Chapter 4. The distribution of small preantral follicles within the ovaries of prepubertal African elephants *(Loxodonta africana)*

The content of this chapter has been published in a different format as an article by FJ Stansfield, JO Nöthling and T Ansari under the title "The distribution of small preantral follicles in the ovaries of prepubertal African elephants (*Loxodonta africana*)" in Animal Reproduction Science 2011; 129:96–103.

4.1. Introduction

When counting follicle numbers in the ovary of any mammal it is rarely possible to examine the whole of the cortex so representative samples of ovarian tissue are studied and counts are extrapolated from them. The distribution of SF in the cortex of the mammalian ovary is considered to be heterogeneous and biopsies of human ovarian cortex have revealed variations of more than two orders of magnitude in the density of primordial follicles (Schmidt 2003). The ovary of the mature African elephant *(Loxodonta africana*) is large at 7 x 5 x 2 cm; (Hildebrandt *et al*. 2000; Sikes 1971), making it particularly pertinent to select a representative sample of the ovary for the estimation of the number of SF in the ovary. Further, the ovary of the elephant has a lower density of SF per unit volume of ovarian cortex than other mammalian species (Stansfield *et al*. 2011b), human (Faddy *et al*. 1992), bovine (McGeady *et al*. 2006), sheep (Gondos 1978), suggesting that it is important to ensure that a sufficiently large sample of ovarian tissue is used for the estimation of the number of small follicles in the elephant ovary.

Historically, protocols involving labour-intensive counting of SF in many serial sections, followed by calculations using assumption-based techniques (Abercrombie 1946; Miller *et al*. 1997), have been employed to determine SF numbers within mammalian ovaries. More recently, however, stereological techniques have been applied to good effect and have lead to greater accuracy due to sound stereological assumptions and improved economy of time (Charleston *et al*. 2007; Miller *et al*. 1997).

Stereology, a design-based technique for counting items in biological tissues, was adopted for this project because stereological counting of follicles does not employ

model-based correction factors and is therefore mathematically sound and reputed to be more accurate than counting in serial sections (Charleston *et al*. 2007). It is also much quicker. Using stereology, Charleston *et al*. (2007) reported a 15–29% variation in the estimated number of non-growing follicles when the number was repeatedly estimated in the same ovary. Charleston *et al*. also achieved an average coefficient of variation of 14% (2–20%) when the number of non-growing follicles was estimated from recounts done on the same human ovaries by the same observer. They further found that counting the follicles in double or three times the number of ovarian slabs than the minimum they deemed necessary did not improve precision, and the coefficient of variation (CV) remained in the range of 14–29%. These results provide a basis for comparison of the precision and repeatability of follicular counts.

In a previous study serial sectioning was used to count SF numbers in the ovaries of young adult elephants aged 9–34 years (Stansfield *et al*. 2011b). However, this method was very laborious and time consuming and it proved difficult to obtain representative samples of cortex for counting in pregnant cows due to considerable distortion of the cortex by the development of the multiple large CL which are a feature of elephant pregnancy (Hodges *et al*. 1997; Short 1966; Smith & Buss 1975). Before counting SF in adult elephants in the luteal phase it is necessary to determine whether one ovary that is not distorted by CL, or part of an ovary, can be reliably used to estimate the follicular reserve in the animal. It is therefore necessary to know the default distribution of the follicle reserve in the African elephant. The present study was undertaken to determine if a systematic difference in follicle density might exist between the left and right ovaries, or between the surfaces and intermarginal and interpolar positions of each ovary, in elephant calves whose ovaries had not been distorted by the presence of pregnancy-associated CL.

4.2. Materials and methods

4.2.1. Specimens and stereology

Twelve sets of ovaries from elephant calves aged 2 months to 4.5 years were collected over a period of three culling seasons (2009–2011) in Savé Valley Conservancy,

Zimbabwe. Ovaries were collected, handled and processed as described in Chapter 2 (also see Figure 4.1a and b).

Recording follicle counts by region

The number of SF in each UCF was recorded (Figure 2.4b) and each UCF was located geographically according to region. The largest region was both ovaries of an elephant combined, the second largest was each ovary on its own, the third largest was each of the two surfaces of each ovary, the fourth largest region was each of the three intermarginal thirds of each ovary and the smallest region was each of the five interpolar fifths of each ovary. These regions were named Elephant (n = 12), Ovary (left or right), Surface (lateral or medial), Intermarginal third (three zones from the mesovarial margin to the free margin) and Interpolar fifth (five zones between the cranial and caudal poles).

