Ontogeny of the ovarian follicular reserve of the African elephant (*Loxodonta africana*)

By

FIONA JANE STANSFIELD

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in the Department of Production Animal Studies

in the Faculty of Veterinary Science,

University of Pretoria

Date submitted: May 2012

Acknowledgements

Working on this PhD thesis for over six years in Zimbabwe has been a long and, at times, isolated period of study and I am greatly indebted to the people in Zimbabwe, South Africa and England who have advised, supported and encouraged me throughout the process. My sincere thanks go out to all involved.

At the outset I express my thanks to my supervisors, Professor Johan Nöthling and Professor John Soley, for both sourcing financial support and for their meticulous statistical analyses and the care they took in arranging the content of this thesis and associated manuscripts. I am most grateful. I also acknowledge the role of the National Research Foundation of South Africa and the University of Pretoria in supporting my research.

I am most grateful to Professor Helen Picton of Leeds University for allowing me to deviate from human embryology to elephant folliculogenesis for my MSc thesis in Clinical Embryology in 2005. This started me on my path towards elephant research.

Dr Chris Foggin and the Wildlife Veterinary Unit listened kindly to initial thoughts on the study and they assisted greatly with obtaining the necessary permits to move specimens from Zimbabwe to England and South Africa for histology purposes.

But for Professor Twink Allen's, kind gesture to allow me the use of Dr Richard Laws collection of elephant ovaries, this study would not have proceeded past the protocol stage as new samples were, at the time, proving so difficult to obtain. His support in word and deed has been immeasurable and his kindness truly appreciated.

To Buzz Charlton and Myles McCallum of Charlton McCallum Safaris who have, faithfully over the years, collected so many of the ovary specimens during their hunting safaris. I thank them sincerely for the uniqueness of the specimens and the great trouble they went to collect and preserve them.

I am indebted to the members and owners of Savé Valley Conservancy for allowing me to collect particularly the young specimens involved in the study and for their constant support and generous hospitality. Without this the thesis would not have been possible.

Dr Sebastien le Bel of CIRAD tolerated my dogged determination to collect specimens from Savé Valley and generously allowed me to work alongside him and take part in his studies involving human wildlife conflict and sustainable usage of wildlife.

I am enourmously grateful to Dr Tahera Ansari and Professor Paul Sibbons of Northwick Park Medical Research Centre, England for their amazing stereological knowledge and kind input. Also to Joey Breedt and Marie Smit at Onderstepoort Veterinary School, and to Sue Gower at the Paul Mellon Laboratory in Newmarket England, for cutting and staining hundreds of 25 µm thick histological sections!

Many friends and family in England have carried me through the highs and lows of the exercise. Lorraine Prothero, Debby Hart, Adie and Ian Wilsher, Dave and Tess McFarlane and Michael and Emma Suddens. They all gave constant and loyal support and, Sandra Wilsher provided humour and thesis preparation survival techniques!

Finally, I offer love and appreciation to my precious son Tristan, throughout most of whose life to date this study has been taking place. For his amazing understanding that "Mum needs to work" and for all the joy he has brought to my life. When prompted by his nursery school teacher as to his mother's occupation, his reply of "My Mummy collects elephant eggs" has brightened many low moments.

Table of contents

List of tables

List of figures

List of Abbreviations

2n2c	Two chromosomes, 2 DNA strands, the genetic constitution of oogonia
2n4c	Two chromosomes, 4 DNA strands, the genetic constitution of primary oocytes
3β-HSD	3β-hydroxysteroid dehydrogenase
5α-DHP	5α-dihidroprogesterone
AMH	anti-mullerian hormone
Ap	Area associated with a point
BCL	B cell lymphoma/leukemia
BV	Biological variation
CE	Coefficient of error
CITES	The Convention in International Trade in Endangered Species
CL	One corpus luteum or more corpora lutea, as would be clear from the context
CN	Corpora nigra
CRL	Crown rump length
CV	Coefficient of variation
D	Dimension, eg. 0–D or 3–D
D1	Diameter 1
D2	Diameter 2
E	For example, E80, embryonic day 80
elPL	Elephant placental lactogen
EP	Early primary follicle, early primary follicles, or the early primary stage of development of a follicle, as would be clear from the context
FGF	Fibroblast-like growth factor
Fig	Factor in germline
FOV	Fields of view
FOX	Forkhead box

FSH	Follicle stimulating hormone
GDF	Growth differentiation factor
GSC	Germline stem cells
H&E	Haematoxylin and Eosin
HEC	Human elephant conflict
IMS	Industrial methylated spirits
KIT	Tyrosine protein kinase
LH	Luteinising hormone
LIF	Leucocyte inhibitory factor
M	Molar tooth eg MII or MVI
min	Minutes
n	Number of a sample
NGF	Non-growing follicle
NP	National Park
Nv	Number in volume
Oct4	Octamer binding transcription factor 4
OSE	Ovarian surface epithelium
PAC	Problem animal control
pZP	Porcine zona pellucida
P	Value of statistical significance
P13k	Phosphotidylinositol 3 kinase
PGC	Primordial germ cell
sec	Seconds
SF	Small follicle or small follicles, as would be clear from the context
SVC	Savé Valley Conservancy
$\sum$	Sum of
$\bar{t}$	Segment thickness

TGF	Transforming growth factor
TP	True primary follicle, true primary follicles or a follicle that is at the true primary stage of development, as would be clear from the context
TPM	True primordial follicle, true primordial follicles, or a follicle that is at the true primordial stage of development, as would be clear from the context
UCF	Unbiased counting frame
Vol.dis	Volume of disector
Vref	Reference volume

SUMMARY

Ontogeny of the ovarian follicular reserve of the African elephant (*Loxodonta africana*)

By

FIONA JANE STANSFIELD

Promoter: Professor J O Nöthling

Co-promoter: Professor J Soley

Department: Production Animal Studies

Degree: PhD

The aim of this study was to define the ovarian follicular reserve of wild African elephants in terms of its type of small follicles (SF), its establishment and distribution throughout the ovaries, and the change in numbers of SF in the embryo and fetus as well as throughout prepubertal and adult life.

The large elephant population in Zimbabwe provided the opportunity to collect ovaries from elephants culled for management reasons and hunted professionally. In total, gross morphological and histological studies were done on the gonadal ridges from 5 embryos (76–96 days post conception) and ovaries from 11 fetuses (4.8–22.2 months), 29 prepubertal females (2 months–10 years), 24 adult females (11–55 years) and 7 aged females (56–70 years). Specimens were fixed in 4% buffered formalin before a series of 25 µm thick sections were cut and examined using stereological protocols to count SF numbers in each section and thereby calculate the follicle reserve of the whole ovary. Prior to counting SF numbers, their distribution throughout the ovary was studied and the repeatability of counts was validated.

Numbers of SF were highest in mid-term fetuses, lower in fetuses during the second half of gestation, even lower in calves younger than 4½ years, whereas the numbers in calves aged 4½–9 years were significantly higher than those in younger calves, and similar to

what they were in late-term fetuses. The numbers of SF were substantially and highly significantly lower in elephant 10–15 years in age compared to calves aged 4½9 years, suggesting a reduction around puberty. Thereafter the ovarian reserve fell steadily until depletion around the age of 70 years. During adult life the ovarian reserve was composed of early-primary (EP) and true-primary (TP) follicles. By 45 years of age only TP follicles remained although these enabled oestrous cyclical activity for many more years; of 7 sets of ovaries recovered from females aged 57–70 years, 6 showed evidence of cyclical activity or pregnancy within the preceding 6 years.

The study shows that EP and TP form the follicular reserve from before birth until 45 years, with TP forming the reserve thereafter, which depletes in some old elephants and persists to maximum life span in others.

Chapter 1. General Introduction

1.1. Classification of elephants

Elephants belong to the order Proboscidea, family Elephantidae, of which there are thought to be two extant species, *Loxodonta africana*, the African elephant (Figure 1.1), of which the oldest fossils known are dated at 1.5 million years (Meredith 2001), and *Elephas maximus*, the Asian elephant (Meredith 2001). Recent debate has failed to draw a conclusion on whether *L. africana* is formed of two sub-species, *L.a. africana* and *L.a. cyclotis*, or whether *L.a.cyclotis* is a separate species (Blanc *et al*. 2007; Debruyne 2005; Roca *et al*. 2005). There is concern that premature allocation into more than one species may leave hybrids in an uncertain conservation status (Balfour *et al*. 2007) as it appears that where their natural boundaries cross, *L.a. africana* and *L cyclotis* may interbreed (Groves & Grubb 2000). Although the natural territory of *L. africana* and *E. maximus* do not overlap, a captive cross species mating has taken place and a live calf born which died 2 weeks later (Eltringham 1997). Pachyderm is another term of classification used to describe elephants, referring to an obsolete order of mammals grouping them with hippopotamuses and rhinoceroses because of their thick skin; the term is still used outside the boundaries of strict biological classification. Elephants' closest living relatives are thought to be *Sirenia* (Dugongs) and possibly *Procavia* (Rock hyrax).

Figure 1.1 The African elephant

a. A fully tusked group of African elephants, two females with two bull calves in Hwange National Park, Zimbabwe
b. A tusked matriarch with tuskless female offspring in Mana Pools NP, Zimbabwe

African elephants were historically found throughout Africa in habitats of deserts, forests, savannas, river valleys and marshes (Estes 1992). As far back as 5000 BC elephants were prized for their ivory in Egypt (Meredith 2001) but, due to climate change associated with the development of the Sahara desert, hunting, poaching and the encroachment of human settlement, elephants are now found only within restricted areas of the continent (Blanc *et al*. 2007). Their ecological importance as a keystone species is undisputed and elephants have a greater impact on their environment than any species other than man (Laws *et al*. 1970). Economically, they have been valued for their ivory which became known as "white gold", the relentless pursuit of which almost brought about the complete extirpation of the African elephant in the early 1900s (Marais & Hadaway 2006). In an attempt to restrict the trade in ivory the African elephant was placed on The Convention in International Trade in Endangered Species (CITES) Appendix III in 1976 and moved to Appendix II in 1977. Finally, in 1989, all African elephant populations were placed in CITES Appendix I, thereby banning international trade in elephants and their parts, including ivory. However, 1997 then saw the down-listing of Botswana, Namibia and Zimbabwe to Apendix II due to their healthy populations. Today there is a world-wide ban on trade in ivory but despite this poaching and an illegal trade in ivory does continue (Lemieux & Clarke 2009). African elephants are now valued by the tourist industry and in areas of overabundance the income generated from trophy hunting is a welcome boost for conservation projects (Lindsey *et al*. 2007). The discrepancy of "too few elephants here and too many there" has led to the systems for their protection in some African countries and to the necessity of reducing their numbers in others. Where there exists an overabundance of elephants alongside a growing human population an inevitable conflict of interests will arise, the solution to which will depend upon the value placed by man (indigenous and foreign) on these incredible creatures.

In Sections 1.2 to 1.4 follows an introduction to the broad field of knowledge on elephant reproduction, ranging from fetal development to reproductive senescence.

1.2. A brief overview of studies related to reproductive processes in the elephant

The reproductive organs of the female African and Indian elephants have been studied since 1734 (Chapman 1880; Forbes 1879; Patterson & Dun 1898; Perrault 1734). These

and other papers were summarized by Neuville (1937) and subsequently reviewed by Perry (1953). The latter's work included studies on ovaries collected from 81 females in Uganda between 1946 and 1950 and is widely accepted as the basis of modern knowledge of the reproductive system of the African elephant. It does, however, have a notable absence of information on folliculogenesis and the ovarian reserve, perhaps because little was known about follicle dynamics at the time. It wasn't until the studies by Zuckerman (1951) and Peters and Levy (1963) that the mechanism of follicle supply became fully appreciated (Gosden 1995).

During periods of culling in Uganda, Tanzania, Kenya and Zambia in the 1960s and 1970s several authors described the reproductive patterns of the elephant (Buss 1966; Cooper *et al*. 1964; Guy 1975; Hanks 1972; Hanks 1973; Hanks 1979; Hanks & Short 1972; Jainudeen *et al*. 1971; Kerr 1978; Laws 1967; Laws 1969; Laws *et al*. 1970; Ogle *et al*. 1973; Sherry 1975; Short 1966; Short 1969; Short & Buss 1965; Sikes 1971; Smith *et al*. 1969; Smith & Buss 1975; Watson & D'Souza 1975; Williamson 1976) but, again, without any description of early follicle development.

Following a growing trend toward a ban on culling elephants in the 1980s and early 1990s very few ovaries have been available for study in recent years and the few additional papers that have been published have been limited to gross descriptions of the ovaries and placentation (Allen 2006; Allen et al. 2005; Allen et al. 2003; Allen et al. 2002; Balke 1988; Gaeth et al. 1999; Glickman et al. 2005; Gunasena et al. 1998; Kidson et al. 1995; Wooding et al. 2005). In addition informative books reviewing historical, behavioural and other aspects of elephant natural history have been published (Moss *et al*. 2011; Spinage 1997; Sukumar 2003).

Endocrine and ultrasonographic techniques have replaced anatomical and histological studies since *post mortem* specimens have not been available for research. While these recent studies have not included small follicle counts they have, nonetheless, established the basic parameters of the elephant's reproductive cycle and are summarized in the review by Hildebrandt *et al*. (2011). The studies have been carried out predominantly on captive elephants in zoos across Europe and North America during investigations into the apparent decrease in fertility of captive females (Proctor *et al*. 2010).

Elephant contraception is an important field of study since, in southern Africa, there exists a need to reduce elephant numbers in isolated populations in order to prevent environmental damage and/or large-scale mortality of elephants due to overcrowding (Bertschinger *et al*. 2008). However, papers reporting studies on immunocontraception in elephants using porcine zona pellucida protein vaccine (pZP) mention the development and regression of follicles without reporting on their numbers.

In view of the longevity of reproductive life in the elephant the establishment of the early follicle population and its loss through life is of considerable interest. Although the view has been challenged in the last 10 years (see review by de Felici 2010) reproductive ageing in mammals is widely accepted to be irreversible due to oogonial stem cells disappearing after birth (Gosden & Lee 2010). However, the debate continues, especially since Zou *et al*. (2009) demonstrated the birth of live young from whole body irradiated mice following transplantation of mesenchymal stem cells to their ovaries in which all indigenous oocytes had been destroyed.

In the light of the above the aim of the current study, as reported in this thesis, was to investigate the establishment of the follicle reserve and the numbers of small follicles constituting this reserve throughout life in the African elephant.

1.3. The gross and microscopic anatomy and physiological functions of the elephant ovary and its follicles

1.3.1. Research pertaining to fetal, neonatal and prepubertal ovaries

In the absence of published data concerning the growth of the ovary in the elephant fetus it is necessary to assume that it develops in the same way as that of other mammals and, folowing sexual differentiation at around 105 days post conception (Glickman et al. 2005; Hildebrandt et al. 2007) is populated at an early stage by oocytes and their attendant granulosa cells. Allen *et al*. (2005) described the ovary of a 650 g elephant fetus (approximately 7 months of gestation) as displaying “a dense accumulation of oocytes in the cortical region, most of which were surrounded by a single layer of flattened pre-granulosa cells”. Cords of these primordial follicles extended towards the medulla with there being no sign of any follicle enlargement or interstitial cell development at this early stage. Interstitial cells, which possess the organelles associated with steroid hormone

synthesis, are present in the fetal gonads of many mammalian species, including humans (Konishi *et al*. 1986) and in some of these species hyperplasia and hypertrophy of interstitial cells results in an enormous increase in size of the fetal gonads which subsequently regress around birth. This enlargement of the fetal gonads occurs during the second half of gestation in the elephant (Allen *et al*. 2005), the horse (Deanesly 1977; Hay & Allen 1975) and grey seal (Hobson & Boyd 1984).

Enlargement of the fetal ovary in mid-gestation may be enhanced by antral formation in numerous growing follicles and their associated thecal layers which become broader and denser along with follicle growth. This occurs in the human (Lintern-Moore *et al*. 1974), the giraffe (Kellas 1958) and, particularly, the elephant (Allen *et al*. 2005) although as gestation continued past 18 months these authors noted a steady decline in the number of antral follicles.

According to Perry (1953), the combined weight of the ovaries reaches 80 g in the fetal elephant but falls to only 20 g in the neonate following regression of the interstitial tissue late in fetal life. Likewise, the ovaries of adult grey seals (*Halichoerus grypus)* weigh 7–27 g whereas those of seal fetuses reach 47 g in total weight (Hobson & Boyd 1984). And in the horse fetus at 250 days of gestation the combined weight of the fetal ovaries may be 50–100 g compared to only 10–20 g in the neonate (Hay & Allen 1975). This late gestation decline in fetal gonad weight in the mare parallels the decrease in concentrations of oestrogens in serum and urine (Cox 1975; Raeside & Liptrap 1975) and it was suggested by Allen *et al*. (2005) that the decrease in weight of the fetal gonad during late pregnancy in the elephant may similarly be associated with the slight decline in maternal serum progestagen concentrations during the same period, as reported by Meyer *et al*. (2004).

Around one year of age each ovary of an elephant calf weighs from 15 to 60 g (Perry 1953), interstitial tissue has become inconspicuous suggesting that the increased volume of the organ is due to growth of the other elements, particularly the connective tissue. The bulk of the cortex is reduced relative to the medulla and now constitutes a well-demarcated peripheral zone containing many fewer follicles than previously (Perry 1953). In the majority of prepubertal elephants the left ovary is heavier than the right and the same disparity is also present in the fetus (Hanks 1973).

1.3.2. Ovarian structures during different phases of the reproductive cycle of adult elephants

1.3.2.1 General morphology of the ovary

The ovary in the adult elephant is generally quoted as measuring, on average, 7 x 5 x 2 cm (Hildebrandt *et al*. 2000; Sikes 1971). The size and shape of the elephant ovary, however vary, resulting in a unique external morphology of each ovary. The surface of the ovary in cycling and pregnant elephants resembles that of the brain, possibly as a result of the restructuring occasioned by the multiple *corpora lutea* (CL) that develop during reproductive life (Hodges 1998; Lueders *et al*. 2011). In an earlier study (Stansfield 2006) it was noted that the ovarian surface in nulliparous animals was usually much smoother than in older multiparous elephants. Perry (1953) suggested that the deeply pitted surface of the elephant ovary may serve to extend the area of surface epithelium and although few in number and shallow in depth the undulations were nevertheless present in all the ovaries studied.

The morphology of the ovary is similar to that of most other mammals. A surface epithelium overlays the *tunica albuginea* beneath which is the cortical layer and, internally, the medulla and central hilus region leading to the mesovarial ligament which attaches the ovary to the uterus. The reproductive status of the animal contributes greatly to its ovarian morphology.

1.3.2.2 An overview of the endocrinology of the oestrous cycle of the African elephants

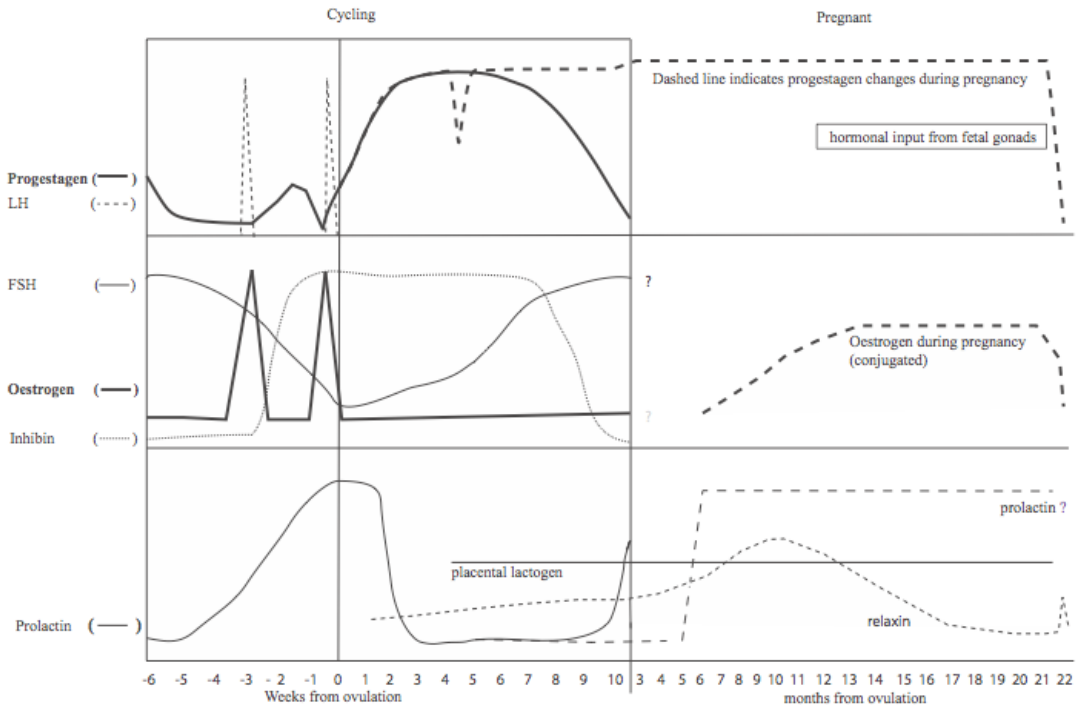
Before describing the structures on the ovaries of the elephant during various stages of the reproductive cycle, this section gives a brief overview of the endocrine changes during the oestrous cycle and pregnancy.

The oestrous cycle lasts 13–18 (16.3 ± 0.4) weeks and consists of a 6–12 (10.5 ± 0.3) week luteal phase and a 4–6 (5.1 ± 0.4) week follicular- or interluteal phase (Brown 2000; Brown *et al*. 2004a; Hildebrandt *et al*. 2011). A diagrammatic representation of the endocrinology of the oestrous cycle and pregnancy is shown in Figure 1.2.

Endocrine dynamics during the interluteal phase: The elevated serum FSH concentrations at the beginning of the follicular phase recruits follicles and stimulates two successive waves of follicular development each culminating simultaneously with one of two distinct, precisely timed, LH surges, the first of which occurs 12–21 days after progestagen concentrations have declined to baseline and the second 19–21 days later (Figure 1.2). This second LH surge induces ovulation about 24 hours later. The two LH surges are qualitatively and quantitatively similar but only the second induces ovulation (Brown *et al*. 1991; Brown *et al*. 1999; Lueders 2010).

Elevated oestrogen concentrations, which may not be measurable in the peripheral circulation during each follicular wave, trigger the LH releases but the follicles in the first wave have been considered functionally not competent to ovulate (Hodges 1998). More recent findings suggest ovulation may take place following the first LH surge but pregnancy does not ensue (Stansfield & Allen 2012). These follicles (with and without ovulation stigmata) are reported to luteinise in response to the first LH surge, thereby forming the luteal bodies destined to become the accessory CL of pregnancy (Lueders et al. 2012).

The concentration of inhibin (which is of follicular origin) in the blood is inversely related to that of FSH and positively correlated with that of progestagen (Brown *et al*. 1991) Inhibin concentrations remain basal until the latter part of the follicular phase, increasing after the first LH surge, peaking before the second LH surge and remaining elevated thereafter until the end of the luteal phase (Taya 2007). This suggests that, in the elephant, not only the granulosa cells of the follicles but also the CL secrete inhibin (Lueders *et al*. 2011; Taya 2007). In the cycling African elephant the concentration of prolactin in the serum is highest during the follicular phase (Brown *et al*. 2004b).



Data extracted from Brown, 2004; Hodges, 1998; Hildebrandt, 2011; Lueders, 2010; Yamamoto, 2011 and Meyer 2004

Figure 1.2 Hormonal changes in the peripheral blood of female elephants during the oestrous cycle and pregnancy (diagrammatic)

Endocrine changes in the luteal phase: Following the 2nd LH surge which stimulates the "fertile" ovulation, progestagen concentration in the blood increases as a reflection of the development and maturation of the CL (Figure 1.2). This is followed by a gradual rise in serum FSH concentration which peaks at the end of the luteal phase. Unlike most other mammals, FSH concentration is maximum at the beginning of the follicular phase and the level then declines to a minimum within 4 days after the ovulatory LH surge (Brown *et al*. 1999). This implies that the early stimulation by FSH is sufficient to provide prolonged stimulation for development of both the follicular waves that constite the follicular phase (Taya 2007). During the luteal phase there is no evidence of follicle development which only recurs once progestagen concentrations have returned to baseline (Hermes 2000; Lueders *et al*. 2010). The circulatory progestagens produced by both the ovulatory CL and the accessory CL of pregnancy are 5α-dihydroprogesterone and other 5α-reduced pregnanes (Heistermann 1997; Hodges *et al*. 1997). Serum oestrogen concentrations remain basal during the luteal phase (Kapustin *et al*. 1996).

Endocrine changes during pregnancy: The luteal phase of the oestrous cycle lasts approximately 10 weeks. During that period the embryo must signal its presence to the mother in order to block cyclic activity. Membranes without any associated embryo have been observed around 3–4 weeks post ovulation (Allen *et al*. 2003; Perry 1974). Serum progestagens increase after ovulation to a peak 3–4 weeks later. They then begin to decline before rising again steeply around 5–6 weeks, after which they remain elevated until mid-gestation. A second slow decline then occurs before they plateau until the end of pregnancy (Meyer *et al*. 2004). They are secreted by the primary and secondary *corpora lutea*, and very likely the secondary rise is stimulated by the luteotrophic action of elephant placental lactogen (elPL) after the first 5–6 weeks (Yamamoto *et al*. 2011), and they are likely supplemented by progestagens secreted by the interstitial cells in the enlarged fetal gonads during the second half of pregnancy; the placenta itself does not produce steroids (Allen 2006; Allen *et al*. 2003). Serum progestagen concentrations begin to fall 3–5 days before parturition (Meyer *et al*. 2004).

Following parturition, the elephant remains in lactation-related anoestrus for at least 22 months (Moss & Lee 2011).

1.3.2.3 Ovarian morphology during post-partum anoestrus

Just before parturition the large *corpora lutea* of pregnancy reduce slightly in size and begin to darken in colour. After birth they steadily reduce further over many months to eventually become small, irregularly-shaped blocks of dark brown or black tissue (*corpora nigra: CN*) distinguished histologically only by the presence of the pigment granules that are responsible for the colour (Perry 1953). Thus, the ovaries of non-pregnant lactating cows are distinguished by the absence of large active yellow or khaki-coloured, homogenous CL. During this post partum period Perry (1953) reported that a number of follicles grew to a diameter of about 5 mm but developed no further. Laws (1969), on the other hand, noted that the mean maximum follicle diameter in anoetrous lactating females was 4–6 mm, with occasional follicles measuring up to 10 mm. Using such ovarian morphology it then becomes possible to identify the reproductive status of a wild, post-partum female during *post mortem* examination with a good degree of accuracy.

1.3.2.4 Morphological changes in the ovary during the oestrous cycle

The possible mode of ovulation in the elephant has been discussed in many previous papers due to the presence of multiple large CL in the ovaries of pregnant females, more than one of which may have prominent stigmata (Hanks & Short 1972; Laws 1969; Perry 1953; Short 1966; Stansfield & Allen 2012). Hodges (1998) concluded that multiple CL, with and without ovulation stigmata, probably formed in successive oestrous cycles, with structural, but not functional, persistence into subsequent cycles as the most likely explanation of the visible evidence. Through the more recent ultrasound studies of Lueders *et al*.(2012) it has now been established that the multiple CL of pregnancy develop from luteinisation of medium sized non-ovulatory follicles after the first of the two LH surges that characterise the interluteal period of the elephant oestrous cycle (Brown *et al*. 1999; Kapustin *et al*. 1996; Lueders *et al*. 2011). In response to the second LH surge that occurs 20–22 days after the first, one larger Graafian follicle (16–21 mm in diameter) ovulates and produces a dominant CL. The several luteal structures that originate from the first LH surge persist in the ovaries (Lueders et al. 2012) and can be seen ultrasonographically to enlarge significantly some 35–50 days into gestation. This probably occurs in response to the commencing secretion of placental lactogen (elPL), a powerful luteotrophin, by the recently implanted trophoblast (Yamamoto *et al*. 2011) and results in a significant secondary rise in progestagen concentrations in maternal blood (Meyer *et al*. 2004).

With regard to the number of CL present in the ovaries of cycling elephants Laws (1969) reported that in 10 females, which had multiple CL in their ovaries but no visible conceptus in the uterus, CL numbers ranged from 3 to 22. It is noteworthy that an embryo aged 4 months weighs <5 grams so can be easily overlooked (Allen 2006). To complement what Laws found, Hanks and Short (1972) observed that non-pregnant adult elephants exhibited a high incidence of very small CL but a paucity of large ones.

Following considerable debate in previous years (Laws 1969; Short 1966; Short & Buss 1965) the maximum size of an ovulatory follicle, as monitored by repeated transrectal ultrasound examination, has been established as approximately 20 mm (Lueders *et al*. 2010). It is possible for the first oestrus after the post-partum lactational anoestrus period to be accompanied by a fertile mating and ovulation with the development of sufficient luteal tissue to maintain the ensuing pregnancy (Perry 1953).

1.3.2.5 Oestrus

Oestrus may last 24–48 hours (Short 1966), or up to 6 days (Skinner & Smithers 1990). A range of pre-copulatory behaviour patterns displayed by the male and female have been noted during more than 30 years of close study of elephant behaviour in Amboseli National Park in Kenya (Poole & Granli 2011).

1.3.2.6 Ovarian morphology following oestrus

Table 1.1 summarises the information obtained by Short (1966) from the ovaries of an elephant shot immediately after she had been observed being mated. This original observation concurs with present-day ultrasound investigations which report that elephants release a single oocyte per cycle while CL originating from 3 different timelines may be observed simultaneously in the elephant ovary (Lueders *et al*. 2010). The significance of the presence of stigmata, which Hodges (1998) observed on 30–40% of CL of apparently similar age is unknown. Lueders *et al*. (2010) suggest that stigmata may not reflect a point of ovulation, whereas Stansfield and Allen (2012) propose that ovulation may take place prior to luteinisation of follicles following the first LH peak.

Table 1.1
Ovarian structures in an elephant shot immediately after mating during oestrus; data from Short (1966)

Right ovary	Left ovary
Corpus luteum 1 (CL1)[a] 5 mm diameter and another (CL2) of 18 mm diameter, each with a clear ovulation stigma	One corpus luteum (24 mm diameter; CL3) and one fresh ovulation point 1–2 days old (9 mm diameter; CL4)
Eight brown regressing *corpora lutea*	Eight corpora nigra
One large Graafian follicle (12 mm)	Nine Graafian follicles (all< 5 mm diam.)
Ten smaller follicles (<5 mm)	

[a] CL 1, 2 and 3 were indistinguishable whereas CL 4 was well vascularised and was made up of two types of cells, one with rounded, reticulate nuclei and the other with densely staining nuclei.

1.3.2.7 Ovarian morphology during pregnancy

Follicles: Perry (1953) and Laws (1969) both recorded the presence of antral follicles in the ovaries of pregnant elephants. Perry (1953) observed that some follicles with antra up to 5 mm in diameter were present in all elephant cows during the first half of gestation ie. until the fetus weighs 8 kg, but no antral follicles occurred beyond this stage, suggesting that pregnancy-associated hormones may suppress follicular growth. Laws (1969) similarly noted that, unlike the follicles measuring 5–7.5 mm in diameter present during early pregnancy, no macroscopically visible follicles persisted by mid-gestation. Likewise, Smith and Buss (1975) reported the mean maximum diameter of Graafian follicles in pregnant elephant as 3.1 mm. A combination of the increased secretion of 5 α dihidroprogesterone and other 5α-reduced progestagens by the accessory CL (Heistermann 1997) and elPL (Yamamoto *et al*. 2011) may underlie this suppression of follicular growth during the second half of gestation.

Corpora lutea The ovaries of pregnant elephant are characterized by the presence of multiple *CL* that measure 5–35 mm in diameter and are frequently, but not always, confined to the ovary that is ipsilateral to the gravid uterine horn (Allen *et al*. 2002; Hodges 1998; Perry 1953; Short & Buss 1965). The luteal tissue is homogenous, yellow-to-khaki in colour and clearly active in terms of progestagen secretion (Hodges *et al*. 1997). The observed increase in weight of the ipsilateral ovary during pregnancy can be attributed almost entirely to the growth of these CL and the increased vascular supply involved in their development (Perry 1953); this author observed at least one CL in all but one of every pair of ovaries he examined throughout gestation. On the other hand Hanks and Short (1972) recorded 22 pregnant elephant that each had CL on only the ovary ipsilateral to the conceptus. Using transrectal ultrasonography, Lueders (2010) recorded that the maximum diameter of both the accessory *corpora lutea* formed by luteinisation of medium sized follicles after the first LH peak of the interluteal period and the ovulatory CL formed after the second LH peak was reached around 40 days after this second LH peak which had stimulated the fertile ovulation.

Several papers have been written about the CL of the elephant, addressing the type of ovulation they may result from and their size, weight and number (Allen 2006; Hanks &

Short 1972; Heistermann 1997; Hodges *et al*. 1997; Hodges *et al*. 1994; Laws 1969; Ogle *et al*. 1973; Short 1966; Short & Buss 1965; Smith *et al*. 1969; Smith & Buss 1975). More recent ultrasound studies (Lueders et al. 2010; Lueders et al. 2012) have gone some way to answering these questions but why so many large CL are produced, and whether they remain necessary and fully functional throughout gestation, still needs to be addressed. The CL can be numerous (Table 1.2) and large (Table 1.3 and Table 1.4) and many of them have a significantly greater diameter than the Graafian follicles from which they apparently originate (Allen 2006).

Table 1.2
Numbers of corpora lutea reported in the ovaries of pregnant elephants

Range of number of corpora lutea	Reference
≥ 50	Perry (1953)
2–42	Laws (1969)
2–26	Hanks and Short (1972)
3–8	Allen *et al*. (2002)

Table 1.3
Weights of luteal tissue reported in the ovaries of pregnant elephants

Weight	Reference
The ovaries of some pregnant elephants contain >200 g luteal tissue	Perry (1953)
Total weight of luteal tissue in both ovaries increased from 19 g initially to 38 g in mid-gestation and declined to 22 g at term	Laws (1969)
All elephants in the first half of gestation have > 17.3 g luteal tissue	Hanks and Short (1972)
Individual CL weighed up to 30 g	Stansfield (2006)

Table 1.4
Range in luteal size reported in pregnant and cycling elephants

Status	CL diameter	Reference
Pregnant	3–6 cm	Perry (1953)
Cycling	2.4–3.3 cm	Hanks (1972)
Cycling	2.3–3.8 cm	Hermes (2000)
Pregnant	3–6 cm	Allen (2006)
Cycling	up to 4.1 cm	Lueders (2010)

Perry (1953) suggested it was possible to distinguish histologically as many as three generations of CL in a single pair of ovaries, the newest having developed before the oldest has regressed sufficiently to have lost its definite form. Lueders *et al*. (2010) now propose that these three generations may represent the anovulatory and ovulatory CL from one oestrous cycle together with the regressing CL from the previous cycle. Regressing CL are commonly termed *corpora rubra* or *corpora nigra (CN)*, to reflect the darkening colour of the degenerating luteal tissue (Smith & Buss 1975).

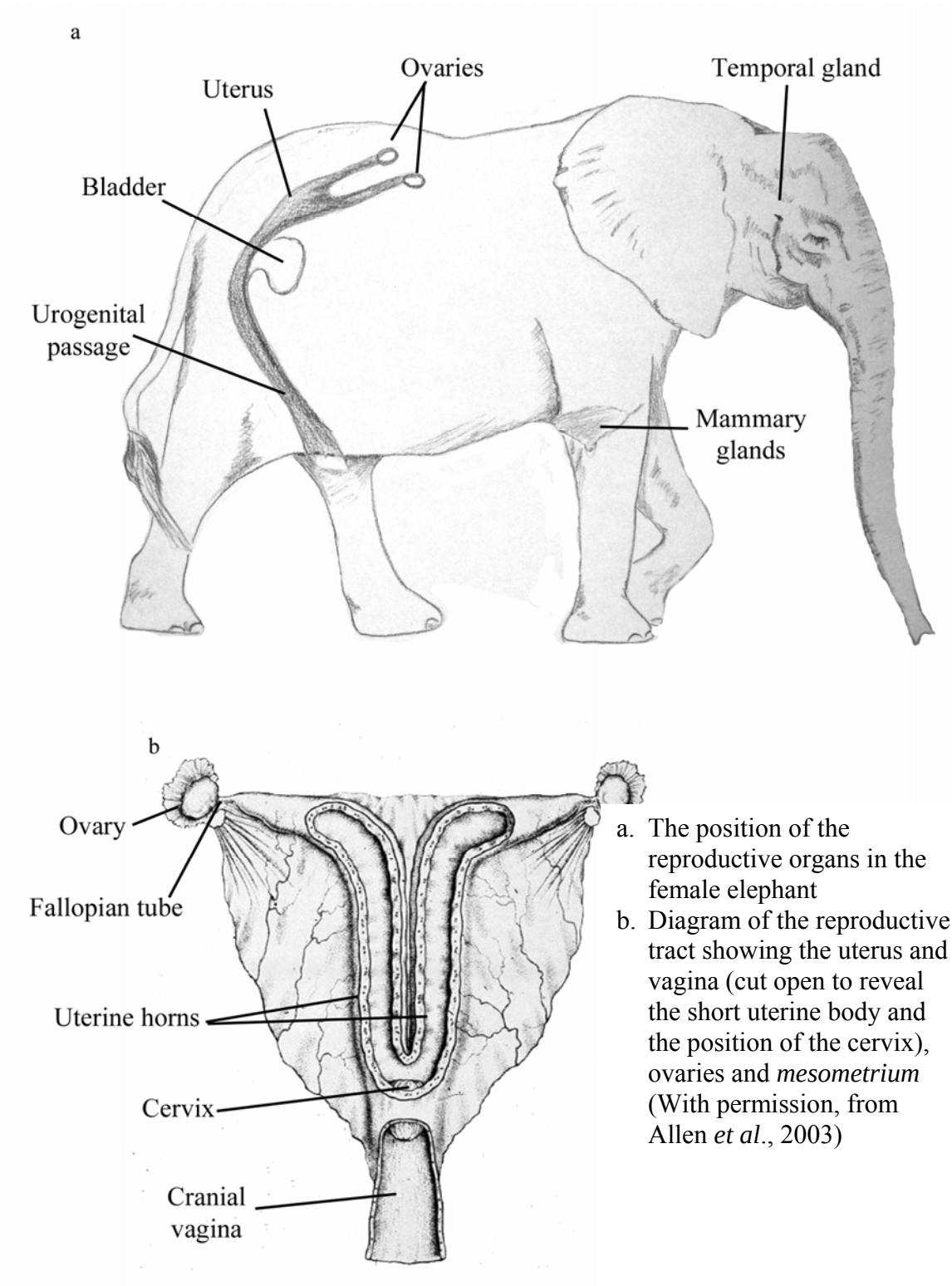
Not surprisingly, the development of the multiple large CL of pregnancy causes extensive remodelling of the ovarian cortex. In a previous study (Stansfield 2006) these large CL were dissected from the ovaries of pregnant elephants prior to serially sectioning the remaining ovarian tissue for histological examination. However, during subsequent follicle counts the expected large numbers of primordial follicles and abundant ovarian reserve could not be identified, which raised the suspicion that the development of the CL had caused sufficient distortion of the ovarian cortex to mask the small follicle populations. The physical impact of the size and number of CL on displacement of the ovarian cortex has not yet been described.

1.4. Further anatomy and physiology of the elephant

1.4.1. Anatomy of the female reproductive system

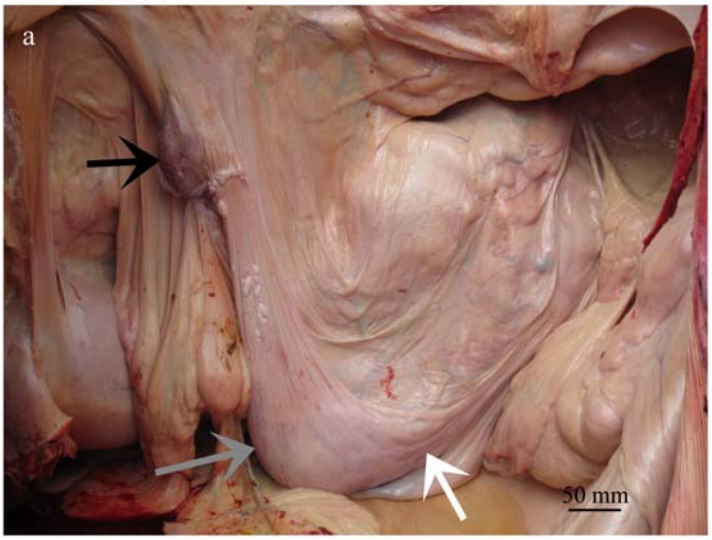
The female reproductive system of the elephant Figure 1.3 and Figure 1.4) comprises two ovaries situated close to the kidneys, comparatively short Fallopian tubes (10–15 cm in

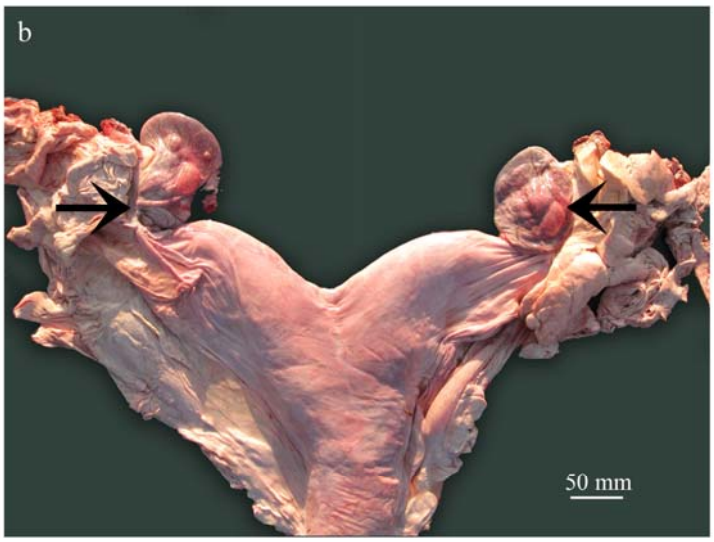
their semi-convoluted state *in situ*), a uterus consisting of two long cornua and a short body leading to a relatively short fibrous cervix. Caudal to the cervix lies the short cranial vagina (30 cm) separated by an almost complete hymen membrane from the long (1.0–1.4 m) urogenital passage, which opens externally at the vulva situated ventrally between the back legs and not in the perineum as in most other mammals (Sikes 1971). The ovary itself is almost completely enveloped by an expansion of the fimbrial funnel of the Fallopian tube. The latter forms a bursa, which is generally referred to as the ovarian sac (Perry 1953), the outer wall of which is covered with peritoneal epithelium while the inner surface is a very luscious mucosa. The ovarian bursa is connected laterally to the ovarian ligament and is open medially. The ovary is anchored to the uterine horn by the ovarian ligament and, cranially, to the body wall by the very short and fibrous suspensory ligament (Perry 1964). Both ligaments are well developed and strong and are contiguous with the hilum of the ovary. The length of the uterine (Fallopian) tube is around 25–30 cm when fully extended which is similar to that of many ungulates (Perry 1964). The lumenae of the uterine horns remain separate for some distance caudal to their point of external fusion and then open into the short common body of the uterus. The embryo probably implants ipsilateral to the side of ovulation, and usually in that part of the uterine horn that is fused externally with the contralateral horn (Sikes 1971).



a. The position of the reproductive organs in the female elephant
b. Diagram of the reproductive tract showing the uterus and vagina (cut open to reveal the short uterine body and the position of the cervix), ovaries and *mesometrium* (With permission, from Allen *et al*., 2003)

Figure 1.3 A schematic representation of the reproductive organs of the female African elephant





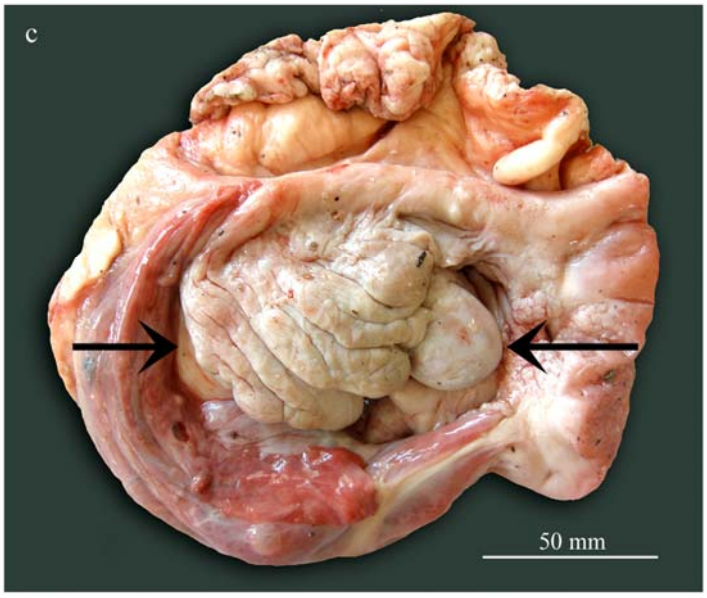


Figure 1.4 The uterus and ovaries of the African elephant

a. Left lateral view showing the ovary (black arrow), uterine horn (grey arrow) and uterine body (white arrow) *in situ*
b. Ventral view of the uterus and ovaries (black arrows) inside the ovarian bursae
c. The ovary (between arrows) attached to the mesovarium (top), with the ovarian sac (bursa) removed medially

1.4.2. Puberty

Laws (1969) classified female elephants on the basis of macroscopic examination of sliced ovaries into; i) immature — ovaries containing neither large follicles nor *CL* or *CN*; ii) pubertal — having no *CL* or *CN* but at least one follicle larger than 5 mm in diameter; iii) mature — having at least one CL or *CN in* the ovary. The age of puberty recorded in wild African elephants is shown in Table 1.5.

