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

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Abstract

Background

Data on the distribution of primordial (single layer of squamous granulosa cells), early primary (some granulosa cells cuboidal) and primary (all granulosa cells cuboidal) follicles, grouped together as small follicles (SF) within the ovary of the elephant is lacking, yet such information is necessary to be able to estimate accurately the total numbers of small follicles in the ovaries of elephant throughout their lifespan.

Aim

To determine if the density of SF differs between ovaries, between the surfaces of an ovary, or between the interpolar and intermarginal zones of an ovary.

Materials/methods

Stereological techniques were employed on 25 μm thick histological sections of the ovaries recovered from 12 prepubertal elephant calves aged 2 months to 4.5 years. Cell densities were calculated using the optical brick method and Cavalieri's principle for volume calculation.

Results

The density of SF (numbers of SF per unbiased counting frame [UCF]) did not differ between the left (1.11 ± 0.39 (mean ± sd)) and right (1.10 ± 0.39) ovaries ($P = 0.82$, $n = 12$), or between the lateral (median 1.24; interquartile range 0.85–1.39) and medial (1.03; 0.76–1.36) surfaces of the ovary ($P = 0.22$, $n = 24$) or among the 5 segments of the ovary between the two poles ($P = 0.20$, $n = 24$). The third of the cortex nearest to the mesovarial margin of the ovary had fewer small follicles per UCF (0.85; 0.51–1.28) than the middle third (1.01; 0.78–1.42; $P = 0.034$), and the third adjacent to the free margin (1.27; 0.79–1.51; $P = 0.0024$), $n = 24$ per group.

Conclusion

Providing a random sample is taken from the full interpolar and intermarginal dimensions of ovary of a non-pregnant elephant, the density of small follicles throughout the cortex may be accurately measured using stereological techniques applied to one of its surfaces.

Keywords

Follicle density; Follicle reserve; Ovary; African elephant

1. Introduction

It is of interest to study oogenesis and folliculogenesis in the elephant in light of the greatly reduced reproductive efficiency exhibited by captive elephant in zoos (Proctor et al., 2010) and the need to manage elephant populations in some countries in southern Africa where pressure from the expanding human population leaves insufficient habitat for continued expansion of the elephants (Balfour et al., 2007). More specifically, it is of interest to determine the numbers of small follicles (SF; primordial, early primary and primary as described by Oktay (1995)) in the ovaries of elephant, as they represent the follicle reserve that supplies the oocytes throughout reproductive life (Gosden and Telfer, 1987).

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 small follicles in the cortex of the mammalian ovary is considered to be heterogeneous (Charleston et al., 2007) 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 African elephant (*Loxodonta africana*) is large (7 cm × 5 cm × 2 cm, Stansfield unpublished data), making it particularly pertinent to select a representative sample of the ovary for the estimation of the number of small follicles 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., 2011), 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] and [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 ([Miller et al., 1997] and [Charleston et al., 2007]).

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 triple the number of ovarian slabs than the minimum they deemed necessary did not improve precision, and the 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 follicle numbers in the ovaries of young adult elephants aged 9–34 years (Stansfield, 2006). 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 corpora lutea which are a feature of elephant pregnancy ([Short, 1966], [Smith and Buss, 1975] and [Hodges et al., 1997]). Before counting SFs in adult elephant in the luteal phase it is necessary to determine whether one ovary that is not distorted by corpora lutea, 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 follicular 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 corpora lutea.

2. Materials and methods

2.1. Tissue collection

Twelve elephant calves were killed humanely by a brain shot from a heavy calibre rifle at close range during routine management culling of complete family groups in the Savé Valley

Conservancy, Zimbabwe. Within 2 h after death the ovaries were recovered and safety-pins of different sizes were attached to denote left and right placement within the body cavity and the lateral versus medial surfaces of the ovary (Fig. 1a). They were photographed and then partially bisected lengthwise before being immersed in approximately 10 volumes of 4% buffered formalin which was replaced after 1 h. The lower jaw of each elephant was recovered and, after being boiled to remove soft tissues, photographed to determine the age of the calf using Laws' molar progression table (Laws, 1966).