In order to determine the repeatability of the follicle counts the numbers of TPM, EP and TP follicles (Oktay 1995) were counted together as SF in the interpolar fifths, intermarginal thirds, and surfaces of each ovary from three elephants on two separate occasions, September 2010 and again in January 2011, the results being recorded by region.

Determining SF density throughout an ovary

The density of SF (the number of small follicles per unit volume) in an ovary ($Density_{ov}$) was calculated using the formula:

$$Density_{ov} = \frac{F_{ov}}{n_{UCF} \times v_{UCF}}$$

Where F_{ov} was the number of SF counted in the ovary, n_{UCF} the number of UCFs observed in the ovary and V_{UCF} the volume of a UCF, calculated as the area of a UCF multiplied by the height of the section studied (15 µm).

4.2.2. Statistical analyses

Due to the low prevalence of TPM and TP in the elephant ovary the data of the 3 types of SF (TPM, EP and TP) were pooled and analyzed as a single group. The number of SF per UCF (follicle density) was used as the response variable to determine the repeatability of

follicle counts in each type of region and to compare the distribution of follicles in different regions. In addition, the actual number of follicles per ovary was used as the response variable to determine the repeatability of the number of SF in an ovary and in an elephant, and to compare the numbers of SF in the left and right ovaries. The repeatability between September 2010 and January 2011 counts was expressed as the coefficient of variation of the number of SF per UCF (Dohoo *et al*. 2009) and the repeatability limit, which represents the width of the 95% confidence interval for two true replicates (Barnhart *et al*. 2007). The coefficient of variation may be compared with that reported by others, such as Charleston *et al*. (2007), thus providing a means of comparing the precision between studies. Dividing the repeatability limit by the estimate (e.g. dividing the repeatability limit of the number of SF in an ovary by the estimated number of SF in the ovary) provides the maximum percentage variation between repeated counts expected in 95% of repeat counts. This percentage variation may be compared to the figures found by others, such as Charleston *et al*. (2007).

The left and right ovaries of the 12 elephant were compared by means of a paired *t*-test with respect to ovarian mass, cortical volume, the proportion of small follicles of each type, the number of UCFs in which SF were counted, follicular density, and the number of SF per ovary. For each of these variables the average over the two ovaries of each elephant was determined and these 12 averages of each variable (one average per elephant) were used to determine which variables, if any, were correlated with age. Pearson's correlation procedure was used for this correlation analysis.

Where data were not normally distributed, non-parametric tests for meaningfully paired observations were used and the data reported as median followed by the 25th to 75th percentile in parentheses or separated from the median by a comma. So, Wilcoxon's signed rank test was used to compare two groups, such as comparing the medial surface with the lateral surface and Friedman's test to compare more than two groups, such as the three zones between the ovarian margins, or the five interpolar fifths. Following the Friedman test, all pairwise comparisons were done by means of Wilcoxon's signed rank test after setting α' for each pairwise comparison according to Ryan's equation, which maintained the experiment-wise level of α at 0.05 (Kirk 1968):

$$\alpha' = \frac{2\alpha}{k(r-1)},$$

where α' is the level of significance required for a particular pairwise comparison, k is the number of groups in the comparison (3 intermarginal zones or 5 interpolar segments), and r is the number of steps from the lower-ranking group in the pairwise comparison to the higher-ranking group, as determined after all groups were ordered in sequence of ascending rank sums (Kirk 1968). Ryan's adjustment resulted in α' varying between 0.0167 and 0.033 for the 3 pairwise comparisons among intermarginal thirds, and between 0.0050 and 0.020 for the 10 pairwise comparisons among interpolar fifths.

Where data conformed to the requirements for parametric tests Pearson's correlation procedure was used for correlation analysis and a paired *t*-test was used to compare 2 groups, in which case the data are reported as mean ± standard deviation (SD). The Wilcoxon's signed rank test for two groups and all parametric analyses were done with STATA statistical package (StataCorp. Stata Statistical Software: Release 11, College Station, Texas), with α set at 0.05. Friedman test was done using StatSource Data Analysis Plus 2.12 (Keller & Warrack 2000), with α set at 0.05. Pairwise comparisons were done in an Excel spreadsheet.