In captivity pregnancy has occurred at ages as young as 3.5 years in the Asian elephant and 7 years in the African elephant (Hildebrandt *et al*. 2011). Pregnancy usually does not occur until 10–12 years of age in the wild (Laws 1969).

Table 1.5

Age of puberty in African elephants

Age (years)	Country	Reference
Mean age at first breeding		
8–12	Uganda	Perry (1953)
11–20	East Africa[a]	Laws (1969)
12	Kenya	Moss (2001)
Mean age at first ovulation		
10–11	East Africa[a]	Laws (1969)
14	Zambia	Hanks (1972)
12–13	Zimbabwe	Sherry (1975)
11 (range 9–15)	Zimbabwe	Williamson (1976)

[a] The mean age of sexual maturity increases with increasing density-dependent physiological, nutritional and social stresses.

1.4.3. Fertility

Following 2 or 3 matings with the dominant bull while visibly in oestrus, the wild African elephant regularly conceives a singleton conceptus, and very rarely gestates twins (Allen 2006; Seth-Smith & Parker 1967). Laws (1969) put the incidence of twinning at 1.35% which is similar to other large herbivores.

Laws (1969) concluded that density-dependent natural regulatory mechanisms operate in elephant populations to induce changes in reproductive rate, predominantly by delaying the age of first calving but also by extending calving intervals (Table 1.6). Hanks (1972) noted peak fertility in cows aged 18–19 years with a reduction from about 40 years of age onwards. He did, however, observe macroscopically visible follicles in all but one of the 5 oldest female elephants in his study. From the published data summarized in Table 1.7 and Table 1.8 it appears that elephant fertility begins to decrease after 40 years of age, with a sharp decline beyond the age of 50 years. Nevertheless, some individuals do continue to breed into their 7th decade. Together these findings indicate that, despite their high level of fertility in younger life, fecundity does decline steadily beyond 50 years of age to leave females with a relatively short post-reproductive lifespan before death occurs as a result of starvation induced by disintegration of the final 4 molar teeth.

The high fertility of the large mating bulls and the mature cows result in high levels of conception and successful pregnancy (Moss 1996). Due to the infrequency of pregnancy loss, high postnatal survival rate and longevity, the African elephant, if not killed by man, is able to increase its population size by a remarkable 8% per annum (Whyte 2001).

Table 1.6
Published data on intercalving intervals in African elephants

Area	Intercalving interval		Reference
	Mean	Range	
East Africa	4	2.8–13	Laws (1969)
Uganda	3.8	3–4	Perry (1953)
Gonarezhou NP, Zimbabwe	3.7	3.3–4.6	Sherry (1975)
Luangwa Valley, Zambia		3.5–4	Hanks (1972)
Tanzania		4–5	Laws (1969)
Tsavo National Park NP, Kenya		6–7	Laws (1969)
Murchison Falls North NP, Uganda		6–7	Laws (1969)
Murchison Falls South NP, Uganda		8–9	Laws (1969)
Hwange NP, Zimbabwe	4		Williamson (1976)
Amboseli NP, Kenya	4.5		Moss (2001)

Table 1.7
Studies reporting on fertility in old African elephants

Published observations	Animals studied	Author
Fertility decreases after the age of 40 years	499	Hanks (1972)
In no case could it be shown conclusively that a female had ceased to breed.	81	Perry (1953)
High fertility in females aged 12–49 years with a sharp decline after 50 years when 32% were either not pregnant or lactating.	1737	Sherry (1975)
No significant peak in fertility between 13 and 49 years of age when approximately 50% of females were pregnant. The rate fell to 17% between 50 and 60 years of age, nevertheless, of the 17 elephants older than 50 years, 15 were lactating.	614	Williamson (1976)
All females aged 56–60 years were reproductively inactive, similar to menopause in women.		Laws *et al*. (1970)
Most females older than 50 years continued to reproduce but their intercalving intervals extended to 4.75 years from a population average of 4.5 years.		Moss (2001)

Table 1.8
Reproductive status of elephants older than 50 years culled in Kruger National Park, South Africa during 1975–1995. Data from Freeman *et al*. (2008).

	Number (and percentage) of elephant				
Age (years)[a]	Studied	Lactating	Pregnant	CL or CN in their ovaries	No ovarian activity (%)
50	8	7 (88)	3 (38)	7 (88)	0
51	12	10 (83)	8 (67)	11 (92)	0
52	12	7 (58)	4 (33)	8 (67)	1 (8)
53	6	6 (100)	2 (33)	6 (100)	0
54	4	2 (50)	1 (25)	1 (25)	0
55	10	7 (70)	5 (50)	4 (40)	1 (10)
56	4	1 (25)	0	3 (50)	1 (25)
57	0				
58	3	2 (68)	1 (33)	0	1 (3)
59	0				
60	5	0	0	2 (40)	3 (60)
Total	64	42 (66)	24 (38)	11 (17)	7 (11)

[a] Aged according to Laws (1966).

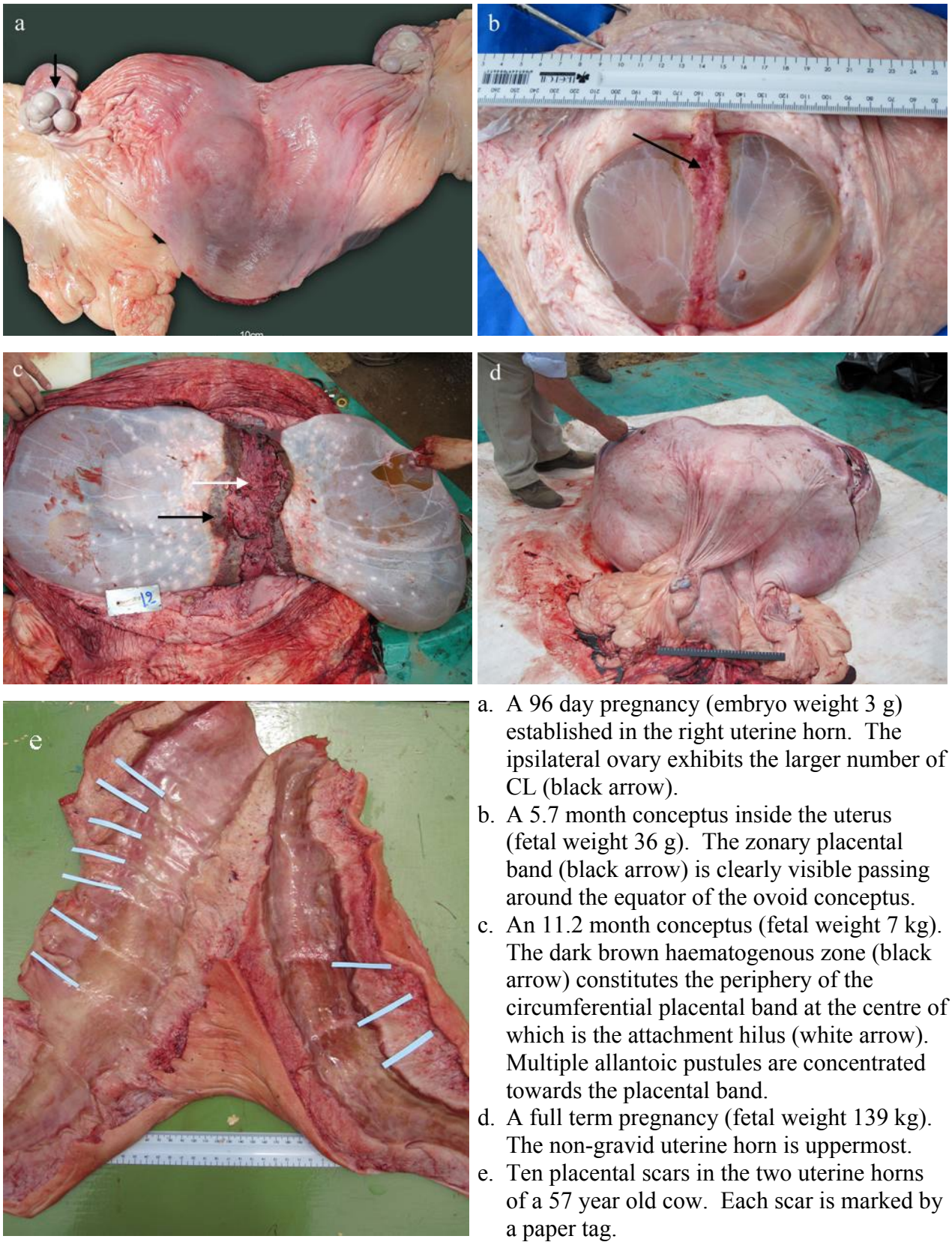
1.4.4. Fetal size

Craig (1984) established a formula for ageing elephant fetuses based on their weight; $t = 106w^{1/3} + 138$, where t refers to the age in days since conception and w the mass in kilograms. This has recently been superceded in the first third of gestation by a formula based on transrectal ultrasonographic measurements of fetal size; age = 28.434 + 54.20 x $(\text{mass})^{1/6}$ (Drews *et al*. 2008; Hildebrandt *et al*. 2007). Maximum fetal weight is approximately 120 kg at 22 months of gestation and a fetus below 8 kg weight is probably in the first half of gestation (Perry 1953).

1.4.5. Placentation

Sometime during the first 10 days after fertilization the embryo enters the uterus and becomes lodged in one of the 4 lateral clefts which cause the endometrial lumen to be star-shaped in cross section (Amoroso & Perry 1964; Perry 1974). During the first 40–60 days of gestation, pregnancy can only be "guessed at" by the presence of multiple large CL on the ovaries and a barely discernible conceptus bulge of only 2–3 cm diameter external protrusion situated near the lateral flexion of the ipsilateral uterine horn (Allen 2006). Between 2 and 3 months of gestation, when the fetus weighs 1–5 g, it is possible to discern the pale ribbon-like thickening which constitutes the developing placental band over the equatorial region of the ovoid conceptus (Allen 2006). A recent paper demonstrated that the African elephant trophoblast secretes a placental lactogen (elPL) from an early stage of gestation (Yamamoto *et al*. 2011). The authors proposed that elPl may stimulate the enlargement and secretory function of the accessory CL of elephant pregnancy and also provide the mitogenic stimulus for placental differentiation and development. In the final stages of gestation, the zonary placental band is 30 cm wide and 13–15 cm deep in its centre (Figure 1.5). It is attached to the endometrium via a narrow (2–3 cm wide) fibrous pedicle or hilus of maternal tissue through which passes endometrial stroma and blood vessels which, together, constitute the maternal component of the placental band (Allen 2006).

In uteri of elephants that calved recently a pronounced scar can be observed in the endometrium passing around the circumference of the previously gravid horn indicating the site of attachment of the placental hilus of the previous conceptus. These placental scars persist throughout life and counting them *post mortem* enables an accurate assessment of the parity status of the individual Figure 1.5e).



a. A 96 day pregnancy (embryo weight 3 g) established in the right uterine horn. The ipsilateral ovary exhibits the larger number of CL (black arrow).
b. A 5.7 month conceptus inside the uterus (fetal weight 36 g). The zonary placental band (black arrow) is clearly visible passing around the equator of the ovoid conceptus.
c. An 11.2 month conceptus (fetal weight 7 kg). The dark brown haematogenous zone (black arrow) constitutes the periphery of the circumferential placental band at the centre of which is the attachment hilus (white arrow). Multiple allantoic pustules are concentrated towards the placental band.
d. A full term pregnancy (fetal weight 139 kg). The non-gravid uterine horn is uppermost.
e. Ten placental scars in the two uterine horns of a 57 year old cow. Each scar is marked by a paper tag.

Figure 1.5 The placenta of the African elephant

1.4.6. Lactation

Perry (1953) observed that lactation overlaps the subsequent pregnancy in the great majority of adult females so that lactation becomes a continuous process throughout reproductive life. Suckling two siblings of different ages simultaneously has been recorded (Douglas-Hamilton 1972). By inference, therefore, the supposed influence of lactation on the presence or absence of Graafian follicles in the ovaries (Smith & Buss 1975) is not absolute.

1.4.7. Ovarian ageing in elephants

Te Velde and Pearson (2002) observed that, in the human female, an almost unique aspect of meiosis is the requirement for individual oocytes to remain meiotically competent for up to 40 years. They further noted that "the only other mammals with a remotely comparable length of female fertility are whales and elephants for both of which there is no information available as to the cause of the eventual cessation of fertility". It appears that elephant oocytes, formed during fetal life as in women, experience meiotic arrest at the dictyate stage so that genetic information is retained within the germinal vesicle until ovulation (Kidson *et al*. 1995). The effects of ageing on elephant oocytes and their chromosomes may be equivalent to that in humans at comparable ages, yet wild African elephants commonly continue to reproduce into their 6th decade and some into their 7th (Hanks 1972; Laws *et al*. 1970; Moss & Lee 2011; Perry 1953; Sherry 1975; Williamson 1976). Human fertility drops off slowly from a peak at around 25 years of age and may decline more sharply from around 37 years, failing completely at menopause around 50 years due to a reduction in the number and quality of oocytes in the ovaries (Faddy 2000; Gosden 1987). Counting the numbers of oocytes that constitute the follicle reserve in the elephants ovary at various times in life would therefore offer an interesting comparison to the existing human studies.

1.4.8. Elephant age classification

The ageing of elephants according to molar progression is described fully Chapter 2. Sykes (1971) provided a useful classification of elephants into various age groups based on ovarian morphology, as shown in Table 1.9. Laws (1969) described the attainment of sexual maturity and these two approaches were used initially in the present study.

Table 1.9
Age classifications of African elephants based on Sykes (1971) and Laws (1969)

Age group	Age (years)	Additional description of ovarian development
Calves	0–5	
Sexually immature juveniles	5–10	Large follicles may be present but no CL or CN.
Pubertal	8–15	No CL or CN but at least one follicle ≥ 5mm in diameter
Sexually mature sub-adults	10–15	At least one CL or a CN is present in the ovaries
Prime adults	15–35	
Senior adults	35–50	
Old	≥50	

1.5. Follicle development in mammals

1.5.1. Origin of the ovarian reserve

1.5.1.1 Primoridal germ cells

The pluripotent cells destined to, among others, give rise to the primordial germ cells, are first recognised in the proximal epiblast, from where they migrate to an extra-embryonic region posterior to the primitive streak, in the extra-embryonic mesoderm, near the site of origin of the exocoelomic cavity. There, some of these cells, now situated at the base of the developing allantois, become committed as primordial germ cells (PGCs). Once the endoderm starts invaginating to form the hindgut these committed PGCs are carried along with the endoderm along the length of the gut. Due to the nature of the invagination of the endoderm the PGCs are initially situated in the ventral aspect of the hind gut, from where they migrate dorsally, around the gut and further to the coelomic angle, and from there laterally to the genital ridge (McLaren 2003). The embryonic gonad develops from the germinal ridge of intermediate mesodermal origin and lies medial to the mesonephros (Langman 1981). Following the arrival of the germ cells into the indifferent gonad, primitive sex cords of the coelomic epithelium grow into the underlying mesenchyme,

invading both the presumptive cortical and medullary regions. Differentiation into ovary or testis depends on the embryonic genotype of the gonadal tissue (Adams & McLaren 2002). Only PGCs that reach the gonadal ridges survive. Those that remain outside in neighbouring tissues and organs undergo apoptosis at various stages of development (McLaren 2001; Zamboni & Upadhyay 1983), perhaps as a defence against their ability to become neoplastic under the influence of certain growth factors (de Felici *et al*. 2005). The chronology of events during gonadal differentiation in different species is shown in Table 1.10.

Table 1.10
Chronology of events during the differentiation of the mammalian gonad in various species (Days post conception)

Species	Cattle	Sheep	Pig	Horse	Dog	Cat	Mouse
Germ cells in the gonadal ridge	<35[a]	30–32	20	21	28		
Gonadal sex differentiation	26	40	52	60	28	28	9
Development of oogonia	57	43	28				
Onset of meiosis	70–82	55–60	40–70	73–90	At birth	40–50	10
First primordial follicles	90	66	64		3 pp	11 days after birth	
Mitosis of oogonia ends	160	82	100		15–17 days after birth	8 days after birth	
	228[b]						
First primary follicles	140	95					
First secondary follicles	210	103			60 days after birth		
First tertiary follicles	230	150		At birth	6 months after birth		
Gestation	280	150	115	336	62	63	20

The table contains combined data from (Adams *et al*. 2008; Latshaw 1987; Russe & Sinowatz 1991; van den Hurk & Zhao 2005).

1.5.1.2 Mitotic division

Primordial germ cells are so named until they reach the gonadal ridge (Byskov & Nielsen 2010). Once there and while they are dividing mitotically in the gonadal ridge, they are called oogonia, or germ cells if the embryo is of indeterminate gender. They become termed oocytes once they enter the process of meiosis. On arrival at the gonad, the final few rounds of mitosis result in the formation of groups of oogonia (2n2c), often called germline cysts or nests and connected by cytoplasmic processes (Pepling 2006). This mitotic proliferation has been observed up to 7.5 months of gestation in domestic cows, the number of oocytes per fetus reaching approximately 2 million (van den Hurk & Zhao 2005). In mice, oogonia proceed through approximately 4 mitotic cycles before entering meiosis (Gosden 1995).

Mitotic multiplication is key to the establishment of the future oocyte population as it is the balance between mitotic production and the apoptotic elimination of oogonia which determines the number of oocytes available for reproductive life in the mammalian female (Aitken *et al*. 2011). The signal for oogonia to stop mitotic division and start meiotic differentiation is unclear but it is thought to be associated with the presence of retinoic acid originating from the mesonephros (Bowles & Koopman 2007; Childs *et al*. 2011).

1.5.1.3 Meiotic division

In cattle oogonia start entering meiosis by 70 days after conception and, following a period of DNA synthesis around 75–80 days (Adams *et al*. 2008), the germ cells become arrested in the diplotene stage of the prophase of the first meiotic division at approximately 140 to170 days (Adams *et al*. 2008; Baker & Hunter 1978) when they are termed primary oocytes (2n4c). In oocytes that do not become atretic, meiosis only resumes following the onset of puberty and immediately prior to ovulation, giving rise to a secondary oocyte with a chromosome and chromatin configuration of 1n2c (Fair 2003). It has been suggested that in animals with longer gestation periods (ie. >40 days) there is a delay in the onset of meiosis and the associated gonadal differentiation which may enable blastemal (pre-granulosa) cells to proliferate, therefore allowing more germ cells to be incorporated into future follicles (Latshaw 1987). The duration of the period during which oogonia enter meiosis is also suggested to be extended with longer gestation

periods; 52–82 days in the ewe, 40–100 days in the sow, 70–170 days in the cow, 70–140 days in the female macaque and 82–160 days in woman. In the hamster, rabbit, ferret and dog the entry into meiosis is post natal (Deanesly 1977). The duration of a meiotic prophase from onset to the time of arrest is estimated to be around one week in the mouse, rat and hamster and 12–15 days in the rabbit, dog, pig monkey and human (Deanesly 1977). Such data is not presently available for the elephant.

1.5.1.4 Formation of the ovarian reserve

Coelomic epithelial cells of mesonephric origin give rise to both the primitive sex cords and the ovarian surface epithelium (Sawyer *et al*. 2002). The primitive sex cords are termed cortical cords peripherally and medullary cords internally and from 7 weeks of gestation in the human, the cortical cords develop and the medullary cords regress (Langman 1981). The genital ducts are also of mesonephric origin and remnants of the mesonephric duct in the female form the rudimentary rete ovarii (Sweeney 1998). Contact between the primary oocytes resident in the gonadal ridge and the pre-granulosa cells derived from either the ovarian surface epithelial cells (Sawyer *et al*. 2002) or the epithelial cells of the rete ovarii (Zamboni 1982), stimulate the pre-granulosa cells to form a basal lamina along their contact with the mesenchymal cells of the immature ovary. In this way the ovigerous cords are formed, consisting of oocytes and their associated pre-granulosa cells and reaching from the outer presumptive cortex to the inner presumptive medulla. The pre-granulosa cells develop cytoplasmic processes which attach to the plasma membrane of the oocyte, and the laying down of a basal *lamina,* to surround these now-termed granulosa cells (Pepling 2012) with the oocyte they enclose, marks the establishment of a primordial follicle, with its oocyte arrested in the germinal vesicle stage (Pepling 2006). This sequence of changes has been confirmed in women (Faddy & Gosden 1995), sheep (Lundy *et al*. 1999), rats (Oktay 1995) (Meredith *et al*. 2000), cows (Wandji *et al*. 1996) (Van Wezel & Rodgers 1996), baboon (Wandji *et al*. 1997), the yak (Cui & Yu 1999), and the monkey (Nichols *et al*. 2005). Oocytes that do not become associated with pre-granulosa cells degenerate (Picton 2001). This occurs in around 90% of the germ cells in the bovine fetal ovary thereby suggesting that the ovarian environment controls the progress through meiosis (Oktem & Urman 2010).

During oogenesis in all species, peak numbers of germ cells are observed around the time of the transition from mitosis to meiosis (Reynaud & Driancourt 2000). At this stage the

mean germ cell store of a cow is 2.1 x 10^6 (Erickson 1966). From their peak at embryonic day 13 (E13) in the mouse, E80 in cattle and E110 in woman, the number of observed germ cells decreases sharply, with two main periods of high germ cell loss; i) the pachytene (recombination) stage of meiosis in the oocyte and ii) the time of formation of primordial follicles (Aitken *et al*. 2011; Reynaud & Driancourt 2000). As a consequence of these losses the number of germ cells enclosed in primordial follicles at birth in the cow is less than 5% of the peak number. Apoptotic death at this time has been associated with anti-apoptotic factors (the BCL2 family of proteins, fibroblast-like growth factor [FGF], leucocyte inhibitory factor [LIF] and KIT/KIT-ligand) and pro-apoptotic factors (Bax protein, transforming growth factor-beta [TGF-ß] and FAS ligand), but the full mechanism has still to be defined (Aitken *et al*. 2011). The reason for this great deletion of the majority of newly formed oocytes so early in life needs to be elucidated. Speculation as to the means of depletion has included, i) death due to defect — involving removal of poor quality oocytes (Pepling & Spradling 2001); ii) death by self sacrifice of individual oocytes to allow pre-granulosa cells to penetrate between remaining oocytes (Pepling & Spradling 2001); iii) provision of nurse cells whereby the pre-granulosa cells of dying oocytes are adopted by near neighbours and, iv) death by neglect where oocytes may not receive the required local stimuli or somatic cell support (Tilly 2001). The highest germ cell survival rate in domestic mammals is found in the pig where approximately half the maximum number of germ cells survive until birth (McGeady *et al*. 2006).

The histological observation of the ovaries of the elephant embryo or fetus during its 22 month gestation may reveal an expanded period of the morphological and physiological development of these organs. Also of special interest in the elephant ovary is the development of antral follicles up to a diameter of 5 mm and large numbers of interstitial cells which, together, result in significant enlargement of the elephant fetal ovary during the second half of pregnancy (Allen *et al*. 2005).

Table 1.11 gives the number of follicles present in the ovarian reserve of various species throughout life.

Table 1.11
Numbers of primordial follicles in the ovary of mammals at varying ages (or number of oocytes during gestation)

Age or stage of gestation	Mouse[a]	Rat[b]	Domestic dog[c]	Domestic cat[d]	Sheep[e]	Horse[f]	Cow[g]	Human[h]
Peak number	250 x 10^3[i]	50–75 x 10^3			9 x 10^6 (75 d)		2.7 x 10^6 (110 d)	7 x 10^6 (5 mo)
Early gestation							16 x 10^3 (50 d)	
Mid gestation					200 x 10^3		107 x 10^3 (170 d)	
Late gestation					82 x 10^3		68 x 10^3 (240 d)	
Birth	7 924	10–15 x 10^3 [j]	700 x 10^3		82 x 10^3[j]		135 x 10^3[j]	1.5 x 10^6
Early life	1 987 (7 d)	924 (4–5 mo)	350 x 10^3		27 018(2 y)[k]			
Puberty		5 180[d]	150 380[d]	74 520	30–50 x 10^3	35 950 (2–4 y)	120 x 10^3 [o]	300 x 10^3
Old age	254 (200 d)		500 (10 y)		10 915[k]		3 x 10^3 [l]	
Max. reproductive life[m]			9	14	16–18	25	25	42
Max. life expectancy[m]			15	20	19	25	30	77+

[a] (Kerr *et al*. 2006); [b] (Meredith *et al*. 2000); [c] (McGeady *et al*. 2006); [d] (Gosden & Telfer 1987); [e] (Gondos 1978); [f] (Driancourt *et al*. 1982); [g] (McGeady *et al*. 2006); [h] (Faddy *et al*. 1992); [i] (Tam & Snow 1981); [j] (van den Hurk & Zhao 2005); [k] (Driancourt *et al*. 1985); [l] (Spicer & Echternkamp 1986); [m] (Cohen 2004)

1.5.1.5 Comparative and gross microscopic description of bovine ovaries

The ovary of the domestic cow and that of the elephant are broadly similar in morphology. Bovine ovaries from mature cows are approximately 3 x 2 x 1.5 cm in sizeand have 5 identifiable cortical zones (Van Wezel & Rodgers 1996) against which the cortical region of the elephant ovary may be compared:

Zone 1; The surface epithelium sitting on a basement membrane. This is a single layer of cells, either cuboidal or elongated, covering the surface of the ovary. Because this superficial epithelium does not revert to mesothelium the ovary is not covered with peritoneum in the adult and ovulation may occur at any point on the ovarian surface (Latshaw 1987). *Post-mortem* studies have revealed that the elephant has a similar surface epithelium to that of the cow (Perry 1953).

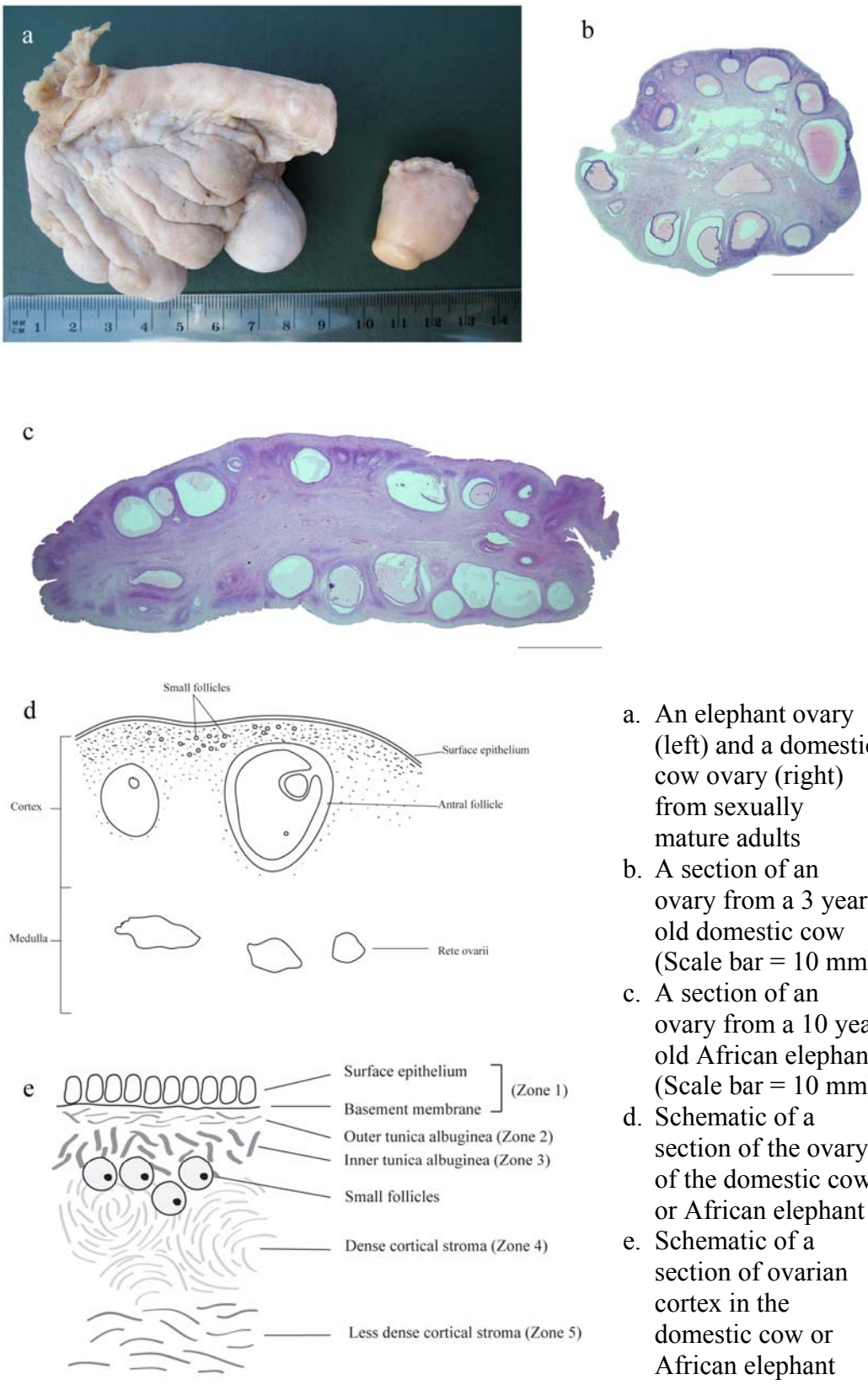
Zone 2; The outer region of the *tunica albuginea*. This is connective tissue composed of spindle-shaped fibrocytes and collagen fibrils generally lying parallel to the epithelium.

Zone 3; The inner region of the *tunica albuginea*. This contains collagen fibrils and also more rounded fibrocytes that are either randomly orientated or are arranged at right angles to the ovarian surface epithelium. The widths of Zone 2 and 3 are extremely variable and Zone 3 is sometimes non-existent. Zones 1–3 have very little blood supply.

Zone 4; This contains large quantities of collagen fibrils, similar to those seen in Zones 2 and 3, but with a much greater density of cells arranged in whorls. Large numbers of primordial and primary follicles are located in the outer region of Zone 4 and at the interface between Zones 3 and 4. Some secondary follicles and small antral follicles are also located in Zone 4 but are often deeper in the ovary than the primordial follicles.

Zone 5; The stromal cells here are less tightly packed and they exhibit a less dramatic whorl arrangement. Large antral follicles may be found here.

The boundary between the cortex and medulla of the ovary is poorly defined. The medulla consists of connective tissue through which a large number of contorted blood and lymph vessels protrude, bunching to form the hilus at the point where they exit the ovary to enter the mesovarial ligament. Preliminary studies indicate that a similar zonal layout to the bovine is present in the elephant ovary (Stansfield 2006).



a. An elephant ovary (left) and a domestic cow ovary (right) from sexually mature adults
b. A section of an ovary from a 3 year old domestic cow (Scale bar = 10 mm)
c. A section of an ovary from a 10 year old African elephant (Scale bar = 10 mm)
d. Schematic of a section of the ovary of the domestic cow or African elephant
e. Schematic of a section of ovarian cortex in the domestic cow or African elephant

Figure 1.6 Similar morphology of the ovarian cortex in the domestic cow and the African elephant

1.5.2. The ovarian reserve and follicle activation

1.5.2.1 Established dogma on the ovarian reserve

It is generally accepted that, in mammals, the resting pool of true primordial follicles containing all the oocytes required during reproductive life (Picton 2001) is formed during fetal life and the follicles are present in the ovary at birth (Gosden 2004; Zuckerman 1951). In cattle, sheep and pigs, follicles are reported to be randomly distributed in the cortex while in dogs and cats they occur in clusters (McGeady *et al*. 2006). Distribution studies need to be carried out to determine the spread of the small follicles within the elephant ovary.

Around 50 years ago the dogma evolved that the process of oogenesis in animals follows a uniform pattern, of which there are two main variants (Bukovsky *et al*. 2005):

i. Oogenesis appears to continue, either continually or cyclically, throughout reproductive life in most teleosts, all amphibians, most reptiles and, conceivably, a few mammals (Bukovsky *et al*. 2005). In lemurs, for example, oogonia persist into adult life (Gosden 1995).

ii. Oogenesis takes place only in fetal- or neonatal gonads, and oogonia neither persist nor divide mitotically during sexual maturity as in cyclostomes, elasmobranch's, a few teleosts, some reptiles, all birds, monotremes and, with a few possible exceptions, all eutherian mammals (Franchi *et al*. 1962).

More recently, however, this concept has been challenged by the immunocytochemical localization of so-called germ line stem cells (GSC) in the epithelial layer covering the outer surface of the ovaries (OSE) of adult mice (Johnson *et al*. 2004) and women (Tilly & Telfer 2009; White *et al*. 2012), and even the birth of live offspring from irradiated mice following transplantation of bone-marrow derived mesenchymal stem cells (MSC) into their oocyte-depleted ovaries (Zou *et al*. 2009). The argument still rages as to whether any post-natal oogenesis which may or may not occur in mice and women could originate from GSC within the ovarian cortex or must derive from migrating MSC (de Felici 2010).

1.5.2.2 Follicle morphology

Descriptions of the morphology of small ovarian pre-antral follicles or small follicles (SF) have been made in several species (Fortune 1994; Gougeon 1996; Hirshfield 1991; Peters 1969; see Table 1.12). From these and other studies it is generally accepted that follicles can be classified in a similar way throughout the range of mammalian species by examination of the shape, size, number and arrangement of the granulosa cells surrounding the oocyte (Griffin *et al*. 2006). For this study the description given by Oktay (1995) and Pepling (2012) was used (Table 1.13) and expanded as described in Section 1.5.4.

Table 1.12
Small follicle classification in mammals

Authors	Species	Description
Block (1951)	Human	Primordial: "Egg cell" surrounded by a single layer of flattened follicular cells. Situated in the outer zone of the ovary. Diameter seldom exceeds 40 µm Growing: Have a distinct cubical epithelium around the egg cell. Diameter >50 µm.
Lintern-Moore *et al*. (1974); Gougeon and Chainy (1987)	Human	B: Flattened granulosa cells B/C: Mixture of flat and cuboidal granulosa cells C: One layer of cuboidal granulosa cells
Richardson *et al*. (1987)	Human	Primordial: An oocyte surrounded by a single layer of flattened granulosa cells Primary: An oocyte surrounded by one or more layers of cuboidal granulosa cells. Follicles transitional between primordial/primary were classified as primordial.
Oktay (1995)	Rat	Primordial: Partly or completely encapsulated by squamous granulosa cells Primary: Single layer of cuboidal granulosa cells
Faddy and Gosden (1995)	Human	Stage1: Small unilaminar follicles one layer of squamous granulosa cells Stage 2: Unilaminar follicles, 1 layer of cuboidal granulosa cells or some squamous cells Stage 3: Growing follicle with enlarged oocyte and 2 or more layers of granulosa cells.
Wandji *et al*. (1996)	Bovine	Primordial: One layer of flattened somatic cells around the oocyte Primary: A single layer of cuboidal granulosa cells around the oocyte

Continued

Table 1.12 (Continued)

Van Wezel & Rodgers (1996)	Bovine	GC: Development cannot be relied upon to indicate the development of the follicle.
Wandji *et al*. (1997)	Baboon	Primordial: One layer of flattened somatic cells around the oocyte Early Primary: Unilaminar with both flattened and cuboidal cells Primary: One layer of completely cuboidal cells.
Lundy *et al*. (1999)	Sheep	Type 1: One layer of flattened granulosa cells. Type 1a – mixed flat and cuboidal granulosa cells Type 2: One to two layers of cuboidal granulosa cells
Cui and Yu (1999)	Yak	Primordial: One layer of granulosa cells Primary: Two layers of granulosa cells
Wright *et al*. (1999)	Human	Primordial: One layer of a majority of flattened pre-granulosa cells Primary: One layer of a majority of expanded cuboidal granulosa cells.
Meredith *et al*. (2000)	Rat	B: Flattened granulosa cells. B/C: Mixture of flat and cuboidal granulosa cells, C: One layer of cuboidal granulosa cells
Barber (2001)	Elephant	Primordial oocytes are surrounded by a single layer of squamous granulosa cells. Follicles become primary as the single layer of squamous cells differentiates into cuboidal cells.
Nichols *et al*. (2005)	Rhesus monkey	Primordial: Flattened granulosa cells Primary: One or several layers of granulosa cells, cuboidal or low columnar

Table 1.13
Small follicle classification (SF)

Type of follicle	Description
True Primordial (TPM)	Oocyte partially or completely encapsulated by squamous granulosa cells
Early Primary (EP)	At least one of the granulosa cells is observed to be columnar (enlarged)[a,]
True Primary (TP)	All the cells in the single layer of granulosa cells show enlargement.

[a] Expansion of the granulosa cells does not occur simultaneously and follicles may be viewed in histological sections which comprise one or more cuboidal cells but still also have some flattened granulosa cells. These follicles have been called "Early Primary" to indicate that some growth has started but they are not yet "True Primary" follicles.

1.5.2.3 Follicle activation

The molecular mechanisms that lead to follicle growth have not yet been established (McLaughlin & McIver 2009) but progress is being made (Reddy *et al*. 2010); they are thought not to depend on gonadotrophic hormones (Oktem & Urman 2010). It is generally understood that the first sign of activation that is visible with the light microscope is a change in the shape of the granulosa cells, from flattened to cuboidal, possibly under stimulation by factors from the oocyte (Rodgers & Irving-Rodgers 2010). Once the oocyte is surrounded by cuboidal granulosa cells it is termed a true primary follicle (TP). Hence, the classification of early follicle growth is rather subjective. The size of the follicles in the resting pool, and the size of primary follicles (TP) are given in Table 1.14 and Table 1.15.

The established theory that only true primordial follicles (TPM) make up the follicle reserve in mammals is based on the premise that follicles arrest with only squamous granulosa cells surrounding the oocyte and any subsequent deviation from this state indicates a commitment to growth which results in the follicles exiting from the reserve (Oktay 1995). However, significantly large numbers of early primary (EP) follicles have been observed in mammalian ovaries, including the elephant (Stansfield 2006), suggesting they may also form part of the ovarian reserve. This assumption is being

reassessed and is summarized well by Gougeon (2010). In healthy young women it is speculated that only one third of all small follicles are at the TPM stage (de Bruin *et al*. 2002), most are at the EP stage and a sizeable proportion have already reached the TP stage. In bovine ovaries it is also suggested that more than 80% of follicles are at the EP or TP stages (Van Wezel & Rodgers 1996). Likewise, Types "B and C" follicles (early primary) comprise 72–80% of the total population of small follicles in mature rats (Meredith *et al*. 2000). In contrast, many papers describe species in which the majority of follicles in the ovary at any one time are primordial (Braw-Tal & Yossefi 1997; Fortune *et al*. 2000; Hirshfield 1989; Oktay 1995; Picton 2001; Wandji *et al*. 1996).

Table 1.14
Diameter of follicles in the resting pool in various mammalian species

	Size of structures (µm)			
	Follicle	Oocyte	Nucleus	Granulosa cells in largest cross section
Elephant TPM[a]	36.1 ± 4.5	30.0	14.5 ± 2.7	5.2 ± 1.3
Elephant EP[a]	38.1 ± 3.9	30.0	13.9 ± 2.5	9.8 ± 1.3
Yak (mature)[b]	40–45		12.5–16	
Bovine				
Bos taurus[c]	<40	29.7		<10 flattened
Bos taurus[d]	35	25		
Bos taurus[e]	30–50	20–35		
Bos taurus[f]	28.9–38	26.8–31.2		
Bos indicus[g]	36.0	28.1		7.3
Human[h]	44	36	19.4 ± 8.5	
Pig[h]	34	29.1		
Hamster[h]	26	23.4		
Mouse[h]	17	12.6		

[a] (Stansfield 2006), [b] (Cui & Yu 1999), [c] (Fortune 2003), [d] (Fair 2003), [e] (van den Hurk *et al*. 1997), [f] (Van Wezel & Rodgers 1996), [g] (Kacinskis *et al*. 2005), [h] (Griffin *et al*. 2006), [i] (Forabosco *et al*. 1991)

Table 1.15
Diameters of various parts of primary follicles (μm)

	Whole follicle	Oocyte	Nucleus	Granulosa cells
Elephant[a]	46.9 ± 4.7	35.0	15.0	15.8 ± 2.8
Bovine				
Bos taurus[c]	40–80	31.1		
Bos taurus[d]	45	30		
Bos taurus[e]	40–60	30–40		
Bos taurus[f]				
Bos indicus[g]	48.5	31.7		14.6
Human [h]	70	42.1		
Pig [h]	64.9	37.4		
Hamster	56	32.2		
Mouse	52.1	28.8		

[a] (Stansfield 2006), [b] (Cui & Yu 1999), [c] (Fortune 2003), [d] (Fair 2003), [e] (van den Hurk *et al*. 1997), [f] (Van Wezel & Rodgers 1996), [g] (Kacinskis *et al*. 2005), [h] (Griffin *et al*. 2006), [i] (Forabosco *et al*. 1991)

1.5.2.4 Oocyte growth

Evidence suggests that oocyte growth follows, rather than precedes, the changes in the granulosa cells (compare the data in Table 1.15 with those in Table 1.16). The analysis of the correlation between oocyte diameter and number of granulosa cells during early growth of the follicle in the domestic cow reveals two distinct and consecutive stages (Braw-Tal 2002). Bovine primordial follicles contain an average of 6 granulosa cells in their largest cross section (Braw-Tal & Yossefi 1997) and a change in the shape of granulosa cells, from flattened to cuboidal, starts when there are at least 7 cells in the largest cross-section or a complete layer of around 20 cuboidal granulosa cells surround the oocyte (Hulshof *et al*. 1992). This is not accompanied by an increase in oocyte diameter. Very similar kinetics of granulosa cell transformation to those in the domestic cow have been reported for humans (Braw-Tal 2002).