2.2. Tissue preparation

In the laboratory each fixed ovary was weighed to the nearest 0.1 g before bisection was completed to yield lateral and medial halves. Starting at the cranial pole and ending at the caudal pole, and cutting perpendicular to the interpolar axis, each half of each ovary was cut into 10 equal transverse segments (numbered 1–10) of about 3–5 mm thickness depending upon the width of the ovary. The segments were cut perpendicular to the ovarian surface and from the mesovarial margin to the free margin (see Fig. 1b). The segments were labeled for ovary (left or right), half of ovary (lateral or medial), intermarginal position identifying the position of observation relative to the cranial or caudal pole and the mesovarial or free margin and interpolar position. A random selection by the roll of a die initiated the use of the 5 odd or 5 even numbered segments from the lateral side of the ovary, whereas, for the medial side, alternate segments were selected and used for analysis (odd numbered segments if the even segments had been used on the lateral side, or even numbered segments if the odd numbered segments had been used on the lateral side). None of the ovaries were >5 cm in the interpolar dimension. The 10 segments selected from each ovary as described above were placed in histology cassettes for processing. Once embedded in wax the segments were sectioned at 25 μm thickness and stained briefly with haematoxylin and eosin (H&E) before being mounted under coverslips.

2.3. Histological and stereological examination

Follicles were observed using a BX41 microscope with an attached C7070 camera (Olympus, www.Olympus.com) and classified according to Oktay (1995) as primordial, early primary and true primary. Numerical density estimation was performed with the aid of a digital microcator (Sylvac, Switzerland) and the Optical Brick stereology tool (Fig. 2a and b) which allowed measurement of the 15 μm dissector height in the *z* axis of the microscope within the 25 μm thickness of each slide. The 25 μm section thickness was chosen in line with the recommendations of Charleston et al. (2007) and because the nuclear diameter of cross sections of elephant SF oocytes ranged from 12.5 to 17.5 μm (Stansfield et al., 2011). Having set the microcator to zero, each SF falling within the *x*, *y* inclusion area of the UCF was focused through in the *z*-axis using a continuous motion (Fig. 2b). The top 5 μm of the *z*-axis formed an exclusion zone; if part of the nucleus of the oocyte of a small follicle was observed in this zone the follicle was not counted. Oocyte nuclei of follicles occurring in the 15 μm dissector height were counted and continuation of these nuclei below the dissector height did not exclude them from the count (Fig. 2b; (Howard and Reed, 2005)). Slides were always examined from the mesovarium towards the free margin of the section in order to take account of the intermarginal spacing. Uniform tessilation of the UCF (at a distance of 1.2 mm) was made over the whole cortical area of the tissue using a random starting point outside the cortex at the mesovarial end of the section (Fig.

2c). The frequency of grid placement was determined as that frequency required for identification of at least 100 SF within the cortical tissue for the ovary being studied (Charleston et al., 2007) which should then yield a CE of approximately 10% (Howard and Reed, 2005)

2.4. Recording follicle counts by region

The number of SF in each UCF was recorded (Fig. 2a) 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 primordial, early primary and true primary follicles (Oktay, 1995) were counted together as small follicles (SF) in the interpolar fifths, intermarginal thirds, and surfaces of each ovary from three elephants on two separate occasions.

2.5. Determining the volume of the ovarian cortex

Cavalieri's principle was used to calculate the volume of the cortex of each ovary.