Charleston *et al*. (2007) concluded that in the human, counting the non-growing follicles in one ovary allows one to estimate the total number of non-growing follicles in the person. Accordingly, the precision with which the number of small follicles in either ovary of an elephant could be used to predict the total number of small follicles in that same elephant was determined using the formula;

$$Error = Absolute\ value\ of \left(1 - \left(\frac{2 \times nSF_{selectedovary}}{nSF_{selectedovary} + nSF_{contralateralovary}}\right)\right)$$

where nSF was the number of small follicles in the particular ovary.

4.3. Results

Across all 24 ovaries examined, 92.0 ± 5.98% (range 86.4–96.2% among ovaries) of SF recorded were EP and the remaining 8.0 ± 5.98% were TP. No TPM follicles were seen. Table 4.1 shows that the left and right ovaries of the 12 elephant did not differ with respect to mass, cortical volume, the percentages of SF that were TPM, EP or TP. Further, Table 4.1 shows that the left and right ovaries were also similar with respect to

the number of UCFs in which SF were counted, the numbers of SF per UCF, and the number of SF per ovary. Table 4.1 also shows that there was a significant positive correlation between cortical volume and age, suggesting that the ovarian cortex tended to be larger in the older elephant calves. The significant negative correlation between SF per UCF and age suggested that the follicular density decreased with age. There was a trend towards a positive correlation (P = 0.06, n = 12) between the number of UCFs examined per elephant and age (Table 4.1). The SF were counted in an average of 172.67 UCFs per ovary (range 113–224), which resulted in a CE of between 8.7–14.8%.

Although not statistically compared among regions, Table 4.2 suggests that there is a trend for repeatability — as expressed in terms of the coefficient of variation and the repeatability limit for repeat counts — to improve as the size of the region increases down the table from Interpolar fifth to Elephant, because the CV as well as Repeatability limit decreased progressively about 20-fold from Interpolar fifth to Elephant, while follicular density remained about the same. As a specific example of this trend, using the repeatability limits from Table 4.2 suggests that two replicate measurements of the density of SF in a particular ovary are expected to agree within 7.5% (0.089 ÷ 1.18), and that of a particular elephant to within 2% (0.024 ÷ 1.17). Similarly, from Table 4.3 it follows that replicate estimations of the numbers of SF in a particular ovary will agree within 16.5% (79 147 ÷ 479 018) and the total number of SF in a particular elephant within 10.5% (100 941 ÷ 958 037).

SF densities (SF per unbiased counting frame) were similar in the lateral (1.24, 0.85–1.39) and medial (1.03, 0.76–1.36) surfaces (P = 0.22, n = 24).

Table 4.1
Mean (±sd) of selected ovarian variables, as well as their correlation with age and their agreement between the left (L) and right (R) ovary of 12 prepubertal African elephants (*Loxodonta africana*) calves aged 2 months to 4.5 years

	L and R ovaries of each elephant combined		L and R ovaries compared		
	Mean per elephant[a]	Correlation[b]	Mean L ovary	Mean R ovary	P[c]
Ovarian mass (g)	10.37 ± 1.47	0.17 (0.60)	10.77 ± 1.86	9.98 ± 0.47	0.18
Cortex vol. (cm^3)	3.17 ± 1.13	0.85 (0.01)	3.18 ± 1.15	3.01 ± 0.55	0.17
Primordial foll.[d]	0		0	0	
Early primary (%)[e]	92.0 ± 5.98	-0.26 (0.41)	91.2 ± 6.3	92.7 ± 5.9	0.08
True primary (%)[f]	8.0 ± 5.98	0.26 (0.41)	8.8 ± 6.3	7.3 ± 5.9	0.08
UCF examined[g]	172.7 ± 31.8	0.56 (0.06)	175.3 ± 33.3	170.1 ± 33.7	0.41
SF per UCF[h]	1.10 ± 0.39	-0.64 (0.03)	1.11 ± 0.39	1.10 ± 0.39	0.82
SF per ovary[i]	393 297 ± 159 438	0.09 (0.80)	410 023 ± 153 365	376 571 ± 156 978	0.22

[a] The values in this column represents the mean of the left and right ovary in each elephant, which was then averaged over elephant (n=12)
[b] Pearson's corellation coefficient (n=12) for pairwise correlation between the mean of each elephant and her age (p-value between parentheses)
[c] Two-tailed P-value for a paired *t*-test (n=12) comparing left and right ovaries, with elephant as subject
[d] True primordial follicles, with flat pre-granulosa cells (there were no true primordial follicles seen at all)
[e] Percentage of all small follicles (primordial, early primary and true primary), where early primary has some cuboidal and some flat granulosa cells)
[f] The percentage of all small follicles that are true primary (having cuboidal pregranulosa cells only)
[g] The number of unbiased counting frames per ovary in which small follicles were counted
[h] The number of small follicles per unbiased counting frame
[i] The number of small follicles per ovary

