Chapter 2. Materials and methods

Materials and methods pertinent to all studies in this thesis are given below. Any methods that were particular to a specific project are given in a brief materials and methods section within each chapter.

2.1. Source of specimens

Starting in 2005, 82 sets of African elephant ovaries were obtained from professional hunting safaris and from problem animal control (PAC) or other ventures under the jurisdiction of The Parks and Wildlife Management Authority of Zimbabwe. These samples proved to be very erratic to source which resulted in a necessary extension of the time period originally allocated to the study. The elephant is a protected species and is presently in Appendix II of the Convention on the International Trade in Endangered Species (CITES) listing in Zimbabwe. However, a quota for hunting tuskless elephant exists in Zimbabwe in an attempt to reduce the number of tuskless individuals in the population and it was from such hunts that the majority of ovaries were collected initially.

In addition to the above, the culling of approximately 60 elephants per year during 2009 to 2011 for management reasons in the Savé Valley Conservancy (SVC) in south east Zimbabwe enabled the collection of further very valuable specimens. Most importantly, these provided ovaries from animals aged 0–9 years which had not been available from the professional hunting safaris. Thus, the project was re-designed so as to use the more recently collected SVC ovaries where possible and to use the long-stored samples to make-up numbers in groups which could not be filled by samples recovered in the recent collections.

2.2. Collection of specimens

2.2.1. Ovary and lower jaw collection

Elephants were killed humanely by professional hunters from the ground with a brain shot delivered by a heavy caliber rifle for adults and a smaller caliber rifle for younger animals. During culling exercises complete family groups were shot to prevent family members remaining, as the management were concerned that such individuals may suffer

stress. During professional hunts individual animals were shot. Within 2 hours of death, the ovaries were removed from the carcass but prior to this wherever possible, safety-pins were attached to each ovary to denote left (small gold pin) and right (large silver pin) placement within the abdomen and also lateral or medial surfaces of the ovary, the pin being placed on the lateral side and this fact noted in the log book. The ovaries were photographed alongside a ruler and after the mesovarial ligament was trimmed away each ovary was partially bisected from the free margin to the mesovarial margin to allow the faster penetration of fixative. Both ovaries were then placed in a single wide-necked glass jar containing 4% buffered formalin. The jar was labeled with the animal's allocated unique number and it was agitated several times during the following hour after which the formalin was completely replaced.

The lower jaw of each elephant was labeled with the animal's unique number. Within 24–48 hours the jaws were skinned and boiled to remove excess soft tissue before being photographed for reference purposes and stored safely either at the skinning shed on the Conservancy or in a locked garage in Harare. The jaws were re-examined approximately 3 months later when they were clean of flesh, which allowed for easier identification of the molar teeth. Finally, since it was not possible for logistical reasons for the author to be present at all the collections from professional hunts, a tracker who accompanied each hunt was trained to collect the ovaries and jaws, and make the body measurements, keeping the process to a minimum. For the culled animals where the author was present it was possible to collect a greater depth of biological data, as listed below.

2.2.2. Body measurements

Carcass measurements were made of shoulder height and back length. Shoulder height was measured by rolling the carcass onto its side before *rigor mortis* had set in. The two front legs were straightened and the front leg on top placed against the one underneath, both perpendicular to the spine. Two stakes were then driven vertically into the ground, one touching the scapula and the other touching the soles of the feet and the distance between them was measured in centimeters as described by Whyte (1996). Due to the large body mass of the dead animal this manipulation could prove difficult in which case a height measurement from the tip of the scapula following the bones of the leg to the sole of the foot was made.

Body length was measured from the caudal margin of the ear at its junction with the head to the base of the tail without following the curve of the spine as described by Laws *et al*. (1975). Female elephants continue to grow slightly in shoulder height throughout their life, but this is barely perceptible (Laws 1969). Their back length, however, increases with age and this dimension can give a good estimation of age (Krumrey & Buss 1968).

In order to age fetuses their body mass was used according to Craig's widely adopted formula (Craig 1984):

$$age = 3 \times mass^{1/2} \times 0.0945 + 138 \text{ or}$$

$$t = 106 \times w^{1/3} + 138, \text{ where t is the age and w is the fetal mass in kg.}$$

For embryos and fetuses up to 200 g, and thereby reckoned to be from 50–200 days of gestation, the formula that Hildebrandt *et al*. (2007) derived from ultrasound investigations was used;

$$Age = 28.434 + 54.2 \times (mass)1/6, \text{ where mass = grams and age = days.}$$

2.2.3. Further data collection

Lactation status was determined by the expression of milk from the mammary gland.

The uterus was removed by transecting it cranial to the uterine cervix, this exposed the lumen of each uterine horn, and a sharp knife was inserted into each lumen and the horn cut open right to its cranial tip. An early pregnancy was revealed by the presence of clear watery fluid and filamentous white membranes (Whyte 1996). If watery fluid and membranes were seen but no embryo, the conceptus was considered to be less than 40 days old (Allen *et al*. 2002). Later pregnancies were revealed by an obvious intraluminal swelling in the uterine horn ipsilateral to the ovary containing all or the majority of the large accessory *corpora lutea* of pregnancy (Allen 2006; Hodges 1998).