To this end, 10 mm square grids were drawn on clear acetate in order to obtain approximately 150–200 points per ovary at the intersection of the grid lines in the cortex. Each tissue section on each slide was photographed at a given distance (8 cm) above a light box and the photographs were subsequently viewed on a computer screen randomly overlaid with the acetate grid. The number of points (intersections of grid lines) falling on the cortical area of the section were counted. If the cortical area was difficult to visualize macroscopically it was delineated using a fine marker pen following microscopic observation. Two rulers placed at right angles in each photograph were used to calculate the area associated with each point. The volume of the cortex was then calculated using the equation; $V_{cortex} = \sum \text{points} \times \text{Ap} \times \bar{t}$ where V_{cortex} is the volume reference for the cortex; $\sum$ points is the sum of the points counted in the cortical area of all sections of the ovary; Ap is the area associated with the point and $\bar{t}$ is the length of the ovary divided by the number of segments that constituted the whole ovary. In this way the total volume of the structure could be reliably estimated with a CE of < 5% (Gundersen and Jensen, 1987). The number of follicles in the ovary was estimated using the formula;
$\text{SF}_{ov} = \frac{\text{SF}_{counted}}{n_{UCF} \times V_{UCF}} \times V_{cortex}$, where SF_{ov} represents the estimated total number of small follicles in the ovary, $\text{SF}_{counted}$ the total number of small follicles counted in all microscope sections from the ovary, n_{UCF} the number of unbiased counting frames examined in all microscope sections from the ovary, V_{UCF} the volume of each unbiased counting frame, and V_{cortex} the estimated total volume of the ovarian cortex.

2.6. Statistical analyses

Due to the low prevalence of primordial follicles and true primary follicles in the elephant ovary the data of the 3 types of SF (primordial, early primary and primary) 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 figure found by others, such 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 SFs were counted, follicular density (number of SFs per UCF), and the number of SFs 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 inerpolar 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 and 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, we determined 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 using the formula;

$$\text{Error} = \text{Absolute value of}\left(1 - \left(\frac{2 \times n\text{SF}_{\text{selected ovary}}}{n\text{SF}_{\text{selected ovary}} + n\text{SF}_{\text{contralateral ovary}}}\right)\right)$$

where *n* SF was the number of small follicles in the particular ovary.

3. Results

Across all 24 ovaries examined, 92.0 ± 5.98 (range 86.4–96.2% among ovaries) of SF recorded were early primary and the remaining 8.0 ± 5.98% were true primary. No true primordial follicles were seen. Table 1 shows that the left and right ovaries of the 12 elephant did not differ with respect to mass, cortical volume, the percentages of small follicles that were primordial, early primary or true primary. Further, Table 1 shows that the left and right ovaries were also similar with respect to the number of UCFs in which small follicles were counted, the numbers of small follicles per UCF, and the number of SF per ovary. Table 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 1). The small follicles 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 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 2 suggests that two replicate measurements of the density of small follicles 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 3 it follows that replicate estimations of the numbers of small follicles in a particular ovary will agree within 16.6% (39,781/239,509) and the total number of small follicles in a particular elephant within 10.5% (50,471/479,018).

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

Although the number of small follicles per UCF did not differ among interpolar fifths (Fig. 3; $P = 0.20$, $n = 24$ ovaries), the 10 subsequent pairwise comparisons revealed that the middle of the 5 regions cut along the interpolar axis (Region 3 in Fig. 3) tended to have a lower number of small follicles (0.81, 0.64–1.40) per UCF than the cranial fifth (Region 1 in Fig. 3) 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 small follicles 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 small follicles 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 $P = 0.09$; Fig. 4).

The error in estimating the total number of small follicles 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 UCF 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 UCF 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 elephant.

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 SFs per ovary and age, suggesting that the number of SFs 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 a previous study in older elephants (Stansfield et al., 2011), the type of small preantral follicle commonly found in these prepubertal animals was the early primary stage, which comprised 92.0 ± 5.98% of all the SF. It might have been expected that the number of primordial follicles in calf ovaries would be higher than the number Stansfield et al. (2011) found in older animals. The present finding that this is not the case further supports our conclusion (Stansfield et al., 2011) that early primary follicles, rather than true primordial follicles, form the ovarian reserve in the African elephant. This contrasts sharply to the figure of 98.2–99.8% of all SF being primordial in structure in the ovaries of prepubertal girls (Schmidt, 2003).