Table 4.2
Coefficient of variation and repeatability limit between repeat counts of the numbers of small follicles per unbiased counting frame (follicle density) in the ovaries of three prepubertal African elephants

	Sep. 2010 count			Jan. 2011 count				
Regions (in order of increasing size down the table)	SF	UCF	SF/UCF	SF	UCF	SF/UCF	Coefficient of Variation	Repeatability limit
Interpolar fifth (n=30)[a]	51.4	42.3	1.21	47.5	40.0	1.20	0.17	0.474
Intermarginal third (n=18)[b]	85.7	70.6	1.18	79.2	66.7	1.15	0.13	0.402
Ovarian surface (n=12)[c]	128.6	105.8	1.20	118.8	100.1	1.19	0.05	0.150
Whole ovary (n=6)	257.2	211.7	1.19	237.5	200.2	1.18	0.03	0.089
Elephant (both ovaries, n=3))	514.3	423.3	1.18	475.0	400.3	1.17	0.01	0.024

SF = average number of small follicles per region; UCF = average number of unbiased counting frames per region; SF/UCF = average number of small follicles per unbiased counting frame.

[a] Each ovary was divided in five fifths along its interpolar axis, resulting in 30 such regions among the 6 ovaries

[b] Each ovary was divided in 3 intermarginal thirds from its mesovarial margin towards its free margin, resulting in 18 such regions among the 6 ovaries

[c] Each ovary was divided in a lateral and medial surface, resulting in 12 such regions among the 6 ovaries

Table 4.3
Coefficient of variation and repeatability limit of repeat counts of the numbers of small follicles in the ovaries of three prepubertal African elephants

	Sep. 2009	Jan. 2010	Coefficient of variation	Repeatability limit[a]
Average number of small follicles per ovary (n=6)	479 018± 121 635	510 392± 133 080	0.059	79 147
Average number of small follicles per elephant	958 037± 240 887	996 841± 305 543	0.043	100 941

[a] The repeatability limit provides the 95% confidence interval for the number of small follicles estimated in repeat counts

Although the number of SF per UCF did not differ among interpolar fifths; P = 0.20, n = 24 ovaries), the 10 subsequent pairwise comparisons revealed that the middle of the 5 regions cut along the interpolar axis (Region 3 in Figure 4.1c) tended to have a lower number of small follicles (0.81, 0.64–1.40) per UCF than the cranial fifth (Region 1 in Figure 4.1c) of the ovarian cortex (1.31, 0.84–1.48; Wilcoxon's signed rank test, two-tailed P = 0.008, n = 24 ovaries). No other pairwise comparison was significant (P > 0.1, n = 24 ovaries).

The numbers of SF per UCF differed significantly among the three intermarginal zones (Friedman's test, P = 0.034, n = 24 ovaries per group). Pairwise comparisons revealed that the zone nearest the mesovarial margin had fewer SF per UCF (0.85, 0.51–1.28) than the zones midway between the margins (1.008, 0.78–1.42; Wilcoxon's singed rank test, two-tailed P = 0.034) or nearest to the free margin (1.27, 0.79–1.51; Wilcoxon's signed rank test, two-tailed P = 0.0024). SF numbers per UCF were similar in the middle zone and that furthest from the mesovarium (Wilcoxon's signed rank test, two-tailed P = 0.09; Figure 4.1d).

The error in estimating the total number of SF in an elephant from the number in either of its ovaries is 10.4% (95% confidence interval 1.3% to 23.5%, n = 12 elephant). There was no correlation between the number of UCFs counted in the right ovary and the error in estimating the total number of SF in an elephant from the number of SF in the right ovary (Pearson's correlation coefficient 0.15, P = 0.64; n = 12). Similarly, there also was no correlation between the number of UCFs counted in the left ovary and the error in estimating the number of SF in an elephant from the number of SF in the left ovary (Pearson's correlation coefficient 0.51, P = 0.09; n = 12). The lowest (113) and second lowest (114) number of UCFs were counted in the same elephant, and the third lowest (126) and fourth lowest (128) number of UCFs were counted in another elephant. In these two elephants with the lowest numbers of UCFs counted the error in estimating the total number of SF in the elephant from the number of SF in one ovary was 4.7% and 4.8%, respectively, which was similar to the average of 10.4% for the 12 elephants.