Furthermore, there is a positive and linear correlation between the number of granulosa cells and oocyte diameter. The first significant change in bovine oocyte diameter is observed in follicles with at least 40 granulosa cells in the largest cross section (4th generation of granulosa cells according to Braw-Tal, 2002). In mice and rats the oocyte starts to grow earlier when there are about 10, mostly cuboidal, granulosa cells in the largest cross-section (2nd generation; Lintern-Moore & Moore 1979). In the human oocyte growth commences at the third generation (Braw-Tal 2002). Comparing the mouse, hamster, pig and human, Griffin *et al*. (2006) found that although the development of the follicle and oocyte was similar across species, the change in the ratio of follicle to oocyte diameter and the proliferation of granulosa cells were all shown to be species specific.

1.5.2.5 Zona pellucida

From immunocontraceptive studies it has been established that the cellular origin and temporal formation of the zona pellucida is species-specific (Fayrer-Hosken 2008). Upon follicle activation a zona pellucida is secreted between the growing oocyte and the granulosa cells and it becomes evident during the primary follicle stage in the rat (Odor 1960), mouse (Wassarman & Josefowicz 1978), rhesus monkey (Zamboni 1974), pig (Sinowatz *et al*. 1995) and cow (Russe 1983). Similarly, contraceptive studies have shown that the zona pellucida also appears during the primary follicle stage in the elephant (Barber & Fayrer-Hosken 2000; Fayrer-Hosken *et al*. 1999).

1.5.3. Continued follicle growth to pre-ovulatory size

At around the secondary follicle stage FSH receptors develop on the granulosa cells and stimulation results in granulosa cell proliferation (Gougeon 1996). At the pre-antral multilayer stage a layer of spindle-shaped stromal cells is recruited outside the basal lamina of the follicle and this forms the *theca interna* which develops LH receptors (Johnson & Everitt 2004). This is usually the maximum degree of development found in the pre-pubertal ovary (Genuth 2004). Gonadotrophin stimulation at puberty results in the production of steroids from both the granulosa cells (oestrogens) and theca cells (androgens). Without such stimulation the follicle will undergo atresia (Gougeon 1996). Granulosa cells also start to exude antral fluid into vesicles which unite to form the antrum of the follicle (Peters & McNatty 1980). With further enlargement cells from the

stroma and an in-growth of blood vessels constitute the theca externa which allows faster access of blood-borne gonadotrophins to the inner parts of the follicle (Young 2010). The antral follicle now stands at approximately 2–5 mm diameter in the human (Genuth 2004).

During the final stage of follicle growth a whole cohort of follicles become "selected" and they increase their diameter by further enlargement of the antrum (follicle diameter 7–10 mm). Eventually, in monotocous species such as the elephant, one follicle becomes dominant and it continues to grow to the point of ovulation (20–25 mm) while the remainder of the original cohort undergo atresia (Findlay *et al*. 2001). During follicle development in cattle the oocyte is extremely active metabolically such that, from primordial to preovulatory size, it is estimated to increase 204-fold in volume (Braw-Tal & Rodgers 1996).

1.5.4. Follicle classification

Considering the widely agreed terminology used in the literature quoted above, the following classifications will be used to define the follicle populations described in the present study:

1.5.4.1 True Primordial follicles

Primordial follicles comprise an oocyte, arrested in the dictyate stage of meiosis I, surrounded by a low number of squamous granulosa cells and enclosed by a basement membrane. A useful definition of a squamous cell is that its height is negligible in comparison to its other dimensions so that it appears as a slender rod which is slightly thickened in the vicinity of its oval nucleus (Bloom & Fawcett 1962). Thus, using this description, a cell that is thicker than the diameter of its nucleus can be described as expanding.

1.5.4.2 Early Primary follicles

According to the definition used for bovine follicles (Van Wezel & Rodgers 1996), some of the granulosa cells of the EP follicle have expanded and become cuboidal, often making the follicle more prolate in shape with a mean of maximum-to-minimum diameter ratio of 1.33 μm. The prolate shape is attributable to a clustering of granulosa cells at the

two opposite poles on the long axis of the follicle (Braw-Tal & Rodgers 1996). For a comparison of EP follicles among various species see Table 1.15.

1.5.4.3 True Primary follicles

TP follicles consist of primary oocytes surrounded by a single layer of cuboidal granulosa cells which begin to express markers of cell proliferation such as proliferating cell nuclear antigen (Wandji *et al*. 1996). The size of TP follicles in a range of species is given in Table 1.15.

1.5.4.4 Secondary follicles

Progression to the secondary follicle stage is characterized by the appearance of a second layer of granulosa cells (Driancourt 1991) which, in the cow, increases to 6 layers when the follicle reaches a diameter of around 150 µm. During the early growth of the secondary follicle, connective tissue fibres become arranged parallel and peripheral to the basement membrane — which encloses the granulosa layer — to form the thecal layer. By the end of the secondary stage, the thecal layer becomes dominated by large, epithelioid, hormone secreting cells and a capillary network (van den Hurk *et al*. 1997). At the same time cortical granules become visible within the oocyte cytoplasm (Fair *et al*. 1997). In *Bos taurus* the diameter of the oocyte is approximately 45–60 µm (van den Hurk *et al*. 1997) and in *Bos indicus* at a follicle diameter of 88.4 µm the oocyte has a diameter of 43.8 µm with approximately 62 granulosa cells in the largest cross-section (Kacinskis *et al*. 2005). Cui and Yu (1999) reported similar measurements in the Yak with follicle diameters of 80–120 µm, oocyte diameters of 40–45 µm and the nucleus of the oocyte at 15 µm.

1.5.4.5 Small preantral follicles

Production of a range of molecules by the granulosa cells creates an osmotic gradient which draws fluid into the follicle across the basement membrane from the thecal vasculature. This creates further expansion of the follicle and considerable remodelling of the granulosa cell junctions is also required to create the follicular antrum filled with follicular fluid (Rodgers & Irving-Rodgers 2010). In cattle, the diameter of small preantral follicles ranges from 81 to 130 µm while the mean oocyte diameter measures

approximately 49.5 µm (Fortune 2003). See Table 1.16 for a comparison of preantral follicles among various species.

Table 1.16
Diameter of various parts of preantral follicles (µm)

	Follicle	Oocyte	Nucleus
Yak [a]	145–180	60–80	25
Bovine			
Bos taurus[b]	131–250	68.6	
Bos taurus[c]	300	65	
Bos taurus[d]	150	60	
Human[e]		80	
Pig[e]	300	90	

[a] (Cui & Yu 1999), [b] (Fortune 2003), [c] (Fair 2003), [d] (van den Hurk *et al*. 1997), [e] (Griffin *et al*. 2006).

1.5.4.6 Small antral follicles

The extent to which the antrum of the follicle expands differs between dominant and subordinate follicles, and among species, with larger animals tending to have larger follicles consisting of 95% follicular fluid (Rodgers & Irving-Rodgers 2010). In cattle, follicles at the mid antral stage have diameters of 3 mm and oocytes 110 µm (Fair 2003). In Yak, follicles of 300–500 µm in diameter would have one complete antrum with oocytes of 60–90 µm and oocyte nuclei of 35–40 µm diameter (Cui & Yu 1999). Oocyte diameter in small antral follicles of 2.5–5.5 mm diameter in elephants has been measured at 155–165 µm (Stansfield 2006).

1.5.4.7 Graafian follicles

A Graafian follicle (or tertiary follicle) is the term used to denote one of a group of growing selectable follicles which may or may not ovulate (Gosden & Lee 2010) whereas an ovulatory follicle is one that has acquired LH receptors on its mural granulosa cells and is destined to ovulate in response to increased levels of pituitary LH (Gougeon 2010).

In cattle the ovulatory follicle reaches a diameter of 15–20 mm and the oocyte 120–130 µm (Fair 2003). Similarly, in women oocytes reach approximately 120 µm in diameter at the time of ovulation (Gosden 2005). Ultrasound studies on Asian and African elephants report the diameter of the preovulatory follicle as 20–21 mm (Hermes 2000; Lueders *et al*. 2010). This is somewhat bigger than the maximum diameter of 13.7 ± 0.7 mm noted in the elephant during the first LH surge early in the interluteal period (Hermes 2000).

1.5.4.8 Atretic follicles

In all mammalian species studied to date the store of primordial follicles decreases between birth and puberty (Gougeon *et al*. 1994). Although follicles start to grow during this period they do not reach ovulatory size due to inadequate gonadotrophic stimulation (Gosden 1995). In sheep, comparison of the populations of primordial follicles in the ovaries of 2- and 8-year-old ewes has demonstrated that 8 primordial follicles disappear from the reserve pool every day between these ages (Driancourt *et al*. 1985). This contrasts with the estimated rate of initiation of growth from the follicle pool (2–3 follicles per day) and suggests that 5–6 follicles die every day within the primordial pool; in cattle this figure is estimated to be 6 per day (van den Hurk & Zhao 2005). Since granulosa cell apoptosis is never visualized in such follicles, it may be assumed that death of the oocyte is the cause of this high rate of loss of primordial follicles. Oocyte death appears to be very fast and pycnotic bodies, the most common marker used to identify a dying cell, are seldom visualized in oocytes (Reynaud & Driancourt 2000).

1.5.5. Small follicles in elephant ovaries

During a pilot study to the present project, Stansfield (2006) noted that non-pregnant adult elephants appeared to be endowed with a relatively low number of small primordial follicles, such that TPM follicles showing no expansion of the granulosa cells numbered less than 0.6% of the total SF population. The majority of SF were present as EP (56–80% of all SF) and TP (19–44% of SF). It was concluded that elephants do not store their oocytes in a true “resting follicle pool” and that constituent follicles have variously developed granulosa cells which range from flat to cuboidal in outline. This point clearly required further investigation and was therefore addressed in the present study reported in this thesis.

1.6. Background to the over-abundance of elephants in Zimbabwe

1.6.1. Elephant numbers in Zimbabwe

Historically, man has reduced elephant numbers. This was recorded as long ago as AD 77 when Pliny noted that the elephant herds in North Africa had been wiped out (Meredith 2001). Furthermore, along with slaves and gold, ivory has, at times, been Africa's major export (Carruthers 2008). Natural curbs on elephant population growth include drought, which kills off particularly the young and the old, lion predation on calves and diseases such as anthrax, which was once wide-spread in Zimbabwe (Hugh-Jones & de Vos 2002). Provision of artificial water sources, a reduction in lion populations due to hunting and human encroachment and effective control of anthrax has meant that elephants nowadays have few natural population control mechanisms during years of average or above average rainfall.

Between 1980 and 2004 Zimbabwe's elephant population increased at a rate of approximately 5% per annum (Table 1.17). If this growth rate has continued since 2004 the present population could number around 120 000. The area of land available for elephants to roam over is 63 784 km^2. This equates to around 2 elephant per km^2 which is well in excess of the widely recommended figure of 0.5–0.6 elephants per km^2 (Foggin 2003). In an effort to control this booming elephant population cropping by shooting was instituted in 1960 and by 1988, some 44 000 elephants, mostly whole breeding families, had been culled. Following cessation of culling activities, between 1989 and 1995, around 2500 more elephants were shot during problem animal control (PAC) operations.

Table 1.17
Estimated elephant numbers in Zimbabwe

Year	Population size	Number culled	Author
1871–1875	"Vast numbers"		Bryden (1889)
1900	4 000		Cumming & Jones (2005)
1930	10 000		Child (2004)
1940	17 000		Child (2004)
1950	25 000		Child (2004)
1980	47 000		Nduku (1991)
1960–1991		46 775	Child (2004)
1991	70 000		Nduku (1991)
2001	88 100		Child (2004)
2004	100 000		Cumming & Jones (2005)
2010	120 000?		

Ecologically, elephants are a keystone species. At densities below 0.25 elephants per km^2 their impact on habitat is limited. Between 0.25 and 1.0 elephants per km^2 structural change occurs in woodland with the result that there are fewer mature trees (Figure 1.7). When the density becomes higher than one elephant per km^2 woodland may be converted into treeless grassland or shrubby coppice states with resulting impacts on the habitats of other herbivores (Kerley & Landman 2006).

The human population of Zimbabwe is also expanding and people are moving into areas previously used by elephants many of which are contiguous with Reserves or National Parks. The value attributed to an elephant depends on the socio-economic background of the observer (Rosa & Joubert 2009). To a tourist on safari an elephant may be a majestic, sentient creature which has as much right to occupy space on planet Earth as members of the human race. To a rural African an elephant may variously be viewed as a source of meat, a threat to his crops and livestock or even to his own life. Hence, many rural Africans regard the elephant as open access property which provides little or no benefit and is a direct competitor. This leads to human-elephant conflicts (HEC) which include crop raiding, granary destruction and loss of both human and elephant lives (Nelson *et al*.

2003). Between 2002 and 2006 more than 5000 cases of HEC were recorded in Zimbabwe which resulted in the killing of 774 elephants during subsequent PAC operations (Campfire 2007). With the populations of humans and elephants both continuing to increase the incidence of HEC can be expected to rise in future years.

In Tsavo National Park, Kenya, some 10 000 elephants—approximately 25% of the population—died of thirst and starvation during a severe drought in 1970-1971 (Meredith 2001). In 2009 a similar situation occurred in Amboseli National Park in Kenya when around 300 elephant died of starvation (Lee 2010) along with 83% of the wildebeest (18 538 reduced to 3098) and 71% of the zebra (15 328 reduced to 4432) populations (Burnham & Gronewold 2010). With the increasing shortage of available land in southern Africa, elephants are being forced into smaller range areas and they can no longer move around the continent, or even the country, as they once did (Whyte 2002). As a first step towards their active management in Zimbabwe boundaries were placed around elephants. If a diverse ecosystem is to be maintained within those defined locations, it is beholden upon man to control expansions of the elephant population that threaten the viability and diversity of that ecosystem. The tragedies in Tsavo and Amboseli show that taking a *laissez faire* attitude will inevitably lead to elephants dominating and changing the ecosystem (even if this may be temporary) to the detriment of other species of both fauna and flora and, eventually, to the elephants themselves. If biodiversity is a priority in Game Parks steps need to be taken to limit the negative impact of elephants on their environment, preferably with the support of valid and current research efforts (Marais & Hadaway 2006).

Figure 1.7 Damage to trees caused by elephants in Savé Valley Conservancy and Mana Pools National Park, Zimbabwe

1.6.2. Attempts to counteract over-abundance

Lotter *et al*. (2008) recorded that "In an ideal world all humans would treat elephants in ways that appropriately acknowledge and respect their moral standing. Elephants would have enough land available to freely live their lives as they see fit and to migrate to other areas when they deem it appropriate. In such a world humans would have no reason to intervene in their lives. However, we do not live in such a world. As a result of the violent history between our species, the exponential growth in human population, and the resultant loss of elephant habitat, conservationists must explore various management options to create the best life possible for elephants within current constraints".

The following management "tools" may be used, alone or in conjunction, depending upon the prevailing aims and views.

1.6.2.1 Passive management options

Laissez-faire: A "nature knows best" approach may be appropriate where ranges are large enough; ie larger than 100 000 km^2 (Cumming & Jones 2005) and elephant populations are self regulating. It is favoured by many on moral and ethical grounds as it is considered non-lethal in terms of human-induced mortality. However, when elephant populations do exceed their available resources the stress and suffering they undergo during drought-induced starvation and mass die-offs may be considerable, inhumane and ethically reprehensible.

Metapopulations: Source-sink migration involves the natural movement of animals to areas of lower population density which may be encouraged by closing down artificial water holes in the high density areas. Difficulties occur when such wildlife areas are surrounded by rural communities. This disrupts the free movement of the elephant through the inhabited land and stimulates HEC leading to PAC. Such dispersal of elephants would inevitably occur slowly as they need to move under their own momentum (Slotow 2005) and considerable community co-operation would be needed to implement such movements.

Fencing elephants out: This may be applicable on a small basis but elephant proof fencing is expensive and not affordable by small rural communities. Biological

alternatives such as bee hives (King *et al*. 2007) and the use of chilli peppers in various forms (Osborn & Parker 2003) may be suitable in some circumstances.

1.6.2.2 Active management options

Translocation: The movement of elephants in whole family groups is considered the least stressful and most successful means of relocation. Past movements of only juvenile animals have resulted in them showing aberrant behaviour towards other species in their new environment (Slotow 2001). Translocated elephants are heavily stressed during the capture and transport processes and they need considerable time to adjust to their new environment (Viljoen 2008). If the animals that remain in the source area also suffer stress, as has been occasionally noted, this will happen whether the missing elephants have been translocated or culled (Scholes 2007). Additionally, the distance moved needs to be substantial to prevent the translocated elephants returning to their origin, as was experienced in Kruger National Park in South Africa (Hofmeyr 2004). With a relocation distance of 500 km, for example, the cost of translocating one elephant was estimated to be US$ 8700 while the cost of translocating 100 elephant reduced to approximately US$ 1000 per elephant (Grobler *et al*. 2008). However, the biggest problem with translocation nowadays is that there is no longer any requirement for additional elephants in any game parks or conservancies within Zimbabwe, South Africa, Botswana or Namibia.

Culling: Culling results in an immediate reduction of the numbers of a population and it reduces the pressure on the ecosystem of the National Park or conservancy. Stress for the culled animals is very brief and that experienced by any remaining associated family groups is no more than when translocation has taken place (Whyte 2001; Whyte 2002). Furthermore, the sale of elephant products from culls (meat, hides, tusks etc) results in the sustainable usage of an elephant population by ranchers and the local rural populations (Lotter *et al*. 2008). The latter need to participate in decisions and benefit from the presence of the elephants on their land, otherwise they view the elephant as direct competition and desire their removal. Culling may however result in a stimulation of the rate of reproduction due to its impact of reducing elephant population density (Laws et al. 1975). More recent studies cite such increases as a response to the complex interaction of resource availability (Caughley 1983; Chamaille-James et al. 2008) rather than specifically due to population density.

Elephants that raid crops do tend to originate from bachelor herds which potentially carry a higher value as trophy animals (Cumming & Jones 2005); therefore problem animal control has serious economic and socio-economic implications which affect both peoples' livelihoods and the financial viability of wildlife management within conservancies, especially with respect to the trophy hunting of elephants. Implementation of a culling programme is an emotive and difficult decision to take but, given the present economic circumstances within Zimbabwe and the aims of maintaining biodiversity within National Parks and conservancies, there is currently no alternative (Foggin 2003).

Reproductive Strategies: Unless the reproductive rate of elephants can be reduced, other tools such as culling or translocation will need to be practiced continually. To this end reproductive strategies are sought to slow down population growth rates. The most promising to date is immunocontraception (Bertschinger *et al*. 2008). Elephant oocytes, along with those of at least 100 other mammalian species, including horses (Kirkpatrick *et al*. 1990), possums (Cowan *et al*. 2003) and white-tailed deer (Kirkpatrick *et al*. 1997), share zona pellucida epitopes with the pig. For this reason, porcine zona pellucida (pZP) proteins have been tested and show the most promise so far as the basis of a vaccine to attempt to limit elephant reproduction by blocking fertilization (Fayrer-Hosken *et al*. 1999). The largest elephant herds in which contraception has so far been successful numbers only 80 head (Delsink *et al*. 2007) and the logistics and cost of the operation, coupled with the need for repeated booster injections, currently prevent its use on larger populations. For example, in Zimbabwe at least 75% of all the breeding females would need to be rendered infertile for at least 10 years to produce a significant drop in the overall population (Foggin 2003). Additionally, contraception does not result in an immediate reduction in population size and a considerable time delay would occur before female elephants that were given the contraceptive treatment would start to die from old age and thereby commence the desired population reduction (Stout & Colenbrander 2004). Following immunocontraception calves would continue to be born for a period of 2 years after the first vaccination since immunisation does not interfere with an existing pregnancy (Delsink *et al*. 2007). Some side effects from the use of this anti-pZP vaccine have been observed (Grootenhuis 2003; Paterson *et al*. 1992; Paterson *et al*. 1996). Although there is concern about the reversibility of the method after long-term usage, which is particularly pertinent for elephants, given their extended reproductive life (Perdok *et al*. 2007), two calves were born from elephant cows in a South African reserve

in 2011 following 7 years of treatment (Delsink 2011). A further major problem with its use in wild elephants is effective and practical delivery of the vaccine (Bertschinger *et al*. 2008).

Long term effects of the pZP vaccine on fertility and also the social effects of the absence of calves on the herd need to be considered. In addition, repeated 16-week oestrous cycles is not a natural reproductive state for the African elephant which would normally only experience one period of overt oestrus and mating every 4 years. The resulting unnatural state of mature bulls being more frequently in contact with female family groups could well disturb social structures and be particularly hazardous for young calves (Poole 1989). These are being addressed by suggestions of rotational treatment (Druce *et al*. 2011) Equally important, perhaps, the ethics of spending such a large amount of money to prevent a species from breeding when the funds could be better used to improve the welfare of the local protein-deficient human population must be borne in mind (Cumming & Jones 2005).

The possibility of the treatment of far fewer elephants by relying on the mate guarding behaviour of dominant bulls has stimulated research into male contraception using gonadotrophin releasing hormone vaccine or dominant bull sterilization (Bokhout et al. 2005).

Alternative reproductive strategies: An alternative strategy for potentially restricting the reproductive capacity of female elephants is to target the earliest stages of oocyte growth in the ovary; namely, the oocytes contained within small follicles (Stansfield *et al*. 2011b). Tens of thousands of primordial follicles populate the ovaries of mammals from which the fertile oocytes will be ovulated during each reproductive cycle. Thus, the number of primordial follicles in her ovaries ultimately determines any female animal's reproductive longevity. The means by which primordial follicles are held in their resting state or are stimulated to grow are not fully elucidated (Gleicher *et al*. 2011). While in this resting stage the follicles are endocrinologically inactive which, together with their residence within a relatively avascular part of the cortex (Oktem & Urman 2010), suggests a local follicular factor, either within the oocyte or in the granulosa cells keeping them inactive. Possible candidates could be oocyte genes, either for stimulating (eg. c-kit, TGF-β, AMH, GDF-9, Figα) or inhibiting (eg. retinoblastoma protein, Wilms tumor suppressor gene, follistatin (Picton 2001). In order to target contraceptive development

towards the early stages of follicle and oocyte development in the elephant it is first necessary to fully understand the concepts of reproductive ageing and ovarian reserves in this species.

The use of hunting and culling programmes as management tools to attempt to limit the growth of elephant populations within Conservancies and Safari Areas in Zimbabwe gave the unique opportunity to undertake the research on folliculogenesis in the elephants ovary which is described in this thesis.

1.7. Objectives of the study

There is a lack of information on the status and dynamics of small ovarian follicles in the African elephant. Such knowledge may be of use in studies targeting the ovary when attempting to achieve contraception in wild elephants and to determine whether the depletion of the ovarian reserve may be a cause of the observed poor fertility in captive zoo elephant. Such knowledge would also permit comparison of their ovarian reserve with that of women who display similar reproductive longevity.

The aim of this study was to define the ovarian follicular reserve of wild African elephants in terms of its type of SF, its establishment and distribution throughout the ovaries, and the change in numbers of SF in the embryo and fetus as well as throughout prepubertal and adult life.

More specifically, studies with the following aims, as reported in subsequent chapters of this thesis, were performed:-

i. To determine the nature of the SF constituting the follicle reserve in the African elephant

ii. To determine distribution of these SF in the ovaries.

iii. To study the development of the germinal ridge in the embryo along with the establishment of the SF reserve in early fetal life.

iv. To monitor the further growth and development of the elephant ovary from mid fetal life to prepuberty, along with its reserve of SF.

v. To quantify the SF reserve between puberty and reproductive senescence, and to determine the timing of such senescence.

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.

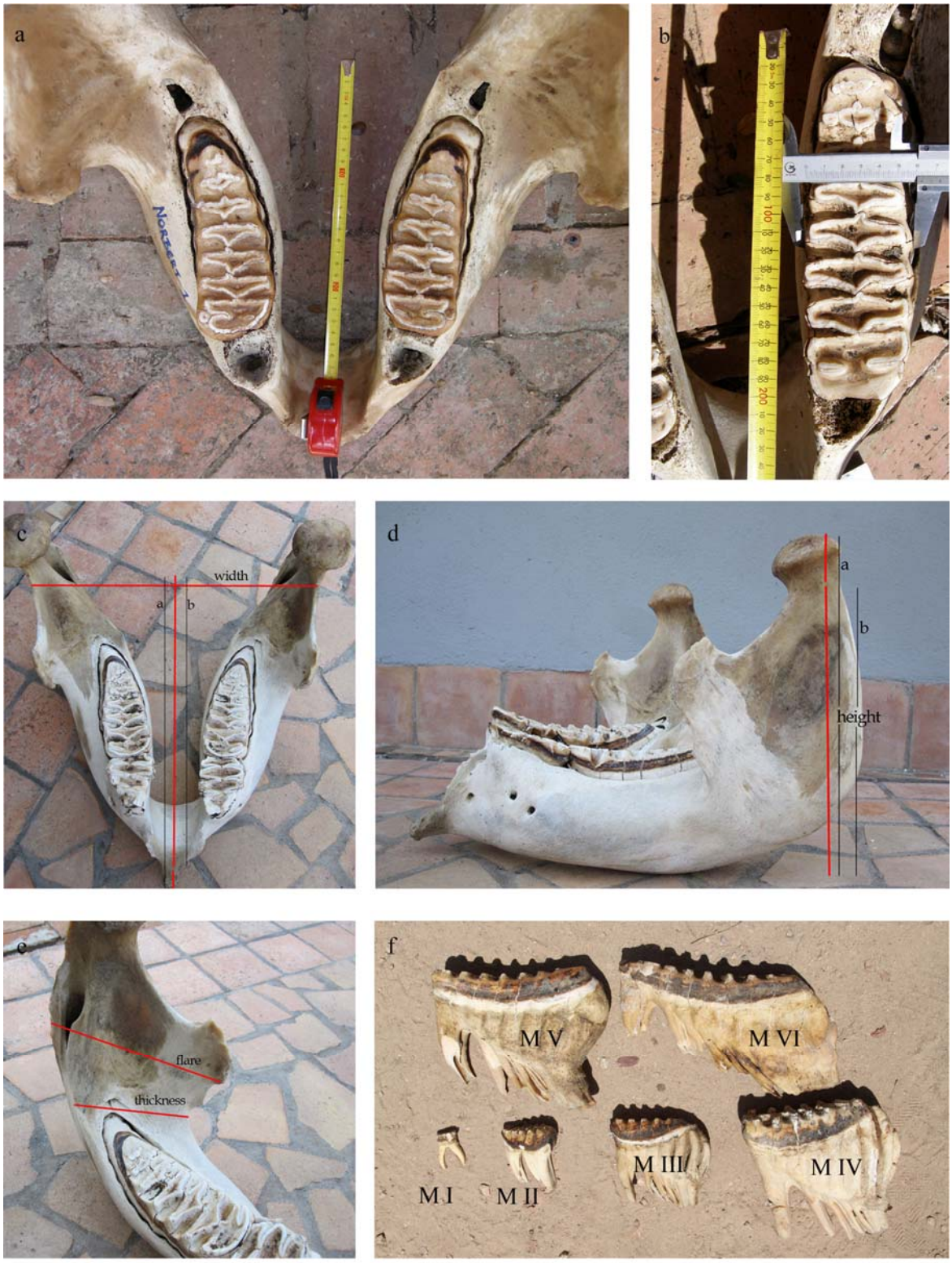


Figure 2.1 The ageing of elephants is achieved by examining the progression of molar teeth through the mandible

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.

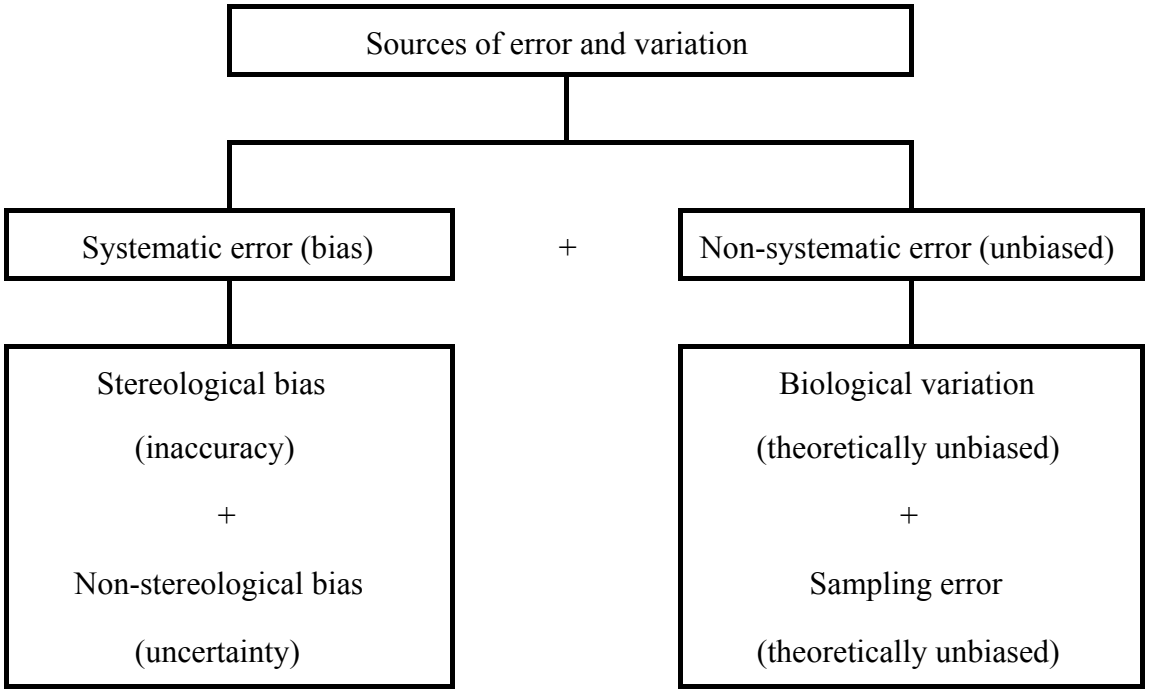


Figure 2.2 Sources of variation and error, according to (Mounton 2002)

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).

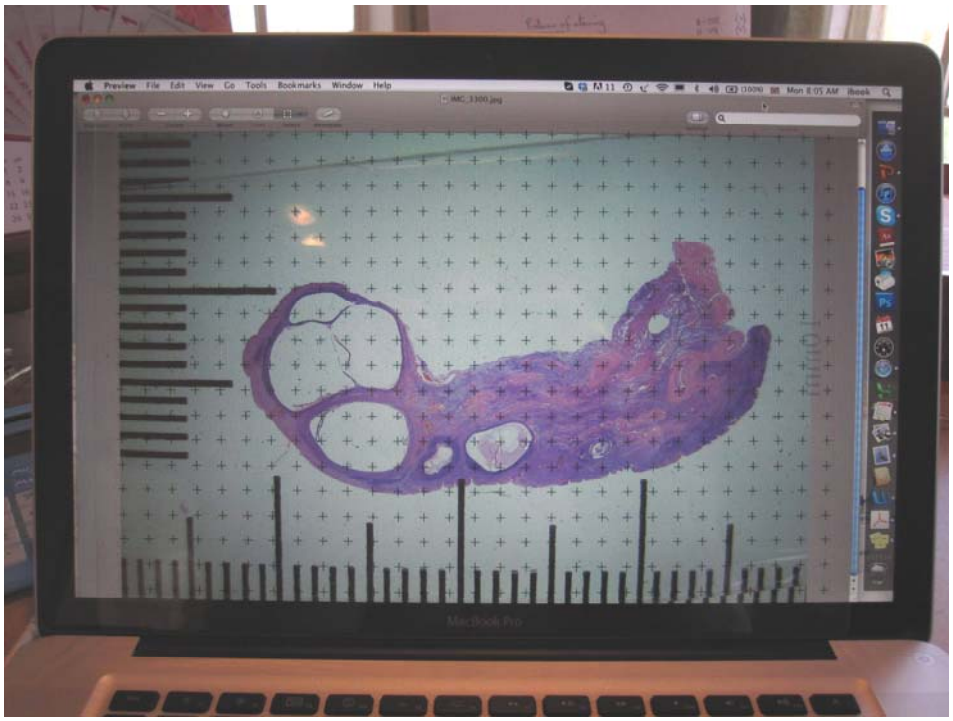


Figure 2.3 An acetate point grid randomly placed over a photograph of an ovarian section to determine the reference volume by means of Cavalieri's principle

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:

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

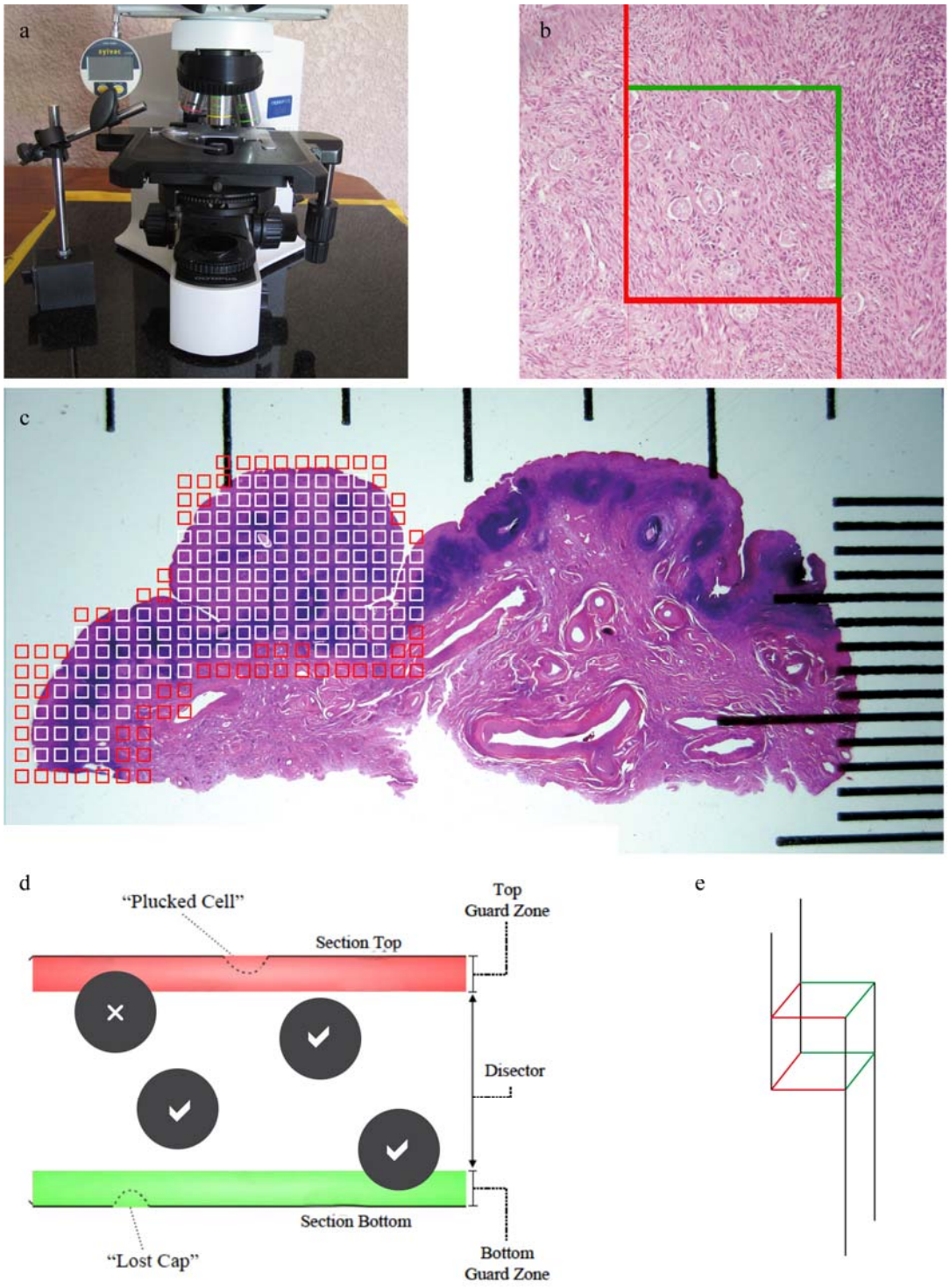


Figure 2.4 Stereology

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.

Chapter 3. Follicle morphology in the ovary of the African elephant and the composition of the ovarian reserve

The content of this chapter has been published in a different format as an article by FJ Stansfield, HM Picton and JO Nöthling and under the title "Early primary- rather than primordial follicles constitute the main follicular reserve in the African elephant (*Loxodonta africana*)" in Animal Reproduction Science 2011; 123:112–118.

3.1. Introduction

For management of the African elephant (*Loxodonta africana*), methods for contraception as well as assisted breeding are required (Brown *et al*. 2004a; Delsink 2006). The duration of the female elephant's reproductive life is about 50 years (Freeman *et al*., 2009; Perry 1953). Thus, together with humans and whales, elephants have an exceptionally long reproductive lifespan which requires that individual oocytes remain meiotically competent for more than 40 years (te Velde & Pearson 2002). This exposes them to prolonged arrested development and, potentially, to structural damage (Faddy & Gosden 1995).

Maternal ageing in women is accompanied by a reduction in oocyte numbers (Faddy *et al*. 1992), increased oocyte aneuploidy (te Velde & Pearson 2002) and a progressive loss of ovarian follicles (Picton *et al*. 1998). Once oocytes have been lost from the ovarian reserve there is little or no renewal and, according to present dogma, the ensuing infertility is irreversible (Gosden & Lee 2010). In 2004 interest was rekindled in the possibility of neo-oogenesis after birth following experiments in mice (Johnson 2005; Johnson *et al*. 2004) and the debate continues (Begum *et al*., 2008; Bukovsky *et al*. 2009; Tilly & Johnson 2007). Knowledge of the ovarian follicle reserve in the wild African elephant may form the basis for investigations on the cause of early reproductive failure (Brown *et al*. 2004a), reproductive senescence (Freeman *et al*. 2009) and the effect of contraceptives on the follicle pool (Perdok *et al*., Delsink *et al*. 2007; 2007; Stout & Colenbrander 2004).

Primordial follicles have been described as the most abundant follicle in the ovary and are commonly referred to as the building blocks of the ovarian reserve (Picton 2001), supplying the female with oocytes throughout reproductive life. Studies in rats

(Hirshfield 1989; Oktay 1995), cattle (Braw-Tal & Yossefi 1997; Wandji *et al*. 1996), humans (Picton 2001) and other mammals (Fortune *et al*. 2000) have shown that the transition from flattened to cuboidal granulosa cells and the accompanying rounding-up of their nuclei signals that the follicle has left the ovarian reserve and is irreversibly committed to growth which may end in ovulation or atresia. The resting pool of follicles, however, may have granulosa cells that range from flat to cuboidal in shape (de Bruin *et al*. 2002; Faddy & Gosden 1995; Gougeon & Chainy 1987; Hirshfield 1992; Lintern-Moore *et al*., 1974; Meredith *et al*., 2000; Moss 2001; Rodgers & Irving-Rodgers 2010; Sawyer *et al*. 2002; Van Wezel & Rodgers 1996). The biological consequences of the differences in somatic cell morphology between species is unclear and very little is known about the follicle population dynamics in species with a long lifespan, such as elephants. Hence, the principal aims of the current study were twofold; i) to compare the dimensions, numbers and abundance of SF at different stages of development; ii) to determine the type of SF that constitutes the follicle reserve in sub-adult and adult wild African elephant.

It is necessary to begin with a detailed description of a normal elephant follicle at each stage of its development and compare this with the extensive literature on the structure of bovine and other mammalian follicles as described in Chapter 1. It is also important to ascertain the composition of the ovarian reserve.

3.2. Materials and methods

The ovaries of 14 African elephant cows shot by professional hunters working under annual authorization granted by The Parks and Wildlife Management Authority of Zimbabwe were used to measure and count early preantral follicles at different stages of development. Subsequently, the ovaries of a further 2 elephants sourced in the same way were used in follicle counts only. Fifteen of these 16 animals were tuskless. The ovaries were collected and handled, and the age for each animal allocated, as described in Chapter 2.

At this early stage of the study prepubertal ovaries (Figure 3.1c) were not available and therefore mature elephant ovaries from both non-pregnant and pregnant animals were used (Figure 3.1a and b).