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 (16.6%). 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 small follicles in an ovary. One may therefore conclude that the error of 16.6% in estimating the number of small follicles 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 small follicles in one ovary of an elephant and doubling that number, provides one with an estimate of the total number of small follicles in the elephant that on average differs by 10.5% from the actual total as determined from counting the small follicles in both ovaries. Further, one may expect that in 95% of instances where the number of small follicles in one ovary from an elephant is doubled, the derived number would be within 1.3–23.5% of the actual total number of small follicles 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 small follicles in an elephant from the number in one ovary is similar to the error of 16.6% inherent in the method of estimation, as derived in the previous paragraph from repeat counts in the same ovary of an elephant. From this follows that, as is the case in the human (Charleston et al., 2007), counting small follicles in one ovary of an elephant and doubling that count provides a reasonable estimate of the number of small follicles in the elephant.

In the two elephant 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 elephant, suggesting the lower number of UCFs resulted in similar accuracy than 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 triple 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.

Our finding that the numbers of small follicles in the left and right ovaries of a prepubertal elephant are similar allows reliable estimation of the number of small follicles 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 small follicles 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.

Acknowledgements

The authors thank the owners and professional staff of the Savé Valley Conservancy for their enthusiastic support and generous hospitality. The authors also thank Professor W.R. (Twink) Allen who kindly read and edited the manuscript.

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Captions

Table 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 elephant (*Loxodonta africana*) calves aged 2 months to 4.5 years

Table 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

Table 3

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

Figure 1a

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 broken arrow runs from the mesovarial margin to the free margin of the left ovary, showing the intermarginal distance, while the solid arrow runs from the caudal pole to the cranial pole, showing the interpolar distance.

Figure 1b

A prepubertal ovary sliced into 10 approximately equal segments prior to sectioning.

Figure 2a

A diagrammatic representation of the placement and counting of follicles associated with a UCF.

Using the UCF rules, 5 follicles would be counted here (*) and the z-axis would then need to be

considered.

Figure 2b

The optical brick

Figure 2c

A diagrammatic representation of the tessellation of UCF over the cortical surface of the ovary (only approximately half the cortical area is demonstrated). Light coloured UCF's would be counted but dark coloured UCF's would not.

Figure 3

Median (± range) numbers of small follicles per unbiased counting frame in each one fifth of the ovarian cortex along the interpolar axis

Figure 4.