With the uterus of non-pregnant elephants an attempt was made to count the number of placental scars in the endometrium in order to give an estimate of the parity status of that animal (Figure 1.5e).

2.3. Estimating the age of elephants

The elephants were initially aged according to molar progression as described by Laws (1966) throughout the present study. However, recent data from Amboseli National Park in Kenya has shown that the ageing criteria proposed by Laws are robust in all but the very last of his 30 differential age groups (Lee *et al.* 2012). Having studied the population of elephants in Amboseli and collected jaws of elephant of known age since 1972, Dr Phyllis Lee and her colleagues estimate that the maximum lifespan of elephants is 70 years rather than the 60+/- 4 years suggested by Laws.

Elephants have a series of 6 molar teeth which pass through each quadrant of the jaw throughout life (Figure 2.1). Studies have shown that molar progression occurs at a given rate per tooth (Jachmann 1988; Laws 1966; Sikes 1966) so that observation of the wear of each tooth gives a good estimate of the animal's age.

Jaws from the hunted and culled elephants used in the present study were collected and placed together for comparison purposes. With the exception of very young calves, when 3 molars may be present in the jaw simultaneously, usually there is only one or two sets of molars in wear at any one time during life and, after the age of 40, only the 6th set of molar teeth are present. The molar teeth are formed in the alveolus in the caudal part of the mandible and they progress rostrally and dorsally during wear until there is virtually no root remaining. The tooth then falls out or is swallowed and it is succeeded by the following molar in sequence. Each molar tooth can be identified by its size, which is most easily assessed by measuring the transverse diameter of the tooth, and also its rostrocaudal length if the full tooth is present in the jaw. Examination of the tooth caudal of, or rostral of the dominant molar in use and also of the one developing in the alveolar pocket can help in molar identification. Once the order of the visible molars has been identified a quick comparison with the progression diagrams drawn by Laws (1966) is a reasonably accurate estimation of the age of the animal. If there was any doubt about which molar was present, the animals shoulder height and back length along with jaw dimensions were also considered, six measurements of each mandible having been made as shown in Figure 2.1c, d and e.

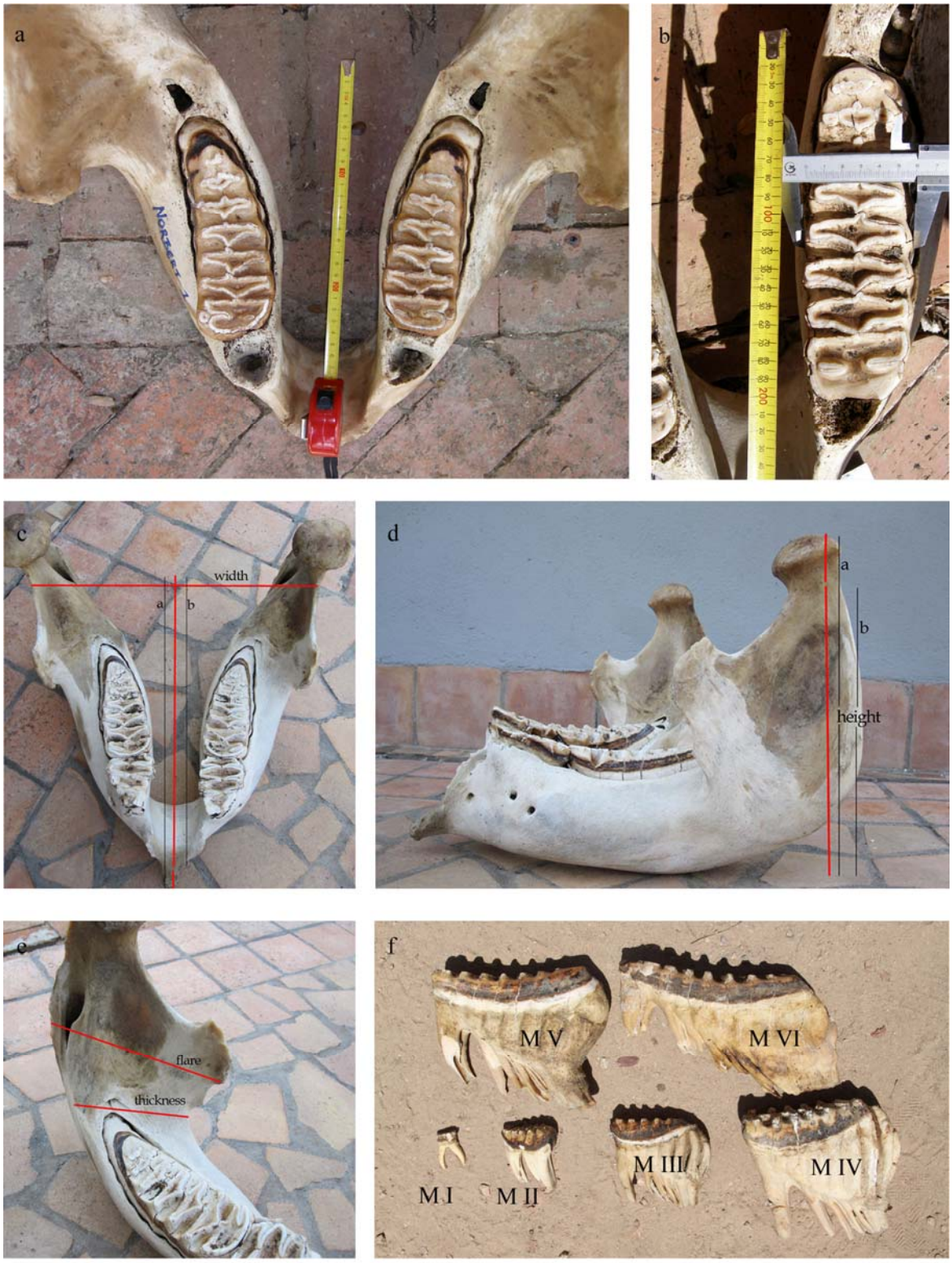

Figure 2.1 The ageing of elephants is achieved by examining the progression of molar teeth through the mandible