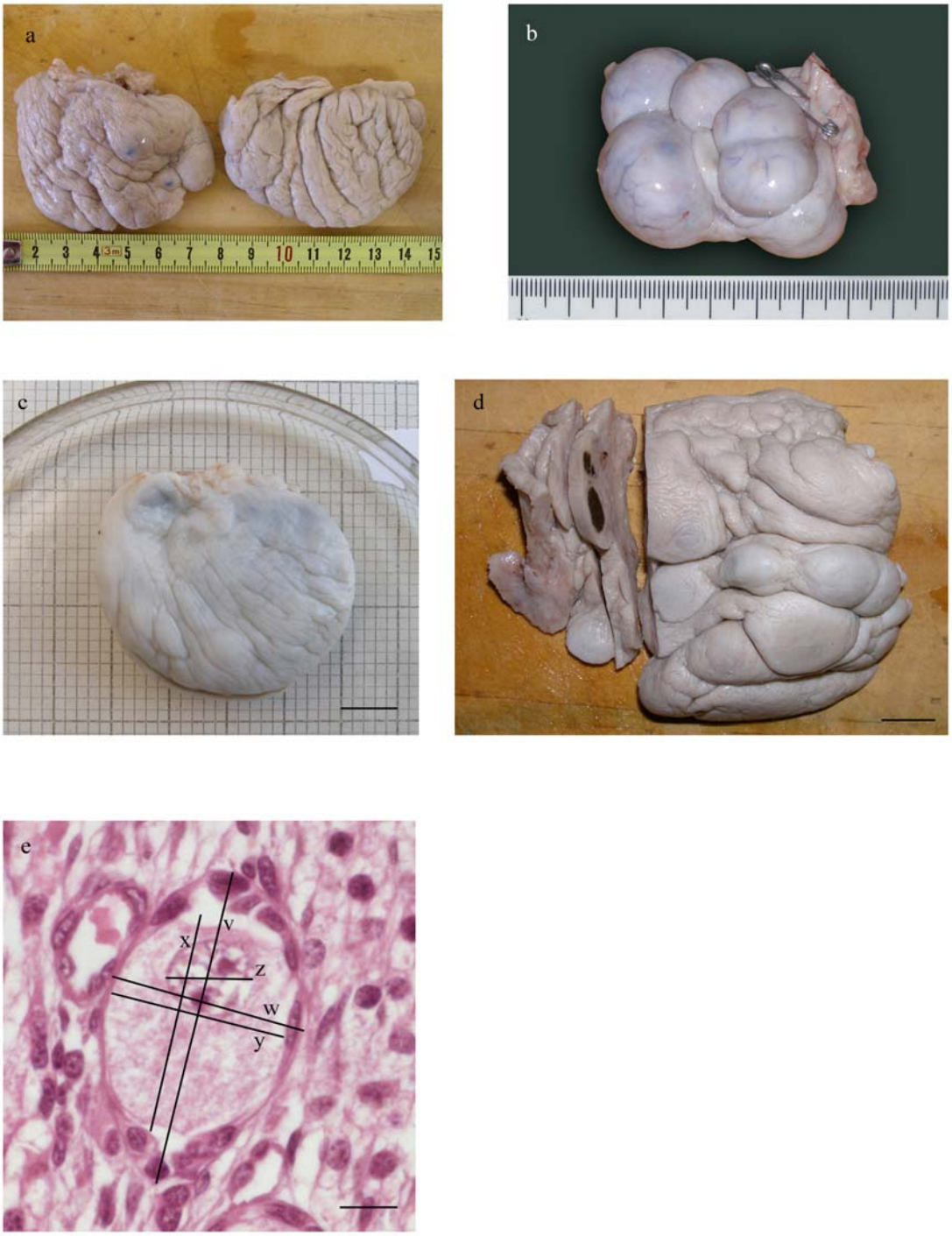


Figure 3.1 The ovaries of elephants and dimensions of measurements of small follicles

a. The 2 surfaces of the same ovary from a non-pregnant elephant.
b. An ovary of a pregnant elephant with the associated many large corpora lutea
c. A small relatively smooth surfaced ovary of a prepubertal elephant; scale bar 10 mm
d. A segment cut from an ovary, scale bar 10 mm
e. The dimensions of measurements in a follicle; the longest dimension and the longest perpendicular thereto, of a follicle (v and w) and its oocyte (x and y), whereas z) shows the nuclear diameter; scale bar 15 µm

Four "sets" of sections, equally spaced across the ovarian surface, were cut from each pair of ovaries (Figure 3.1d). Each set consisted of 100 serial 4 µm sections extending from the mesovarial margin to the free margin of the ovary and perpendicular to the *tunica albuginea*. Every 5th section was mounted on a glass slide and stained with haematoxylin and eosin (H&E; Sigma-Aldrich, South Africa) before being examined using an Olympus BX41 microscope (Wirsam Scientific, Johannesburg, RSA). The SF were classified as TPM (Figure 3.2), EP and TP as described in Table 1.13 and the numbers of follicles in each category were counted in each stained tissue section. To avoid double counting only those follicles in which the oocyte displayed a clear nucleus were counted in each section. From these counts the number of each type of SF was estimated in the total population according to Gougeon and Chainy (1987) and with a correction factor according to Abercrombie (1946) as described by Gosden and Telfer (1987).

Coincidentally with counting SF numbers, a range of measurements were made using an ocular micrometer fitted to the microscope. These included, i) follicle diameter, calculated as the average of the maximum diameter and the largest perpendicular diameter thereto (Wandji *et al*. 1997); ii) oocyte diameter, measured as above and, iii) nuclear diameter, where only one dimension was measured because all their nuclei appeared round in cross-section; (Figure 3.1e). The number of granulosa cells surrounding the cross-section of the follicle being studied was also counted. Approximately 100 SF were measured per animal and the slides from an animal on which follicle measurements were made were selected randomly. On each selected slide all the follicles of which the section through the nucleus was approximately equatorial, often denoted by the presence of the nucleoli, were measured.

A set of serial sections were also cut in which each consecutive slide was stained in order to obtain a full visual representation of sections through the depth of small follicles.

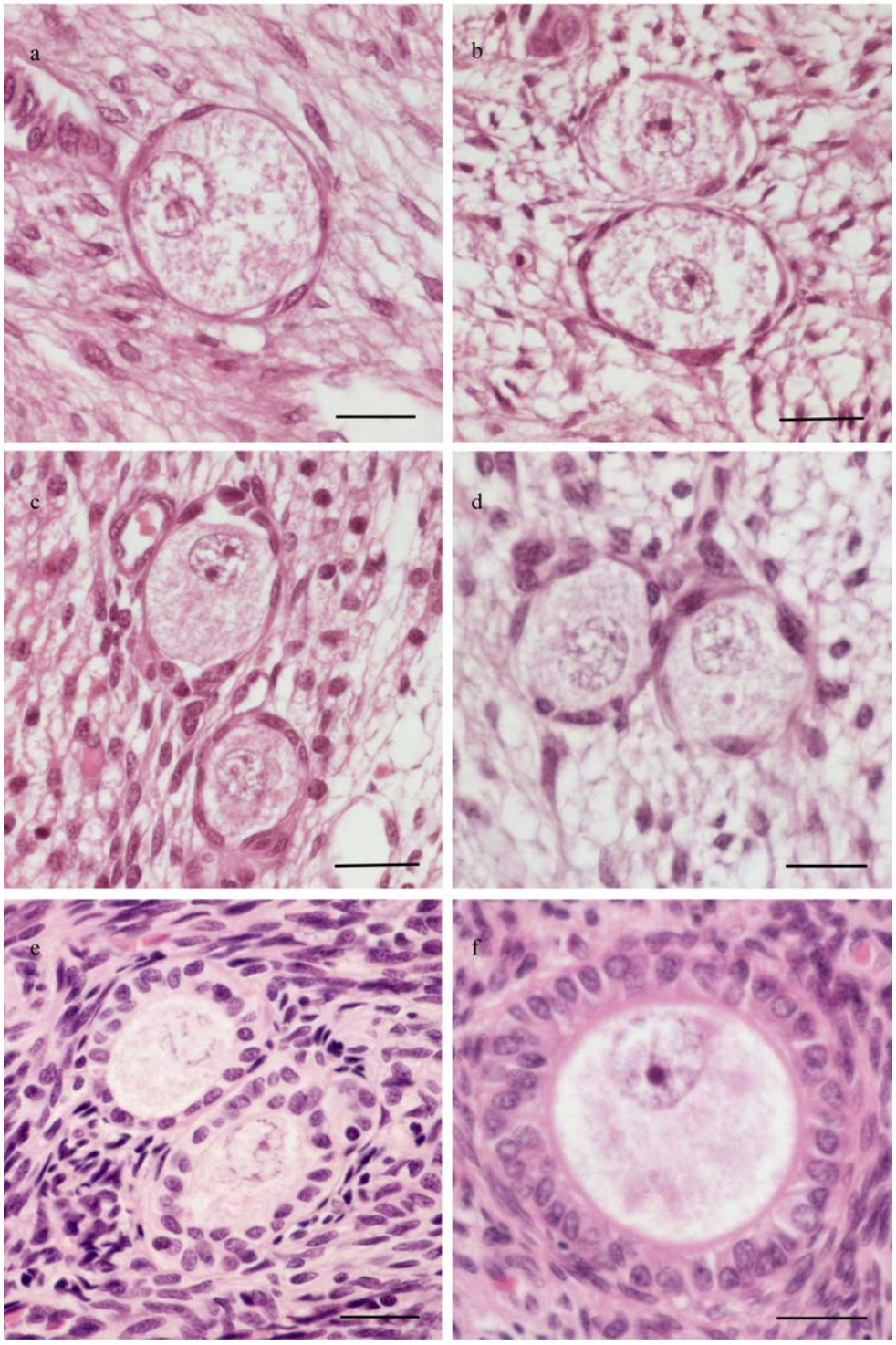


Figure 3.2 Small follicles in elephant ovaries

a. True primordial follicle; scale bar 15 µm
b. Early primary follicles; scale bar 20 µm
c. Early primary follicles; scale bar 20 µm
d. Early primary follicles; scale bar 15 µm
e. True primary follicles; scale bar = 15 µm
f. A transitional follicle developing a second layer of granulosa cells; scale bar 20 µm.

3.2.1. Statistical Analysis

Wilcoxon's signed rank test was used to compare i) the numbers of TPM and TP follicles and, (ii) the numbers of TP and EP follicles. The Wilcoxon's rank-sum test was used to compare the ages of elephant with CL in their ovaries and the numbers of SF in those elephant with CL to those without. The same statistical test was used to compare the numbers of EP follicles in the 7 elephants that were 19 years or younger with those in the 6 elephant aged 25 years or older.

Comparisons of TPM, EP and TP follicles with respect to their dimensions and the number of granulosa cells surrounding them were made using the Kruskal-Wallis test. If the medians differed, Wilcoxon's rank-sum test was used to compare TPM follicles to EP follicles and EP follicles to TP follicles. The skewness and kurtosis test was used to determine whether variables were normally distributed or not ($P < 0.05$). Variability is indicated as mean ± standard deviation for normally distributed variables and as median (25–75th percentile) for non-parametric variables. All statistical analyses were done using STATA 11 (StataCorp, Texas, USA).

3.3. Results

Ovarian morphology was recorded and ovarian follicle dynamics were quantified in 16 elephants aged 9–34 years. In all these animals the TPM population constituted less than 2% of the total number of SF counted, whereas 75.8 ± 11.8% were EP and 23.8 ± 11.8% were TP follicles. In each elephant the number of TPM was the lowest, the number of EP follicles the highest and the number of TP follicles intermediate between the two (Table 3.1). Overall, this ranking was highly significant ($P < 0.001$, Table 3.2).

Table 3.1
The number of small follicles in the ovaries of each of 16 African elephants aged 9–34 years, and with or without one or more large *corpora lutea* (CL) in their ovaries.

Age (y)	Ovaries with CL	Primordial	Early primary	True primary	Total small follicles	ID[a]
9	0	0	26 421	811	27 232	27
10	2	179	10 276	1 360	11 815	14
13	0	72	16 623	13 671	30 366	2
16	0	254	30 137	11 527	41 918	3
17	0	0	57 488	2 498	59 986	26
18	0	292	17 900	4 174	22 366	1
19	2	0	10 287	3 367	13 654	5
20	1	47	25 320	6 952	32 319	8
20	1	0	5 418	1 603	7 021	11
20	0	55	10 334	5 334	15 723	6
25	2	0	2 853	849	3 702	7
25	1	0	2 196	775	2 971	10
25	0	224	11 813	4 944	16 981	4
30	1	69	5 920	4 842	10 831	12
32	0	103	18 615	4 250	22 968	17
34	0	0	2 195	792	2 987	16

[a] Elephant identification number

Table 3.2
Numbers of small follicles (SF) in the ovaries of 16 African elephants.

Percentile	Primordial	Early primary	True primary
25th	0	5 669	1 105
Median	51[a]	11 074[c]	3 771[b]
75th	141	21 968	5 139

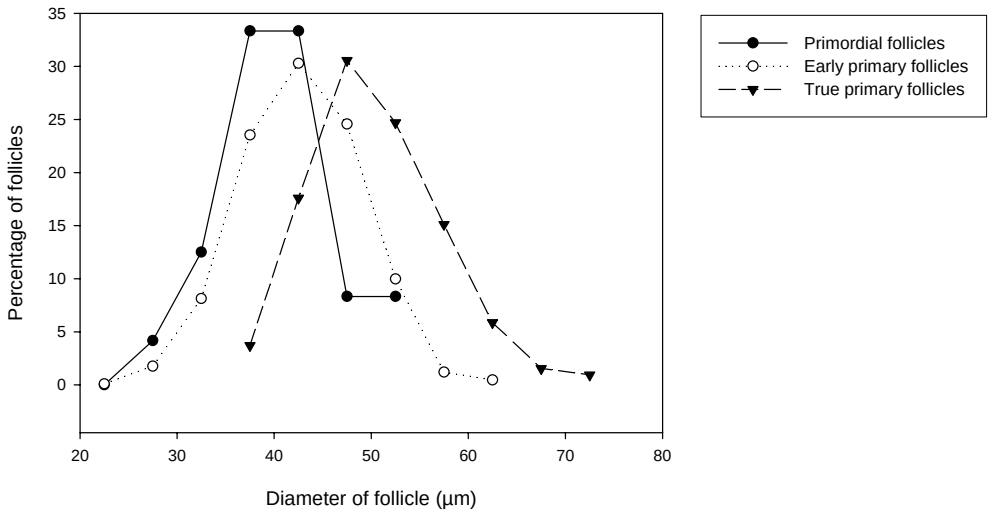
a < b < c, p < 0.001, n = 16 for each comparison

The mean age of the 16 elephants was 20.8 ± 7.3 years. The 10 elephants of 20 years or younger had an average age of 16.2 ± 4.16 years and the 6 elephants aged 25 years or older had an average age of 28.5 ± 4.04 years. The 10 younger elephants had a median population of 17 262 (10 287–26 421) EP follicles, which was significantly more than the median of 4 387 (2 196–11 813) counted in the 6 older animals (P = 0.04).

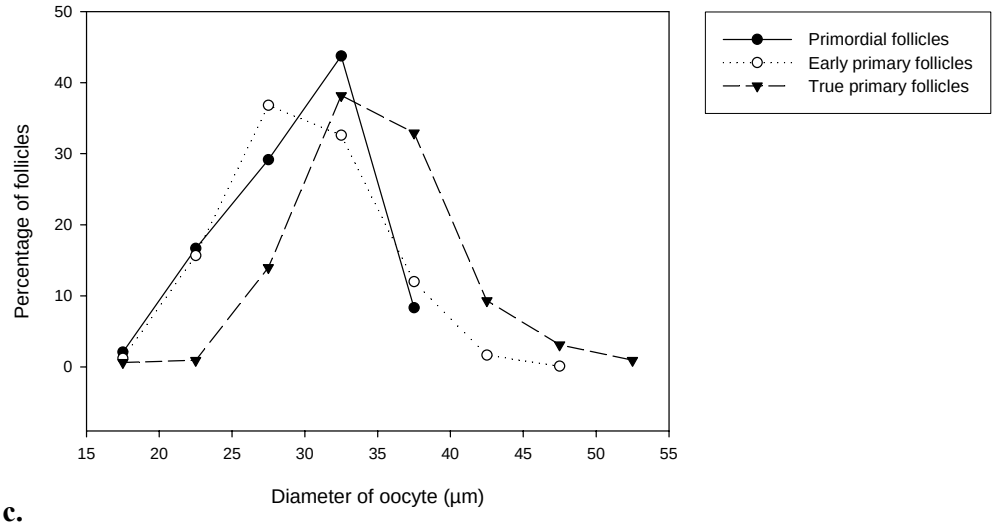
Seven elephants had one or more CL in their ovaries and 9 had none. The median age of elephants with CL was 20 (19–25) years, which was similar to the median age of 18 (16–25) years in the elephant without any CL (P = 0.56). Nevertheless, the 7 elephants with CL in their ovaries had 5 920 (853–10 287) EP follicles, which was appreciably fewer than the 17 900 (11 813–26 421) in the ovaries of the 9 elephants without CL (P = 0.04).

There was a progressive increase in follicle diameter and the number of granulosa cells surrounding the oocyte going from TPM to EP follicles and on to TP follicles (P = 0.004: Table 3.3; Figure 3.3a, b, c). However, the diameter of the oocyte, as well as that of its nucleus, only increased when EP follicles developed to become TP follicles (P <0.01). Follicle measurements also indicated a prolate shape for most follicles (Table 3.4).

a.



b.



c.

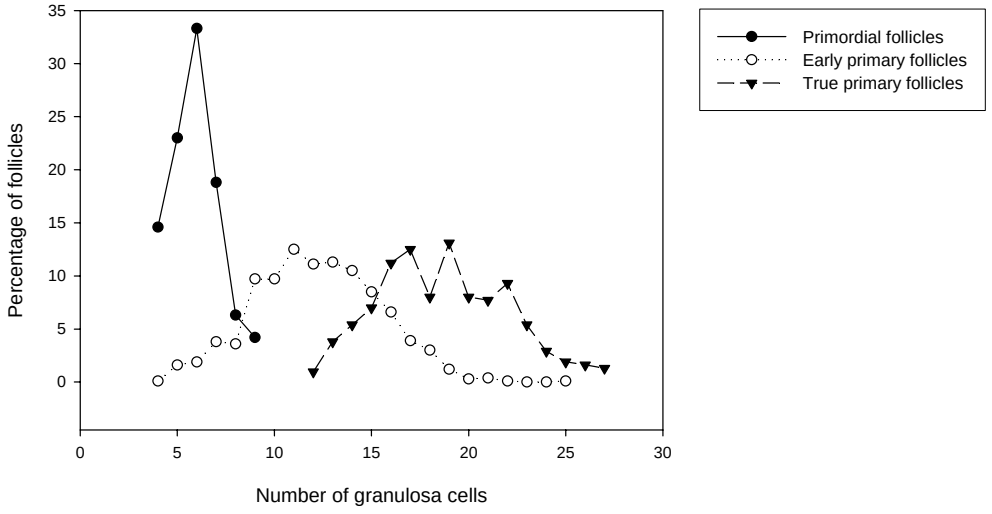


Figure 3.3 Comparison of follicle diameter, nuclear diameter and numbers of granulosa cells of small ovarian follicles in the African elephant

Table 3.3
Dimensions of the various types of small follicles (SF) in the ovaries of 14 African elephants and the numbers of granulosa cells surrounding them.

		Percentile	
Follicle type	Median	25th	75th
True primordial (n = 48)			
Follicle diameter (µm)	40.6[a]	36.3	43.8
Oocyte diameter (µm)	31.3[d]	28.1	33.1
Nucleus diameter (µm)	14.4[f]	12.5	15.6
Number of granulosa cells	6[h]	5	7
Early primary (n = 1092)			
Follicle diameter (µm)	43.8[b]	38.8	47.5
Oocyte diameter (µm)	30.0[d]	27.5	33.8
Nucleus diameter (µm)	15.0[f]	12.5	16.3
Number of granulosa cells	12[i]	10	14
True primary (n = 324)			
Follicle diameter (µm)	50.0[c]	46.3	55.0
Oocyte diameter (µm)	35.0[e]	32.5	38.9
Nucleus diameter (µm)	15.0[g]	12.5	17.5
Number of granulosa cells	19[j]	16	21

$a < b$ ($P = 0.004$), $b < c$ ($P < 0.001$), $d < e$ ($P < 0.001$), $f < g$ ($P < 0.01$), $h < i$ ($P < 0.001$), $i < j$ ($P < 0.001$)

Table 3.4
Ratio between the mean diameters of follicles and oocytes and the two largest perpendicular diameters of oocytes and follicles for each small follicle (SF) class

Follicle stage	Oocyte			Follicle		
	Ratio	D1 (µm)[a]	D2(µm)[b]	Ratio	D1(µm)	D2(µm)
Primordial	1.2:1	33.1	28.0	1.3:1	45.4	36.3
Early primary	1.3:1	34.3	26.4	1.3:1	48.4	38.3
True primary	1.3:1	40.2	31.0	1.3:1	57.1	44.9

[a] D1 represents the maximum diameter of the structure
[b] the largest diameter perpendicular to D1.

Far fewer follicles had developed to secondary follicles or later. The mean and range for these classes of follicles is given in Table 3.5. The zona pellucida was first observed at the late transitional stage (one complete ring of granulosa cells with a second ring that is not yet complete surrounding the oocyte) when the follicle was approximately 60–100 µm in diameter. Thecal cells were noticeably starting to accumulate in the late secondary stage when the oocyte measured 140–300 µm. The antral follicles measured had diameters up to 3.4 mm with an oocyte diameter of 165 µm. Preovulatory follicles measured approximately 21 mm. Signs of atresia, such as pycnotic oocytes, were not noted in the very small follicles but plenty of atretic antral follicles were observed.

Table 3.5
Dimensions of growing follicles (transitional to early antral) in the ovaries of 14 African elephants and the numbers of granulosa cells surrounding them.

Follicle type	Mean	Range
Transitional (n = 45)		
Follicle diameter (µm)	79.9	54–118
Oocyte diameter (µm)	49.2	28–90
Nucleus diameter (µm)	19.8	15–22.5
Number of granulosa cells	31.5	18–57
Secondary (n= 24)		
Follicle diameter (µm)	178.0	116–300
Oocyte diameter (µm)	82.4	40–143
Nucleus diameter (µm)	23.3	20–30
Number of granulosa cells	68.6	43–115
Transition to Antral (n=51)		
Follicle diameter (µm)	180	175–300
Oocyte diameter (µm)	100	70–143
Number of granulosa cells	100	74–115

3.4. Discussion

The current study suggests that EP, not TPM, follicles constitute the main follicular reserve in African elephants; around 75% of the SF were at the EP stage while <2% could be classified as TPM. The study also showed that reserve follicles undergo significant growth during transition from the EP to the TP stage. Further, the study suggests that young adult elephant cows aged 28.5 ± 4.0 years have fewer SF than cows aged 16 ± 4.2 years.

3.4.1. Follicle classification

Each SF consisted of an oocyte surrounded by a single layer of granulosa cells of varying thickness. The oocyte contained pale eosinophilic cytoplasm and a dark staining nucleus within which a prominent and darkly stained nucleolus was sometimes visible. A description of atretic SF was difficult to make as the precise identification of an atretic follicle of this size remains undecided (Reynaud & Driancourt 2000). These early follicles were classified depending on the expansion of the granulosa cells, a descriptive system which has been used widely throughout mammalian species (Oktay 1995; Pepling 2012); i) primordial (TPM; oocyte surrounded by a single layer of squamous granulosa cells; Figure 3.2a); ii) early primary (EP; oocyte surrounded by a single layer of granulosa cells of which most are squamous but at least one is cuboidal; Figure 3.2b, c, d); or, iii) true primary, (TP; when the oocyte is surrounded by a single layer of granulosa cells all of which are cuboidal; Figure 3.2e).

In the elephant the transitional stage of follicle development proceeds from the TP follicle stage, starting at a mean follicle diameter of 50–60 µm and with around 20–25 granulosa cells in the circumference of the largest cross section of these follicles (Figure 3.2f). It is during the late transitional stage with a follicle diameter of approximately 60–100 µm that the *zona pellucida* first becomes visible (1–3 µm thick) under the light microscope; this finding differs with that made during contraceptive studies in elephants in which Barber *et al*. (2001) reported that the *zona pellucida* appeared following granulosa cell differentiation at the primary stage of development. This difference may be explained by the fact that the transitional stage of follicle growth (ie one complete ring of granulosa cells with a second ring that is not yet complete) was not described in the paper by Barber *et al*. (2001) in which transitional follicles were combined with primary follicles. The present study therefore defines more precisely the stage at which the *zona pellucida* becomes visible on H&E stained sections viewed under a light microscope around the elephant oocyte.

Progression to the secondary follicle stage in the elephant is characterized by the appearance of a second complete layer of granulosa cells when the mean follicle diameter is around 120 µm and some 55–60 granulosa cells are present in the circumference of the largest cross section. Similar to the situation in the domestic cow (Driancourt 1991), the layers of granulosa cells in the elephant increase to 6 and the follicle reaches a mean

diameter of 150 µm in the cow and 180 µm in the elephant before an antrum begins to form. Mean oocyte diameter in secondary follicles was recorded as 82.4 µm (n=24) with around 69 granulosa cells in a cross section in the elephant compared with 45 µm (Fair 2003) to 60 µm (van den Hurk *et al*. 1997) in Bos taurus and 88.4 µm in diameter and approximately 62 granulosa cells in the largest cross-section in Bos indicus (Kacinskis *et al*. 2005). During the early growth stage of a secondary follicle in domestic cattle, connective tissue fibres are arranged parallel to the basement membrane that surrounds the peripheral layer of granulosa cells to form a thecal layer. By the end of the secondary stage the theca is dominated by large epithelioid (hormone producing) cells and a capillary network (van den Hurk *et al*. 1997). At the same time, cortical granules are formed within the oocyte cytoplasm and become visible microscopically (Fair *et al*. 1997). The thecal layer was also first observed during the late secondary stage of follicle development in the elephant in the present study.

In cattle small preantral follicles measure 81–130 µm and oocytes measure 49.5 µm (Fortune 2003). The transition from pre-antral to antral follicle takes place in the elephant at a follicle diameter (taken at the basement membrane) of approximately 300 µm and an oocyte diameter of approx 140 µm. The mid-antral stage follicle prior to final recruitment in the cow measures 3 mm and the oocyte 110 µm (Fair 2003). At a similar follicle diameter the elephant oocyte measures approximately 140 µm with an upper range of 165 µm.

In cattle the ovulatory follicle reaches a diameter of 15–20 mm and the oocyte attains 120–130 µm (Fair 2003). In women oocytes measure approximately 120 µm at ovulation (Gosden 2005). Ultrasound studies have noted preovulatory follicle size to reach 20.2 mm (n=11) in the Asian elephant (Lueders *et al*. 2010) and 21.0 mm in the African elephant (Hermes 2000). The latter author reported that mean peak follicle diameter for the anovulatory surge was 13.7 ± 0.7 mm with a range of 10–19 mm. The largest elephant oocyte encountered in the present study measured 165 µm in diameter and it existed within a 3 mm diameter follicle.

Atretic follicles of secondary stage and smaller were rarely seen in the ovaries examined in this study, although plenty of atretic antral follicles were observed.

3.4.2. The ovarian reserve

The established theory that only TPM make up the follicle reserve in mammals is based on the premise that follicles arrest only when squamous granulosa cells surround the oocyte. Any subsequent deviation from this state indicates a commitment to growth which results in the follicles exiting from the reserve (Oktay 1995). Three alternative theories to explain the apparent shape of the granulosa cells and their relation to follicle activation can be mooted. First, the follicle pool is formed with follicles showing variously shaped granulosa cells. These may originate from different regions of the fetal ovary such as the mesonephros (squamous cells) or the surface epithelium (low cuboidal cells) or indeed, from neighbouring pyknotic oocytes (Hirshfield 1992; Sawyer *et al*. 2002). Second, all follicles start as primordial follicles but some show slow growth in the follicle reserve over many years (Fortune 2003; van den Hurk & Zhao 2005). Third, the rounding up of granulosa cell nuclei is not related to the increase in the number of granulosa cells and is therefore not an indicator of follicle activation (Van Wezel & Rodgers 1996). In the youngest elephant ovaries examined in the present study fewer than 2% of the total SF population were TPM, thereby suggesting that one, or a combination of all three theories is, relevant to elephant folliculogenesis.

The molecular mechanisms that lead to follicle growth have not yet been established (Oktem & Oktay 2008; Westergaard *et al*. 2007), although the morphological changes involved have been described in a number of species. The present study suggests that, in African elephants, TPM possess few attendant granulosa cells, with significantly more being acquired during their transition to EP stage. Whether this increase in the number of granulosa cells is the first indication of follicle activation in the elephant remains to be confirmed. Similar kinetics have been reported in bovids (van den Hurk & Zhao 2005), women (Gougeon & Chainy 1987) and mice (Lintern-Moore & Moore 1979).

The present findings indicated that the total number of SF in the African elephant reduces with increasing age, which concurs with the generally held view in other mammalian species (Telfer & McLaughlin 2007). In each of the 16 elephants studied there were fewer TPM than either EP or TP follicles, with EP follicles always being the most populous, thereby suggesting that EP, and not TPM, are the likely reservoir for the replenishment of TP follicles in the elephant.

Some 300 000 small follicles have been recorded in the ovaries of young pubertal women (Faddy *et al*. 1992), 120 000 in the ovaries of pubertal heifers (McGeady *et al*. 2006) and 30 000–50 000 in the ovaries of ewes, compared with the mean of only 21 253 small follicles counted in the ovaries of three 13–16 year old elephants in the present study (elephants reach puberty between 8 to 16 years of age (Hanks 1972; Laws 1969). A similar comparison may be made at 30–34 years of age when a mean of 8982 follicles were counted in 3 elephants compared to 90 000 counted in the ovaries of similarly aged women (Faddy 2000). This suggests that, either, counting follicles in the very large elephant ovary using serial sectioning is susceptible to error, or, despite her extended reproductive lifespan, the African elephant retains relatively few follicles in her ovarian reserve. Although the method used to count follicle numbers in the study has been superseded and its possible shortcomings noted (Charleston *et al*. 2007; Meredith *et al*., 1999; Miller *et al*. 1997), the counts have nevertheless given an accurate ratio of small follicle classes in the elephant ovary and they have indicated that this ratio differs from those reported in many other mammals.

The previous assumption that TPM make up the vast majority of the resting follicle pool across the species is being reassessed. In healthy young women it is speculated that only a third of all SF are at the TPM stage, most are at the EP stage and a sizeable proportion have already reached the TP stage (de Bruin *et al*. 2002). Similarly, in bovine ovaries more than 80% of follicles are also at the EP or TP stages (Van Wezel & Rodgers 1996) and Type B/C follicles (EP) comprise 72–80% of the total population of SFG in mature rats (Meredith *et al*. 2000). In marked contrast, there are many species in which the majority of follicles in the ovary at any one time are TPM in development (Braw-Tal & Yossefi 1997; Fortune *et al*. 2000; Hirshfield 1989; Oktay 1995; Picton 2001; Wandji *et al*. 1996).

If elephants were to rely on TPM follicles as their resting pool, according to the rules of reproductive ageing ascribed to other mammalian species (Gosden 1995), their ovaries would soon be devoid of potentially fertilisable oocytes, thereby resulting in reproductive senescence relatively early in life. The longevity of reproductive lifespan recorded in the female elephant of around 50 years suggests that either she has a particularly parsimonious turnover of her follicle reserve, with far lower levels of follicle attrition

than those found in other mammalian species, or the somatic cells in follicles that form the reserve can vary morphologically from flat to cuboidal.

3.5. Conclusions

Findings from this study suggest that the ovarian reserve in the elephant includes both TPM and EP follicles. It becomes important to investigate follicle dynamics in the African elephant throughout its reproductive lifespan, and also during fetal and postnatal life, to determine the starting point of the follicle pool and to examine ovaries before their cortices become distorted by development of large structures such as the corpora CL of pregnancy. The composition and dynamics of the SF population can then enable an accurate description of the age-related decline in ovarian oocytes in this species which has, in all likelihood, the longest reproductive lifespan of all land mammals.

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.

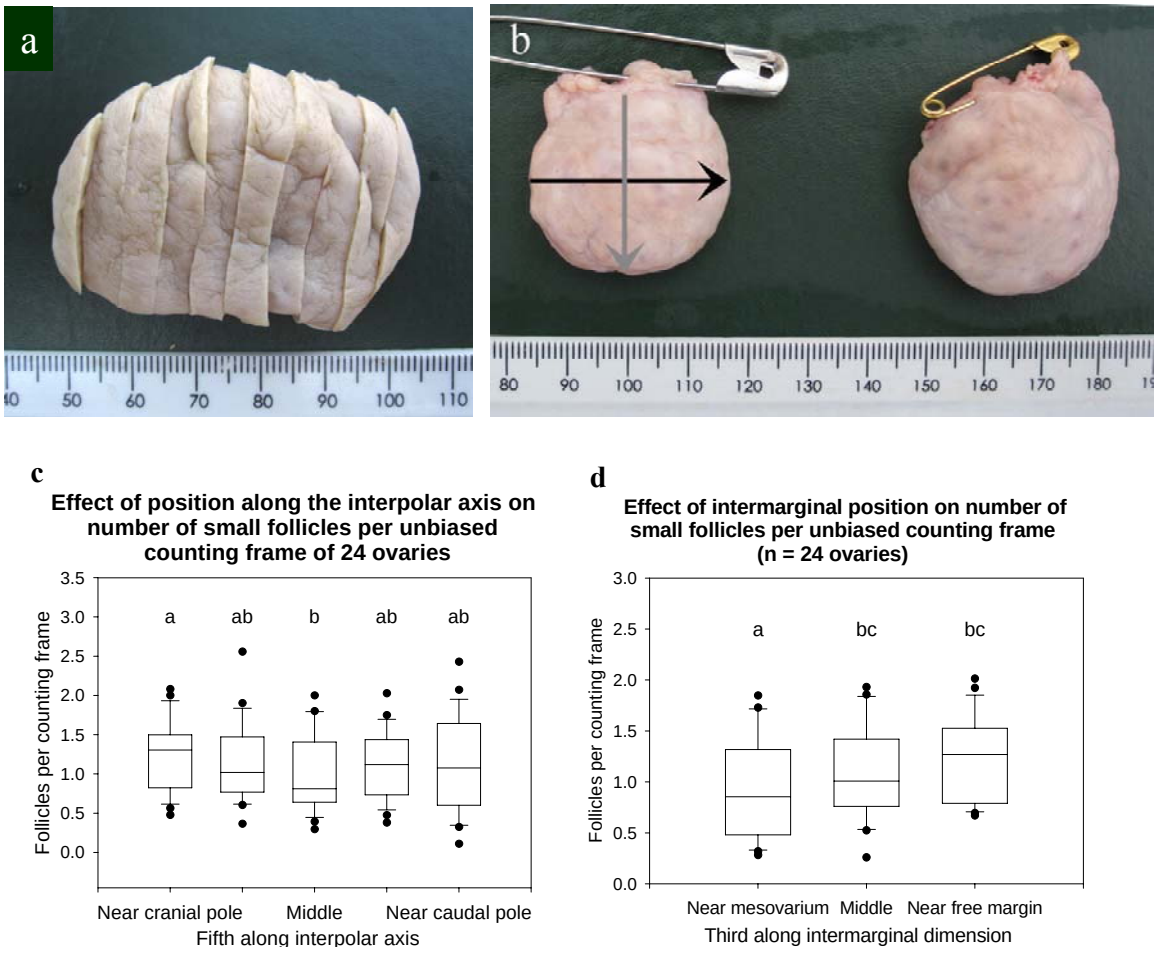


Figure 4.1 Assessment of the effect of position along the interpolar axis and along the intermarginal dimension on the number of small follicles per unbiased counting frame in the ovary of the African elephant

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.

Chapter 5. Development of the germinal ridge and ovary in the African elephant (*Loxodonta africana*)

5.1. Introduction

The elephant has the longest gestation period (656 days) of all land-based mammals (Meyer *et al*. 2004; Moss & Poole 1983), and demonstrates a remarkably flat fetal growth curve during the first half of this period (Craig 1984). Two previous studies have examined embryonic development in the elephant. The first highlighted the aquatic ancestry of elephantids as evidenced by the development of the trunk, the internal placement of the testes in the male, the unusual structure of the pleural cavity in postnatal life and the presence of fish-like nephrostomes in the embryonic and fetal kidney (Gaeth *et al*. 1999). The second mapped embryonic and early fetal development during the first 200 days of gestation by comparing trans-rectal ultrasound scans of the conceptus in zoo elephant with known mating dates to 22 elephant fetuses recovered during culling operations in South Africa (Hildebrandt *et al*. 2007). Using this information a formula was produced which may be used to age embryos and fetuses from the time they are first measurable ultrasonically at 50 days through to 202 days of gestation. This is thought to improve on the previous formula utilizing fetal weight that was devised by Craig (1984) and improves the ability to perform studies on fetal development.

Existing dogma holds that the complement of oocytes for adult reproductive life is established during fetal life (Gosden 2004). The process is similar throughout the mammalian kingdom although time scales and gestation lengths vary greatly. Oogenesis begins with the migration of primordial germ cells (PGCs) from their extra-embryonic origin in the yolk sac to the indifferent gonadal ridge which develops as an outgrowth from the mesonephros (McLaren 2003). Having divided mitotically during their journey the PGCs arrive at the gonadal ridge where they lose their pluripotency and are now termed oogonia (Byskov & Nielsen 2003), or simply germ cells if the sex of the embryo is not obvious. Mitotic multiplication is key to the establishment of the future oocyte population as it is the balance between mitotic production and apoptotic elimination of oogonia which determines the number of oocytes available for reproductive life in the female mammal (Aitken *et al*. 2011). The signal for oogonia to stop mitotic division and start meiotic differentiation is unclear but it is thought to be associated with the presence

of retinoic acid originating from the mesonephros (Bowles & Koopman 2007; Childs *et al*. 2011). The length of a single meiotic prophase to the time of arrest is estimated to be around a week in the mouse, rat and hamster and 12–15 days in the rabbit, dog, pig monkey and man (Deanesly 1977). However, the duration of the period in which oogonia enter meiosis is long and is suggested to be related to gestation length; eg. 52–82 days in the ewe, 40–100 days in the sow, 70–170 days in the cow, 70–140 days in the macaque and 82–160 days in man (Deanesly 1977). In the hamster, rabbit, ferret and dog, entry into meiosis is post natal (Deanesly 1977). The mitotic-meiotic transition is accompanied by the formation of follicles. Oocytes that are not captured within follicles become atretic (Picton 2001). In most species these follicles are termed primordial follicles, being surrounded by a layer of flattened granulosa cells (Pepling 2012). As previously described by Stansfield *et al*. (2011b) the small follicle pool or reserve in elephants from birth onward is composed of EP and TP follicles which together have been termed SF, as defined in Table 3.3. There are almost no TPM in the ovaries of elephant of all ages (see Chapter 3 onwards). During oogenesis in all species, peak numbers of germ cells are observed around the time of the transition from mitosis to meiosis (Reynaud & Driancourt 2000). From their peak at embryonic day 13 in the mouse (E13), E80 in cattle and E152 in woman, the number of germ cells decreases sharply, with two main periods of high loss; i) the pachytene (recombination) stage of meiosis in the oocyte and, ii) the time of formation of primordial follicles (Aitken *et al*. 2011; Bendsen *et al*. 2006; Reynaud & Driancourt 2000). As a consequence of these considerable losses the number of oocytes enclosed in primordial follicles at birth in the cow for example is less than 5% of the peak value. The highest survival rate of germ cells in domestic mammals occurs in the pig where approximately half the original number of germ cells survive until birth (McGeady *et al*. 2006). There has been considerable speculation over the past decade on whether post natal neo-oogenesis can occur to replenish oocyte stocks in prepubertal and adult life (for a review see de Felici 2010).

The present study was undertaken to monitor the development of the elephant ovary and its germ cells, from its early indifferent stage prior to 100 days of gestation until it begins to enlarge greatly in mid-pregnancy due to hypertrophy and hyperplasia of interstitial cells in the medulla and the development of multiple antral follicles.

5.2. Materials and methods

Four early embryos of indeterminate gender harvested during the first 100 days of gestation and 4 pairs of ovaries from female fetuses between 4.8 and 11.2 months of gestation were collected from pregnant elephants culled in family groups. An additional embryo of indeterminate gender aged 87 days was kindly donated by Dr R M Laws from his collections in the 1960's (Laws 1969), and two paraffin-embedded samples of fetal ovaries aged 11.3 and 13.5 months were available from a previous study (Allen *et al*. 2005). Crown rump length (CRL) was measured from the vertex of the skull to the base of the tail (Arey 1966) and this figure, together with its weight (g) was used to age each embryo or fetus against the formulae described by Hildebrandt *et al*. (2007) and Craig (1984).

Within 2 hours of death the whole embryos or the ovaries of the fetuses were immersed in 4% v:v neutral buffered formalin; weights and CRLs were measured following fixation. The embryos were placed whole into cassettes and serially sectioned along the sagittal plane at 5 µm thickness due to the small size of the gonad which was an unknown factor before sectioning took place. Every 5th section was mounted on a slide and stained with haemotoxylin and eosin (H&E). The four sets of fetal ovaries were bisected and placed in cassettes before 10 uniformly spaced 25 µm transverse sections were cut using a microtome, placed on slides and stained with H&E. The slides were examined for morphological development and the germ cells counted using stereological protocols.

For embryos 1–5 every 5th serial section was stained and an unbiased counting frame (UCF) was tessellated over the whole area of the tissue in each slide in order to count the number of germ cells, the latter being identified by a nucleus of 10.0–12.5 µm in diameter, which clearly contrasted with nuclear diameters of about 5 µm of the smaller cells in the region. The formula of Abercrombie (1946) was used to calculate the number of germ cells as previously described by Mouton (2002). For the sets of fetal ovaries stereological calculations were carried out using the unbiased brick to determine density and Cavalieri's estimator to determine volume.

5.3. Results

5.3.1. Milestones in embryonic development

The earliest specimen studied at 76 days (2.5 months; Figure 5.1, Table 5.1) exhibited a "cauliflower floret" shaped gonadal ridge on the ventral surface of the relatively large mesonephros on each side of the abdomen (Figure 5.2a). Coelomic epithelium formed a dense outer lining of the presumptive gonad (Figure 5.2a, b) and penetrated inward as cortical cords between the mesothelial stromal cells. Germ cells with an overall diameter of around 12.5 µm and a nuclear diameter of around 10.0 µm were observed within the stroma of the still indifferent gonad (Figure 5.2b) which demonstrated a two-dimensional profile in the sagittal plance of approximately 1200 µm x 350 µm. Immediately medial to each gonadal ridge, both cranially and caudally, PGCs of indeterminate status were also visible (Figure 5.2c).