Number of small follicles per unbiased counting frame in the intermarginal zones

Figure 1a

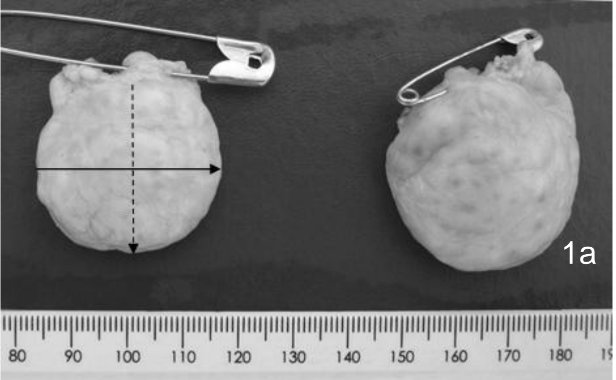


Figure 1b

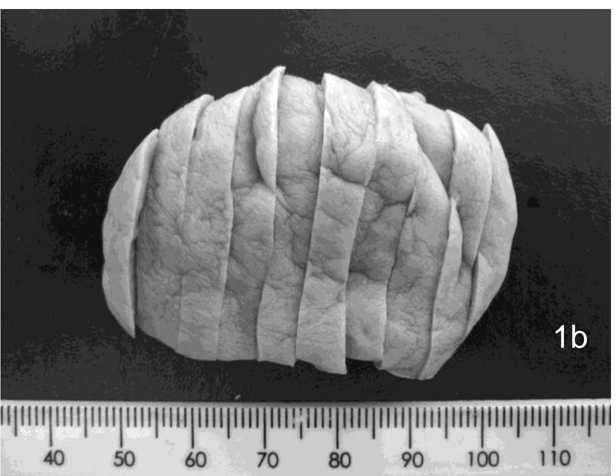


Figure 2a