Continued

Figure 2.1 (continued)

a The mandible of a 25 year old female showing the lower M V molars. Note the socket at the rostral part where M IVs have been lost and the open alveolus at the caudal part where M VIs are being forming (molars not visible).

b Measurement of rostrocaudal length and transverse diameter of an M V using ruler and callipers.

c, d and e. Mandible measurements of width, length (x2), height (x2), flare and thickness.

f. A series of the six molar teeth of the female African elephant.

g. The mandible of a 50 year old cow has been bisected at the chin and the medial surface of the right side removed. The M VI is being erroded rostrally and the alveolar pocket is filled with spongey bone with no further tooth development.

h. Comparison of the half mandibles of a 50 year old cow (top) and a 33 year old bull (bottom) showing the sexual dimorphism in size. The largest remaining tooth in both jaws is M VI.

i. The mandible of an aged cow with almost no remaining M VI molars.

2.4. Histology and stereology

2.4.1. Establishment of the protocol

Although larger antral follicles can be identified and counted using transrectal ultrasonography (Hildebrandt *et al*. 2000), small pre-antral follicles in which the resting oocytes are held are only visible under the microscope, requiring the examination of histological sections.

The primary aim of the first study of this thesis (Chapter 3), was to determine the composition of the follicle reserve in the elephant (Stansfield *et al*. 2011b). A model based approach, using serial sectioning of segments taken from the ovarian surfaces, based on the method of Block (1951) and described in detail by Stansfield (2006), was used. In addition, the number of SF per ovary was also estimated. The number of follicles counted in these sections was multiplied by the inverse of the sampling fraction to obtain a raw estimate of the total number of SF within the ovary. A correction factor (assuming that the nucleus had a particular size, shape and orientation) was then used in an attempt to counteract the tendency to over-count nuclei that might occur in more than one section (Abercrombie 1946; Floderus 1944). These correction factors are problematic as it is not possible to determine if they improve the estimate or not (Abercrombie 1946; Miller 1999). Having noted the relatively low number of follicles that were being calculated in the elephant in the first study of this thesis (Chapter 3), it was decided for the reasons given below that stereology would be used to make follicle counts in future studies, and to count the number of small follicles in the ovaries of the animals used in the first study again, this time using stereology. Design-based stereology does not employ model based correction factors and therefore it may be suggested that it has greater mathematical soundness (Charleston *et al*. 2007). However, it is not possible to say definitively which protocol (serial sectioning or stereology) yields the most accurate results (Charleston *et al*. 2007).

Small follicles are known to be distributed heterogeneously within the cortex of mammalian ovaries (Charleston *et al*. 2007). The aim of Study 2 in Chapter 4 (Stansfield *et al*. 2011a) was to determine if the distribution of follicles differed between ovaries, between the surfaces of an ovary, among intermarginal positions or among interpolar positions. Based on the result of Study 2 it was initially planned that, in further studies,

the number of ovarian sections made per animal could be reduced from 20. Due to the low density (small number per unit volume) of SF in the elephant ovary and prior knowledge (Study 1, reported in Chapter 3) that the number of follicles are expected to decrease with advancing age, the number of ovarian sections examined per animal was kept at 10 per ovary and therefore 20 per animal.

Due to the impact of the multiple large CL associated with pregnancy on the overall shape and size of the ovary of pregnant elephants (Figure 3.1b and Figure 7.1a) it was decided for the stereological studies to use only ovaries without the large CL. In addition the following ovaries were considered for use if sufficient of the above described ovaries were not available; i) ovaries with only small CL associated with early pregnancy, or following the first LH peak, ii) ovaries from pregnant animals which contained no CL, having come from the side contralateral to the gravid horn. Furthermore it was planned that the initial samples would ideally originate from young calves which in turn necessitated an extension to the period of specimen collection since prepubertal ovaries could only be collected from a cull situation which took place for the first time in 2009.

Unless otherwise stated in each individual study, the following protocol was used for histological preparation and for stereological examination.