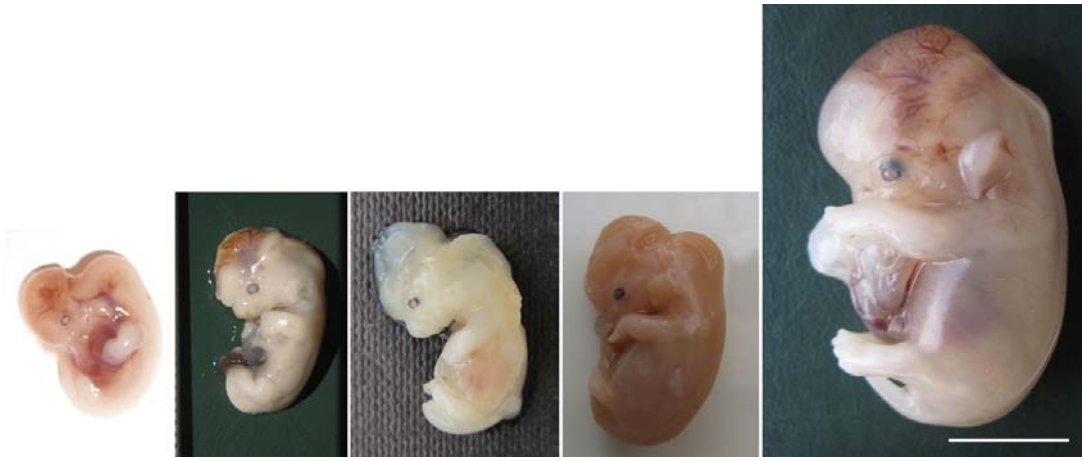


Figure 5.1 African elephant embryos aged (from left to right) 76, 81, 82, 87, and 96 days post conception (scale bar = 10mm)

Table 5.1
Mass, crown-rump length (CRL) and estimated ages of the 5 elephant embryos

			Estimated age in days		
Embryo	Mass (g)	CRL (mm)	Based on mass[a]	Based on CRL[b]	Used in the study
1	0.47	14	76	76	76
2	0.50	16	77	78	81
3	0.55	23	78	87	82
4	0.75	25	80	89	87
5	3.00	34	94	98	96

[a]Age according to mass as formulated by Hildebrandt *et al*. (2007)
[b]Age according to CRL as formulated by Hildebrandt *et al*. (2007)

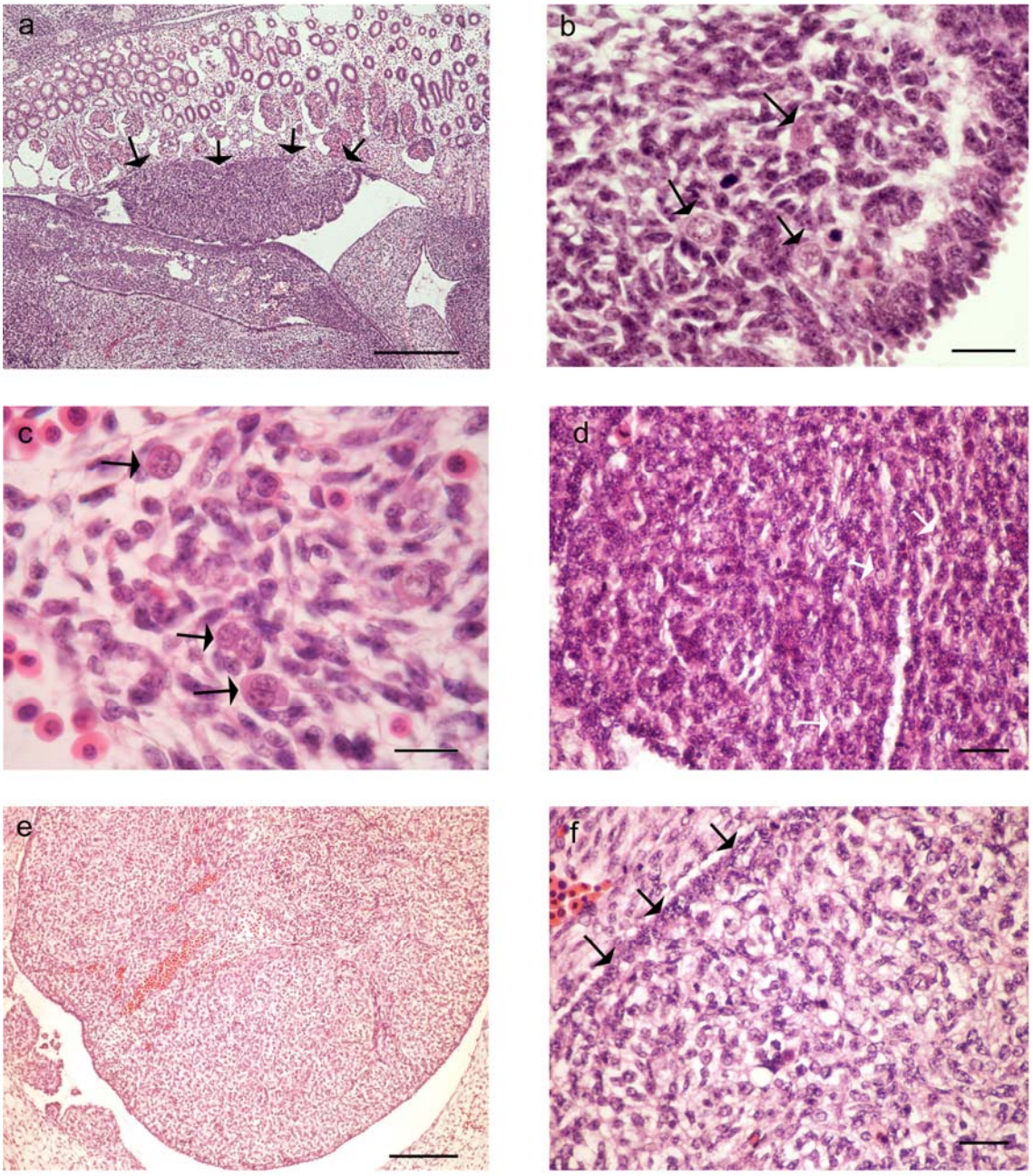


Figure 5.2 Development of the gonad of the African elephant embryo

a. The gonadal ridge of a 76 day old embryo; black arrows mark the boundary of the gonad and mesonephros. Scale Bar 350 µm.
b. Germ cells (black arrows) within the gonadal ridge of the 76 day embryo. Scale bar = 20 µm.
c. Mitotic primordial germ cells (black arrows) observed in the tissues dorsal and medial to the gonadal ridge in the 76 day embryo. Scale bar = 20 µm.
d. At 82 days post conception the germ cells (white arrows) are smaller and more difficult to identify between the blastema. Scale bar = 40 µm.
e. At 96 days post conception the gonad has become a roughly spherical independent organ with a single point of attachment to the mesonephros. Scale bar = 125 µm

Continued

Figure 5.2 (continued)
f. A higher magnification of (e). Very few germ cells were distinguishable from the other cells within the blastemal mass of the stroma. Black arrows mark the epithelial cells of the gonadal surface. Scale bar = 40 µm

Sagittal sections of Embryo 2 (81 days or 2.7 months; Figure 5.1) were cut but were not suitable for morphological study due to delayed fixation and poor staining. The embryo was however useful for ageing criteria using external morphology and measurements (see Table 5.1).

At 82 days (2.7 months; Embryo 3) development of the gonad was well advanced although it was still attached in two places to the mesonephros. It measured 750 x 1140 µm at its largest diameter in the sagittal plane and lacked visible internal organization. Some large germ cells, with nuclear diameters of around 10.0–10.5 µm and therefore similar to those in the 76 day old Embryo 1 (Table 5.2), were observed while other germ cells of smaller (7.5 µm) nuclear diameter were also visible. These smaller cells, possibly mitotic were difficult to distinguish from the invading coelomic epithelial cells, precursors of the pre-granulosa cells (Figure 5.2d).

At 87 days (2.9 months; Embryo 4) the gonad measured 670 x 1240 µm in the sagittal plane and was attached to the mesonephros by a thin stalk, presumably the precursor of the mesovarium. The surface epithelium comprised a layer of cuboidal epithelial cells with some interspersed germ cells. The germ cells were distributed throughout both gonads although they were now less obvious than during earlier developmental stages. No PGCs were observed in tissues outside the gonads.

Table 5.2
The size of germ cells in the elephant embryonic and fetal gonad, and the number of granulosa cells surrounding the oocyte of different types of small follicles

ID no.	Age months (days)	Type of cells or follicles	Diameter (µm)			Number of granulosa cells
			Follicle	Oocyte	Nucleus	
Embryo 1	2.5 (76)	Germinal cells[a]	-	12.5	10.0	-
Embryo 2	2.7 (81)	nd	-	nd	nd	-
Embryo 3	2.7 (82)	Germinal cells		15.0–16.0	10.0-10.5	-
Embryo 4	2.9 (87)	nd	-	nd	nd	-
Embryo 5	3.2 (96)	nd	-	nd	nd	-
Fetus 1	4.8	Oogonia	-	15.0–20.0	7.8–10.0	-
Fetus 2	5.3	Oogonia	-	16.0-25.0	10.0-12.5	-
Fetus 3	5.9	Oogonia	-	17.5–25.0	10.0–12.5	-
		SF	35.0–37.5	21.5–23.0	14.0–16.5	8–11
Fetus 4	11.2	EP	47.5–65.0	45.0–55.0	20.0–22.5	12–14
		TP	55.0–105	60.0–80.0	20.0–22.5	21–30

[a] Germinal cells not yet identifiable as oogonia or prespermatogonia, because gonadal sex differentiation has not yet occurred. Nd = no data.
PGC = primordial germ cells, SF = small follicles, EP = early primary follicles, TP = true primary follicles

At 96 days (3.2 months; Embryo 5) the gonads were relatively large, rounded structures with a two-dimensional profile of approximately 1500 x 1175 µm in the sagittal plane. Very few germ cells could be distinguished from the pregranulosa and other cells within

the blastemal mass of the stroma (Figure 5.2e and Figure 5.2f) but blood vessels could be seen penetrating the organ from the point of attachment to the mesonephros. No sexual differentiation of the gonad was apparent.

5.3.2. Milestones in fetal development

Using the two established formulae, Fetus 1 (Figure 5.3a) was aged at between 4.8 months (Hildebrandt *et al*. 2007) and 6.2 months (Craig 1984). Phenotypically the fetus was clearly female. The two ovaries had volumes of 39 and 46 mm^3 respectively (Table 5.3 and Figure 5.3b). Their surface epithelium (OSE) consisted primarily of low cuboidal mesothelial cells which was multilayered in places. The OSE was separated in the most part from underlying pockets of round cells by a layer of fusiform mesenchymal cells. Surface epithelial cells were rarely observed to extend into the developing gonad. The pockets of large round cells were present throughout the ovary (Figure 5.3c). They were isolated from the surrounding stroma by tracts of fibroblast-like cells with elongated nuclei. No basal lamina was visible beneath the surface epithelial layer. The round cells were of two sizes. The larger, more plentiful ones were the oogonia in germline cysts or nests (Pepling 2006), still attached to each other by cell bridges and the smaller ones were possibly the pre-granulosa cells which, although in close proximity to the oogonia were not yet adherant to them in any sort of follicle formation. The nuclei of the oogonia were typically 7.8–10 µm in diameter although some were as large as 15 µm. By contrast, the smaller pre-granulosa cells had nuclear diameters of only around 5 µm. The nests of oocytes occurred throughout the ovary with the exception of the area of attachment of the ovary to its suspensory ligament (the hilum), which was composed of loosely packed stromal cells and some blood vessels. With the latter exception, stromal cells were more densely associated in the central ovary giving the impression that fibroblasts were streaming from the point of attachment peripherally around the nests of oogonia. The peripheral part of the ovary had fewer stromal cells between the oogonia nests.

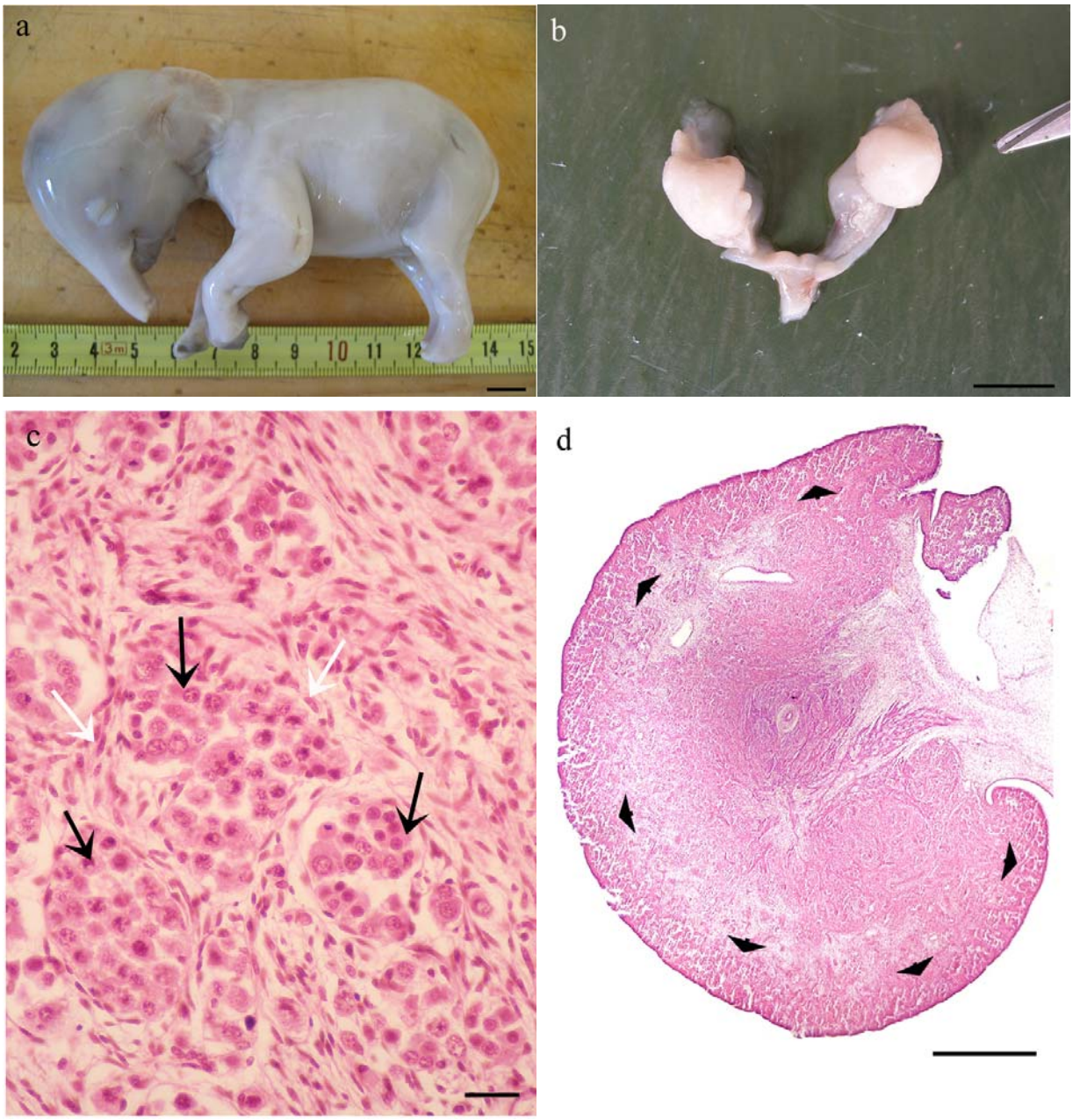


Figure 5.3 Photographs and photomicrographs of the gonads of elephant fetuses at 4.8 to 5.9 months of gestation

a. A 4.8 month old elephant fetus without the lower half of its trunk. Scale bar = 10 mm.
b. The ovaries and uterus of the 4.8 month fetus. Scale bar = 3 mm.
c. Pockets of oogonia (black arrows) surrounded by fibroblasts (white arrows) present throughout the ovaries of the 4.8 month embryo. Scale bar = 30 µm.
d. Cross section through the ovary of a 5.3 month elephant fetus, with the inner limit of the developing cortex marked by black arrow heads. Scale bar = 1 mm.

(Continued)

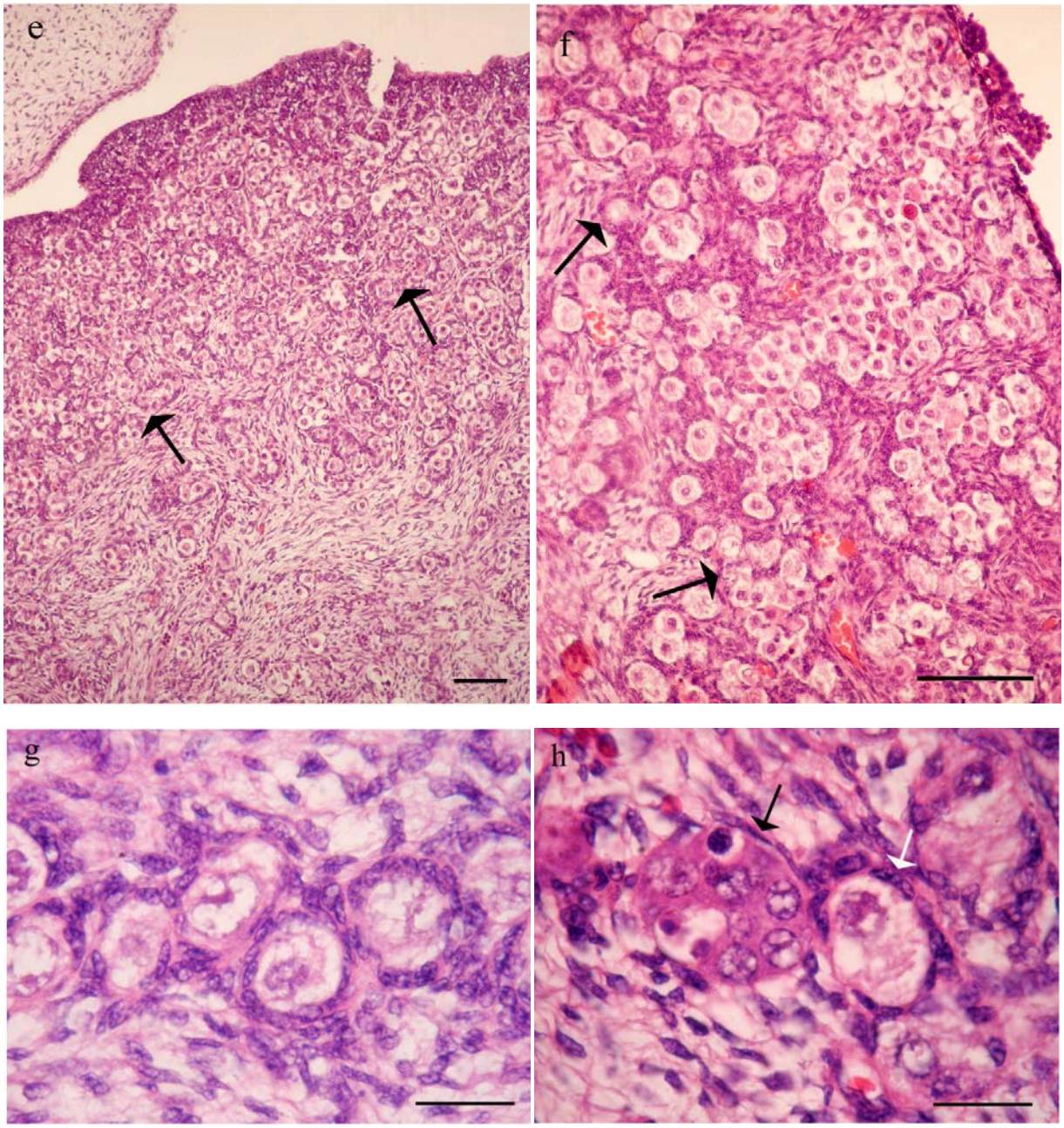


Figure 5.3 (continued)

e. Higher magnification of the ovary in (d) showing oocytes in the cortex, the cortico-medullary border (black arrows) and in the medulla. Scale bar = 100 µm.
f. Oogonia within the ovigerous cords of a 5.9 month old fetal ovary. The cords reach from just below the surface epithelium to the presumptive cortico-medullary border (black arrows). Scale bar = 100 µm.
g. Newly formed follicles in the same ovary as (f). The string of follicles extends from the end of ovigerous cords from which the follicles finally "bud-off". Note the number and shape of the pre-granulosa cells. Scale bar = 30 µm.
h. A small follicle (white arrow) in the medulla of the 5.9 month fetal ovary, alongside an ovigerous cord containing oogonia (black arrow). Scale bar = 30 µm.

Table 5.3
Ovarian volumes (mm^3) of elephant fetuses

Fetus	Age (m)	Ovary[a]	Cortex	Medulla	Whole ovary
1	4.8	1			39
		2			46
2	5.3	1	17.7	26.7	44
		2	19.2	35.7	55
3	5.9	1	35	50	85
		2	33.5	46.9	80
4	11.2	1	925	1719	2644
		2	622	1684	2306

[a] 1=left ovary, 2=right ovary

Fetus 2 (Figure 5.5b) was aged between 5.3 months (Hildebrandt *et al*. 2007) and 6.6 months (Craig 1984). The mean volume of its ovaries was 50 mm^3 per ovary. The OSE consisted of 3–4 layers of very densely packed cells which protruded as cords of cells in a wave like manner into the presumptive cortex. Oogonia were separated from the OSE and each other by the more plentiful epithelial cells which may originate from the OSE (Sawyer *et al*. 2002) or the *rete ovarii* (Zamboni *et al*. 1979). Approximately two thirds of the oogonia fell within the developing cortical region. From the hilum fibrocytes or fusiform mesenchymal cells branched out in tree-like fashion (Figure 5.3d) in "cell streams" as described by Zamboni *et al*. (1979) to invade all parts of the ovary right up to the OSE in places. In the 25 µm sections these cell streams were commonly observed to be associated with darker staining endothelial cells of blood vessels. At times these elongated cells could be observed running parallel to the periphery of the ovary in the region consistent with the placement of the future cortico-medullary border. The first small follicles formed were sparsely dispersed in the central ovary and individually or in strings along the presumptive cortico-medullary border (Figure 5.3e). Many nests of oogonia remained undeveloped in the hilum near to the mesovarial attachment and inner medulla, and these centrally placed oogonia were less densely arranged than in Fetus 2.

At 5.9 (Hildebrandt *et al*. 2007) to 7.2 months (Craig 1984) the OSE of Fetus 3 (Figure 5.5c) was very densely packed with cells and no basement membrane was visible. Just under this layer (Figure 5.3f) the oogonia were contained within ovigerous cords for

approximately two thirds of the depth of the presumptive cortex which now, typically, measured 500–875 µm. Moving deeper into the cortex, new SF could be observed still linked to neighbours within the cords (Figure 5.3g). The cortico-medullary border contained newly formed follicles but very few oogonia remained in this region. Follicles with an early primary configuration appeared to "bud off" the end of the ovigerous cords and be released into the medulla, either individually or in strings. SF were also observed within the medulla but none of these had developed beyond the primary stage (Figure 5.3h).

At 11.2 months of gestation (Craig 1984) the OSE in Fetus 4 (Figure 5.5e) had become less densely packed and it was composed of only 1–2 layers of cuboidal cells. No naked oogonia were visible and all the oocytes were now contained within follicles of early primary and true primary configuration of 47.5–75.0 µm diameter. The cortex was typically 460 µm deep and still contained some ovigerous cords. Differentiation between the cortex and medulla had become more obvious due to vascularisation and development of interstitial tissue in the medulla. In addition, follicles in the central medulla had started to grow to multilaminary stages while those in the cortex remained as SF Figure 5.4a and b). Within the medulla the first antral follicles had begun to form and these showed diameters of 1.0–1.6 mm. Interstitial tissue was visible and vascularisation had increased. These fetal ovaries had a mean weight of 2.5 g.

At 13.5 months of gestation the mean weight of the ovaries had increased to 14.1 g. Dense accumulations of interstitial cells were present in the medulla and these were interspersed with cords of elongated stromal cells. The stromal tissue of the cortex was now less dense than in the younger fetus and SF were visible close to the OSE. Some growing follicles, both transitional and secondary (Chapter 3, Table 3.5) were seen in the medulla but there was no obvious arrangement of smaller follicles being more peripherally positioned. The antral follicles were well supplied with blood vessels and their theca cells adjoined the interstitial cells.

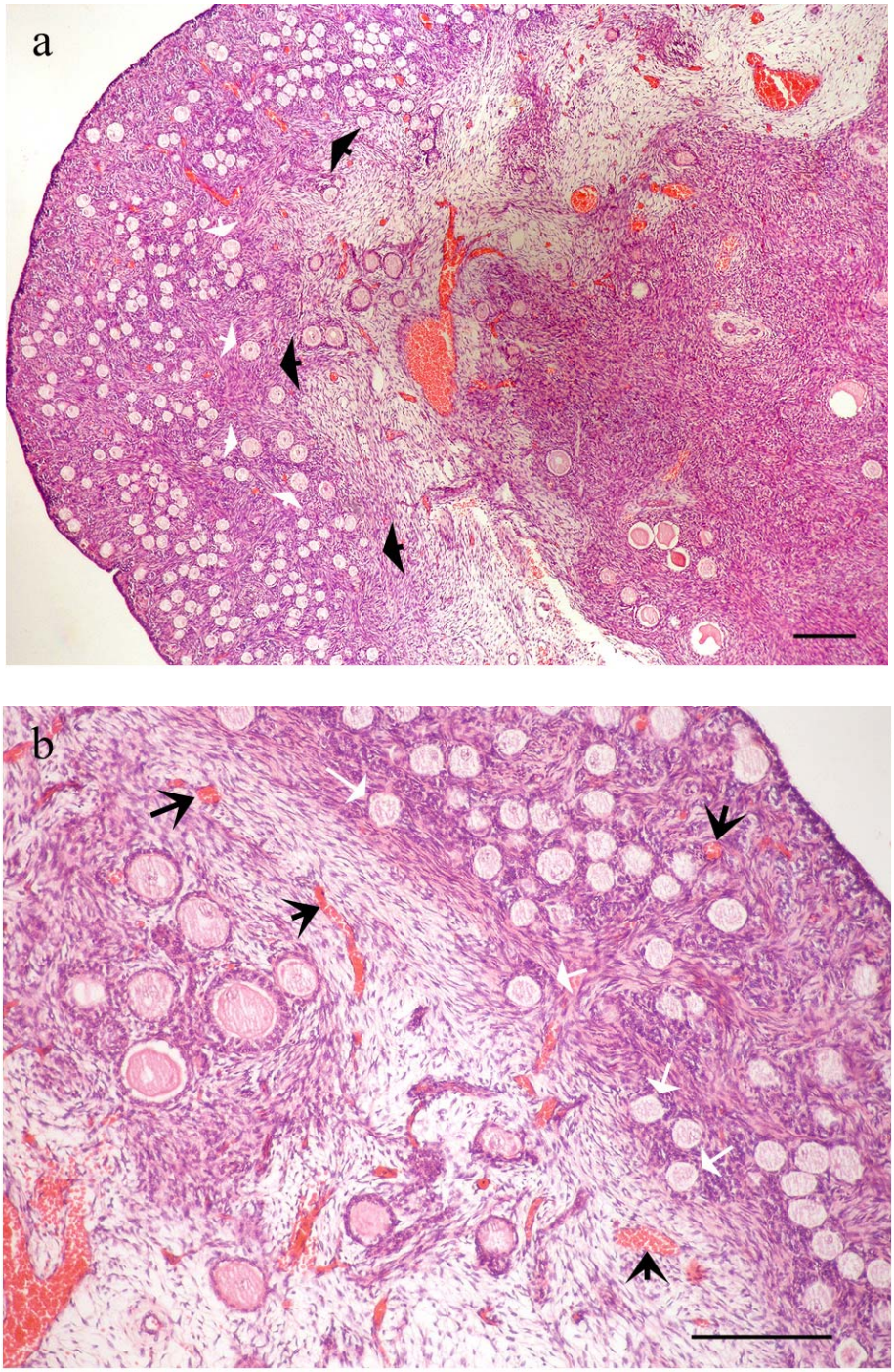


Figure 5.4 Ovarian sections from a mid-term (11.2 month) African elephant fetus

a. The cortex and the medulla are now well defined regions. Black arrowheads mark the inner limits of the cortex. Some small follicles in the inner cortex have initiated growth (white arrows). Scale bar = 100 µm
b. Small follicles within the outer cortex are "non-growing". Follicles within the medulla have started to grow. Some SF along the cortico-medullary border may be destined to progress further inward in which case they will become committed to growth (white arrows). Vascular supply can be seen within the cortex and medulla (black arrows). Scale bar = 100 µm.

5.3.3. Germ cell counts

A count of germ cell numbers gave values of 156 and 162 for the two gonadal ridges of Embryo 1. The difficulty of identification of germ cells due to the similarity in their size and morphology to the somatic cells in the remaining embryos made it impossible to calculate meaningful values for them. The results for the fetuses are given in Table 5.4.

Table 5.4
Numbers and distributions of oogonia and small and growing follicles in the ovaries of elephant fetuses

Fetus	Age (months)	Oogonia		Follicles				
				Small		Growing		Total
		Number	c:m[a]	Number	c:m	Number	c:m	Total
1	4.8	1 272 139		0		0		1 272 139
2	5.3	1 079 080	63:37	55 720	32:68	0		1 134 800
3	5.9	1 322 487	100:0	727 390	81:19	0		2 049 877
4	11.2	0		4 003 960	100:0	460 126	0:100	4 464 086

[a] Percentage in cortex:percentage in medulla

Figure 5.5 Elephant fetuses of different ages

Continued

Figure 5.5 (continued)

a. A 4.1 month elephant fetus (male). Scale bar = 2 cm
b. Fetus 2, a 5.3 month female. Scale bar = 2 cm
c. Fetus 3, a 5.9 month female. Scale bar = 2 cm
d. An 8 month fetus (male). Scale bar = 3 cm
e. Fetus 4, an 11 month female. Scale bar = 5 cm
f. A 17 month female fetus (see Chapter 6). Scale bar = 10 cm
g. A 20 month female fetus (see Chapter 6). Scale bar = 20 cm
h. A 22 month fetus (male). Scale bar = 20 cm

5.4. Discussion

5.4.1. Developmental stage and age of the embryos

Although each measurement and description of the gonad and ovaries are independent observations, the pattern of change over time is suggestive of development and, in the absence of longitudinal studies, provides the only currently available data.

The ages of the 5 embryos used in the study were calculated initially from the ageing formula devised by Hildebrandt *et al*. (2007) based on embryonic weight (Table 5.1). However, the visual appearance of Embryos 1, 2 and 3 suggested strongly that they were more widely separated in age than the 2 days ascribed by the Hildebrandt *et al*. (2007) ageing formula. Accordingly, photographs of them were compared with photographs included in the Hildebrandt *et al*. (2007) paper. As a starting point, Embryo 2 appeared developmentally closer in age to the 81 day old embryo (EF4) in the Hildebrant *et al*. (2007) paper so it was ascribed an age of 81 days, despite its having a weight-age of 77 days. Embryo 1 appeared less developed morphologically than Embryo 2 so it was allotted its CRL- and weight- indicated age of 76 days. Embryo 3's weight suggested an age of 78 days and its CRL an age of 87 days. A small amount of tissue was missing from its dorsal spine area so its true weight might have been higher than the figure recorded in Table 5.1. Also it showed a less pronounced degree of flexure than its neighbouring embryos which might have exaggerated its CRL age. Since this embryo was clearly more advanced than Embryo 2 and appreciably less advanced than Embryo 4, it was allocated an age of 82 days. The weight and CRL predictions for Embryo 4 were 80 and 89 days respectively and, since it was clearly more developmentally advanced

than Embryo 3, it was allocated an age of 87 days. Similar calculations for Embryo 5 gave ages of 94 and 98 days respectively (see Table 5.1) so it was allocated an age of 96 days. It weighed a full gram less than the embryo of 97 days (EF6) described in the Hildebrandt *et al*. (2007) paper.

5.4.2. Morphology of the gonadal ridge and developing ovary

In mammals the germ cells must be resident in the primordial gonad before sexual differentiation takes place. PGCs start entering the gonad during the 7th week of gestation in the human embryo (Langman 1981) and are thought to continue this invasion over a period of 14 days (Byskov & Nielsen 2003). At this age a human embryo is developmentally similar to the youngest elephant embryo in the present study. In this elephant (Embryo 1), the PGCs were observed external to the indifferent gonad and germ cells within the gonad. The number of PGCs that migrate from the extra-embryonic yolk sac is not known in the elephant but in this embryo 162 and 156 germ cells were counted within the two gonadal ridges. In Embryos 3 and 4 germ cells were still visible within the gonad but did not appear to have increased greatly in numbers from Embryo 1. This could have been due to the germ cells proceeding through a series of mitotic divisions and reducing in size as they did so, oogonia having higher mitotic activity than PGC's (Oktem & Urman 2010). No further germ cells were seen in tissues external to the gonad in Embryos 3 and 4 which suggests that Embryo 1 may have been nearing the end of the migratory phase of the PGCs, or possibly, the age difference between Embryos 1 and 4 was greater than the 4 days suggested by the ageing formula of Hildebrandt *et al*. (2007) or the 11 days allocated in the present study. Embryo 5 at 96 days of age was calculated to be 18 days older than Embryo 1 and may therefore have entered the period of sex differentiation, previously suggested to occur around the age of 105 days at an embryonic weight of 9 g (Glickman et al. 2005). This could not be confirmed by light microscopic examination of the gonad, since it was a homogenous cluster of cells which must, necessarily, have been both somatic and germinal in nature. A specific staining method for PGCs and oogonia is required to be able to determine the developmental stage of the gonad and count the germ cells at this age.

Fetus 1 at 4.8 months of age exhibited relatively large ovaries attached to a comparatively small and underdeveloped uterus. In terms of follicle formation it approximated to a 55 day old sheep fetus as described in detail by Sawyer *et al*. (2002). Oogonia in various

stages of mitosis were present throughout the ovary within germline cysts, groups of germ cells thought to be formed by successive incomplete divisions of a single cell (Gondos & Zamboni 1969). This cyst formation is suggested to be well conserved between vertebrates and invertebrates during evolution (Pepling *et al*. 1999). These cysts or nests were surrounded by streams of fibroblasts associated with which were a few smaller round cells of unknown origin destined to become the pre-granulosa cells and later granulosa cells surrounding oocytes to form early follicles. Dogma argues that these pre-granulosa cells originate from the *rete ovarii* in a similar way to the Sertoli cells of the testes in the male originating from the *rete testis* (Gosden 1995; Peters & McNatty 1980). However, Sawyer *et al*. (2002) proposed their origin in the sheep to be the surface epithelium as has been mooted previously by others (Gosden 1995). No follicle development was observed in Fetus 1.

The ovaries of Fetus 2 (5.3 months gestation) paralleled closely the developmental stage of a 75 day old sheep fetus (Sawyer *et al*. 2002). Low numbers of SF were present within the cortico-medullary region while the remainder of the cortex was packed with oogonia within the newly formed ovigerous cords. Although these cords seemed to be forming in the same manner as described in sheep (Sawyer *et al*. 2002) the basal lamina, if present at all, was very difficult to discern on the H&E sections. No attempt was made to identify the basal lamina in PAS-stained sections (Junqueira & Carneiro 2005). The new follicles were EP in nature with constituent follicular cells that were either flat or cuboidal. True primordial follicles are rarely seen in the elephant ovary although "comet-like" follicles with a prolate shape, as described in sheep by Sawyer *et al*. (2002) are observed commonly. Some SF and oogonia in nests were also visible throughout the medulla, but in much lower numbers than in the presumptive cortex. The latter germ cells are comparable to the "medullary germ cells" described in the fetal sheep ovary by Sawyer *et al*. (2002). Unlike the medullary germ cells in the sheep fetus, the medullary oocytes in the elephant fetus do associate with somatic pre-granulosa cells to create follicles. If, in the elephant fetus, these pre-granulosa cells derived from the OSE it could be speculated that their invasion into the central ovary had occurred very early, around the time of cortical cord growth because of their current placement far from the OSE.

In all species studied to date, follicle formation begins in the innermost part of the ovary and spreads gradually outwards towards the periphery (Byskov & Nielsen 2010). In the

elephant, follicle formation occurred concomitantly on the presumptive cortico-medullary border and within the central region of the ovary. Peripherally placed follicles emerged from ovigerous cords as described by Sawyer *et al*. (2002) while the centrally placed follicles arose more independently from the branched cell nests. The suggestion that meiotic onset is either stimulated by a medullary factor, or is restricted by a cortical factor (Pepling 2006) is still pertinent but it does not explain the spasmodic initiation of folliculogenesis throughout the presumptive medulla. The boundary between the cortex and the medulla is not static during adult life and may be described as the mid point between the follicles of the cortex and the blood vessels of the medulla (Fawcett 1997). However this definition is not consistent with events in the elephant fetal ovary as the cortex is vascularised and follicles occur within the medulla. Nevertheless, a definite cortico-medullary border does become apparent in the elephant fetal ovary from around 5 months of gestation (Figure 5.3e, Figure 5.4a and b).

In Fetus 3 SF formation was advanced and the pycnotic nuclei of presumptive atretic oogonia and oocytes were observed as "black spots" in the sections, thereby highlighting the ongoing balance between atresia and mitosis within the pool of germ cells in the fetal ovary. The ratio between oogonia and SF within the cortex of Fetus 3 was close to 2:1 and these ovaries approximated to the 90 day of gestation fetal ovary in sheep (Sawyer *et al*. 2002).

By mid-pregnancy (11.2 months; Fetus 4) the follicle pool was contained within the more clearly defined cortical region of the ovary while the follicles within the medulla had started to enlarge to give rise to the first antral follicles observed. The follicle reserve within the cortex is thought to be held in a "dormant" state by the presence of local factors (Adhikari & Liu 2009). These inhibitory molecules for follicle activation may not be present in the medulla of the elephant fetal ovary so that the follicles enter primary recruitment and start to grow. In the medulla sections of ovigerous cords were still apparent in association with streams of stromal cells and the dark staining interstitial tissue that comes to play such a prominent role in the elephant fetal gonad throughout the second half of pregnancy was now visible (Stansfield & Allen 2012). At 13.5 months the considerable enlargement that is typical of the elephant fetal ovary during the second half of gestation had started as a result of the now clear increase in volume of interstitial tissue.

The results of this study have shown that ovarian development in the elephant fetus mirrors that of the human, sheep and other well-studied mammals up to mid gestation (Sawyer *et al*. 2002). While the source of the pre-granulosa cells could not be determined accurately the evidence supports a mesonephric origin, as described by Zamboni *et al*. (1979), rather than an epithelial origin as described by Sawyer *et al*. (2002). Quirke *et al*. (2001) observed that mesonephric and mesothelial cells of the presumptive medulla stain positively for the steroidogeneic enzyme 3ß hydroxysteroid dehydrogenase (3ß–HSD) while those of the presumptive cortex do not during early gestation in the sheep. The granulosa cells in the mid-gestation elephant fetal ovary stain positively and precisely for 3ß–HSD (F J Stansfield and W R Allen unpublished observations), as do the Sertoli cells in the elephant fetal testis of the same age (Allen *et al*. 2005). However the contribution of the surface epithelium cannot be ruled out. A further theory (Byskov & Nielsen 2010) suggesting that the oocyte may be capable of stimulating neighbouring somatic cells to differentiate into granulosa cells is an attractive alternative.

5.4.3. Germ cell counts

Only 318 germ cells were counted in the undifferentiated gonad of Embryo 1. This is many fewer than the 11 000 germ cells calculated to reside in the gonad of the undifferentiated mouse fetal gonad (Tam & Snow 1981) although similar to the 450–1400 germ cells in the human embryo (Witschi 1948). The germ cell counts may already have been reduced by the initiation of mitosis producing the smaller sized oogonia that were not positively identified. Similar counts were not attempted in the other 3 embryos due to the difficulty in distinguishing precisely the mitotic germ cells from their surrounding pre-granulosa cells (Figure 5.2d and f). Accurate counts using the pluripotent markers Oct4 and VASA to identify oogonia are planned.

The distribution of follicles within the fetal ovaries is listed in Table 5.4. It was interesting to find that the number of oogonia remained at around 1–1.3 million between 4.8 and 5.9 months of gestation when they were dividing mitotically and also entering into meiosis to form small follicles. Thus entry into meiosis as determined by the development of the first follicles, was first observed around 5 months of gestation and it persisted through to 11 months. Although oogonia were not visible at 11.2 months in Fetus 4, examination of the ovaries of another 11.3 month old fetus from a previous study (Allen *et al*. 2005) revealed a small number of oogonia within and directly below the

OSE. From this observation it may be assumed that Fetus 4 marked the end of the 6 month period of mitotic-meiotic transition. Such a conclusion would concur with the observation by Deanesly (1977) that the period of entry into meiosis is extended in animals with longer gestation periods. This interval is 22 days longer in the elephant than in women although, given the considerable duration of gestation in the elephant it might have been expected to be longer.

As in other species the peak number of germ cells counted in the elephant fetal ovary (4.5 million) occurred during the period of mitotic-meiotic transition with the likelihood that it is toward the end of the latter period (Table 5.5). The 450 000 growing follicles that were counted in the medulla of the 11 month fetus were destined to grow to the mid-antral size (5 mm) before becoming atretic. Such atresia reflects a considerable loss of genetic material although this figure as shown in Table 5.5, is still well below the numbers of follicles recorded to be lost in other species. Antral follicles have been observed to develop in the fetal ovaries of other species, most notably the giraffe (Benirschke 2007a; Kellas 1958), but the biological reason and stimulus for such growth is unknown.

Considerable inter-animal variation in the number of follicles containing the lifetime reserve of oocytes is evident in other species (Schmidt 2003) and this also occurs in the elephant. Although such variation suggests that the results obtained from the relatively small number of samples examined in the present study may contain "significant error" due to this natural variation, the numbers do nevertheless follow the progression measured in other mammals.

In conclusion some developmental milestones in the ovary of the elephant embryo and fetus have been described for the first time. Migration of PGCs into the indifferent gonad terminates around 76 days of gestation, entry into meiosis and first follicle formation commences around 5 months when many granulosa cells have already assumed a cuboidal outline. Peak numbers of follicles are present at mid gestation towards the end of the 6 month mitotic-meiotic transition period. It appears that the cortex of the elephant fetal ovary at mid-gestation (11 months) has already reached a developmental status of the ovaries of many other mammals at full term. Ovarian development during the second half of gestation in the elephant fetus is directed more towards the medulla and its clearly significant role in steroid hormone synthesis.

Table 5.5
Number of small follicles in the reserves of different mammalian species

Stage of gestation	Mouse[a]	Rat[b]	Domestic dog[c]	Sheep[d]	Cow[c]	Human[e]	Elephant
Peak number	250 x 10^{3}[f]	50–75 x 10^{3}[g]	NA	9 x 10^{6} (75 d)	2.7 x 10^{6}(110 d)	7 x 10^{6}(5 mo)	4.5 x 10^{6}(11 mo)
Early gestation	NA	NA	NA	NA	16 x 10^{3}(50 d)	26–250 x 10^{3}(42 d)[h]	1.2 x 10^{6}(4.8 mo)
Mid gestation	NA	NA	NA	170–200 x 10^{3}(90 d)	107 x 10^{3}(170 d)	9.0 x 10^{6}(110 d)[i]	4.5 x 10^{6}(11 mo)
Late gestation	NA	NA	NA	82 x 10^{3}(135 d)	68 x 10^{3}(240 d)	NA	850 x 10^{3} [j]
At birth	7 924	10–15 x 10^{3}[g]	700 x 10^{3}	82 x 10^{3}[g]	135 x 10^{3}[g]	1.5 x 10^{6}	560 x 10^{3}[j]

[a] (Kerr *et al*. 2006), [b] (Meredith *et al*. 2000), [c] (McGeady *et al*. 2006), [d] (Gondos 1978), [e] (Faddy *et al*. 1992), [f] (Tam & Snow 1981), [g] (van den Hurk & Zhao 2005), [h] (Bendsen *et al*. 2006), [i] (Mamsen *et al*. 2011), [j] Chapter 6, NA = not available

This chapter is to be submitted in a slightly different form to an accredited scientific journal for consideration for publication.

Chapter 6. Growth and development of the ovary and small follicle pool from mid fetal life to pre-puberty in the African elephant *(Loxodonta africana)*

6.1. Introduction

The number of follicles in the ovarian reserve of mammals is established during fetal life or early in neonatal life (Gosden 2005). From this reserve a number of follicles are recruited each day, the majority of which become atretic while a very few develop to the point of ovulation in each oestrous cycle (Peters & McNatty 1980). The biological norm is therefore to show a steady loss of SF throughout life with the resulting depletion of the follicle reserve (Faddy 2000). This dogma has been challenged in recent years and the debate on neo-oogenesis and the existence of post-natal germ-line stem cells (GSC) which can transform into meiotic, fertile oocytes continues (de Felici 2010; Johnson *et al*. 2004; White *et al*. 2012).