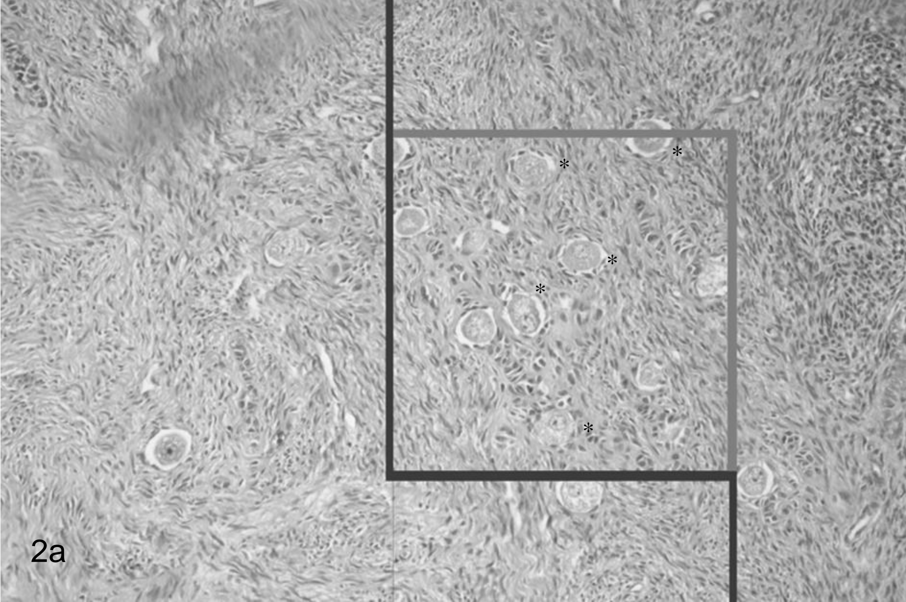


Figure 2b

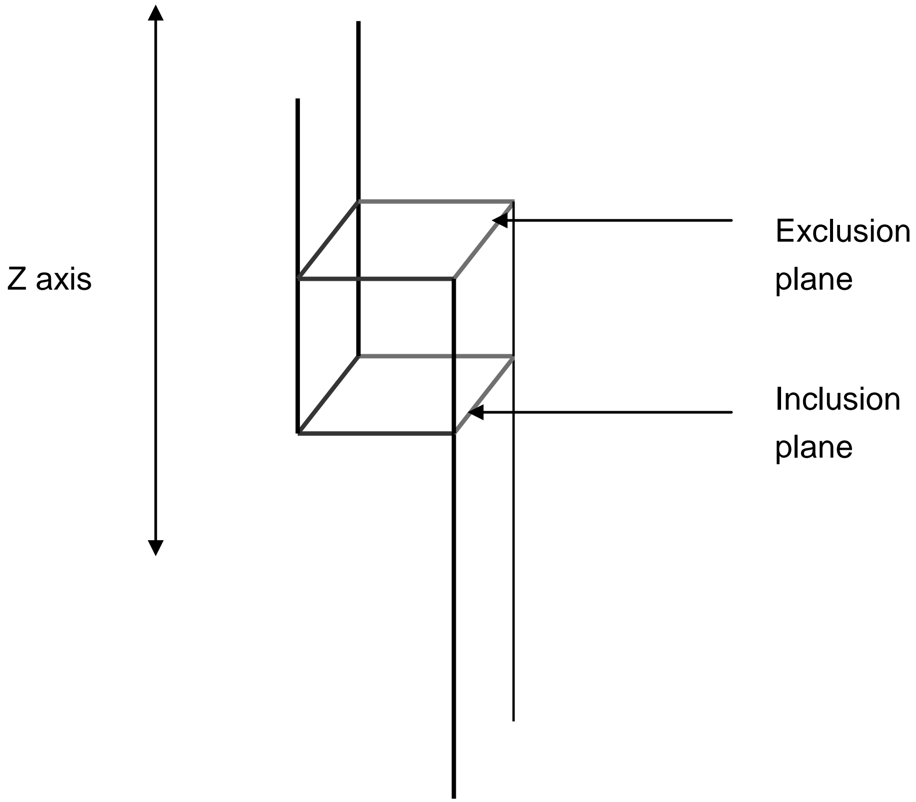


Figure 2c.

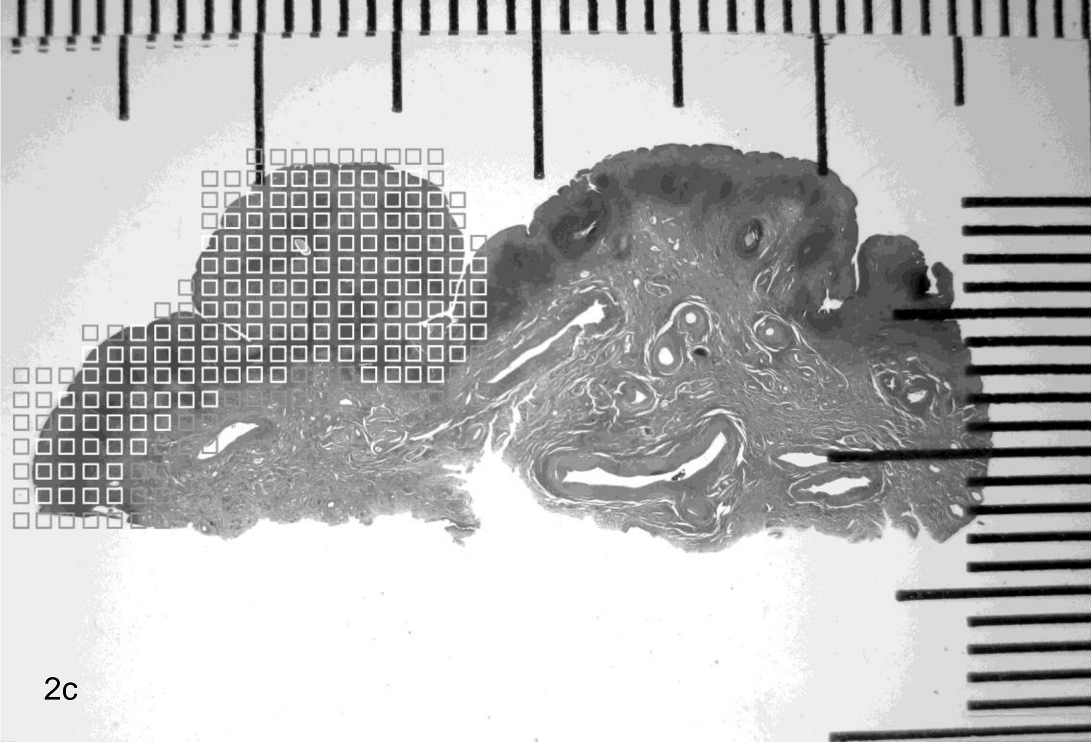
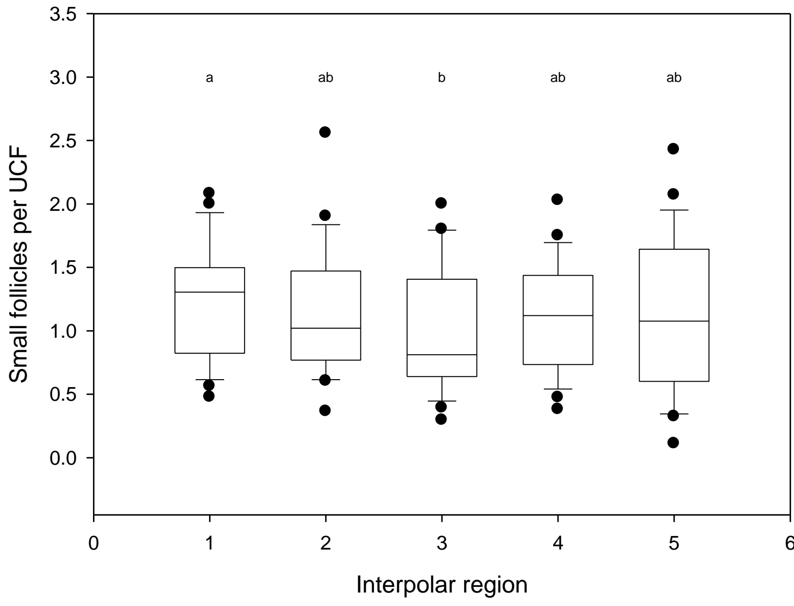