2.4.2. Histology

In the laboratory the whole, fixed ovaries were weighed to the nearest 0.1 g and each was fully bisected into a lateral and medial half; the surfaces of the half ovaries were re-photographed with identification numbers in the photographs. In cases where the orientation of the ovary within the body was not known (mostly with the hunted animals), the surfaces of the ovaries were named as described in the randomization protocol in section 2.4.2.1. Following bisection each half ovary was then cut in a transverse plane into 10 equal segments, each about 3–5 mm thick. With larger ovaries, up to 17 segments of approximately 5 mm thickness were cut and 10 representative sections, evenly distributed throughout the width of the ovary, were used. The segments were cut perpendicular to the ovarian surface and from the mesovarial margin to the free margin (Figure 3.1d). Each segment was identified for ovary (left or right), surface of ovary (lateral or medial) and interpolar position (position relative to the cranial or caudal pole), whereas the orientation of the section permitted identification of intermarginal position

(position relative to the mesovarial- and free margins). Any remaining tissue segments were placed on a safety pin in their correct sequence and stored in formalin.

The cut segments were placed in histology cassettes with the mesovarial margin toward the hinged edge of the cassette with the aim of having all sections placed in an orderly manner on microscope slides. A simplified code system was written on the cassette and the codes given corresponding labels recorded in an Excel spreadsheet. A random selection using the roll of a die determined whether the 5 odd, or the 5 even numbered segments from the lateral side of the ovary would be used; for the medial side, the alternative, (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) were selected and used for analysis. The 5 odd- or 5 even-numbered segments from each side of each ovary were placed in histology cassettes for processing, giving 20 segments per animal. Following embedding in wax (see section 2.4.2.1 for processing and embedding protocol) the segments were sectioned at 25 µm using a microtome (for the reasons described below in section 2.4.3.5). One section was cut for examination from each segment, this section being selected once a full surface of the tissue was exposed following repeated sweeps of the microtome. Ideally the sections would be cut at a uniform distance but in practice this depended upon the skill of cutting a good complete section at the right place. A specially adapted protocol was used to stain these thick 25 µm sections with haemotoxylin and eosin (see section 2.4.2.1 for staining and mounting protocol). They were then mounted under a coverslip using DPX mounting medium, (Leica, Germany).

2.4.2.1 Standard procedures used during histological preparations

Randomizing Protocol

This protocol was used to select the labelling of ovary surfaces during preparation of segments in November 2009 when the orientation of the ovaries within the body was not known.

The two ovaries of each animal were placed on a bench-top randomly, left and right. A table of random numbers was used in which the identification number of the animal being studied was identified in the first column. Reading across to the second column, if the first digit there was odd then the ovary placed on the left would be designated Ovary A,

and the ovary on the right Ovary B. When labelling surfaces of each ovary the second column of the random table was referred to. If the first digit was odd then the top surface of the ovary was labelled Side 1 and the lower one Side 2.

Embedding and sectioning protocol

A Shandon automated processor (Thermo Fisher Scientific, Runcorn, Cheshire) was used to pass the cassettes through a series of graded isopropanol baths under vacuum to dehydrate the tissues and then move them into wax (also under vacuum). The program was as follows:

Chemical	Duration (min)
Formalin (NBF)	15 min at 30 ^{o}C
Alcoholic formalin	15 min at 30 ^{o}C
Isopropanol	60 min (x2) at 45 ^{o}C
Isopropanol	90 min (x3) at 45 ^{o}C
Isopropanol	105 min at 45 ^{o}C
Wax	90 min (x2) at 62 ^{o}C
Wax	150 min at 62 ^{o}C

Consumables supplied by R&L Slaughter, Upminster, Essex. UK.

The paraffinized specimens were removed from the automated processor and were placed with cut surface downwards on the bottom of the mold. The labeled tissue cassette was placed on top of the mold as a backing. The wax blocks were then cooled for about 20 minutes till hard and subsequently removed from the molds.

Blocks to be sectioned were not cooled before cutting as this made sectioning at 25 µm more difficult. The 25 µm sections were cut with a microtome (Microm-HM 335E, Raymond A Lamb, Eastbourne, Sussex, UK). The resulting ribbon of sections was floated on the surface of a 52 ^{o}C water bath and the chosen section was floated onto the

surface of a Menzel Superfrost slide (V W R Laboratory Supplies, Essex, UK) with the free margin of the ovarian section at the label end of the slide. A soft wet brush was used to gently brush over the edges of the paraffin section to anneal it to the slide and the section was blotted with damp filter paper to aid adhesion. The slide with its paraffin section was placed in a 65 ^{o}C oven for 20 min to bond the section to the glass and then stored at room temperature over night.

Staining and mounting protocol

A staining protocol employing shorter periods of staining was used on the 25 µm sections so that the tissue morphology was clearly visible. A normal 5 µm staining protocol led to the section being very dark and difficult to examine.

Protocol as supplied by the Department of Surgical Research, Northwick Park Institute for Medical Research, Harrow, London, UK:

Step number and action		Incubation Time
1. Deparaffinize sections in	Xylene 1	10 min
	Xylene 2	10 min
2. Rehydrate sections in	100% IMS[a] for	2 min
	95 % IMS	2 min
	75 % IMS	2 min
	Running water	2 min
3. Stain with Harris' Haematoxylin solution		45 seconds
4. Rinse in running tap water		5 min
5. Differentiate in acid alcohol to remove excess haematoxylin		3 x 3 seconds
6. Rinse in running tap water		5 min
7. Stain with 0.5% aqueous Eosin		5 seconds
8. Rinse in running tap water.		1 min
9. Rehydrate sections in	75 % IMS	2 min
	95 % IMS	2 min
	100% IMS	2 min
	Xylene 2	2 min
	Xylene 1	2 min
10. Mount sections using DPX mounting media. Use excess amounts (~3x more than 5µm sections) to ensure section penetration and allow correct sealing of the tissue to prevent tissue damage. Air dry overnight.		