Interstitial cells are found in the fetal gonads of many mammalian species, including the elephant (Allen *et al*. 2002); human (Konishi *et al*. 1986), spotted hyena (Lindeque *et al*. 1986), guinea pig (Guraya 1978) and rock hyrax (Kayanja & Sale 1973). And in some—notably the horse (Hay & Allen 1975) and the Common and Grey seals (Amoroso *et al*. 1965)—hyperplasia of these cells causes a significant increase in fetal gonadal volume. In the female fetus in most of these species, the interstitial cells multiply independently of follicles within the presumptive ovarian medulla. In the elephant (Allen *et al*. 2005) and giraffe (Benirschke 2007a; Kellas 1958), however, interstitial cell hyperplasia and hypertrophy not only takes place independently but also in association with antral follicle development during the second half of gestation. In the elephant, this results in the fetal ovaries becoming markedly larger than the fetal testis (Allen et al. 2005; Hanks 1971). Interstitial cells have been studied in fetuses up to the age of 21 months and have been shown to be steroidogenically active, with the potential to synthesise progestagens from cholesterol and pregnenelone (Allen *et al*. 2002). The fate of interstitial cells after birth of the elephant fetus has not been examined.

In this chapter a description is given of the development of the elephant ovary during the second half of fetal life and the first nine years of postnatal life. Formation of its pool of

SF and the growth, regression and steroidogenic potential of the medullary interstitial cells is also described. This period of study forms a logical division based on ovarian development particularly in regard to the development and regression of interstitial cells, followed by maturation of the ovary to the point of puberty.

6.2. Materials and methods

6.2.1. Tissue recovery

Pairs of ovaries were recovered from 6 fetuses in the second half of gestation and 29 prepubertal elephants aged 2 months to 9 years. Apart from these, a 7th pair of ovaries recovered for morphological and immunohistochemical studies only, from a fetus aged at 11.2 months and a block sample from a 13.5 month old fetus collected during a previous study (Allen *et al*. 2005) were examined. Each ovary was photographed and partially bisected along the sagittal plane before being fixed and sectioned. The lower jaw of each elephant was boiled to remove soft tissue, photographed and later used to estimate the age of the animal (Laws 1966; Lee *et al*. 2012).

6.2.2. Tissue preparation

In pregnant females gestation stage was calculated from fetal weights using the formula devised by Craig (1984). Tissue preparation for haematoxylin and eosin (H&E) staining is described in Chapter 2

6.2.3. Immunohistochemical staining

Normal 5 µm sections cut from 3 widely spaced segments of the fetal and prepubertal ovaries were stained with H&E to study the morphology of the tissues and immunocytochemically to study the steroidogenic potential of both the interstitial cells and the granulosa and *theca interna* cells surrounding the follicles. For this purpose the paraffin embedded sections were immersed in a 56 °C oven overnight to remove the wax before they were placed in a pre-heated (68 °C) bath of high pH antigen unmasking solution (Dako Ltd, Ely, Cams, UK) and heated to 97°C for 20 minutes. After cooling the slides were rinsed in neutral buffer and transferred to a Dako Autostainer in which a computer controlled indirect staining method was performed using a mouse monoclonal

3ß-hydroxysteroid dehydrogenase (3ß-HSD) primary antibody (3ß-HSD[37-2]:SC-100466; Santa Cruz, Biotechnology Inc. Ca USA). A biotinylated goat anti-mouse serum (BA – 9200; Vector Laboratories, Burliname, California, USA) diluted 1:200 was used as the secondary antibody. Incubations with both the primary and secondary antibodies lasted 30 minutes and blocking reagents, buffer, substrate, chromogen and nuclear stains were all as supplied by Dako Ltd. After staining the slides were removed from the machine, dehydrated, cleaned and mounted in DPX.

6.2.4. Histological and stereological examinations

Elephant ovaries are relatively large compared to those of other mammalian species studied to date using stereology (Hansen *et al*. 2008; Miller 1999; Myers *et al*. 2004) and the present protocol was tailored to take account of the low density of small preantral follicles within the ovaries of elephant calves (Stansfield *et al*. 2011a). The stereology was carried out in two steps, i) calculation of the volume of the cortical area of the ovary using Cavalieri's Principle and, ii) calculation of the number of follicles per unit volume (cm^3) of the ovary using the optical brick method as described by Howard and Reed (2005). The data did not meet the requirements for parametric tests, and the Kruskal-Wallis test was used to compare medians, with α set at 0.05. NCSS Statistical Software 2004 (NCSS, Kaysville, UT, USA) was used for statistical analysis.

6.2.5. Classification of prepubertal calves into 3 physiological groups

Prior to counting SF numbers the cohort of 29 calves was divided into 3 groups based on macroscopic observation of the ovarian sections; Group 1 (about 2 months to 2 years of age, n = 9) exhibited a large volume of interstitial tissue persisting in the ovaries ; Group 2 (about 2.5–4.5 years of age, n = 9) showed much smaller amounts of interstitial tissue and Group 3 (about 4.5–8 years, n = 11) had no interstitial tissue and the ovaries were markedly larger than those placed in the other two groups. There were two animals of about 4.5 years, one of which fell into Group 2 and the other into Group 3.

6.3. Results

6.3.1. Fetal ovarian morphology (11–20 months of gestation)

The combined mass and volume of the ovaries of each elephant fetus and calf are shown in Figure 6.1 while Figure 6.2 shows that the proportion of the ovarian volume consisting of interstitial cells increases with fetal age, whereas the proportion contributed by antral space declines during late fetal development and the proportion consisting of cortex remains constant.

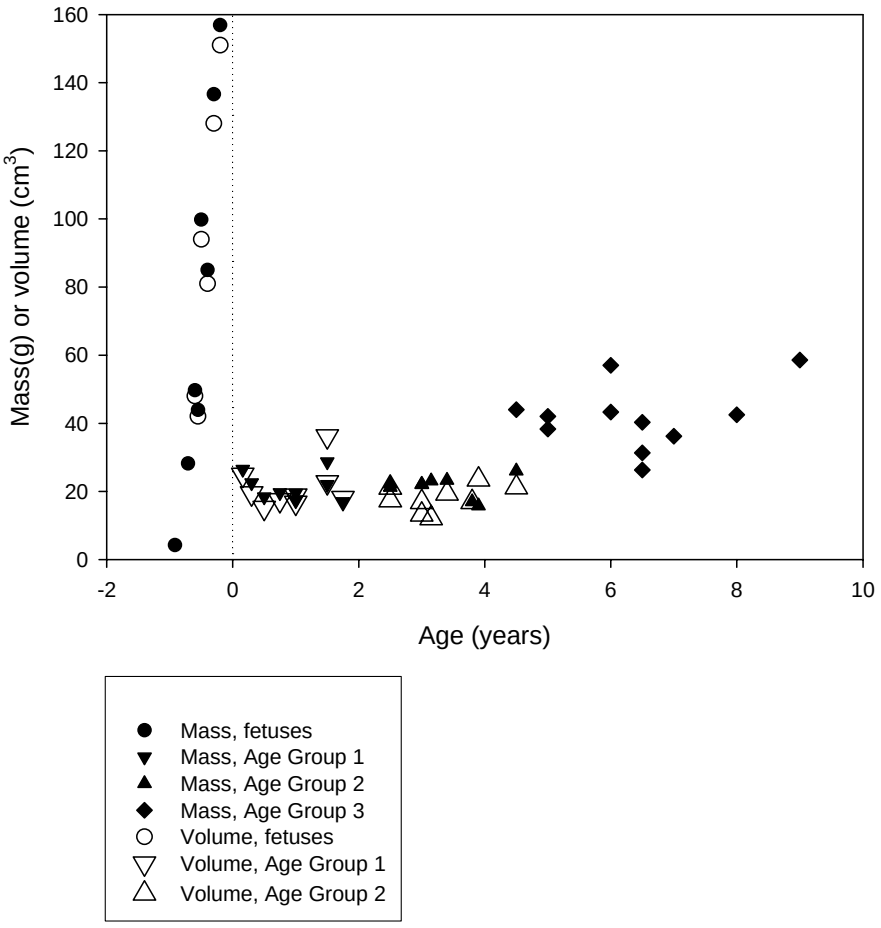


Figure 6.1 Combined weights and volumes of the ovaries of African elephant fetuses from mid-gestation onwards and of calves up to 9 years of age

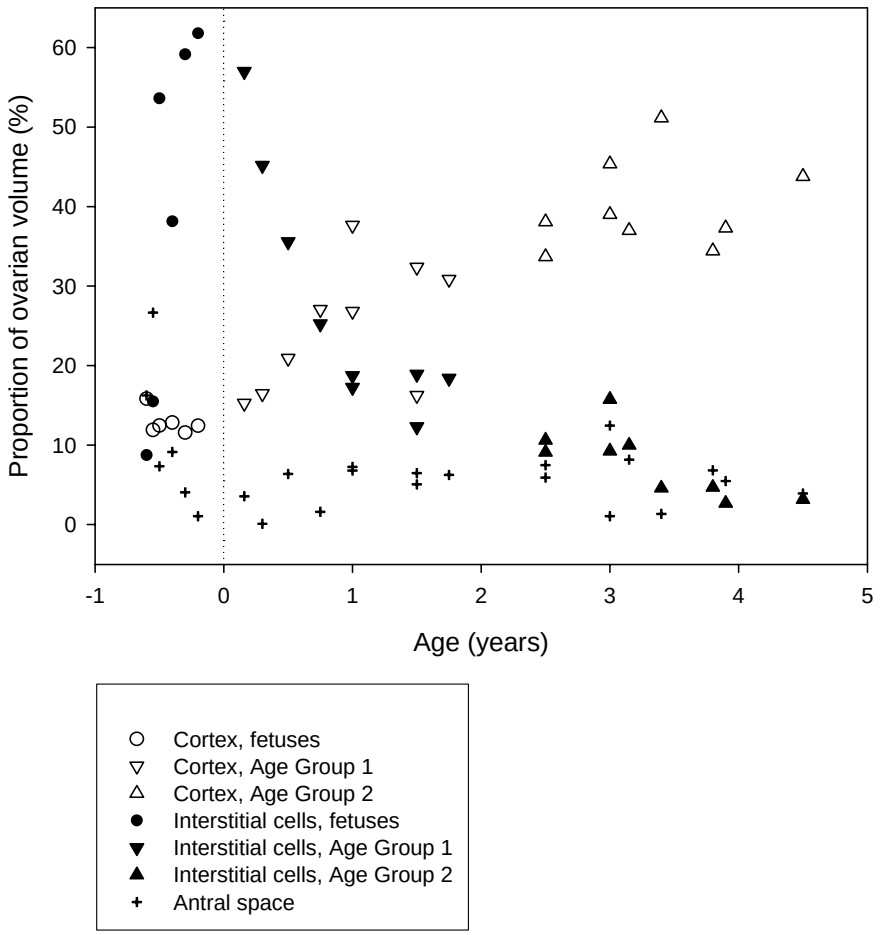


Figure 6.2 Relative contributions of cortex, interstitial cells and follicular antra to the volumes of the ovaries of elephant fetuses from mid-gestation onwards and of calves up to 4.5 years of age

A description of the ovaries from the 11.2 month old fetus is given below to describe the basic morphology of the gonad before the enlargement associated with the second half of gestation has commenced. In this 11 month fetus at mid-pregnancy the left ovary had a mass of 0.25 g and the right 0.27 g. The cortex and medulla were well defined (Figure 6.3a), the cortex being approximately 450 µm in depth and composed of remnants of ovigerous cords, small follicles and cortical stroma consisting of fibrocytes and mesenchymal cells (Figure 6.3b). The ovarian surface epithelium consisted of a monolayer of cuboidal cells with occasional oogonia visible juxtaposed to these epithelial cells (Figure 6.3c). In some sections the *tunica albuginea* formed a narrow layer of constituent cells running parallel to and just beneath the ovarian surface. Follicles present in the cortex were predominantly early primary in development (Figure 6.3d) and

measured 50.0–65.0 µm, (oocyte 45.0–55.0 µm, nucleus 20.0–22.5 µm). A vascular network was clearly visible deep in the cortex. The presumptive medulla was strongly demarcated by the presence of dark staining interstitial cells which had no particular association with follicles (Figure 6.3a). Large blood vessels and *rete ovarii* were also present within the medulla. Growing small follicles (Figure 6.3b), up to the stage of small antral follicles with a diameter of 1.6 x 1.0 mm, were present within the medulla but these were spread more sparsely than follicles in the cortex.

In the fetus at 13.5 months of gestation the left ovary weighed 14.0 g and the right 14.2 g and antral follicles were present within the medulla and along the cortico-medullary border (Figure 6.4a). The medulla appeared more highly vascularised and now contained prominent accumulations of darkly staining interstitial cells (Figure 6.4b). These were polyhedral in shape with dark staining nuclei and eosinophylic cytoplasm (Figure 6.4c) and were appreciably larger than those of the granulosa cells or other stromal cells. The medullary interstitial cells appeared to be continuous with the *theca interna* cells of growing and atretic antral follicles which now reached diameters of 1–2 mm. Within this presumptive medulla were patches of undifferentiated light-staining adipose-rich, vacuolated mesenchymal cells.

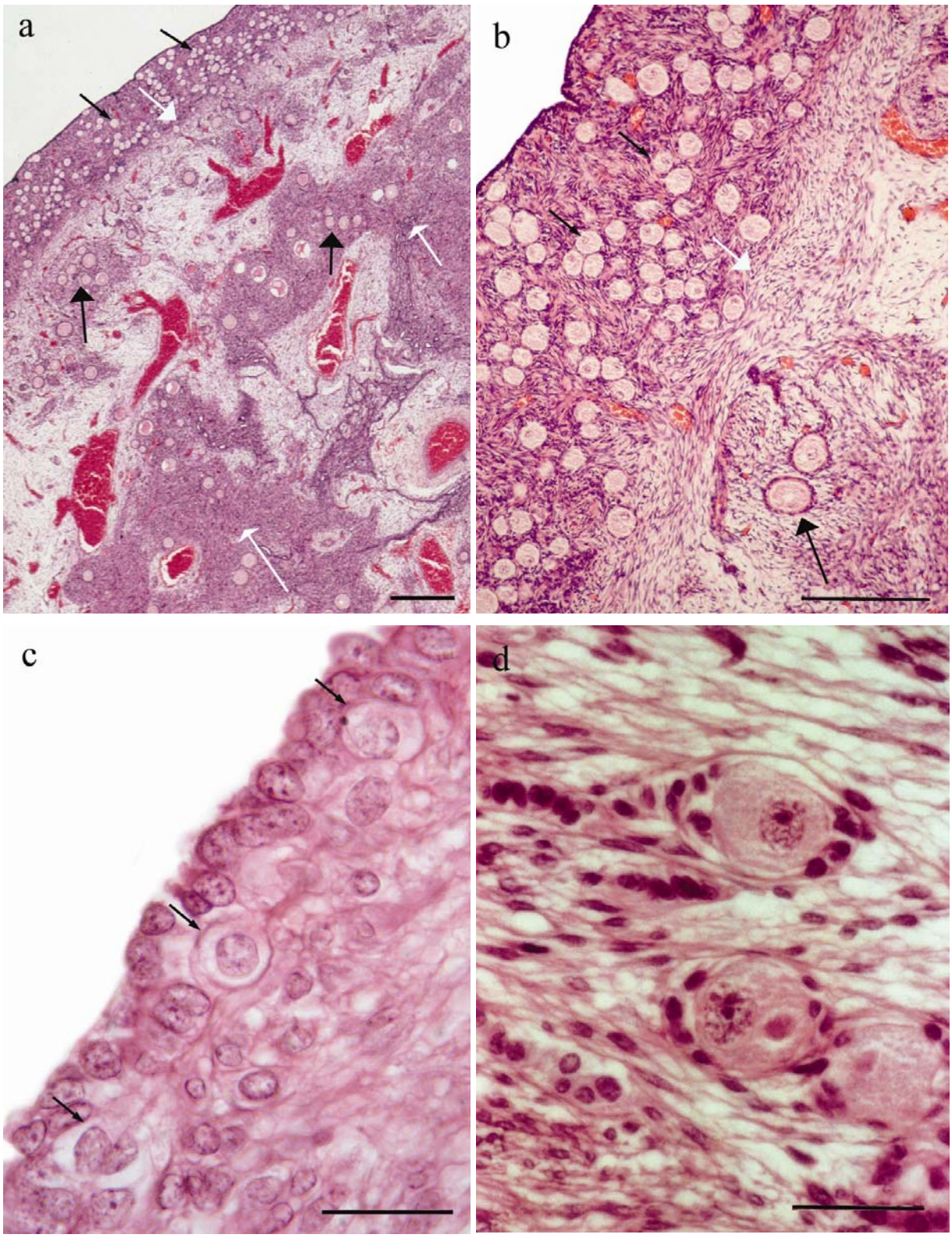


Figure 6.3 Sectioned ovary of an elephant fetus at 11 months of gestation

Continued

Figure 6.3 (continued)

a. Section from the surface epithelium to the central medulla showing small follicles throughout the cortex (small black arrows). The longer white arrow indicates the cortico-medullary border. In the medulla the black arrows mark groups of pre-antral follicles and the shorter white arrows areas of interstitial tissue (scale bar = 500 µm).
b. Higher magnification of the cortex and cortico-medullary border (white arrow). Small follicles within the cortex are indicated by small black arrows and growing follicles within the medulla by the larger black arrow (scale bar = 250 µm).
c. The surface epithelium of the ovary consisting of a monolayer of cuboidal cells juxtaposed to which in places are cells with the size and appearance of oogonia (black arrows; scale bar = 20 µm).
d. Early primary follicles showing the typical prolate shape (scale bar = 40 µm).

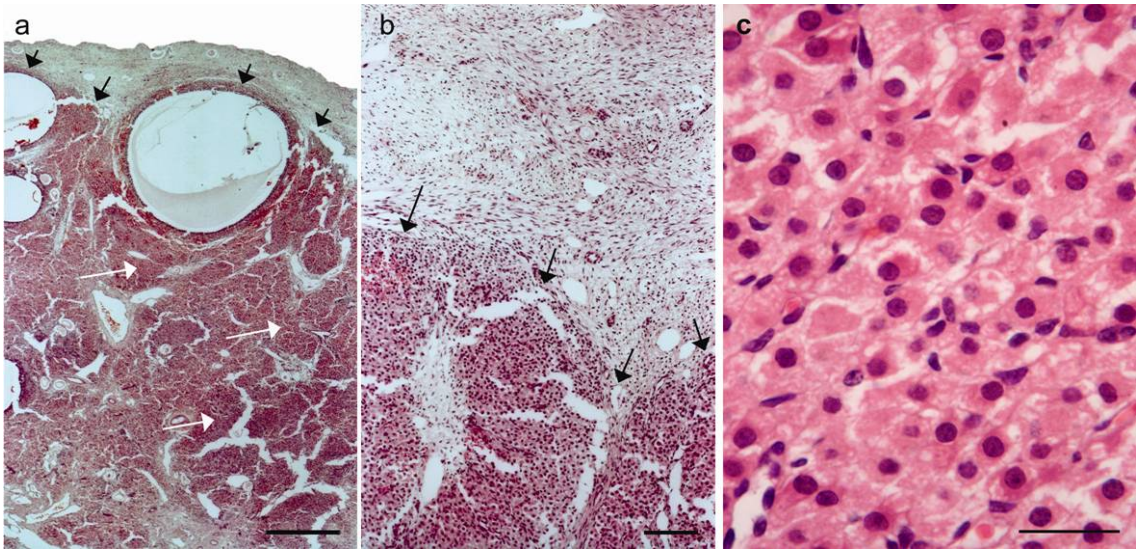


Figure 6.4 Sections of the ovaries of an elephant fetus at 13.5 months of gestation

a. The black arrows indicate the cortico-medullary border. The white arrows indicate interstitial tissue (scale bar = 700 µm).
b. The border between cortical tissue at the top of the photograph and interstitial tissue at the bottom (scale bar = 350 µm).
c. Higher magnification of the interstitial cells shown in b (scale bar = 20 µm).

The cortical region of the fetus at 15.2 months (Figure 6.5a) contained oocytes that typically showed a prolate association of granulosa cells similar to those in the early primary follicles shown in Figure 6.3d. These formed a narrow band beneath the surface epithelium. EP follicles measured 37.5–46.0 µm, the oocytes 25.0–31.0 µm and their nuclei 15.5–17.5 µm. The left ovary weighed 27.5 g and the right 21.5 g.

Maximum follicle growth within the specimens studied was observed around 16.7 months of gestation (Figure 6.5b) when antral follicles of 3–5 mm diameter were present in the medulla to give a total antral volume of around 11.2 cm^3 in both ovaries combined. The well-vascularised cortex had a depth of 400–500 µm and the ovarian surface remained smooth.

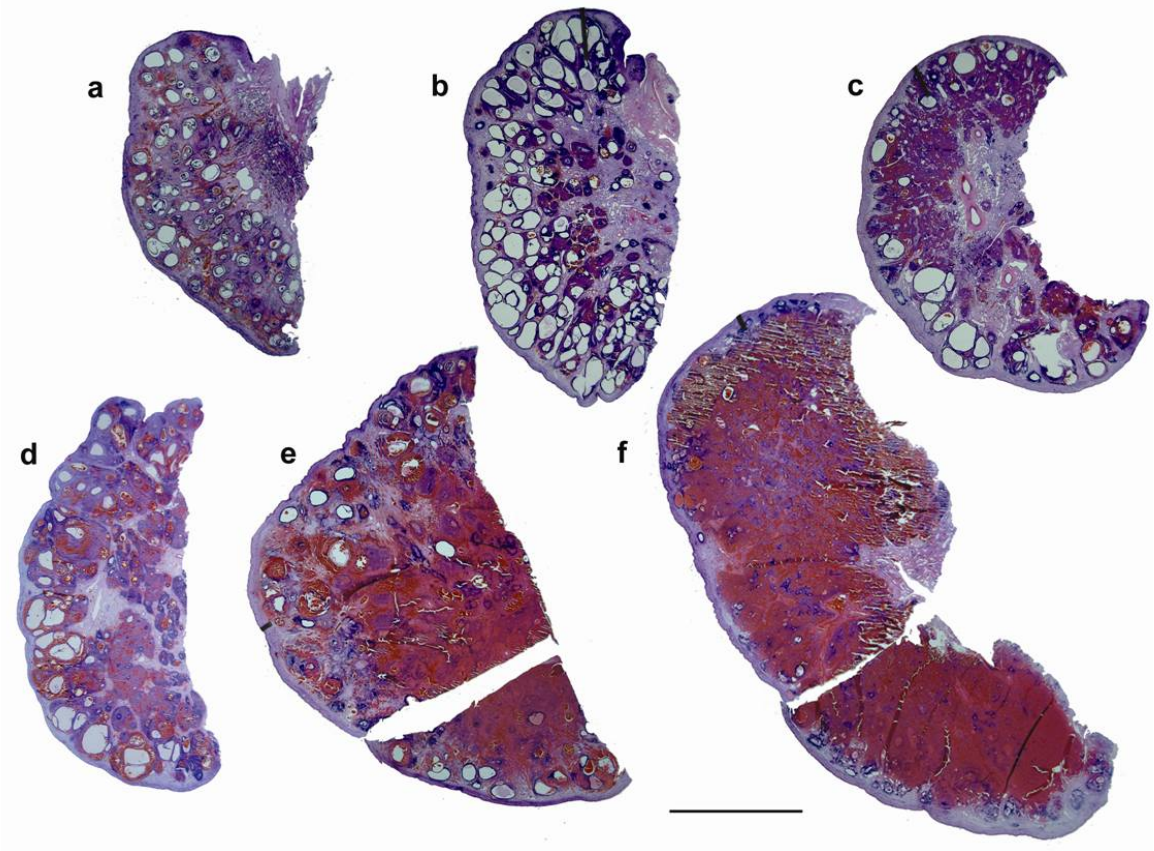


Figure 6.5 Photographs, taken above a light box, of 25 µm thick sections of fetal elephant ovaries recovered between 15 and 21 months of gestation (Scale bar = 10 mm)

a. 15.2 months; the cortical region surrounds a central medulla filled with small growing and antral follicles and dark staining interstitial tissue.
b. 16.7 months; showing maximum recorded antral follicle development.
c. 17.1 months; there are fewer antral follicles than in (b) and more interstitial tissue is apparent.
d. 17.5 months; increasing vascularisation of the ovary is apparent.
e. 19.3 months; ovarian size has increased significantly from (d) due to hypertrophy and hyperplasia of interstitial cells.
f. 20.2 months; the fetal ovary is approaching maximum size. Few antral follicles remain and, as in e), the interstitial tissue is partially obscured by extravasated red blood cells.

At 17 months (Figure 6.5c and Figure 6.5d) of gestation distinctly fewer antral follicles were visible in the central medulla where highly vascularised groups of interstitial cells now predominated. These were distributed throughout the stroma of the medulla and they contributed up to 54% of the volume of the ovary (Figure 6.2). Dense patches of interstitial cells or theca cells marked atretic antral follicles and glassy remnants of basal membranes (not shown) could also be seen. The cortex was typically 375–875 µm deep.

At 19 months of gestation distinctly fewer and smaller antral follicles were present in the medulla compared to the ovaries from younger individuals and these were confined to the border of the cortex which was typically 375–1125 µm deep. The bundles of interstitial cells appeared less densely crowded together than in earlier samples (Figure 6.5e).

Blood oozed from the cut surface of each purple-coloured ovary recovered from the fetus at 19 months and the one at 20.2 months of gestation, illustrating the intense vascularization of the medulla in particular at these later stages (Figure 6.6a and Figure 6.6b). Many fewer antral follicles were present and these were smaller than in the ovaries of younger fetuses, and were now distributed only along the cortico-medullary border (Figure 6.5f). Interstitial cell bundles were widely spread throughout the medulla and in the fetus at 20.2 months of gestation they occupied approximately 93.3 cm^3, which was about 60% (Figure 6.2) of the total volume (151 cm^3) of the two ovaries combined (Figure 6.1). The combined cortical volume of the two ovaries of the 20-month old fetus was 18.8 cm^3 which was some 2.5 times larger than that of the 15-month fetus (7.6 cm^3) although the proportional contribution to ovarian volume was the same because the ovarian volume of the 20-month fetus was much larger than that of the 15-month fetus (Figure 6.2).

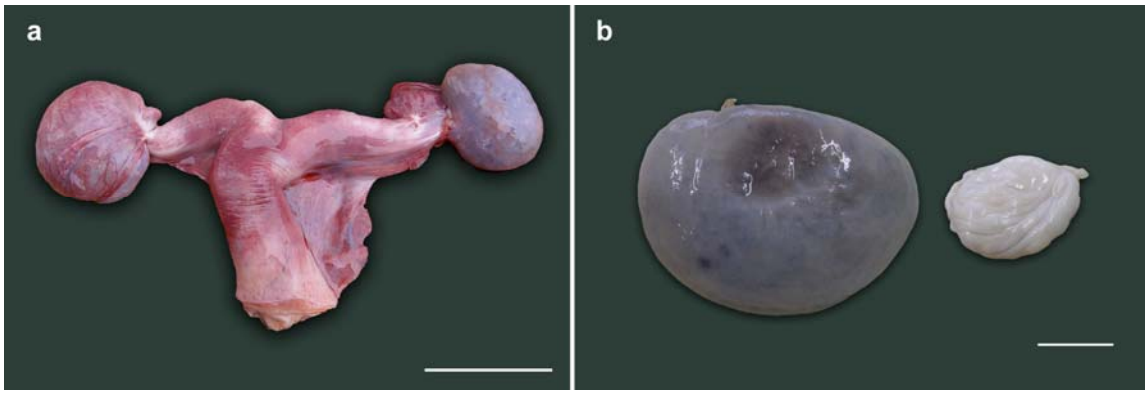


Figure 6.6 The ovaries of two late-stage African elephant fetuses

a. The uterus and ovaries of an elephant fetus at 17.5 month of gestation. The ovary on the left is still enclosed within its ovarial sac (Scale bar = 50 mm).
b. Ovary from a 20.2 month old elephant fetus on the left and from a 6 month old calf on the right, showing great shrinkage around the time of birth due to a significant reduction in the amount of interstitial cells (Scale bar = 20 mm).

6.3.2. Prepubertal calf ovarian morphology

In the youngest female calf, examined at 2 months of age, ovarian weight (13.1 and 13.4 g) and volume was dramatically lower than those of late fetal life (Figure 6.1), predominantly because of a significant reduction in the volume of interstitial cells and the organisation of extravasated blood (Figure 6.6b). The cortical tissue appeared denser than in fetal life, contained many fewer blood vessels and ranged from 450 to 1000 µm in depth (Figure 6.7a). The distinctly fewer and smaller (2 x 2 mm) antral follicles along the cortico-medullary border occupied <2% of the ovarian volume. By two months after birth the islands of interstitial tissue which, along with blood vessels and remnants of atretic follicles occurred throughout the medulla, were smaller than those observed during late fetal life and occupied only 14 cm^3 per animal compared to 93 cm^3 in a 20-month old fetus. Furthermore, more stromal cells were observed among the interstitial cells than was the case in younger animals. At 4.5 years of age interstitial tissue had reduced to 3% of ovarian volume (Figure 6.2). Cortical depth varied from 2 to 3 mm and became further extended by the presence of antral follicles.

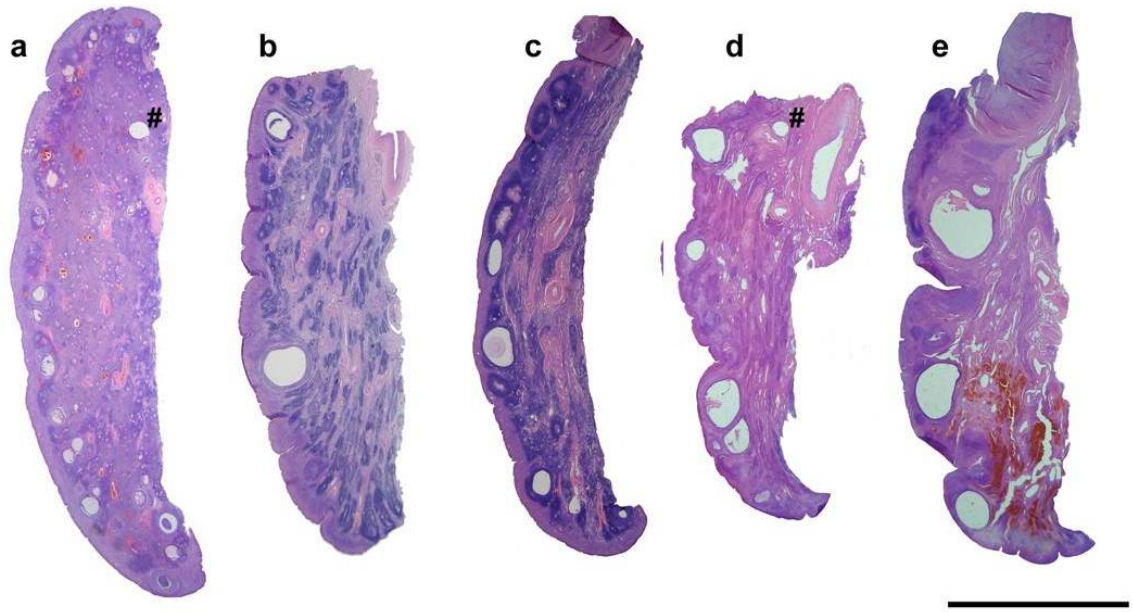


Figure 6.7 Photographs of 25 µm thick sections of the ovaries of prepubertal elephant calves (Scale bar = 10 mm)

a. At 2 months of age abundant interstitial tissue is visible within the medulla. Several small antral follicles are seen on the cortico-medullary border. These are now regarded as being in the cortex even though they may bulge into the medulla. Artifacts marked with a # are pin holes.
b. At 1 year of age the cortex is deeper than in (a) and isolated larger antral follicles may be seen. Interstitial tissue is present in streaks within the medulla.
c. At 1.5 years of age, interstitial tissue is still visible within the medulla and antral follicles occur more frequently than in (b).
d. By 3 years of age the ovary is settling into its adult pattern of antral follicles residing wholly within the true cortex.
e At 4.5 years the ovary is now starting to enlarge due to a considerable increase in the volume of the cortex.

During the early months after birth scattered deposits of haemosiderin were observed throughout the ovary reflecting the previously mentioned intense vascularisation of the tissue in late fetal life. The distribution of tissues within the postnatal ovary is detailed in Figure 6.2. By 6 years of age interstitial tissue occupied <1% of ovarian volume and the general morphology of the ovary had settled into its "adult" form (Figure 6.7e).

6.3.3. Immunohistochemical staining of interstitial cells

The whorls of interstitial cells in the fetal ovary stained with increasing intensity (Figure 6.8a) during the second half of gestation for 3β-hydroxysteroid dehydrogenase (3β–HSD),

the steroidogenic enzyme necessary to convert pregnenelone to progesterone and other progestagens, as described previously by Allen *et al*. (2002).

Initially, from around 11 months of gestation the cytoplasm of the 3β–HSD positive cells stained evenly and diffusely but, again as gestation advanced, groups of interstitial cells were observed in which the staining pattern within the cytoplasm became very intense and granular (Figure 6.8b). The thecal cells of the growing preantral and antral follicles (from about 400 µm in diameter) also stained positively but less intensely. Of great interest was the finding that the interstitial cells continued to stain strongly for 3β–HSD activity after birth (Figure 6.8c). The incidence and intensity of staining declined steadily during the first 2 years of postnatal life although there were still small patches of 3β–HSD positive interstitial cells visible at 2.5 years of age (Figure 6.8d). Of interest also was the 3β–HSD positive staining of the cytoplasm of the granulosa cells surrounding the small follicles (Figure 6.8e) which began around 16 months of gestation. Prior to this only the oocyte cytoplasm had stained lightly. This cyctoplasmic staining of the granulosa cells continued in the SF present in the cortex, and those in the medulla, during fetal life and up to at least 5 years after birth. With progression to the secondary stage of follicle development and onward, the granulosa cells no longer stained positively.

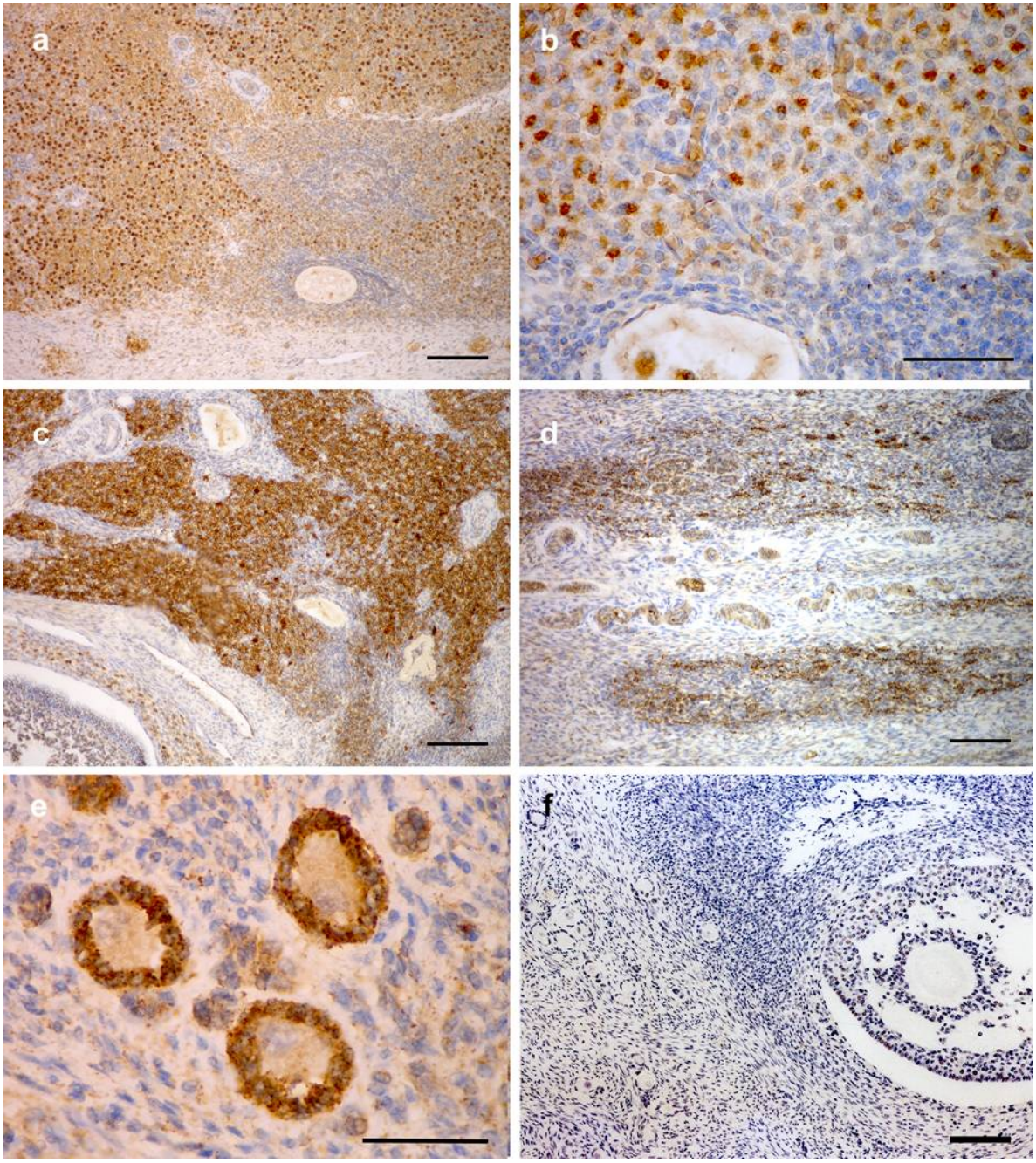


Figure 6.8 Sections of the ovaries of elephant fetuses and calves stained immunocytochemically with an anti-3β–HSD antibody

a. Interstitial cells in the ovary; fetus at 17.5 months of gestation (scale bar = 350 µm).
b. Higher magnification of the interstitial tissue showing the intense, granular staining of 3β–HSD (scale bar = 40 µm).
c. Positively stained interstitial tissue in a 2 month old calf (Scale bar = 350 µm).
d. 3β–HSD staining of groups of interstitial cells in the ovary of a 2.5 year old calf (scale bar = 350 µm).
e. The granulosa cells of small follicles within the cortex (and medulla in the fetal ovary) stained precisely and strongly for 3β–HSD activity in all the samples examined between 16 months of gestation and 5 years of age (scale bar = 15 µm).

Continued

Figure 6.8 (continued)
f. Negative control showing the complete absence of staining following replacement of the primary anti-3β–HSD antibody with an unrelated mouse monoclonal antibody (scale bar = 350 µm).

6.3.4. Ovarian weight

Figure 6.5 demonstrates the change in ovarian size with age. Ovarian mass differs by age group ($P<0.001$), the ovaries of fetuses at 15–20 months of gestation and elephants 4½–8 years of age are larger than those of elephants between 2 months and 4½ years in age.

6.3.5. Follicle number

Figure 6.9 shows the numbers of SF per elephant for fetuses and calves of different ages. No TPM follicles were seen. EP follicles (SF with a single layer of granulosa cells, of which most are flat and few cuboidal) were abundant and constituted between 63 and 99% of all SF. TP follicles (SF with a single layer of cuboidal granulosa cells) were less abundant; the maximum observed was 419 717 per animal (Figure 6.9). The number of EP follicles as well as the total number of SF depend on age ($P = 0.01$), being highest in fetal life and in Group 3 (Table 6.1), whereas the number of TP follicles tends to remain more constant ($P = 0.06$) but increases with age as a percentage of total SF. Without including outliers the numbers of TP as a percentage of SF were: Group 0 ≤2.6%, Group 1 ≤5%, Group 2 ≤12%, Group 3 ≤16%. The number of TP was lower in calves aged 2.5–4.5 years than in calves aged 4.5 years or older (Table 6.1). Cortical volume increased with age in prepubertal calves (Figure 6.10).