Figure 3

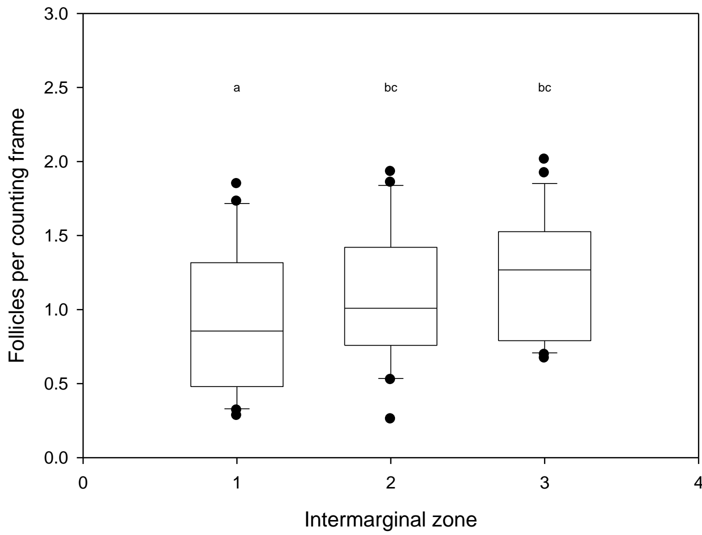
Boxplot of the numbers of small follicles per unbiased counting frame in each one fifth of the ovarian cortex along the interpolar axis (the box shows the interquartile range and the median, the whiskers the 10th and 90th percentiles, and the dots the more extreme data)



1 = cranial fifth and 5 = caudal fifth in the 24 ovaries examined. Groups marked with different superscripts differ from each other, $P<0.05$.

Figure 4.

Boxplot of the number of small follicles per unbiased counting frame in the intermarginal zones (the box shows the interquartile range and the median, the whiskers the 10th and 90th percentiles, and the dots the more extreme data).



1 = zone nearest to the mesovarial margin, 3 = the zone nearest the free margin in 24 ovaries. Regions with different superscipts differ significantly, P<0.05)

Table 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 elephant (*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. (ml)	1.77±0.61	0.72 (0.01)	1.87±0.70	1.67±0.55	0.07
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]	197561±78255	0.08 (0.80)	206492±77566	188631±84711	0.18

[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, with the 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] The percentage of all small follicles (primordial, early primary and true primary) that are early primary (having some cuboidal and some flat pre-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 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	1.154	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 follilces 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 three 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 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	239,509±60818	249,210±70,893	0.063	39,781
Average number of small follicles per elephant	479,018±120444	498,420±152,772	0.043	50,471

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