[a] IMS = industrial methylated spirits
All consumables were supplied by R&L Slaughter, Upminster, Essex. UK with the exception of the stains which were supplied by Raymond A Lamb, Eastbourne, Sussex, UK.

2.4.3. Stereological examination

Design-based stereology enables the estimation of 3-dimensional quantities from 2-dimensional images and it has now become the norm for counting items in biological tissues. It provides a set of tools that are inherently imbued with lack of bias and can therefore be "taken off the shelf" and used in any situation (Howard & Reed 2005).

2.4.4. Sampling and bias

In scientific work involving microscopy it is rarely possible to examine the whole of an object of interest, this requires a sample of some description to be taken in order to estimate the number of objects of interest. This must be implemented without sampling or systematic bias.

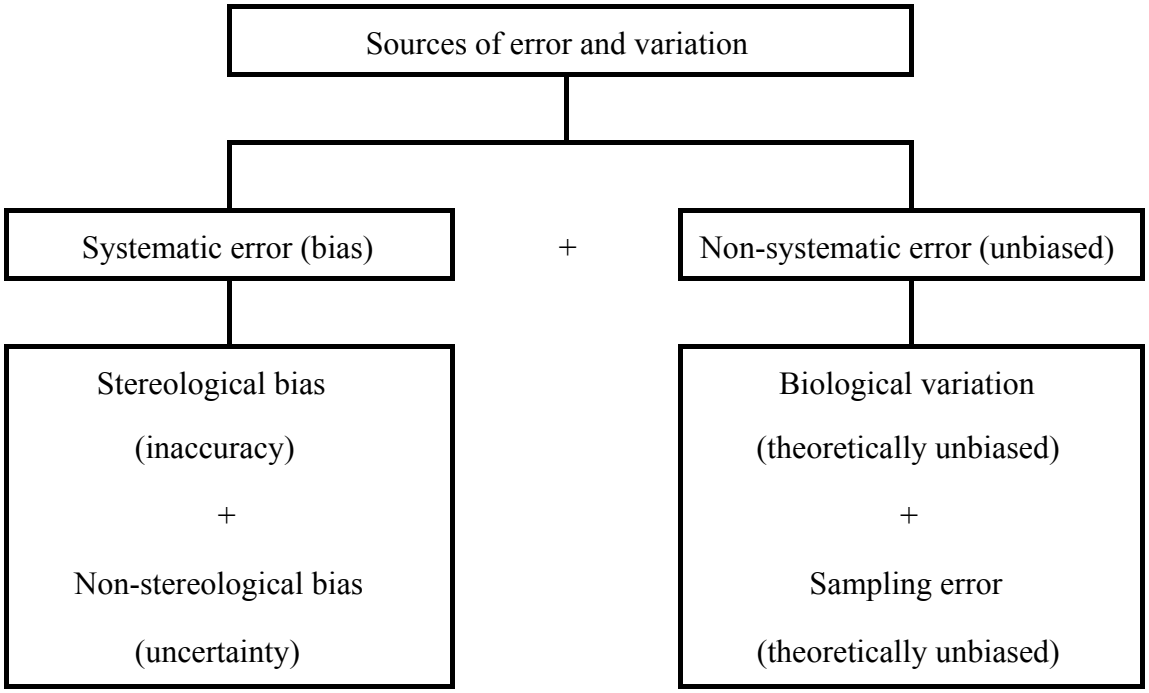

Figure 2.2 Sources of variation and error, according to (Mounton 2002)

When sampling from a population every member thereof should have an equal probability of being selected in order to minimize non-systematic (unbiased) error and thereby the apparent magnitude of biological variation (Figure 2.2). To achieve this the sample should be random, starting in a random position and making uniform selection thereafter. Such uniform random sampling should be employed at every level of sampling hierarchy, therefore at no time within the defined reference space should anything be chosen. Improved precision of a result may be achieved by greater sampling frequency at a high level of sampling hierarchy, for example looking at more individuals in a population or looking at more segments per individual (Table 2.1). It has become a rule of thumb in many biological stereological studies to count no more than 200 areas of interest per

animal at the lower levels of sampling hierarchy as counting more than this does not greatly improve the accuracy of the estimate (Howard & Reed 2005).

Table 2.1
Hierarchy of experimental variability (Howard & Reed 2005)

Hierarchy	Variability
Variability among individuals (biological variability; BV)	70%
Variability among segments	20%
Variability among sections	5%
Variability among fields	3%
Variability among measurements	2%
Total observed experimental variability	100%

In addition to natural variation, the second major non-systematic error is sampling error, or coefficient of error (CE) which is the standard deviation of the sample divided by the mean of the sample, ie variability within the sample. This value reflects on the variability of the estimated mean with respect to the population mean.

2.4.4.1 Systematic bias

Stereological tools are theoretically capable of delivering unbiased results (Howard & Reed 2005) and should therefore be free of systematic errors. Errors may, however, occur due to inefficient implementation of the protocol; for example, improper calibration of equipment or incorrect mathematics (Mounton 2002). According to Mounton, accuracy of results is affected more by the care with which methods are employed than by working harder in counting more objects of interest in a given section. A correct stereological protocol must be followed in order to produce unbiased results.