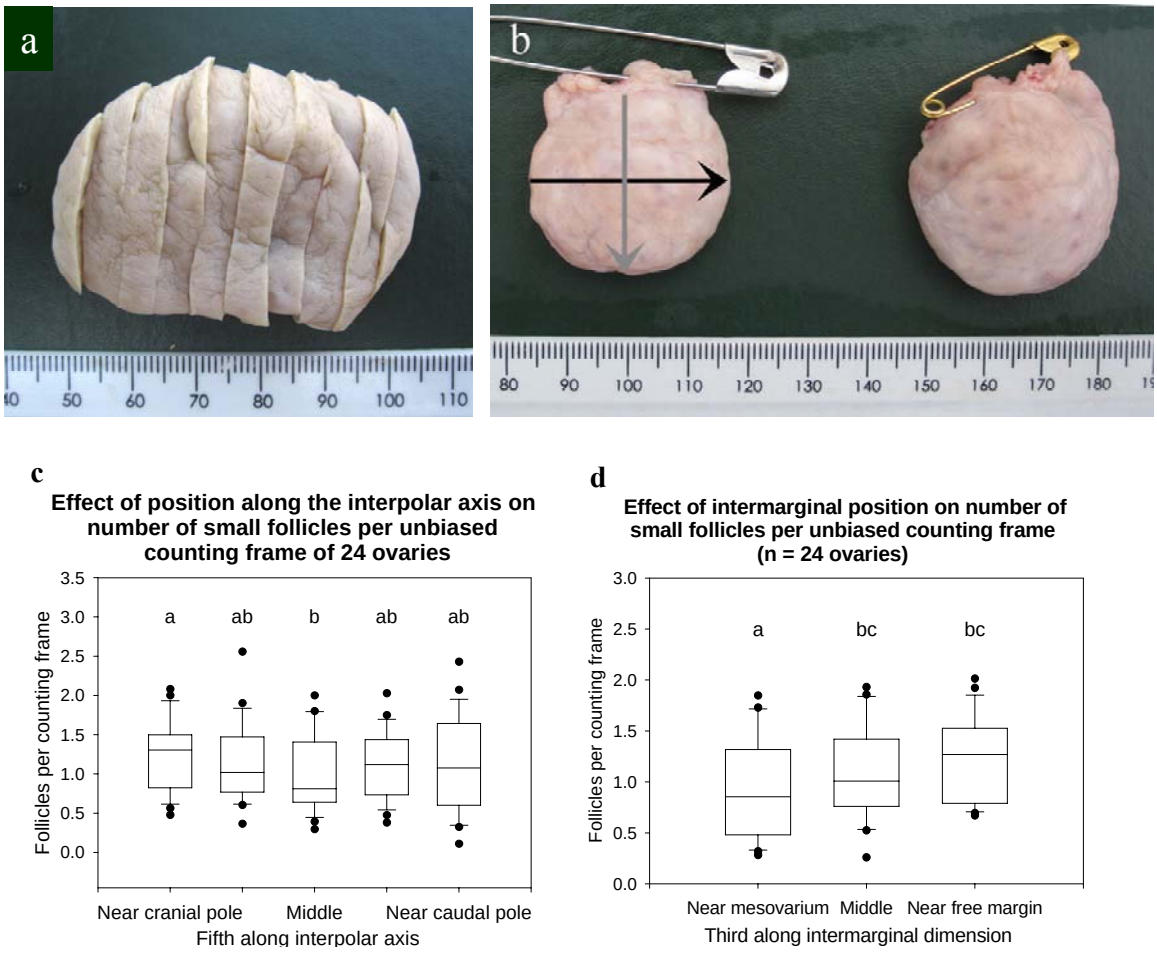

Figure 4.1 Assessment of the effect of position along the interpolar axis and along the intermarginal dimension on the number of small follicles per unbiased counting frame in the ovary of the African elephant

a. A prepubertal ovary sliced into 10 approximately equal segments prior to sectioning
b. Ovaries of a 2 year old elephant, with size and colour of pin indicating placement of ovary (left or right) and the position of the pin indicating the lateral surface of the ovary. The grey arrow runs from the mesovarial margin to the free margin of the left ovary, showing the intermarginal distance, while the black arrow runs from the caudal pole to the cranial pole, showing the interpolar distance.
c. The box shows the interquartile range and the median, the whiskers the 10th and 90th percentiles, and the dots the more extreme data. Groups with different letters above differ ($P < 0.05$)
d. As for c.