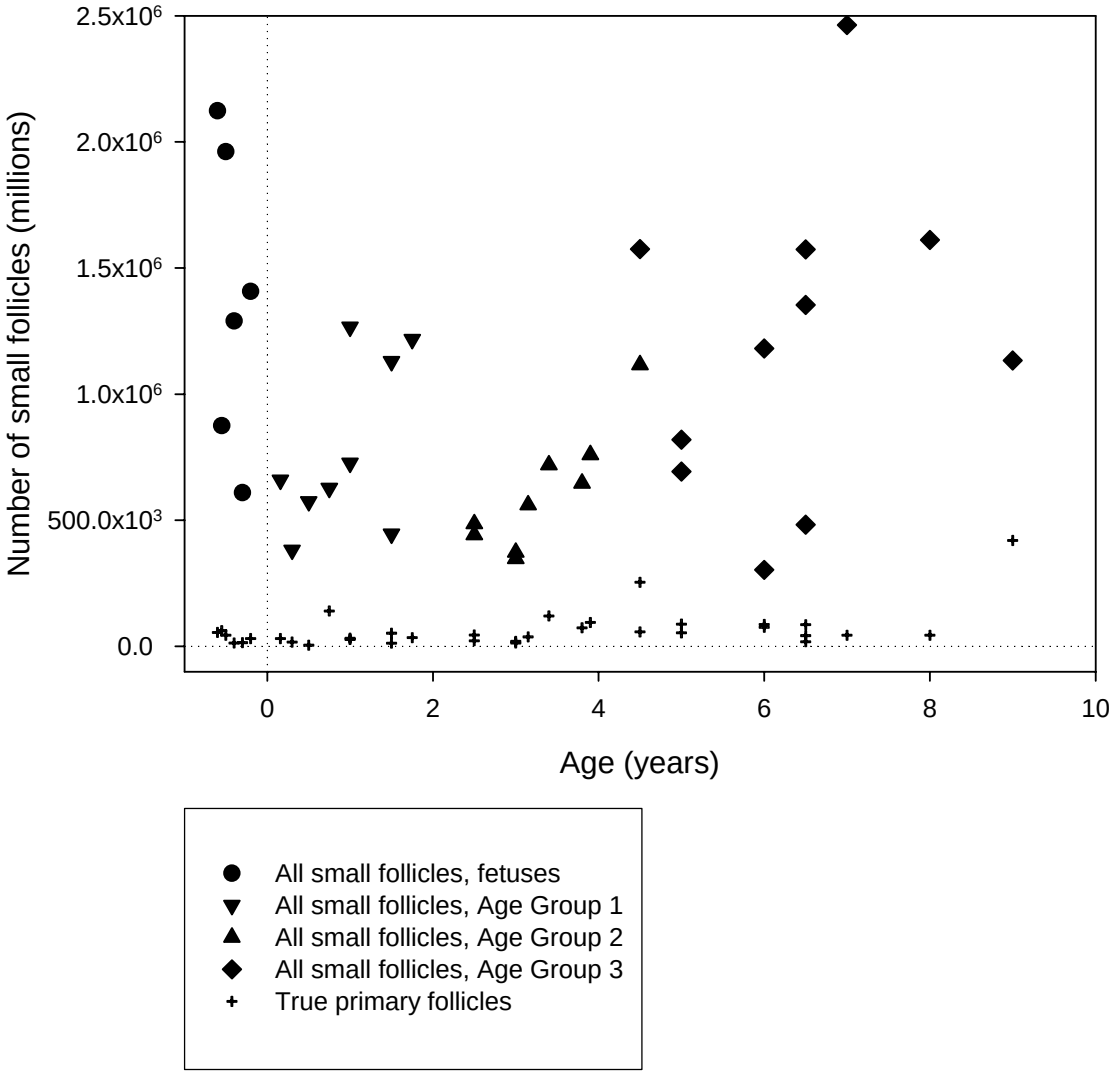


Figure 6.9 The number of small follicles in the ovaries of elephant fetuses and prepubertal calves in relation to age

Table 6.1
The median (95% confidence interval) combined number of small follicles in both ovarian cortices of African elephant fetuses and prepubertal calves

			Type of small follicle		
Age group	Age (years)	n	Early primary	True primary	All
0	-0.6 to –0.16[a]	6	1 336 570 (596 440–1 957 260)[b]	37 506 (13 390–62 875)	1 348 101 (609 623–2 123 103)[b]
1	0.16–2	9	627 680 (433 039–1 181 875)	31 298 (12 827–52 498)[b]	658 978 (445 867–1 216 548)
2	2.5–4.5	9	523 065 (359 989–664 542)[cd]	45 268 (19 857–95 113)	561 023 (373 157–759 654)[cd]
3	4.5–9	11	1 093 978 (463 416–1 530 106)[e]	76 118 (43 085–88 172)[c]	1 180 948 (482 007–1 575 054)[e]

[a] Ages below zero refer to fetuses and indicate the fractions of a year before birth
[b,c,d,e] Within a column, medians marked with [b] differ from those marked with [c] and those marked with [d] differ from those marked with [e] ($P<0.05$)

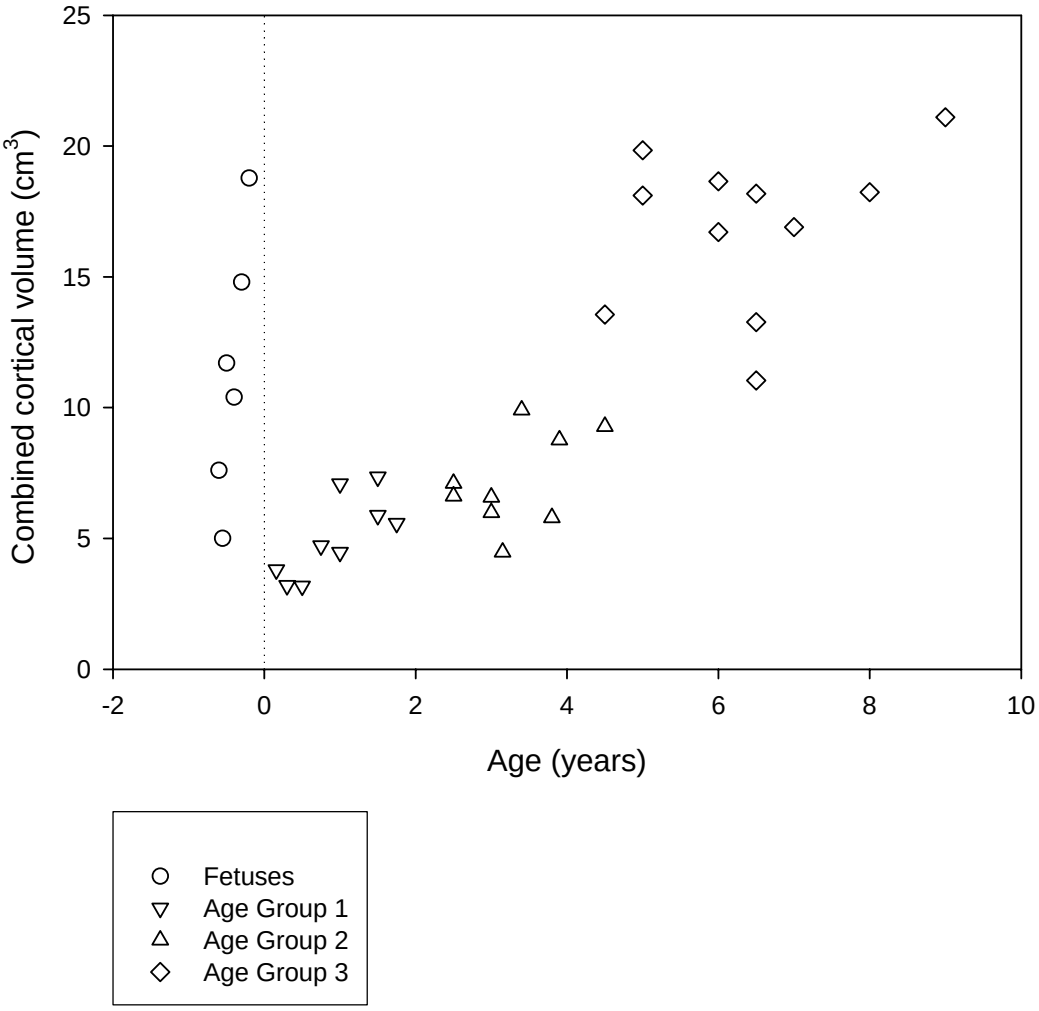


Figure 6.10 Cortical volumes of fetal and prepubertal ovaries of African elephants

6.4. Discussion

6.4.1. Late fetal and prepubertal ovarian morphology

The foregoing described the morphological development and differentiation of the ovaries of the African elephants and the numbers of small follicles they contain, from 11 months of gestation to a prepubertal age of 9 years. Although each measurement and description of the ovaries are independent observations in different individuals, the pattern of change

over time is suggestive of development and in the absence of longitudinal studies provides the only currently available data.

At 11 months gestation very few naked oogonia remain identifiable within the cortex of the fetal ovary and nearly all the germ cells are present as meiotic oocytes arrested at the dictyate stage of prophase I (Kidson *et al*. 1995) and surrounded by a single layer of granulosa cells which vary in shape from flat to cuboidal in the same follicle. These follicles within the cortex appear to form the follicle reserve for future reproductive life. The many follicles observed within the medulla of the elephant fetal ovary do not share this future and are destined for growth — beginning around 12 months gestation — to small antral stages before the onset of atresia which may occur at any developmental stage thereafter up to mid antral size. Fetal ovarian weight and volume increase from 11 months with the onset of interstitial cell hyperplasia and the growth of medullary antral follicles. By 15 months, as described previously by Allen *et al*. (2005), the increase is significant. The maximum number of antral follicles is observed around 16 months following which they decline to occupy less than 2% of ovarian volume at birth. Meanwhile, the volume of interstitial tissue continues to grow toward a maximum point thought to occur just prior to birth (22 months), although this could not be confirmed in the present study as the oldest set of fetal ovaries collected was at 20.2 months of gestation. Loss of small follicles and pre-antral follicles has been observed in other mammalian species during fetal life (Gosden 1995). The reasons for this loss are speculated upon (Pepling & Spradling 2001; Tilly 2001) and are now generally referred to as natural wastage. The present findings suggest, however, that the elephant fetus may employ its medullary follicles for a useful purpose; ie the production of greater amounts of interstitial tissue as discussed below. In turn, the rising steroid production within the fetal ovary may halt the further development of antral follicles.

How and why the small follicles within the cortex are protected from recruitment to pre-antral growth and the fate of atresia is not known, nor is it known in other species what causes activation of some TPM and not others during fetal and post natal life (McLaughlin & McIver 2009). What seems likely from this elephant study is that recruitment is intimately related to local environment, this being stimulative in the medulla and restrictive in the cortex during the second half of fetal life.

During postnatal life a remarkable feature of the elephant ovary is the persistence of interstitial tissue within the medulla, otherwise it develops in a manner similar to the bovine (Van Wezel & Rodgers 1996) and other mammals.

6.4.2. Interstitial cells

Interstitial cells within the mammalian ovary are not static components (Kingsbury 1914). They differentiate from stromal fibroblasts, either independently (primary) or when they become associated with growing follicles (Mori & Matsumoto 1970; Peters & McNatty 1980) and they revert to this cell type, either independently or following follicle atresia (Peters & McNatty 1980).

In equids, interstitial cells in both the fetal testis and fetal ovary begin to multiply rapidly in the absence of any follicle or seminiferous tubule growth from around day 80 of gestation. This interstitial cell hyperplasia and hypertrophy continues unabated to days 220–250 of gestation when the fetal ovaries weighing 50–100 g each are considerably heavier than the now-inactive maternal ovaries (Cole *et al*. 1933; Hay & Allen 1975). The tightly packed epithelioid interstitial cells secrete large quantities of 19-carbon androgen precursors, including androstenedione and dehydroepiandrosterone (DHEA; (Bhavnani *et al*. 1969; Bhavnani *et al*. 1971) and the very unusual 3-ß hydroxy-5,7 pregnanedien-20-one and 3-ß hydroxy-5,7 androstadien-17-one (Tait *et al*. 1983). The latter are then aromatised by the placenta to produce the relatively enormous (µg/ml) quantities of both phenolic (oestrone and oestradiol) and Ring B unsaturated (equilin and equilenin) oestrogens that are present in the blood and urine of pregnant mares during the second half of gestation (Cox 1975; Raeside & Liptrap 1975). These appear to be important for growth and development of the very precocious equine fetus at birth (Pashen & Allen 1979). Similar interstitial tissue development results in ovarian hypertrophy in the grey and common seals, also without antral follicle formation (Amoroso *et al*. 1965). In both horses and seals the interstitial tissue declines rapidly in late gestation so that the gonads have shrunk to their normal prepubertal size at birth (Amoroso *et al*. 1965; Gonzalez-Angulo *et al*. 1975; Hay & Allen 1975).

As described by Hanks (1971) and Allen *et al*. (2005), and confirmed in the present study, gonadal enlargement also occurs in the elephant fetus during the second half of gestation due to a similar hyperplasia and hypertrophy of primary interstitial cells augmented by

secondary interstitial tissue that persists following atresia of antral follicles. And as also demonstrated in the present study, these hypertrophied primary interstitial cells stain for the steroidogenic enzyme, 3β–HSD which indicates that they are capable of synthesising progestagens. Indeed, Allen *et al*. (2002) demonstrated that slices of elephant fetal gonad incubated with tritium-labelled cholesterol or pregnenelone secreted appreciable quantitites of 5-α dihydroprogesterone (5α-DHP) and other 5αpregnane derivatives into the culture medium. More recently, Yamamoto *et al*. (2011) demonstrated the secretion of placental lactogen (elPL) by elephant trophoblast tissue and speculated that this chorionic hormone may be the essential luteotrophic stimulus for the enlargement of the fetal gonads and their synthesis of progestagens to assist the accessory corpora lutea in the maternal ovaries to maintain the pregnancy state. The highly vascularised nature of the fetal ovary from 18 months of gestation to term, in addition to supporting the metabolic activities of the enlarged ovary, would also help to transport the progestagens being synthesized by the interstitial tissue to the fetal, and hence to the maternal circulation to boost the supply of progestagens for pregnancy maintenance.

An unexpected and interesting finding in the present study was the persistence of 3β–HSD positive nests of interstitial cells in the ovaries of female elephant calves after birth. Such steroid-secreting tissue accounted for some (30–40%) of the total volume of the ovary during the first 6 months of life and it declined slowly thereafter to disappear completely only at around 4.5 years of age. It seems reasonable to speculate that continued secretion of progestagens by these interstitial cells during early post natal life may act locally to suppress any significant growth of antral follicles during the period, and indeed, very little antral follicle development occurs in the first 1–2 years of life in the elephant calf. A few antral follicles develop later in the second year of life, coincidentally with the disappearance of the interstitial tissue and the rate of antral follicle growth increases markedly from 4.0 to 4.5 years onwards when the whole ovary begins to increase in size. Further research is planned to explore the steroid output of these post-natal cells.

Also of interest was the 3β–HSD positive staining of the granulosa cells of SF from 16 months of gestation till the oldest studied sample at 5 years of age (Figure 6.8e) as indeed do the Sertoli cells of the testis at the same age (F J Stansfield and W R Allen, unpublished data). Light staining for 3β–HSD has also been described in the pig (Garrett

& Guthrie 1999), the sheep (McNatty *et al*. 2000) and the human (Gougeon 1996) ovary. It is highly irregular to obtain such a precise and specific staining for 3β–HSD in the granulosa cells of SF during what is considered to be a gonadotrophin independent stage of follicle development (Berne *et al*. 2004)). In the current study, the granulosa cells of follicles beyond the secondary stage of development did not stain positively.

It is normal in mammalian ovaries for the production of androgens to take place exclusively in the theca cells of the developing follicle (Johnson & Everitt 2004) and theca cells are usually first observed when the follicle has more than 2 rows of granulosa cells. In the rat these theca cells are capable of producing steroids just prior to antrum formation (Teerds & Dorrington 1993; Young 2010). In this study 3β–HSD stained theca cells of small antral follicles starting around 400 µm in diameter were observed from 16 months of gestation to 5 years of age.

6.4.3. Follicle numbers

The stereological measurement of the numbers of small follicles in the ovaries of 29 prepubertal elephant calves and 6 late gestation fetuses revealed a wide between-animal variation in numbers (range 303 084–2 456 741) during the period; the close agreement in follicle numbers between the two ovaries of each animal gave great confidence as to the validity of the counting method (Stansfield *et al*. 2011a). Natural variation in SF numbers between animals of similar age is commonly observed in many mammalian species. For example, Schmidt *et al*. (2003) revealed variations of more than two orders of magnitude following cortical biopsies of human ovaries and Hansen *et al*. (2008) showed similar results in a review of small follicle numbers in 122 women from birth to 51 years of age.

The number of SF fell during late fetal life, as observed in many mammalian species (Johnson & Everitt 2004). At birth and during the first 9 months of life in young post natal elephants (n = 4) approximately 560 919 (SD 124 609) follicles were calculated to be present in the ovaries. Yet in the period approaching puberty (age 6–9 years, n = 8) the mean number of SF was 1 261 593 (SD 676 417). These follicles were evenly distributed between the left and right ovaries as observed previously (Stansfield *et al*. 2011a) and follicle density decreased coincidentally with the age-associated increase in cortical volume of the ovary (Figure 6.10). No true primordial follicles were observed so,

as in the previous studies (Stansfield 2006; Stansfield *et al*. 2011b), the SF pool was taken as being composed basically of EP and TP follicles.

This very unexpected finding of a significantly larger (P = 0.017) mean number of SF in the ovaries of the elephant calf groups between birth and 9 years of age, especially between 4 and 9 years (P = 0.014), raises a number of interesting questions as to how such an unusual situation might occur. Possible explanations include genetic diversity and post natal oogenesis. The additional possibility of error in calculating volume was also examined closely.

6.4.3.1 Biological variation

Relatively few animals could be included in the study (n = 6 fetuses and 29 calves) and biological variation may indeed have impacted on the apparent increase in numbers of follicles counted after birth (Forabosco & Sforza 2007; Wallace & Kelsey 2010). It is known that in women the age of menopause, brought on by exhaustion of the follicle reserve, is highly heritable and related to the size of the follicle reserve at birth (Hansen *et al*. 2008). It may be speculated that the ovarian follicular reserve in elephants is similarly heritable.

Paternally, natural variation could involve the presence of different dominant mating bulls as sires of the calves above and below 4 years of age. From a maternal perspective it is noteworthy that nearly all of the original 670 elephants which constituted the founding population of the Savé Valley Conservancy were introduced during 1991 and 1992 from a closed population in nearby Gonarezhou National Park and no further translocations have been made during the ensuing 20 years while population size has increased by natural breeding alone to an estimated 1500 animals. Predominantly, family groups were introduced originally to the Conservancy so the population is biased towards females with few mature mating bulls.

Given that the SVC population was only founded 20 years ago, reproduction may have proceeded in such a way that genetic transmission occurred more within the family groups as they were originally constituted, rather than among family groups. If this were so, and if the genetic capacity towards the establishment of the ovarian follicular reserve differed among founding family groups, such differences among family groups may have

persisted to the time the experimental subjects were culled. If the latter was the case and given that the calves used in the current study were from different family groups, then the effect of genetic differences among family groups may explain the observed higher number of SF in the older calves.

6.4.3.2 Volume calculations

The estimation of the volume of the ovarian cortex may be suspected as a source of error to produce the higher, instead of the expected lower numbers of SF in the calves older than 4½ years compared to those 2½–4½ years. The formula for the derivation of the number of SF per ovary in an elephant calf may be simplified to $N = C \times \frac{V}{S}$, where

N is the total number of SF per ovary,

C is the number of SF counted in all unbiased counting frames in which SF were counted

V is the estimated volume of the ovarian cortex and

S is the combined volume of all unbiased counting frames in which SF were counted. From the formula it appears that the number of SF in the ovary depends on the estimated volume of the ovarian cortex.

Having found a larger average value of N for older calves than younger calves, the question arises whether biased estimation of N may have caused a spuriously higher number of SF in the ovaries of older calves. N in either the older calves or the younger calves or both groups may only be biased if either the value of C is biased or if the value of $\frac{V}{S}$ is biased in either or both groups of calves.

C may be biased if areas that are actually part of the cortex — and may therefore contain SF — are considered to not be part thereof. In this way the numbers of SF in fewer unbiased counting frames will be counted during tessellation of the counting frame, resulting in a lower value of C. At the same time both V and S will be lower by the same proportion than they should be, implying that the value $\frac{V}{S}$ would remain the same as it would be if the cortex was demarcated correctly.

Changing *V* and *S* by the same proportion, for example by considering areas outside of the cortex as falling inside the cortex (or considering areas that are actually inside the cortex as falling outside thereof) for both, the determination of *V* and *S*, will not affect the value of $\frac{V}{S}$ and would therefore not cause bias. The only cause of bias in the value of $\frac{V}{S}$ is if different criteria were used to firstly determine the margin of the ovarian cortex on a histology section for the determination of the number of points on the unbiased grid falling in the cortex and to secondly determine which unbiased counting frames fall in the cortex and which not during tessellation of the unbiased counting frame over the cortex on a histology section. The number of points of the unbiased grid falling on the cortex were determined on the same section as the one used for tessellation of the unbiased counting frame over the cortex and where the position of the cortex was unclear it was drawn on the section. The methods used were therefore such that it seems unlikely that the value of $\frac{V}{S}$ was biased between age groups.

There exist no reason why the accuracy with which the cortex was demarcated would differ between the group of calves older than 4½ years compared to the demarcation in younger calves, suggesting that this is an unlikely cause for bias.

Another factor that may cause bias by affecting the value of *C* is if SF were more easily observed in older calves than in younger calves, causing some SF to go unnoticed in younger calves. There exists no reason to suspect that this was the case. Once again, it seems unlikely that the value of *C* was biased among age groups.

Overall, the above arguments suggest that faulty estimation of the number of SF is an unlikely explanation for the higher number of SF seen in older calves compared to younger calves.

6.4.3.3 Post natal oogenesis

The third possibility of some form of post natal oogenesis occurring in the elephant is particularly intriguing. As mentioned in the Introduction, the localization of germ-line stem cells (GSC) of adult mouse and human ovaries (Johnson *et al*. 2004; Tilly & Telfer 2009) and the birth of pups following transplantation of female germ-line stem cells (FGSC) to the ovaries of irradiated mice (Zou *et al*. 2009) has raised significant, although

still controversial, doubts about the finality of meiotic arrest in the ovaries of all mammals during fetal life. It remains possible that GSC may persist in, or migrate to, the epithelium covering the outermost surface of the cortex of the elephant ovary during early post natal life which could, due to some hitherto unknown stimulus, multiply mitotically within the cortex before entering meiotic arrest as in fetal life prior to acquiring an outer layer of persisting granulosa cells to form new SF and so boost the reserve of these structures.

6.5. Conclusion

In conclusion the small follicle populations of the late-fetal and prepubertal ovaries of the African elephant were described along with the changing morphology of these organs. The changes noted represent a series of events that have been recorded only in the elephant and the giraffe species to date (Benirschke 2007b). The expansion of the interstitial tissue of the fetal ovary and its continued presence in early post natal life may well contribute to the control of follicle development in these early years. Further research is required to determine the reasons behind the 3β-HSD staining of the granulosa cells of small follicles in the specimens in this study, and also the variation of numbers of small follicles in the ovaries of prepubertal calves.

The content of this chapter has been submitted in a slightly different format to an accredited journal and is currently under review.

Chapter 7. The progression of small follicle reserves in wild African elephants (*Loxodonta Africana*) from puberty to reproductive senescence

7.1. Introduction

The accepted dogma for the ovarian reserve in mammals is a steady loss of small follicles through natural attrition and ovulation during pre- and post-pubertal life (Gosden 1987), leading to full depletion and reproductive senescence in the majority of individuals that achieve their maximum lifespan (Cohen 2004).

Elephants are one of the longest lived land mammals and it is therefore of interest to study their longevity of reproduction. Behavioural studies (Moss & Lee 2011) and information from culling exercises (Freeman *et al*. 2008; Hanks 1972; Laws 1969) support the concept of a long reproductive life but there has been no research into the capacity of the ovary to supply viable oocytes throughout life. A useful comparison may be made with women in whom the process of reproductive ageing is determined by depletion of the ovarian pool of non-growing follicles (Hansen *et al.* 2008). Women exhibit peak fertility during their mid-twenties after which there is a general decline and a constantly increasing rate of loss of small follicles until menopause and the consequential cessation of fertility at an age of 51±8 years (Faddy & Gosden 1996), following which they may experience a considerable post-reproductive lifespan. The ability of the human oocyte to be able to maintain a state of meiotic arrest for up to 50 years has been praised (te Velde & Pearson 2002) although this may be surpassed by the elephant oocyte's ability of over 60 years (Moss & Lee 2011). It is also of interest to make a comparison of the ovarian reserve in wild elephants with that of captive elephants in zoos in Northern Hemisphere countries (when *post mortem* specimens become available), the fertility of which is severely compromised (Brown *et al*. 2004a).

The first aim of this study was to determine whether there is a progressive decline in the number of small follicles (SF) in the ovaries of African elephants from puberty through sexual maturity and into old age. The second was to determine whether a depletion of the follicular reserve poses a constraint on fertility of old African elephants.

7.2. Materials and methods

7.2.1. Animals

Thirty-one pairs of ovaries were obtained from post pubertal elephants during managerial off-takes within private conservancies and from professional hunting safaris in Zimbabwe. Only the ovaries of non-pregnant elephants (Figure 7.1b) were used in the study due to the distortion of the cortex caused by the development of multiple large corpora lutea during pregnancy (Figure 7.1a).

Nine of the elephants were killed during managerial off-takes. They were shot in family groups with no prior knowledge of the composition of the family groups. Criteria for selection of the family was a distinct grouping of 10 to 17 elephants within the Conservancy in an area of overabundant population or one of human-elephant conflict. All the members of the family group were shot with medium to heavy caliber rifles by professional hunters. Ovaries collected from hunting safaris (n = 21) were from tuskless females placed on hunting quota by the Parks and Wildlife Management Authority of Zimbabwe, in an attempt to reduce the number of tuskless elephant in the national population. One set of ovaries was collected from a semi-domesticated elephant that died of natural causes.

The animals were aged according to Laws (1966) and Lee (2012). Additional data was collected on lower jaw dentition and on shoulder height and back length of each animal for ageing purposes.

The elephants were divided among 5 age groups: Sub-adults (10–15 years), when most elephants are between puberty and first calving (Laws 1969); Young Prime Adults (16–25 years); Older Prime Adults (26–35 years); Senior (36–50 years) and Old Adults (51–70 years).

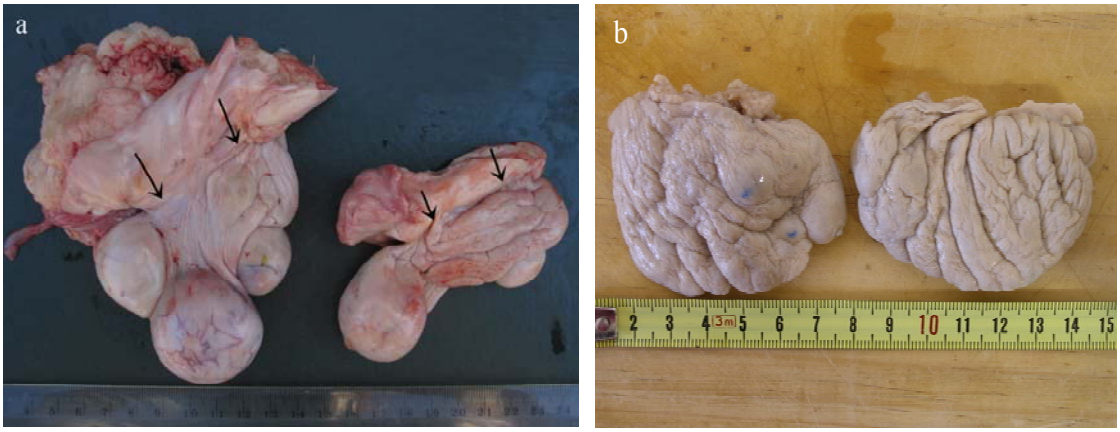


Figure 7.1 Ovaries from a pregnant and a non-pregnant African elephant cow

a. The two ovaries from a pregnant elephant cow (11 months gestation) showing the multiple corpora lutea of pregnancy, the ovary ipsilateral to the pregnancy is on the left. Arrows denote the attachment of the ovaries to the mesovarium.
b. The two sides of a bisected ovary from a non-pregnant cow.

7.2.2. Collection and processing of specimens

Collection and processing of the ovaries are described in Chapter 2. Briefly, the ovaries were removed from the carcass within 2 hours of death and fixed in 4% neutral buffered formalin. Subsequently, twenty 25 µm thick sections were cut from the ovaries of each animal using a uniform random sampling method (Howard & Reed 2005) and stained with haemotoxylin and eosin. A stereological protocol using Cavalieri's Estimator for volume and the unbiased brick for density calculations, as first described by Gundersen & Jensen (1987), were used.

7.2.3. Estimation of the age of *corpora nigra* (CN)

CN form as a result of degeneration of *corpora lutea* and remain in the ovaries for up to 77 months (Smith & Buss 1975). A sample group of 3 elephants aged 30–35 years old with CN in their ovaries were selected (some were pregnant and were not otherwise used in the current post-pubertal age group study) and the maximum size of CN in their ovaries measured. Age was defined as the interval between the origin of the CL that transformed into a CN and the time at which the ovaries were collected. Taking an elephant that was 21 months pregnant at the time of death as an example, the age of the cohort of CN that included the largest one was estimated as follows: Assuming that the cohort of CN

remained from the beginning of the previous pregnancy (Hodges 1998; Stansfield & Allen 2012); knowing that gestation length is 22 months (Moss & Poole 1983). Assuming that the duration of post-partum anoestrus is 24 months (Laws 1969; Williamson 1976), and knowing that the elephant was 21 months pregnant at the time of death (Craig 1984), it follows that the estimated age of the cohort of CN is 22 + 24 + 21, which amounts to 67 months.

7.2.4. Statistical analysis

Where the data did not meet the requirements for parametric tests medians of more than two groups were compared with the Kruskal-Wallis test or, for two groups, with the Wilcoxon Rank-sum test. When data met the requirements for a parametric test, a t-test was used to compare two groups. After the Kruskal-Wallis test, the Kruskal-Wallis z-test with Bonferroni's adjustment was used to compare pairs of age groups. For all comparisons α was set at 0.05.

The relationship between the numbers of SF and age were analysed in two phases: In the first, the age groups as they were prospectively defined were compared. In the second, visual appraisal of the numbers of SF in elephant against age led to a post hoc comparison of the number of SF of older prime adults with young prime adults in order to determine whether the follicular reserve was lower in the older group.

The variance of the number of TP follicles among age groups was compared by means of the Modified-Levene-Equal variance test. The number of TP in elephant of the different age groups were compared by means of a Kruskal-Wallis test, followed by pairwise comparisons using the Kruskal-Wallis z-test with Bonferroni's adjustment.

The coefficient of variation for the number of SF counted in each of the two ovaries was determined (Dohoo *et al.* 2009). NCSS Statistical Software 2004 (NCSS, Kaysville, UT, USA) was used for statistical analysis.

7.3. Results

7.3.1. The relationship between the number of small follicles and age

Age affected the number of SF per elephant (P<0.001). Figure 7.2 suggests that the number of SF per elephant is more variable in sub-adults and young prime adults than in older elephant. Pairwise comparison among age groups show that sub-adults and young prime adults have higher numbers of small follicles per elephant than old elephants (Table 7.1). A post-hoc comparison between young prime adults and older prime adults revealed that the young prime adults had more SF than the old prime adults (P = 0.01).

Table 7.1
The number of small follicles (SF) per elephant of different age groups

		Number of small follicles		
Age group (years)	n	Median	25th Percentile	75th percentile
Subadults (10–15)	6	253 455[a]	171 142	556 949
Young prime adults (16–25)	6	386 974[a]	162 257	613 635
Older prime adults (26–35)	6	105 054[ab]	44 776	154 750
Senior adults (36–50)	6	57 920[ab]	45 133	79 307
Old (51–70)	7	11 113[b]	0	50 837

[a, b] Medians not sharing a common superscript differ (P < 0.05)

Table 7.2 shows the number of SF in each of the 7 old elephant in the study. Two of the old elephant had no SF whereas a third had none in one ovary and a mere 3020 in the other. Five of the 7 had fewer SF than each other elephant in the study except one 29-year-old elephant that had no SF in one ovary and 2392 in the other.

Excluding the 2 elephants that had no SF in either ovary and the 2 that had no SF in one ovary and a low number (2392 or 3020) in the other, the CV of the difference between the number of SF in the two ovaries of an individual was 11.1% (n = 27), showing that the numbers of SF in the two ovaries of an elephant were quite similar.

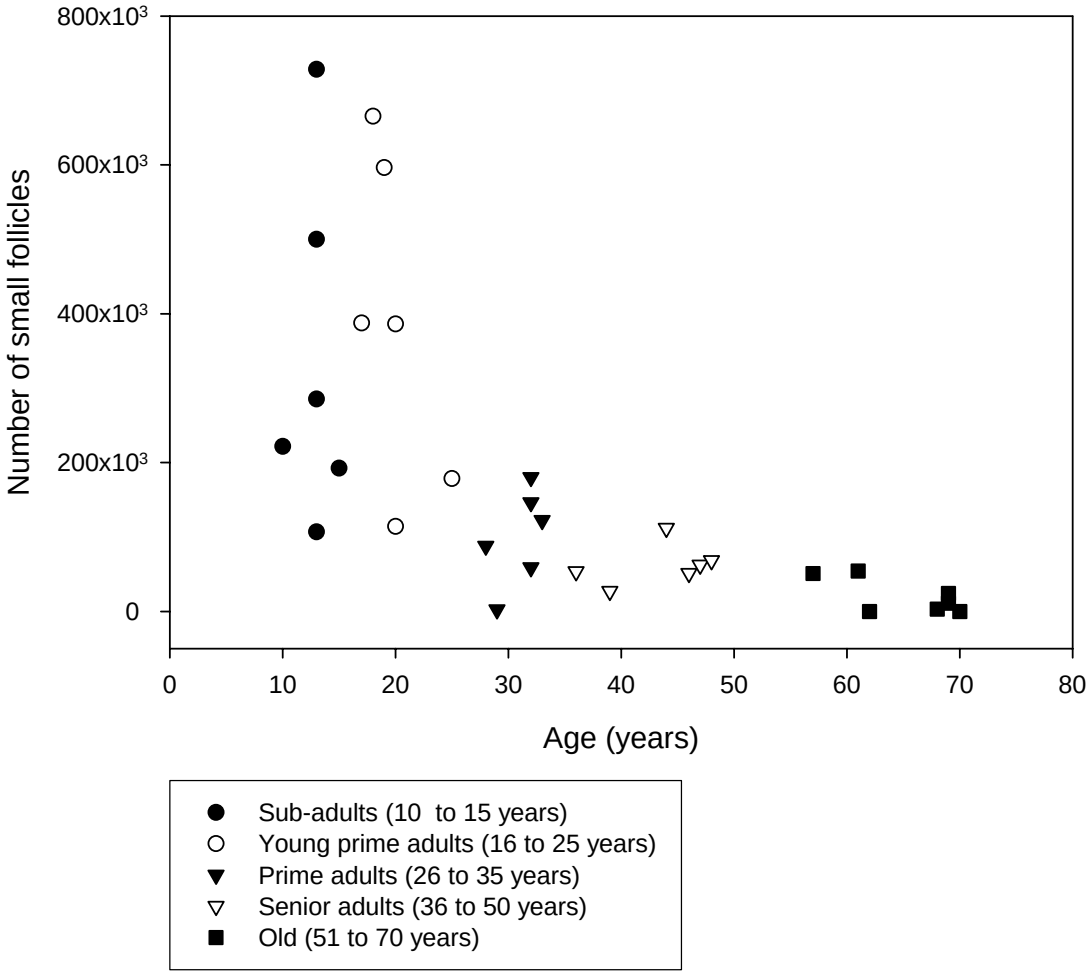


Figure 7.2 The total number of small ovarian follicles (SF) in elephant of different ages

Table 7.2
The number of small follicles (SF) in the two ovaries combined, as well as other signs of current or recent ovarian activity in 7 old African elephants

			Diameter of the largest structure (mm)[a]			
Elephant	Age (years)	Small follicles	Graafian follicle	Corpus luteum	Corpus nigrum	Lactation status
10–48	57	50 837	8		15	Lactating
19	61	54 337			11	Lactating
31	62	0	6		8	Lactating
54	68	3 020[b]			0	Lactating
09–14	69	24 278	6		15	Lactating
79	69	11 113	8		11	Lactating
64	70	0			5	Lactating

[a] An empty cell indicates that an elephant had no structure of the type that was visible with the naked eye
[b] All the small follicles occurred in one ovary, while the other had none

7.3.2. The relationship between the type of small follicles and age

Irrespective of their age, all but two elephants had 150 000 or fewer TP (Figure 7.3, Table 7.3) while EP follicle numbers were initially much higher than TP but became depleted by 45 years of age to leave only TP forming the follicle pool for the remainder of reproductive life.

The number of TP was similar among all age groups (median 46 770, 25th percentile 20 475, 75th percentile 89 120, $P = 0.18$), as was the variance ($P = 0.64$). The pairwise comparisons among groups revealed no differences ($P>0.05$).

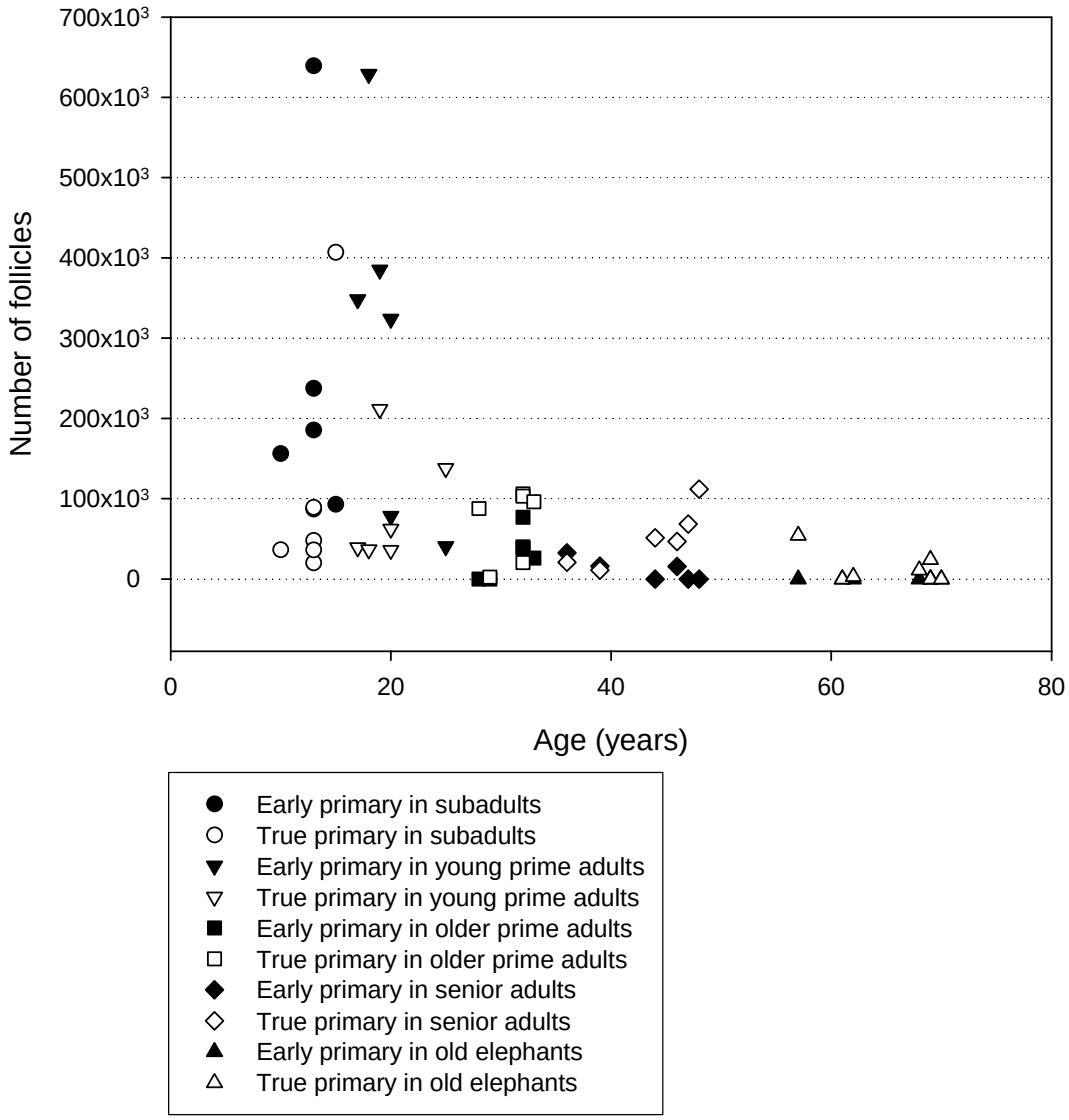


Figure 7.3 The numbers of early primary (EP)- and true primary (TP) follicles in the ovaries of African elephants of different ages

Table 7.3
True primary follicles (TP) as a percentage of total small follicles (SF) in the ovaries of elephants of different age groups

		Percentage		
Age group (years)	n	Median	25th Percentile	75th percentile
Subadults (10–15)	6	17.7	15.3	34.5
Young prime adults (16–25)	6	23.8	9.0	45.9
Older prime adults (26–35)	6	75.6	51.6	100
Senior adults (36–50)	6	87.5	40.5	100
Old (51–70)	5[a]	100	100	100

[a] Two of the 7 old elephant had no small follicles, and the percentage of all small follicles that were true primary did not apply to them

7.3.3. The relationship between reproductive status or tusklessness and the number of small follicles

The appearance of their ovaries suggested that the elephants belonged to one of four reproductive statuses: i) no antral follicle development (Figure 7.4a) and no CL but sometimes with CN, indicating post-partum anoestrus (Perry 1953); ii) few antral follicles (Figure 7.4b); iii) many antral follicles (Figure 7.4c) and iv) CL present (Figure 7.4d).

In the 10–15 year age group, 3 nulliparous females had a mean of 504 469 (SD 221 531) SF in their ovaries compared to a mean of 173 666 (SD 59 715) in the 3 females that were post calving ($P = 0.067$). Otherwise there was no relationship between either reproductive status ($P = 0.31$) or the tuskless state ($P = 0.46$) on follicle numbers.

7.3.4. Reproductive status of the old elephant

Table 7.2 shows that 6 of the 7 oldest animals in the study showed signs of recent ovarian activity in the form of antral follicles (Figure 7.4e), CL or large CN. A comparison of CN size in three 30–35-year-old cows is given in Table 7.4. The oldest animal, aged 70 years, had seemingly inactive ovaries with no SF reserve and no antral follicles present (Figure 7.4).

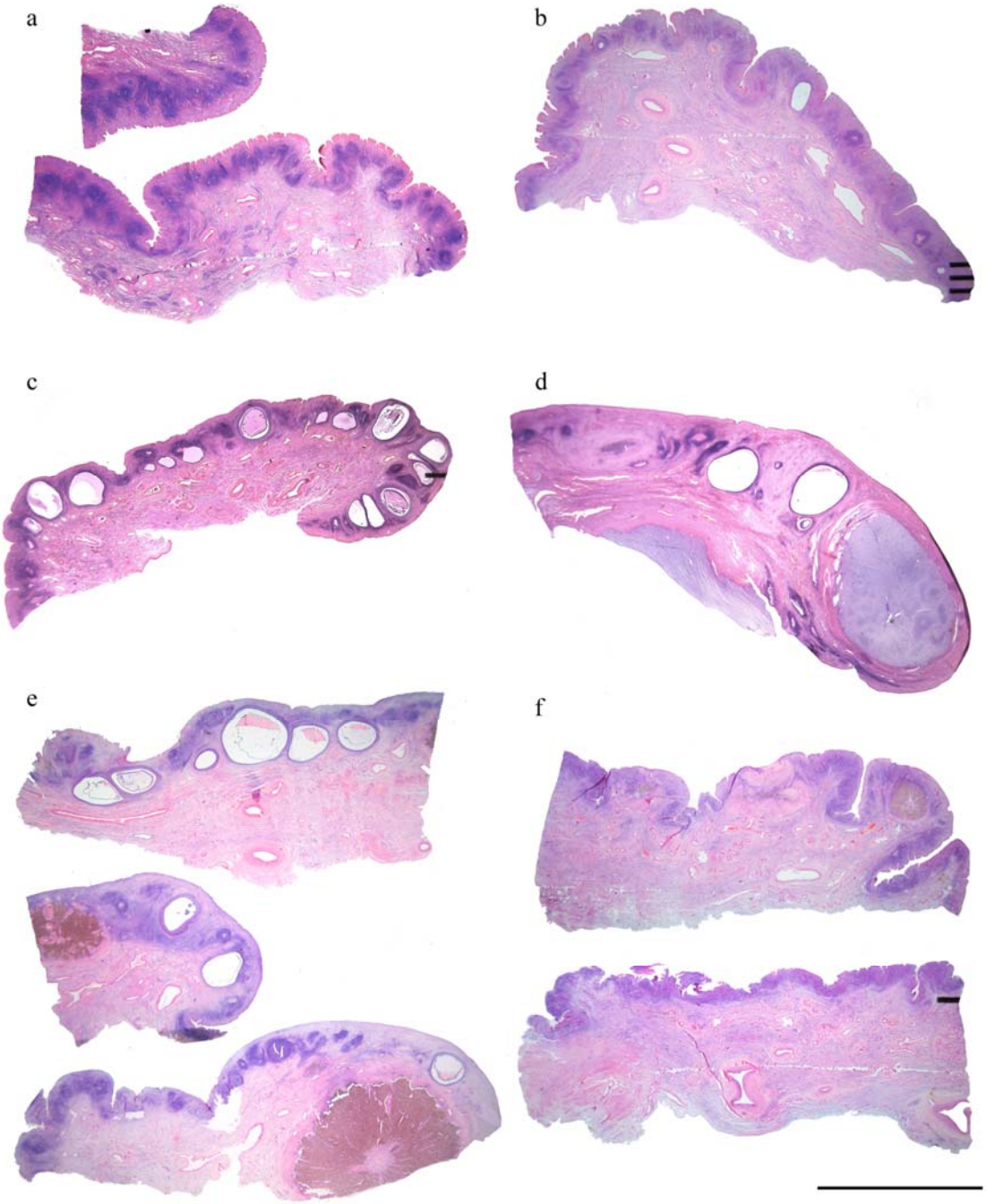


Figure 7.4 Photomicrographs of 25 µm sections cut in a transverse plain, perpendicularly to a longitudinal bisection of the ovary, revealing the cyclical changes within the cortex of the elephant ovary

a. An inactive-looking cortex from a cow in post-partum anoestrus.
b. Section showing a small degree of antral follicle development.
c. Section showing a large degree of antral follicle development.
d. Section showing antral follicles and a small corpus luteum.
e. Three segments from the 2 very active ovaries of a 69-year-old elephant.
f. Segments from the ovaries of a 70-year-old elephant which, apart from a small luteal remnant, are inactive. Scale bar = 10 mm.