2.4.4.2 Probes and reference space

Stereology employs estimators which are rules used in conjunction with a theoretically unbiased geometic probe (Mounton 2002). The probe used depends upon the type of sample of study (Table 2.2).

Table 2.2
Types of probes used during stereological studies

Sample for study	Type of parameter	Type of probe to use
Total number	0-D	3-D (dissector volume)
Total surface	2-D	1-D (line probe)
Total length	1-D	2-D (planar probe)
Total volume	3-D	0-D (grid point)

In the present study grid points (0-D) were used with Cavalieri's Estimator to estimate a volume (3-D) called the Vref (the reference volume within which the stereological count is being made) of the fundamental sampling unit (FSU), for example the cortex of the ovary. A dissector (unbiased brick; 3-D) was used to count numbers of SF to give Nv (number of SF in the reference volume) according to Howard and Reed (2005) . It is the employment of the 3-D unbiased brick that requires the use of the 25 µm thick sections in order to be able to work within a volume and not a planar unit.

In biological systems the definition of the reference space is critical and therefore when making between animal comparisons the use of total quantities should be reported, for example, quote follicles per animal (Gundersen & Jensen 1987; Howard & Reed 2005), failure to comply with this has been termed the 'reference trap'.

2.4.4.3 Cavalieri's Estimator to determine *Vref*

Cavalieri's principle was used to calculate the volume of the cortex of each ovary. This was done in 3 stages as described by Browne *et al*. (1995):

i) The ovary was sliced into segments as described in 2.4.2. Assuming that the ovary was destined to be cut into segments each with thickness *t* mm, the position at which the first cut would be made was randomly chosen within the first *t* mm from the left edge of the ovarian surface. Subsequent slices were then made at a uniform distance *t* mm apart, thereby allowing equal opportunity for any section throughout the ovary to be chosen and therefore capture the full variability of the parameter in the sample estimate.

ii) The total area of each slide mounted section was estimated using the point counting method described below. The chosen section became representative of the segment it was cut from.

iii) If the distance between the segments is equal then the volume of the object may be estimated by multiplying the distance between the segments ($\bar{t}$) by the total surface area calculated from the sections.

In order to calculate cortical volumes, 10 mm square grids were printed on clear acetate (printed grids were supplied by The Department of Surgical Research, Northwick Park Institute for Medical Research, Harrow, London, UK) in order to obtain approximately 150–200 points at the intersection of the grid lines falling on the cortex of the ovary (Gundersen & Jensen 1987). Each tissue section on its slide was placed on a light box and photographed at a distance of 4 cm above the 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) that fell 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. A ruler placed next to each section, so that it became incorporated in the photograph of the section, was used to calculate the area associated with each point. The volume of the cortex was then calculated using the equation

$$Vref_{cortex} = \sum points \times Ap \times \bar{t}$$

where, $Vref_{cortex}$ = Volume reference for the cortex; $\sum$ points = the sum of the points counted in the cortical area of all sections of the ovary; Ap = the area associated with the point and $\bar{t}$ = 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 was reliably estimated with a CE of <5% (Gundersen & Jensen 1987).

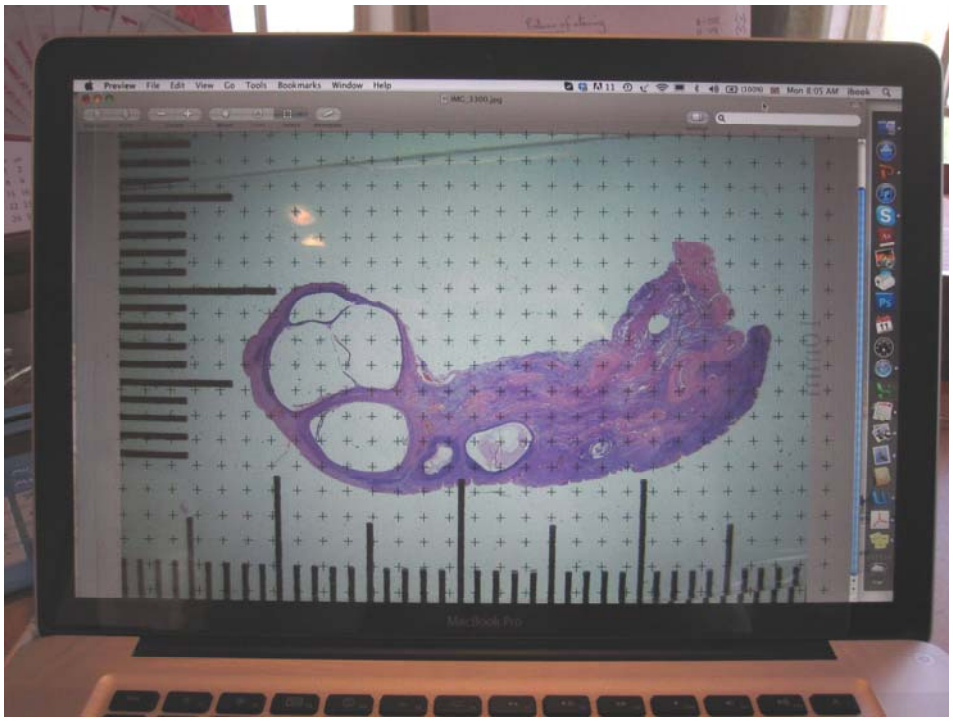

Figure 2.3 An acetate point grid randomly placed over a photograph of an ovarian section to determine the reference volume by means of Cavalieri's principle