4.4. Discussion

This study shows that the density of follicles in the ovaries of prepubertal African elephant calves is not influenced by either the placement (left or right) or the surface (lateral or medial) of the ovary. However, some variation in follicle densities exists between the poles and the margins of the ovary.

There was no significant correlation between the number of SF per ovary and age, suggesting that the number of SF per ovary did not significantly change over the age spanned in the current study. Yet, the volume of the ovarian cortex increased with age while the follicular density decreased. Seen together, these correlations suggest that the decrease in follicular density may be due to expansion of the cortex, rather than due to an absolute decrease in the number of small follicles.

In agreement with the previous study in Chapter 3 in older elephants (Stansfield *et al*. 2011b), the type of SF commonly found in these prepubertal animals was the EP stage, which comprised 92.0 ± 5.98% of all the SF. It might have been expected that the number of TPM follicles in calf ovaries would be higher than the number found in older animals. The present finding that this is not the case further supports the conclusion (Stansfield *et al*. 2011b) that EP, rather than TPM follicles, form the ovarian reserve in the African elephant.

From the repeat counts in six ovaries it appears that the determination of the density of small follicles in an ovary is more repeatable (7.5%) than estimating the actual number of small follicles in an ovary (16.5%). Presumably this is because the estimation of the cortical volume (which is required to derive the number of small follicles per ovary) adds another source of variability to the estimation of the number of SF in an ovary. One may therefore conclude that the error of 16.5% in estimating the number of SF is inherent in the method of estimation. This level of precision is similar to the lower limit of the 15–29% range reported by Charleston *et al*. (2007) for repeat counts on the same ovary in the human.

Taking the number of SF in one ovary of an elephant and doubling that number, provides one with an estimate of the total number of SF in the elephant that on average differs by 10.5% from the actual total as determined from counting the SF in both ovaries. Further, one may expect that in 95% of instances where the number of SF in one ovary from an elephant is doubled, the derived number would be within 1.3–23.5% of the actual total number of SF in the elephant as determined by counting the follicles in both ovaries. This mean error of 10.5% (95% confidence interval 1.3–23.5%) in estimating the total number of SF in an elephant from the number in one ovary is similar to the error of 16.5% inherent in the method of estimation, as derived in the previous paragraph from repeat counts in the same ovary of an elephant. From this it follows that, as is the case in

the human (Charleston *et al*., 2007), counting SF in one ovary of an elephant and doubling that count provides a reasonable estimate of the number of SF in the elephant.

In the two elephants in which the lowest numbers of UCFs were counted the error in estimating the number of SF in the elephant from the number of SF in one ovary was similar to the mean error over 12 elephants, suggesting the lower number of UCF resulted in similar accuracy as the higher numbers counted in other ovaries. This finding supports that of Charleston *et al*., (2007) who found that counting small follicles in double or three times the minimum amount of tissue they deemed necessary did not improve the precision of the estimate of the number of small follicles in an ovary.

The finding that the numbers of SF in the left and right ovaries of a prepubertal elephants are similar allows reliable estimation of the number of SF per elephant in cases where only one ovary is available, even if it is not known whether it is from the left or right side of the animal, which of its surfaces is medial or lateral and which of its poles is cranial or caudal. The number of SF in the ovaries of pregnant animals can also now be estimated by using the ovary contralateral to the gravid uterine horn, which usually contains many fewer, if any, of the large accessory CL which are such a prominent feature of elephant pregnancy (Allen *et al*. 2003).

During culling of elephant for management purposes the collection of scientific samples is not usually a priority which can result in a significant delay until access is gained to the reproductive tract. The present finding that SF distribution is similar between the two ovaries will allow for early post-mortem excision of one ovary without the need for removal of the complete reproductive tract and it will be unaffected by the side of recumbency of the carcass. The collection of this uppermost ovary is relatively quick and simple via a small flank incision behind the last rib once that panel of skin has been removed.

It is now safe to conclude that future studies on small preantral and antral follicles in the ovaries of African elephants can be carried out confidently in the knowledge that the ovarian reserve in prepubertal individuals of this species is distributed uniformly between the ovaries and between the surfaces of each ovary.