canine oestrous cycle. Res. Vet. Sci. 14, 173–179. https://doi.org/10.1016/S0034-5288(18)33909-2.

Bonney, R.C., Moore, H.D., Jones, D.M., 1981. Plasma concentrations of oestradiol-17β and progesterone, and laparoscopic observations of the ovary in the puma (*Felis*

concolor) during oestrus, pseudopregnancy and pregnancy. J. Reprod. Fertil. 63, 523–531. https://doi.org/10.1530/jrf.0.0630523.
Callealta, I., Ganswindt, A., Goncalves, S., Mathew, A., Lueders, I., 2018. Detection of Simonsiella spp. in the vagina of lions and leopard in oestrus. Reprod. Domest. Anim. 53 (6), 1605–1608. https://doi.org/10.1111/rda.13298.
Callealta, I., Ganswindt, A., Malan, M., Lueders, I., 2019. Non-surgical artificial insemination using a GnRH analogue for ovulation induction during natural oestrus in African lions (*Panthera leo*). Theriogenology 139, 28–35. https://doi.org/10.1016/j.theriogenology.2019.07.022.
Callealta, I., Lueders, I., Luther, I., Ganswindt, A., in press. Positive reinforcement conditioning as a tool for frequent minimally-invasive blood and vaginal swab sampling in African lions (Panthera leo). J. Appl. Anim. Welf. Sci.
Callealta, I., Lueders, I., Callealta, J., Ganswindt, A., submitted-a. Comparative blood and fecal steroid patterns during the ovarian cycle of African lions (Panthera leo), and endocrine response to ovulation induction with a GnRH analogue. Horm. Behav. (under review).
Cline, E.M., Jennings, L.L., Sojka, N.J., 1980. Analysis of the feline vaginal epithelial cycle. Fel. Pract. 10, 47–49 Record number: 19802258458.
Herron, M.A., 1977. Feline vaginal cytologic examination. Fel. Pract. 7, 36–39.
IUCN, 2019. The IUCN Red List of Threatened Species. Last accessed: July 2019. https://www.iucnredlist.org.
Johnston, S.D., Root Kustritz, M.V., Olson, P.N.S., 2001. Vaginal cytology. In: Johnston, S.D., Root Kustritz, M.V., Olson, P.N.S. (Eds.), Canine and Feline Theriogenology, first ed. Saunders, Philadelphia, pp. 32–40.
Liche, H., Wodzicki, K., 1939. Vaginal smears and the oestrous cycle of the cat and lioness. Nature 144, 245–246. https://doi.org/10.1038/144245b0.
Malandain, E., Rault, D., Froment, E., Baudon, S., Desquilbet, L., Begon, D., Chastant-Maillard, S., 2011. Follicular growth monitoring in the female cat during estrus. Theriogenology 76 (7), 1337–1346. https://doi.org/10.1016/j.theriogenology.2011.06.002.
Mills, J.N., Valli, V.E., Lumsden, J.H., 1979. Cyclical changes of vaginal cytology in the cat. Can. Vet. J. 20 (4), 95–101 PMC1789534; PMID: 570881.
Mowrer, R.T., Conti, P.A., Rossow, C.F., 1975. Vaginal cytology: an approach to improvement of cat breeding. Vet. Med. Small Anim. Clin. 70, 691–696 PMID: 1041070.
Porter, R.W., Cavanaugh, E.B., Critchlow, B.V., Sawyer, C.H., 1957. Localized changes in electrical activity of the hypothalamus in estrous cats following vaginal stimulation. Am. J. Physiol. 189 (1), 145–151. https://doi.org/10.1152/ajplegacy.1957.189.1.145.
Putman, S., Brown, J., Franklin, A., Schneider, E., Boisseau, N., Asa, C., Pukazhenthi, B., 2015. Characterization of ovarian steroid patterns in female lions (*Panthera leo*) and effects of contraception on reproductive function. PLoS One 10, e0140373. https://doi.org/10.1371/journal.pone.0140373.
Schmidt, P.M., Chakraborty, P.K., Wildt, D.E., 1983. Ovarian activity, circulating hormones and sexual behavior in the cat. II. Relationships during pregnancy, parturition, lactation and the postpartum estrus. Biol. Reprod. 28, 657–671. https://doi.org/10.1095/biolreprod28.3.657.
Schramm, R., Briggs, M., Reeves, J., 1994. Spontaneous and induced ovulation in the lion (*Panthera leo*). Zoo Biol. 13, 301–307. https://doi.org/10.1002/zoo.1430130403.
Schutte, A.P., 1967. Canine vaginal cytology – I Technique and cytological morphology. J. Small Anim. Pract. 8, 301–317. https://doi.org/10.1111/j.1748-5827.1967.tb04554.x.
Seal, U.S., Plotka, E.D., Smith, J.D., Wright, F.H., Reindl, N.J., Taylor, R.S., Seal, M.F., 1985. Immunoreactive luteinizing hormone, estradiol, progesterone, testosterone, and androstenedione levels during the breeding season and anestrus in Siberian tigers. Biol. Reprod. 32, 361–368. https://doi.org/10.1095/biolreprod32.2.361.
Shille, V.M., Lundström, K.E., Stabenfeldt, G.H., 1979. Follicular function in the domestic cat as determined by estradiol-17 β concentrations in plasma: relation to estrous behavior and cornification of exfoliated vaginal epithelium. Biol. Reprod. 21 (4), 953–963. https://doi.org/10.1095/biolreprod21.4.953.
Swanson, W.F., 2006. Application of assisted reproduction for population management in felids: the potential and reality for conservation of small cats. Theriogenology 66, 49–58. https://doi.org/10.1016/j.theriogenology.2006.03.024.
von Heimendahl, A., England, G.C.W., 2010. Determining breeding status. In: England, G.C., Heimendahl, A.V. (Eds.), BSAVA Manual of Canine and Feline Reproduction and Neonatology, second ed. Quedgeley, Gloucester, pp. 44–50.
Stanton, L.A., Sullivan, M.S., Fazio, J.M., 2015. A standardized ethogram for the Felidae: a tool for behavioral researchers. Appl. Anim. Behav. Sci. 173, 3–16. https://doi.org/10.1016/j.applanim.2015.04.001.
Valle, G., Toledo, J., De Figueiredo, C., 2006. Detection of *Simonsiella* species in the vagina of a bitch in heat. Vet. Rec. 159, 156–157. https://doi.org/10.1136/vr.159.5.156.