2.4.4.4 Optical Brick (or unbiased brick) to obtain density of follicles.

An unbiased counting frame (UCF; supplied by The Department of Surgical Research, Northwick Park Institute for Medical Research, Harrow, London, UK) marked on thin plastic and measuring 8 x 8 mm was placed within the optic path of the microscope and was therefore visible on the slide. The UCF comprised red and green lines (Figure 2.4b) that enclosed a square measuring 0.8 x 0.8 mm. on the focal plane of the section. The object of interest (in this case the oocyte nucleus) was counted if it lay completely within the UCF or if it crossed a green (inclusion) line, but not a red (exclusion) line. UCF's were only counted if cortical tissue filled more than half the frame (Figure 2.4c).

The optical brick counting rules extend the UCF to a 3D counting rule that is applicable for particles of any shape and size (Gundersen & Jensen 1987). The optical brick has 3 surfaces that are "acceptance surfaces" (the right, the back and the bottom (see Figure 2.4e) and 5 surfaces that are rejection surfaces (the left, front and upper surfaces of the brick, as well as two vertical surfaces of which the upper margins are represented by the tails of the counting frame (see Figure 2.4e). The single counting rule is: a particle is counted by the brick if it intersects the brick and does not intersect a rejection surface.

The unbiased brick requires that the observer focuses up and down throughout the extent of the brick and verify that the particles being counted do not touch a forbidden surface (Figure 2.4d), also that some part of the particle does fall inside of the unbiased brick or touches an acceptance surface (www.stereology.info.com 2010).

The following formula was used to calculate the *Nv* (Howard & Reed 2005):

$Nv = \frac{\sum follicles}{\sum FOV \times vol.dis}$, where ∑ follicles = the sum of all the follicles counted in the ovary.

∑ FOV = the sum of the fields of view (number of times the UCF was placed on the cortical area), vol. dis = the area of the UCF multiplied by the height of the section studied (15µm)

In order to give a CE of <10% it has been calculated that around 50 UCFs need to be counted per ovary (Mounton 2002). A CE rising above 10% indicates that the level of events (ie number of follicles being counted) has fallen and the number of UCFs needs to be increased.

Elephant ovaries are larger than those of other mammalian species that have been studied using stereological protocols (Hansen *et al*. 2008; Miller 1999; Myers *et al*. 2004) and the present protocol was tailored to take account of the low density of SF within the ovaries of elephant calves. This included counting a greater than normal number of unbiased counting frames in order to maintain the coefficient of error (Mounton 2002).

In post pubertal animals where the density of follicles was much lower than in younger animals, the UCFs were counted adjacent to each other. By doing this, the uniform random sampling was replaced by sampling the whole population within the sections selected. However this still started at a random point.

Finally the Nv or density must be multiplied by the reference volume to give the number of small follicles estimated within the cortical area for the ovary, using the formula below:

$$Nv = \frac{\sum follicles}{\sum FOV \times vol.dis} \times Vref_{cortex}$$

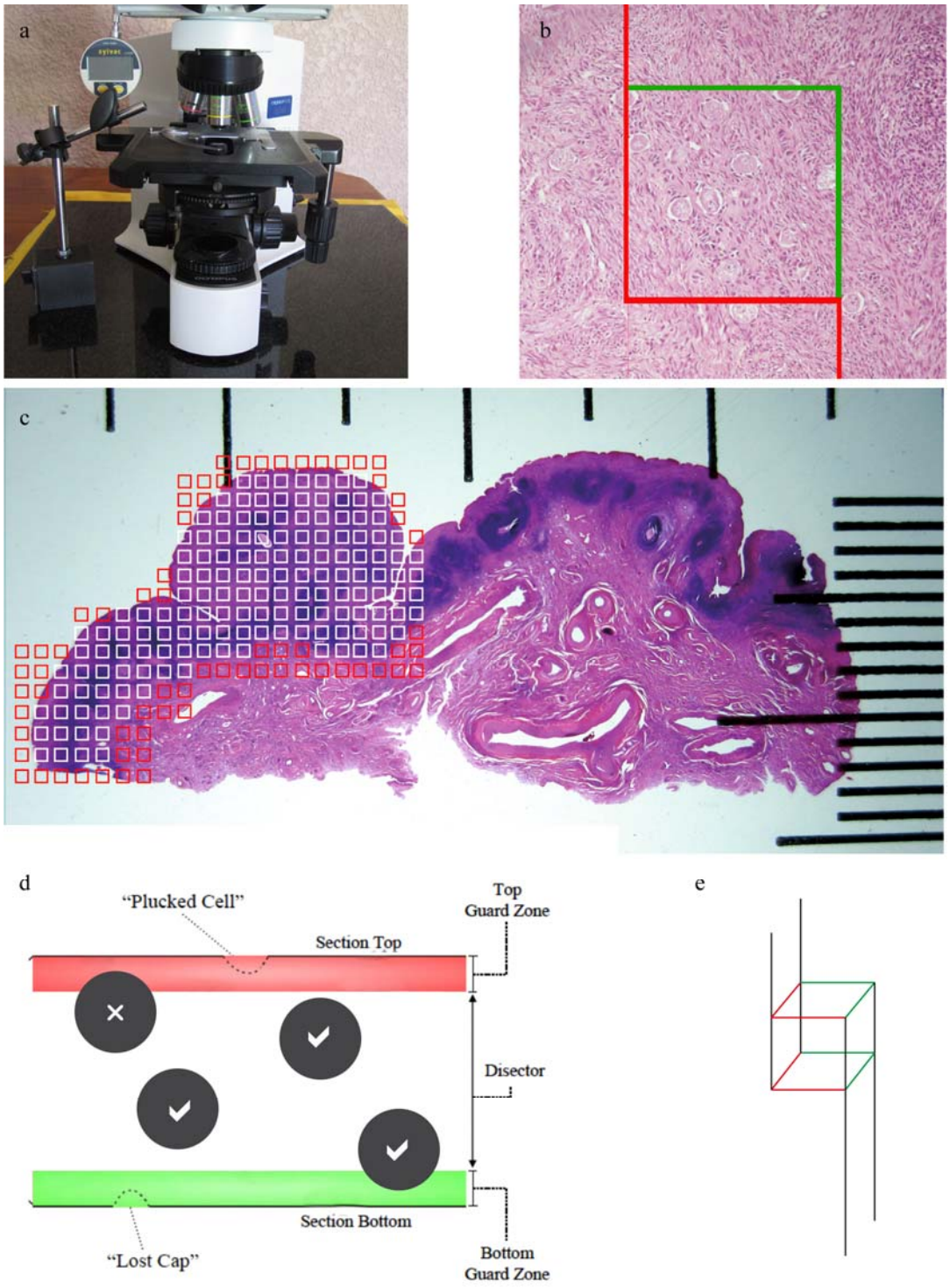

Figure 2.4 Stereology

a. Microscope with microcator.
b. Unbiased counting frame.
c. Illustration showing tesselation of UCF over the cortical area of half an ovarian section.
d. Illustration of the z- height of the disector.
e. Unbiased brick showing acceptance surfaces (green) and rejection surfaces (red).

2.4.4.5 Stereological protocol

Follicles were observed using a BX41 microscope (Wirsam Scientific, Johannesburg 2092, South Africa) with an attached C7070 camera (Wirsam Scientific, Johannesburg 2092, South Africa) and classified according to Oktay (1995) as true primordial (an oocyte surrounded by a single layer of granulosa cells, all of which are flattened), early primary (an oocyte surrounded by a single layer of granulosa cells, some of which are flattened and some cuboidal), and true primary (an oocyte surrounded by a single layer of granulosa cells, all of which are cuboidal). Numerical density estimation was performed with the aid of a digital microcator (Figure 2.4a; Sylvac, 1023 Chrissier, Switzerland) and the Optical Brick stereology tool (Figure 2.4e) which allowed measurement of the 15 µm dissector height in the z axis of the microscope within the 25 µm thickness of each slide, so giving a 5 µm guard zone at the top and bottom of the slide (Figure 2.4d). The 25 µm section thickness was chosen in line with the recommendations of Charleston *et al*. (2007) and because the diameter of nuclear cross sections of elephant SF oocytes is 12.5–17.5 µm (Stansfield *et al*. 2011b). 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. The top 5 µm of the z-axis (guard zone) formed an exclusion zone (such guard zones avoid sampling near the upper and lower edges of the sections where the tissue may be altered due to the histological sectioning process) and if part of the nucleus of the oocyte of a SF was observed in this upper 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 (into the lower guard zone) did not exclude them from the count (Howard & Reed 2005). Sections were always examined from the mesovarium toward the free margin of the section in order to take account of the intermarginal spacing. Uniform tessellation of the UCF (at a distance of 1.2 mm, or adjacent UCFs in the older animals) 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 (Figure 2.4c).

The number of fields of view (number of times the UCF was placed on the cortical area) was recorded on a log sheet together with the number of each type of small follicle falling within the allowed area of the frame and the placing of the frame within the intermarginal distance of the ovary.

Antral spaces, corpora nigra and corpora lutea

The occurrence of a UCF falling within an antral space was recorded as it was noted that in sections where there were a large number of antral follicles these could influence the results since although antral follicles fall within the cortex of the ovary, there is nil chance of a follicle being found within an antral space. However this was found not to be the case. There was no significant difference in results obtained between a) including antral spaces—using the sum of all points falling on the cortex for the Cavalieri's estimate (see below) and all the UCF, including those falling in the antral spaces for the optical brick, and b) without antral spaces where the sum of all points included only those that fell in cortical tissue (not antral space) and also excluding the UCF falling in antral spaces. Following this observation all the calculations carried out included antral spaces. The same calculation issues would apply to large corpora lutea or corpora nigra, in primiparous and older animals, that also fell within the cortical area.

The records were subsequently transferred onto a Microsoft Excel worksheet for the stereological calculations and the results then entered into a commercial stastistical software programme for statistical analysis. The statistical analyses, as well as the soft ware packages used are described with each individual study.