Table 7.4
Comparative data of *corpora nigra* (CN) of known age in African elephants

			Corpora nigra	
Elephant ID	Age (y)	Status at death	Diameter (mm) [a]	Age (months) [b]
80	34	21 months pregnant [c,d]	5	67
11–17	30	5.7 months pregnant [c,d]	10	52
		Dam of 2.5 y old calf [e]		
11–12	32	Dam of 1.5 y old calf [e]	14	40

[a] The diameter of the largest CN in the elephant
[b] Estimated age of the cohort of CN that includes the largest one, assuming that the original CL formed at the beginning of the previous gestation and a 24-month lactation anoestrus preceded the current pregnancy
[c] Aged by fetal weight (Craig 1984)
[d] Assuming a 24-month lactation anoestrus preceded the current pregnancy
[e] Aged by calf jaw

7.4. Discussion

7.4.1. The follicle reserve after puberty

Natural variation in follicle numbers was found to be lower in the sexually mature age groups than during pre-pubertal life. The coefficient of variation for the numbers of SF in the two ovaries of an elephant was 11%, suggesting that the numbers of SF in the two ovaries of an elephant are quite similar.

This study shows that the size of the follicular reserve in African elephants depends on age. Comparing the follicular reserve of sub-adults (10–15 years of age), young prime adults (16–25 years of age), prime adults (26–35 years of age), senior adults (36–50 years of age) and old elephant (51–70 years of age) reveals that old elephant have lower follicular reserves than sub-adults and young prime adults, but similar to older prime

adults and senior adults. When, led by the data, only the young prime adult elephant group and the older prime elephant group were compared, it was found that the former had significantly more SF than the latter. Although not statistically significant, the median number of SF also became numerically lower from older prime adults to senior adults and finally to old animals, but larger numbers of elephants per age group need to be examined to determine whether or not a significant trend underlies this numerical pattern. Assuming that follicular reserves of the elephants included in the current study are representative of those of African elephants in general, it follows that the follicular reserve shows a significant decline during the third decade of life and that it is not uncommon for old elephant to have either no follicles remaining in their ovaries or a small number, in the order of 10 000 or fewer.

The old elephants in the current study did not die of old age. Hence, the current study does not answer the question whether or not the ovaries of all African elephants eventually become depleted of their follicle reserve. The current study does, nevertheless, suggest that it is not uncommon for old elephants, in their 7th decade of life, to have no follicular reserve remaining and that depletion of the follicular reserve would constrain fertility of old African elephants. This study also suggests that although the follicle reserve may finally become exhausted in African elephants it does not do so until very late in the total lifespan, which is brought to a close by erosion of the last set of molar teeth. This is well beyond the 45 year age of average life expectancy of a female elephant noted in the long term study in Amboseli Game Park in Kenya by Moss and Lee (2011).

As a result of natural variation such sustained fertility will not exist in all aged animals. Some individual had unusually low numbers of SF. For example, one 29-year-old individual had a total of only 2400 oocytes remaining in her ovaries. In this study, this low level of reserve was otherwise only seen in animals older than 57 years of age.

7.4.2. The change in follicle numbers around puberty

In Chapter 6, using the same methods and animals from the same population as those of the current study, SF were counted in 8 elephants, aged 6–9 years, which are the years just before puberty. They had 1 261 593 (SD 676 417) SF, which was substantially and highly significantly more than the 339 091 (SD 232 134) of the 6 sub-adults, aged 10–15

years, which were examined in the current study (one-tailed t-test, P = 0.003). This comparison between studies suggest a sharp drop in the number of SF from around puberty until the age of 15 years, which is when sub-adults usually calve for the first time.

7.4.3. A switch in type of small follicle constituting the reserve

Irrespective of their age, all but two elephants had 150 000 or fewer TP. EP numbers were initially much higher than TP but became depleted by 45 years of age to leave only TP forming the follicle pool for the remainder of reproductive life.

When sub dividing the population of SF into EP and TP the current study showed that the numbers of TP in the ovaries of postpubertal elephants was below 200 000 (Figure 7.3). True primary follicles, expressed as a percentage of the small follicle pool, increased from below 2.6% in fetal life to levels reaching 7% in calves aged 0–3.4 years and levels reaching 16% in young animals aged 3.5–9.5 years (Chapter 6) The current study showed that, irrespective of their age, postpubertal elephants generally had 150 000 or fewer TP. In spite of their fairly constant number, the TP constituted a progressively larger proportion of all SF until the proportion reached 100% by about 45 years. This change in ratio while the number of TP remained fairly constant is due to a gradual reduction in the number of EP. It is not known whether the number of EP decreases mainly due to atresia or due to conversion to TP, or due to both processes. EP are no longer present from around the age of 45 and for the remaining reproductive years only TP type follicles hold the oocytes.

TP follicles have been classed as growing follicles or non-growing follicles in other mammals (Hansen *et al.* 2008; Picton 2001), the point of follicle activation being determined by the expansion of the first granulosa cells from a squamous to a cuboidal shape. As previously noted, elephant ovaries contain almost no TPM throughout life, their follicle pool or reserve being composed entirely of EP and TP. It is not known whether the SF designated as EP and TP in elephant are resting and non-growing, or whether they are growing very slowly. If the TP in elephant older than about 45 years are growing, they have to do so sufficiently slowly to ensure that the stock of TP present in the ovaries at 45 years of age may last a further 20–25 years.

The number of SF in the single nulliparous semi-domesticated elephant (age 28) included in the current study was within the usual range for her age, although all these follicles showed a TP morphology. Being nulliparous, the state of her follicle reserve may be compared to captive nulliparous zoo elephants that fail to breed beyond 30 years of age.

7.4.4. The value of the current study with respect to understanding infertility of Zoo elephants

The population of elephants maintained in zoos in North America and Europe is not self sustaining (Brown *et al*. 2004a; Dow *et al*. 2011; Proctor *et al*. 2010). In USA, 12% of adult females exhibit irregular oestrous cycles and 31% do not cycle at all.

It has been suggested one of the reasons for cessation of reproduction may be a direct result of ovarian ageing (Hermes 2000). That is, loss of the oocyte pool due to increased cyclicity in nulliparous zoo elephant. For example, a captive female elephant is likely to ovulate as many oocytes by the age of 12 years as a wild female does in her entire lifetime (Hermes 2000). Constant cyclicity is also known to lower the age of menopause in women (van Noord *et al.* 1997).

Data from the current study provides useful information to address this particular point. Only a tiny fraction of the original ovarian endowment of oocytes find their way to ovulation, with follicular atresia causing the demise of the bulk (Krysko *et al.* 2008). Such loss of oocytes takes place every day and continues throughout pregnancy and lactation in all mammalian species studied to date (Gougeon 2010). In women for example, according to a model of non-growing follicle numbers, at the peak of follicle activation at around 14 years of age a mean of around 880 (range 100–7 500) non-growing follicles per month enter the growth phase (Wallace & Kelsey 2010).

In the present study transitional and secondary follicles were observed in the ovaries of non-pregnant elephant at all ages except the very old animals in which few follicles still existed. Although these transitional and secondary follicles were not counted they appeared rare. Specimens from pregnant elephants in a previous study (Stansfield 2006) showed similar results. Although the presence of transitional and secondary follicles indicate early follicle growth from the reserve of EP and TP, further study is required to investigate their perceived rareness. In the light of this perceived rareness, the suggestion

that more SF are activated from the reserve in nulliparous non-pregnant elephants is not necessarily correct, particularly because the early stages of initiation of follicle growth are thought to be independent of gonadotrophic stimulation (Berne *et al.* 2004). Small follicles are constantly being "activated" in a paracrine controlled environment, taking more than 120 days to pass from the primordial to the secondary stage in women. Growth to the pre-antral stage takes a further 85 days, at which point the follicles become gonadotrophin sensitive and may be recruited. Then follows the 8–12 day period of selection and dominance of one follicle some days prior to ovulation (Eshre 2005).

It would therefore be of interest to compare the number of oocytes within the ovaries of captive elephants with the cross sectional results reported here in wild elephants. To this end, the ovaries of the one semi-domesticated, nulliparous elephant aged 28 years (named MM) could be studied. The total number of oocytes in her ovaries (88 000) was within the acceptable range for her age but it was notable that she exhibited only TP follicles. Only one other wild elephant under 45 years of age had no reserve of EP follicles in her ovaries. She was aged 29 years and had only 2392 TP. Elephant MM had shown no oestrous activity throughout her life and was dominant in her "family group"over a 27-year-old bull and a 19-year-old cow which had similarly shown no breeding activity. At post mortem her ovaries contained a recent ovulation stigma, antral follicles up to 9 mm in diameter and some CL and small CN. In contrast, the wild 29-year-old mentioned above that showed the mentioned low ovarian reserve had inactive-looking ovaries with no antral follicle development at all. She did, however, exhibit some large CN from the pregnancy that produced the 2-year-old calf by her side. One further wild elephant was of interest in that she had an inverted number of TP:EP ratio (92 961 EP in relation to 406 967 TP) and was nulliparous at 15 years of age. She showed plenty of antral follicle development and some small CN from previous cyclical CL.

These isolated observations raise the question of whether prolonged nulliparity in the elephant may prematurely exhaust the EP pool in their ovaries. Although MM had a good supply of SF these were all at the TP stage and therefore equivalent to a wild elephant of around 45 years of age when some authors have reported a slowing in reproductive rate of wild elephants (Hanks 1972; Laws *et al.* 1970; Sherry 1975). Whether MM is representative of the follicle status of a mature nulliparous animal needs to be addressed

by examining more ovaries from captive deceased animals when they become available at *post mortem* studies.

MM forms an important observation since although she was nulliparous she was in lean body condition at the time of death and had experienced a natural diet and a relatively stress free daily life, in marked contrast to the normal experience of zoo elephants. However, her social status was not normal as the 3 companion animals had been captured as calves during culling operations and had been hand-reared together throughout their lives. It is likely that her familiarity with her companions prevented her overt expression of oestrus although it did not suppress ovarian cyclicity (Freeman 2004).

7.4.5. The relationship between tusklessness and the follicular reserve

In African elephant inherited bilateral tusklessness is sex-linked in females (Steenkamp *et al.* 2007) and increasing numbers of tuskless animals within Zimbabwe is thought to be a result of selective ivory poaching. Apart from the current study, which showed no effect, no report has been found on the effect of tusklessness on ovarian function.

7.4.6. Reproductive senescence

Turning to the oldest samples collected, 7 sets of ovaries from elephants aged 57–70 years were available for this study (Table 7.2). Low numbers of SF were counted in the ovaries of all these animals thereby causing the coefficient of error to be above normally acceptable levels when using the same stereological protocol as for younger animals. Due to this very low number of SF, and some cases in which no follicles at all were counted, it was decided it would be fruitless to cut and examine any greater number of sections (Charleston *et al.* 2007). When comparing the three 57–62-year-old individuals with the four 68–70-year-old individuals, a higher number of SF was counted in the younger animals. Nevertheless, the four oldest animals still had from zero to 24 278 (median 7067) SF, which is appreciably higher than the average of only 1000 oocytes remaining in the ovaries of women when they approach menopause at around 51 years of age (Faddy & Gosden 1996).

The structures left behind from the large CL of pregnancy become increasingly dark brown in colour and are termed *corpora nigra*. They have been suggested to persist for at

least 77 months from the time of CL formation (Smith & Buss 1975) and therefore could be used as a guide to the interval since last calving. The size of the CL of pregnancy vary greatly as will the CN that persist from them (Lueders *et al*. 2011; Stansfield & Allen 2012). However, data from CN of known age presented in Table 7.4 suggests that not many years had past since 6 of the 7 oldest females studied had produced large CL of pregnancy.

The present finding of sustained reproductive capacity into old age is supported by behavioural studies on elephants in Amboseli National Park in Kenya where age-specific fecundity has been recorded in a population of elephants studied closely since 1972 (Moss 2001; Moss & Lee 2011). This has shown that, of 38 females in the study group with well known histories that had reached 50 years of age, most continued to reproduce and only 9 appeared to have stopped breeding, with 7 years having past since they had last given birth. Twelve females in the group over 60 years of age had given birth, with the most recent calving intervals averaging 4.75 years compared to 4.5 in the general population. This difference of 0.25 years represents less than the time period of one oestrous cycle thereby indicating a remarkably constant level of fertility over a period of some 50 years. This contrasts with women, where fertility rates begin to fall from the early 20's. Although there may be numerous causes of the difference in maintenance of fertility rates between elephant and women and the answer is not known, one may be that a different regulation of follicle reserve takes place in the elephant compared to women.

This remarkable reproductive longevity of African elephants has been observed and documented across Africa including South Africa (Freeman *et al.* 2008), Uganda (Laws 1969; Perry 1953), Zambia (Hanks 1972), and Zimbabwe (Sherry 1975; Williamson 1976). The only other long-lived mammals capable of supplying oocytes right up to death at age of maximum life expectancy are the baleen whales which may live and breed till over 100 years of age (Mizroch 1981).

7.5. Conclusion

In conclusion, this study shows that the size of the follicle reserve in the African elephant depends on age with a noticeable decline in the number of SF after the age of 25. Despite the trend for a depletion in SF throughout life, some old elephant still had low reserves of

SF in their ovaries at the time of culling. The EP follicles that constituted the follicle reserve in younger animals were no longer visible in the ovaries of elephant after the age of 45, and TP follicles only formed the reserve for their remaining years of life.

The content of this chapter will be submitted in a slightly different format to an accredited journal.

Chapter 8. General Discussion

8.1. Main findings on the ontogeny of the follicular reserve in the African elephants

The aim of the present study was to determine the type, distribution and establishment of the follicular reserve by counting the numbers of SF in the ovaries of wild African elephants throughout embryonic, fetal, prepubertal and adult life. Oogenesis, folliculogenesis and development and regression of the elephant ovary through fetal, pre-pubertal and mature life were described in the preceding chapters and ovarian reserve was seen to approach depletion or be depleted around the time of maximum life expectancy of 70 years. The salient findings of the study may be summarised as follows:

- PGCs arrived in the elephant embryonic gonad around 70–76 days post conception and sexual differentiation of the gonad occurred around 90 days.
- The period during which oogonia stopped dividing mitotically and converted to oocytes entering meiosis started at 5 months and ended at 11 months after conception.
- Follicles first formed following the onset of meiosis in oocytes at around 5 months of gestation and the number of SF peaked in the fetus at around 4.5 million in mid-gestation, towards the end of the 6-month mitotic-to-meiotic transition period.
- By mid gestation the cortex of the fetal ovary had reached a developmental stage described for full-term in the fetuses of other species
- During the second half of gestation the fetal ovary was dominated by growing antral follicles and increasing numbers of interstitial cells within the medulla.
- Antral follicle growth was maximum in the fetal gonad around 17 months of gestation but maximum interstitial tissue did not occur until around the time of birth.
- Around the time of birth the ovaries shrank considerably from late gestation size, although persisting interstitial cells continued to stain positively for 3β–HSD as did the granulosa cells of the small follicles.

- Two types of SF occurred in elephant ovaries from the very earliest stages of follicles studied; EP and TP. No TPM were observed during stereological studies.
- The number of SF at least remained static, or may have even increased during prepubertal life.
- The number of SF declined significantly post-pubertally, around the time of first calving ($P = 0.003$).
- The number of SF in older prime adult elephants (26–35 years of age) was significantly lower than in young prime adults (16–25 years), suggesting a sharp decline in numbers during prime adulthood, after which they reduced more slowly and steadily to 70 years of age — the maximum age studied — which is near the end of the expected natural lifespan.
- After having been the predominant type of SF in younger elephant, EP were absent in elephant older than about 45 years of age, suggesting that they had become depleted.
- TP, which were present in similar numbers in all age groups after puberty, constituted the follicle reserve from 45 years onwards and were absent in some elephant towards the end of life, suggesting that they had become depleted.

8.2. Scope of inference from the salient findings

This study is the first to describe the lifetime ovarian reserve of the African elephant. From the results obtained there appears to be a large degree of natural variation in the numbers of SF present within the ovaries of the elephant at a given age. Results obtained from such a variable population would benefit from an increased sampling number which may happen in the future when more specimens become available. Low specimen numbers relate particularly to the embryo and fetal samples which therefore may not be representative of the species as a whole, however they are the best guide lines available at the moment.

Such studies are necessarily cross-sectional in nature, longitudinal studies on the development and change in number of SF would require multiple ovarian biopsies over time and are not practical in a wild species such as the elephant. The inference of a

change in SF with time between different animals is at the moment, the best way to obtain relevant data on the age-related changes of the small follicle reserve which may permit drawing conclusions on the species as a whole and changes over time. The short comings of this approach, however, have been noted.

Despite low numbers of specimens and a large degree of natural variation, the results of this study do indicate that elephants develop their ovarian reserve during fetal life and gradually deplete it after birth, follicles remaining in the ovaries into old age.

Surprising findings such as the post natal, prepubertal increase suggested in the number of SF need to be supported by further research.

8.3. Follicle counting methods, stereology versus serial sectioning.

The first experiment, reported in Chapter 3, embarked on a description of the pool of SF in the elephant ovary and used serial sectioning techniques to count follicle numbers in these early specimens. However, the relative paucity of follicles counted led to all the remaining follicle counts for further studies being carried out using modern unbiased stereology tools, during which the counts for 8 of the animals included in Study 1 were repeated, this time using stereology. An additional benefit of using stereology was the ability to view a greater depth of each follicle and the shape of its granulosa cells. It is proposed this is the reason that no true primordial follicles were viewed in the stereological studies—all follicles viewed stereologically had some cuboidal granulosa cells.

When the results for both the serial sectioning and the stereology were plotted together (Figure 8.1) it was apparent that although the pattern of decline with increasing age was similar for the two methods, the numbers generated from the serial sections were several orders of magnitude lower than those generated from stereology. A similar difference was also noted by Mamsen *et al*. (2011) when comparing stereological calculations of fetal oocyte numbers with those obtained by volumetric studies (Baker 1963). Because stereology is based on mathematically sound techniques it was concluded that results obtained by stereological methods would be more accurate and that the reduced follicle counts derived from serial sectioning likely arose from incorrect volume calculations. To

the author's knowledge the present study is the first in which serial sectioning and stereology were used on the same ovaries.

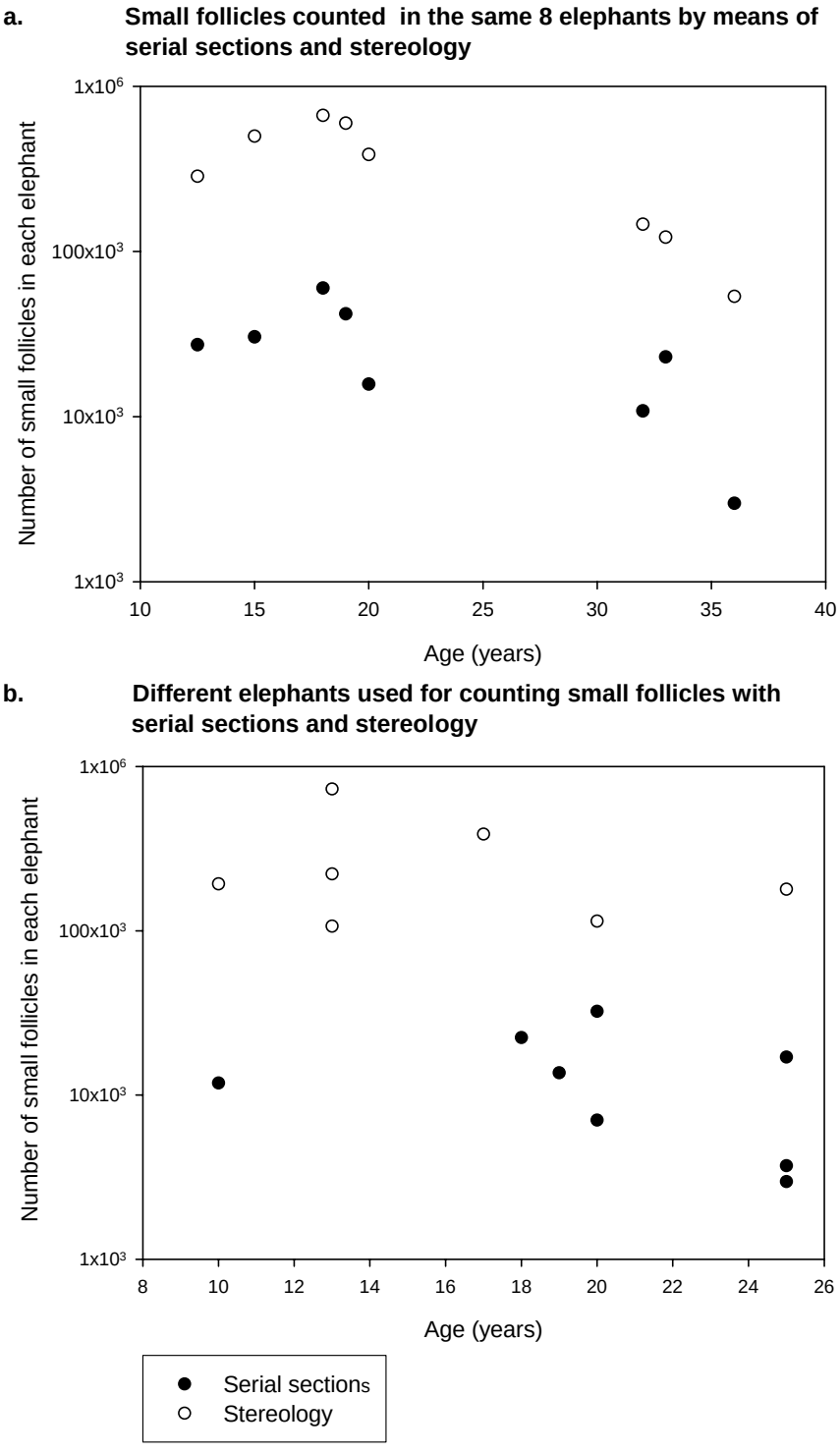


Figure 8.1 The numbers of SF in elephant ovaries counted by serial sectioning and stereology

8.4. The distribution of small follicles in the ovary of the African elephant

The distribution of SF within the cortex of the elephant ovary, after birth, is similar to that found in most other mammals with the exception of the equids (McGeady *et al*. 2006; Schatten & Constantinescu 2007). In horses and their relatives there is an inversion of the cortex and medulla of the ovary — such that the cortex occupies the superficial region of the ovary in a small area at the ovulation fossa only, with the medulla almost completely surrounding the cortex where the small follicles are resident. The SF within the cortex of mammals are reported to be found in the peripheral cortex (Bloom & Fawcett 1962; Genuth 2004; Junqueira & Carneiro 2005) as was also seen in the elephant.

Studies of the distribution of SF in mammalian ovaries are uncommon, one report states that, "in cattle, sheep and pigs, follicles are reported to be randomly distributed in the cortex, while in dogs and cats they occur in clusters" (McGeady *et al*. 2006). Following an extensive search of the literature, other reports were found that relate to biopsies of human ovaries (Lass *et al*. 1997; Poirot & al 2002; Qu *et al*. 2000; Schmidt 2003; Sharara 2004). In these reports follicle counts, and the variation between the biopsies from one ovary were stated; for example, Qu *et al*. (2000) quoted a variation between zero and 106 follicles per mm^3 in cortical specimens. They therefore deduced that SF were unevenly distributed throughout the cortex of the ovary. To the author's knowledge there has been no other study performed on mammalian ovaries which has determined the regional distribution of SF such as outlined for the elephants in Chapter 4.

8.5. Insights gained in the type of follicles constituting the follicle reserve in the African elephant

The definition of the ovarian reserve and the identification of the timing of follicle activation are important bench marks when modeling the follicle dynamics of a species (Faddy & Gosden 2010).

The reserve of SF in the elephant is not typical of that reported in most other mammals (Findlay 2010). In the serial sectioning study (Chapter 3), true primordial follicles formed less than 2% of the follicle reserve, while in stereological studies (Chapter 4 onwards) no TPM were found (see Section 7.3). The majority of follicles in young elephants were present as early primary (EP) with a smaller number of true primary (TP),

however, the percentage TP content of the reserve increased throughout life (Chapter 4 onwards). These two follicle classes were combined as SF and examined together in the current study as the follicle reserve, although this may not necessarily be the pool of resting follicles. The data given in Table 3.3 and Table 3.5 suggests that follicle activation, following primary recruitment and subsequent irreversible commitment to growth, in the elephant is not marked by an increase in height of flat granulosa cells but rather by an increase in the diameter of the oocyte and its nucleus, and an increase in the number of granulosa cells surrounding the oocyte. In the elephant this occurs at the development to the transitional stage. From this it may be surmised that TP form part of the resting pool of follicles.

Although TP should be seen as part of the resting pool of follicles, it was noted in fetal ovaries that at least some of the granulosa cells surrounding new follicles were flattened which, together with the low percentage of SF in the fetus that are TP, suggest that TP develop from EP, and they are not primarily formed as TP. The rate at which this takes place is not known.

TP were present at below a maximum of approximately 30 000 during late fetal life and up to 3 years of age. Between 3 and 9 years of age the number was below a maximum of approximately 50 000. Around puberty the number of TP increased. The finding that the TP are present at similar numbers (fewer than 150 000) regardless of age following puberty again suggests that they form the terminal part of the growth phase of small follicles, and are indeed either growing very slowly or have entered growth from the EP stage to the TP stage where further growth has been halted again.

Table 8.1
Approximation of the small follicle component of the ovarian reserve throughout life in the African elephant.

		Number of SF per elephant	
Age group (years)	Percentage of all SF that are TP	TP	EP
Fetal life	2.6	<30 000	672 000
Post-natal life			
0–3.4	7	<30 000	315 000
3.5–9.5	16	<50 000	505 000
10–24	20	<150 000	144 000
25–44	75	<100 000	20 000
45+	100	<50 000	0

In addition, the finding that only TP follicles are present for a period of up to 20 years in ovaries of elephants over 45 years of age suggests that the TP observed in the follicle pool of younger elephant are indeed resting or growing sluggishly.

It is not known whether the EP reserve is reduced due to atresia or due to growth to TP which may, apart from replenishment from developing EP, develop further or undergo atresia while at the TP stage. Death in small follicles is difficult to identify as small follicles can vanish within 12 hours of the onset of apoptosis (Faddy & Gosden 2010). Whichever process is taking place in the elephant, there is a drop in the number of EP following puberty and again following 25 years of age, after which the majority of the follicle reserve is made up of TP, further suggesting that, in the elephant, TP do indeed belong to a stage of follicle development prior to follicle activation.

A further observation from the fetal studies in relation to follicle growth is that, in the fetus at 11.2 months of gestation, the follicle, oocyte and nuclear diameters were greater than observed either before (5.9 months) or after this stage during gestation (15.2 months). They were also greater than observed in mature animals (Table 8.2). Byskov

and Nielsen (2010) point out that all first generation oocytes in the mammalian ovary begin to grow immediately on reaching the diplotene stage of meiosis I, and are therefore committed to further growth and wastage. It could be speculated that the follicles present and measured in the 11.2 month aged fetus are these very first follicles (their oocyte and nuclear diameter suggest that they are growing) and result in the antral follicles formed during the second half of pregnancy.

Table 8.2
Diameters and number of granulosa cells for small follicles (SF), early primary follicles (EP) and true primary follicles (TP) and their oocytes at various stages through life

	Elephant fetuses			Mature elephants	
	SF in a 5.9-month fetus	EP in a 11.2-month fetus	EP in a 15.2-month fetus	EP	TP
Follicle diameter	35.0–37.5	50.0–65.0	37.5–46.0	43.8	50.0
Oocyte diameter	21.5–23.0	45.0–55.0	25.0–31.0	30.0	3.05
Nucleus diameter	14.0–16.5	20.0–22.5	15.5–17.5	15.0	15.0
Granulosa cells (n)	8–11	12–14	9–14	12	19

(Data are from Table 3.3 and Table 5.2, and section 6.3.1).

8.6. Insights gained in the cessation of reproductive life

Female African elephants have a natural life expectancy of about 65–70 years, when death follows the wearing away of the last sets of molar teeth as shown in Figure 2.1i (Lee *et al*. 2012). Three of the four elephants 68 years or older in the current study still had between approximately 3 000 and 20 000 TP, suggesting that they may still have had the ability to ovulate and reproduce. Further, in contrast to Hanks (1972), Laws *et al*. (1970) and Sherry (1975), but in agreement with Moss, (2001), Perry (1953) and Williamson (1976), all but one of the old females in the present study were showing cyclic ovarian activity or signs of that having been the case shortly before the animals were killed (Table 7.2). It can therefore be concluded that the elephant has the ovarian

capacity to remain fertile through to very late age and virtually up to the time when death occurs naturally. This being so, female elephants do not experience a "menopausal" cessation of reproduction and do not have a significant period of post-reproductive life. The observations by Moss (2001) that the calving interval in the very oldest females observed in Amboseli National Park in Kenya extended to only 4.75 years from a population average of 4.5 years shows a remarkably consistent and long-lived fertility. In addition, the differentiation of a 49-month intercalving interval following the birth of a female calf (n = 11) compared to a 63 month interval following birth of a male calf birth (n = 11) may be due to demands of the faster growing male calf (maternal investment) impacting on body reserves (Moss & Lee 2011), rather than the longer calving interval being due to an inherent difference in fertility among individuals. Similar constancy of reproductive potential has been recorded in the olive baboon to 21 years, with a maximum lifespan of 27 years, and the African lion to 14 years, with a maximum lifespan 17 years (Packer *et al.* 1998). In ageing females natural variation will ensure there are always exceptions to this general rule.

The reasons for an extended reproductive life arouse speculation. In a meta-analysis of 42 species, 83% showed evidence for natural periods of post-reproductive lifespan (Cohen 2004), the most extended of which are the human and various species of toothed whales (Johnstone & Cant 2010; Ward *et al*. 2009). Perhaps it is significant that older female elephants make better mothers and the offspring of matriarchs have the greatest survival potential (Lee & Moss 1986). Large family groups created as a result of member longevity are also instrumental in calf survival. Male elephants have a reproductive output that is unlike most other mammalian species (Hollister-Smith *et al.* 2007). For example, they have little reproductive success before 25 years of age and they only contribute significantly to the gene pool beyond the age of 40 years (Poole *et al.* 2011); their continued growth throughout life and the sexually active period of musth are determinants of this strategy (Poole *et al.* 1984). The selection force for late-age fertility must therefore be high and longevity, in association with extended reproductive performance are highly desirable traits. Female elephants produce relatively few offspring (a maximum of 10–12) during their 70 year lifespan and it is therefore crucial that the "best" males, in terms of size and reproductive fitness, are selected by the females for breeding; older females have been observed to exercise mate choice for themselves and also for their family members, preferring the older, larger bulls (Moss & Poole 1983).

Elephants have an absolute limit placed on their lifespan by their dentition. When the 6th and last sets of molar teeth are worn down the animal can no longer masticate food, causing body condition to decline followed by death due to starvation. Evolution appears to have accurately matched the female elephant's oocyte store with her dentition, thereby allowing both to fail simultaneously in most, if not all, individuals.

8.7. The value of the current study with respect to understanding and improving strategies for contraception of African elephants

Regardless of which management strategy is employed, the basis for all operations to regulate reproduction in wild elephants is knowledge of their anatomy, physiology and reproductive biology (Hildebrandt *et al.* 2004). This study provides a baseline description of the SF reserve throughout the life of the elephant against which the results of other studies may be compared. For example, to determine the effect of immunocontraceptive treatment on the SF pool.

With the new information on the follicle reserve of the elephant throughout life generated by the present study, a novel and humane contraceptive technique to target the ovarian follicle reserve may now be proposed. It is known from studies in mice that a synergistic and coordinated suppression of follicular activation provided by multiple inhibitory molecules is necessary to preserve the dormant follicle pool (Adhikari & Liu 2009). Loss of function of any of the inhibitory molecules (Phosphatase with TENsin, PTEN; Foxo3a, p27 and Fox l2) leads to premature and irreversible activation of the primordial pool and depletion of the follicle reserve (Adhikari & Liu 2009). The PTEN gene encodes a phosphatase enzyme that negatively regulates the phosphotidylinositol 3 kinase (PI3K-Akt) signaling pathway (Reddy *et al.* 2010). PTEN deletion in the oocyte increases protein kinase B (Akt) phosphorylation and nuclear export of downstream Foxo3 proteins (John *et al.* 2008). Studies on human ovarian cortical tissue using a PTEN inhibitor and a P13k activating peptide induced dormant primordial follicles to develop into large antral follicles (Li *et al.* 2010). According to Adhikari & Liu (2009), the use of several small molecular compounds capable of inhibiting the function of the kinases and phosphatases mentioned above could trigger initiation of follicle growth. Whether this method would be feasible for use on live animals has yet to be investigated but the idea of targeting and

depleting the ovarian reserve in older females would be an attractive contraceptive method.

Contraceptives for wild animals should be efficacious, allow remote delivery, be reversible, produce no deleterious short- or long-term health effects, should not change social behaviour and group integrity, must not pass through the food chain, should be safe if administered during pregnancy and should be affordable (Bertchinger *et al.* 2008). Such a therapy to deplete the ovarian reserve of oocytes would ideally be administered as a single treatment to the older age range of the female population, the starting age being dependent on the required rate of population growth reduction. It would clearly not be reversible in the individual animal but by selecting the older females in the population which had already produced calves, their genes would not be completely lost to the pool. It may be noted, however, that the proportion of calves born over time from cows with proven good fertility and reproductive longevity would decrease. Although other safety factors would need to be demonstrated, the potential use of synthetic enzymes to complete the task as suggested above appears attractive. The great advantage of such a method of contraception would be maintenance of the social family structure; namely, calves would still be born into the family group. Furthermore, matriarchs and older females, although themselves rendered sterile, would still remain within the herd as repositories of knowledge (McComb *et al.* 2001) and as "allomothers" for their offspring's calves (Lee 1987). The additional benefit, not experienced with immunocontraception, as the most promising contraceptive practice to date, is that in addition to maintaining family structure and cohesion, the treated cows would no longer come into oestrus.

Contraceptive methods that induce sterility in females cannot quickly reduce overall numbers in an over-abundant population. They could, however, be used in combination with other management tools if necessary to reduce population growth rates.

8.8. Does the longevity of reproductive life in the elephant offer insights into reproductive senescence in humans, or *vice versa*?

Elephants and humans exhibit some interesting similarities in their ageing and reproductive characteristics and such comparisons were an important driver of the present study. For example;

a) Both species live for around 70 years and both exhibit extended gestation periods.

b) The human and elephant show marked similarity in the age of puberty and first conception. Although, due to improved nutrition, the age of menarche in Western human societies has been reduced from 16 to 12 years, it remains at around 16.5 years in undeveloped human societies with the age of first pregnancy at 18 years (Jansen 1995; Wood 1989). In elephants, these parameters vary appreciably among populations; Moss and Lee (2011) report that the elephant of the Amboseli Park in Kenya females can undergo their first ovulation between 7 and 22 years of age, have a mean age at first conception of 11.3 years and a median age at first calving of 14.2 years (Moss & Lee 2011).

c) Humans and elephants both show a long reproductive life. In the Hutterite sect in North America contraception is condemned and Tiertze (1957) recorded that Hutterite women entered their last pregnancy at 40.9 years of age. And in a more recent study in Israel, Laufer *et al*. (2004) noted that natural pregnancies and deliveries after 45 years of age accounted for only 0.2% of all deliveries. Important to note, as recorded in Chapter 7, is that female elephants continue to conceive well into their 6th decade of life with the ability to conceive into their 7th , which exceeds the reproductive lifespan of women. However, premature cessation of reproduction is experienced by many elephants in captivity. For example Hildebrandt (1997) noted an apparent 10–15 year window of ovarian cyclicity after the occurrence of the first pubertal oestrus in 200 elephants until a dramatic decline in reproductive fitness commenced, particularly in nulliparous cows.

The considerable natural individual variation in follicle numbers in the ovaries of both women and female elephants leads to some speculation on the interpretation of figures obtained for the elephant due to the relatively low sample numbers available, once the 22

month fetal and 70 year lifespan are plotted on one graph. Figure 8.2 (shown on a larger scale in Figure 8.3 and Figure 8.4) compares the numbers of small follicles per elephant counted in the present study with data obtained from a robust mathematical model of the human follicle reserve (Wallace & Kelsey 2010). Discrepancies still exist in the fetal model for the human with one report showing peak follicle numbers of 600 000 at 18–20 weeks gestation (Wallace & Kelsey 2010) and another showing 10 000 000 follicles at 20 weeks of gestation (Mamsen *et al.* 2011). In the light of these big differences in the human figures, the surprising findings in the postnatal, prepubertal elephants could be interpreted in two ways. First, and according to the results recorded in Chapter 6, it appears that SF in the elephant ovary at birth are similar to those in the newborn human infant. Then from birth to 9 years of age in the elephant there occurs a small but nonetheless significant increase — rather than the expected decrease — in the number of follicles, followed by a sharp fall in numbers after puberty. This could be interpreted as the new formation of SF by a hitherto unknown mechanism in calves between the age of 4½ and 9 years. Second — if one were to assume that the significanly lower number of SF in calves younger than 4½ years compared to the numbers in near-term fetuses and calves older than 4½ years was due to a Type 1 statistical error (even though the probability thereof is only 1%) — then one may argue that the follicle number fell gradually throughout prepubertal life from a maximum of 600 000 at birth, generally much in line with the equivalent human loss curve. A definitive result on this intriguing question will have to await either a larger cohort of prepubertal elephant ovaries or perhaps the immunocytochemical localization of germline stem cells in the pre-pubertal elephant ovary, giving rise to the possibility of some form of post-natal neo-oogenesis as postulated recently to be able to occur in the ovaries of both humans (White *et al.* 2012) and mice (Zou *et al.* 2009).

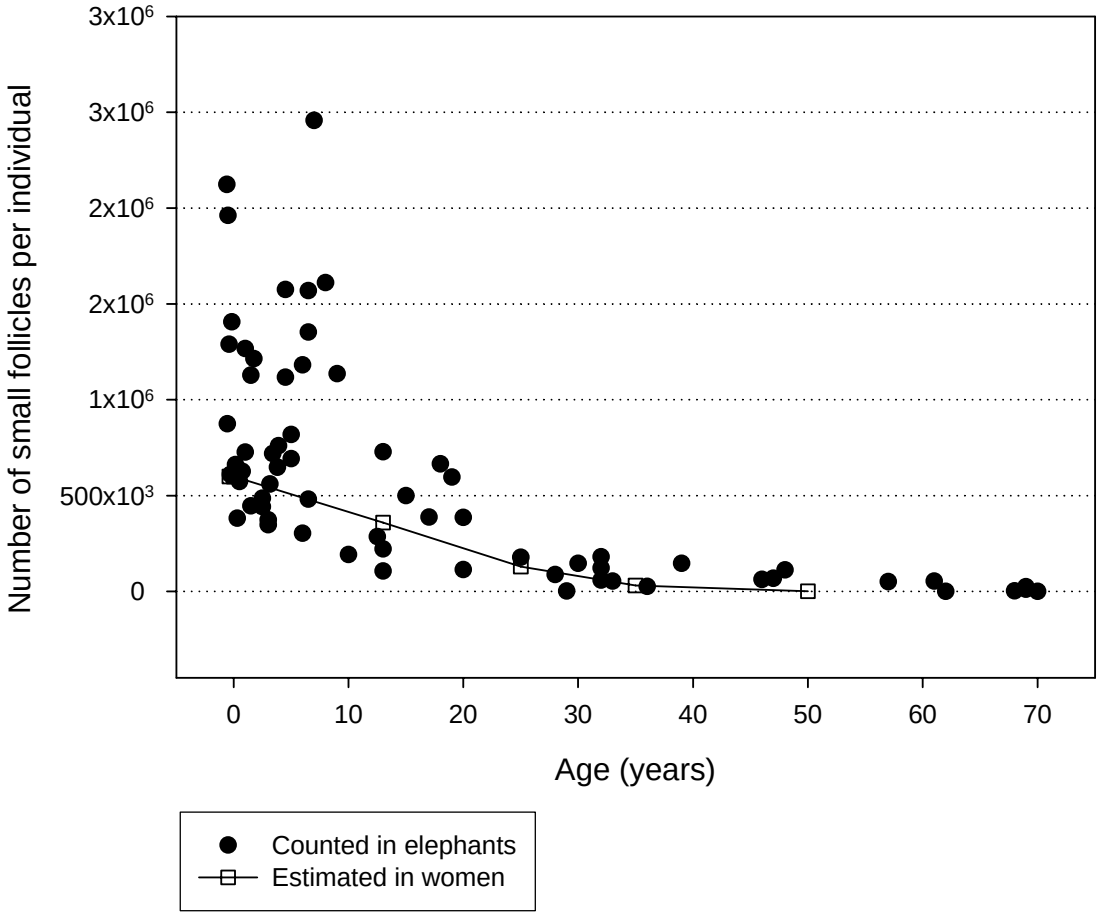


Figure 8.2 Comparison of the numbers of small ovarian follicles per elephant found in the current study and the mean number of non-growing ovarian follicles per woman, as modelled by Wallace and Kelsey (2010), from approximately mid gestation to the cessation of reproductive life

Regardless of prepubertal follicle numbers, following the period of puberty to first calving, the numbers of SF in the elephant ovary mark the upper limit of those in the human ovary and, from mid-life onwards, follicle numbers gradually reduce in both species, more quickly in women who experience menopause at an average of 51 years of age, and more gradually in elephants in which the follicle pool remains viable into the 6th decade of life. The new power model for non-growing follicles (NGF) in humans suggests that the reduction in numbers is due to a constantly accelerating rate of decay (Hansen *et al.* 2008) rather than a sudden increase in the rate of decay from the age of 38 years as proposed from the previously popular model by Faddy and Gosden (1996).

However, it is interesting to note from the earlier observation by Faddy *et al*. (1992) that without the accelerated increase in the rate of exponential decline in follicle numbers that they predicted from the originally proposed 38 year age point, the follicle endowment would last for a further two decades which is now seen to closely match the limited elephant data obtained in the present study. It has been accepted generally that the reduction in follicle numbers in women is not biphasic as described above (Faddy 2000; Hansen *et al*. 2008; Wallace & Kelsey 2010) as it would imply a biologically implausible process. However, it may be suggested that, compared with the elephant, the trajectory of depletion displayed by women's ovaries is more closely represented by the human differential equation model proposed by Faddy (2000) and championed by Coxworth and Hawkes (2010) than the power model published by Hansen *et al*. (2008).

Knowing now that follicles remain present in the elephant ovary right up to the time of maximum life expectancy it will be of interest in the future to study the ultrastructural integrity of the aged elephant oocyte and compare this with its human equivalent. Perhaps such examination will offer useful insights into the apparently greater viability of the elephant germ cell.

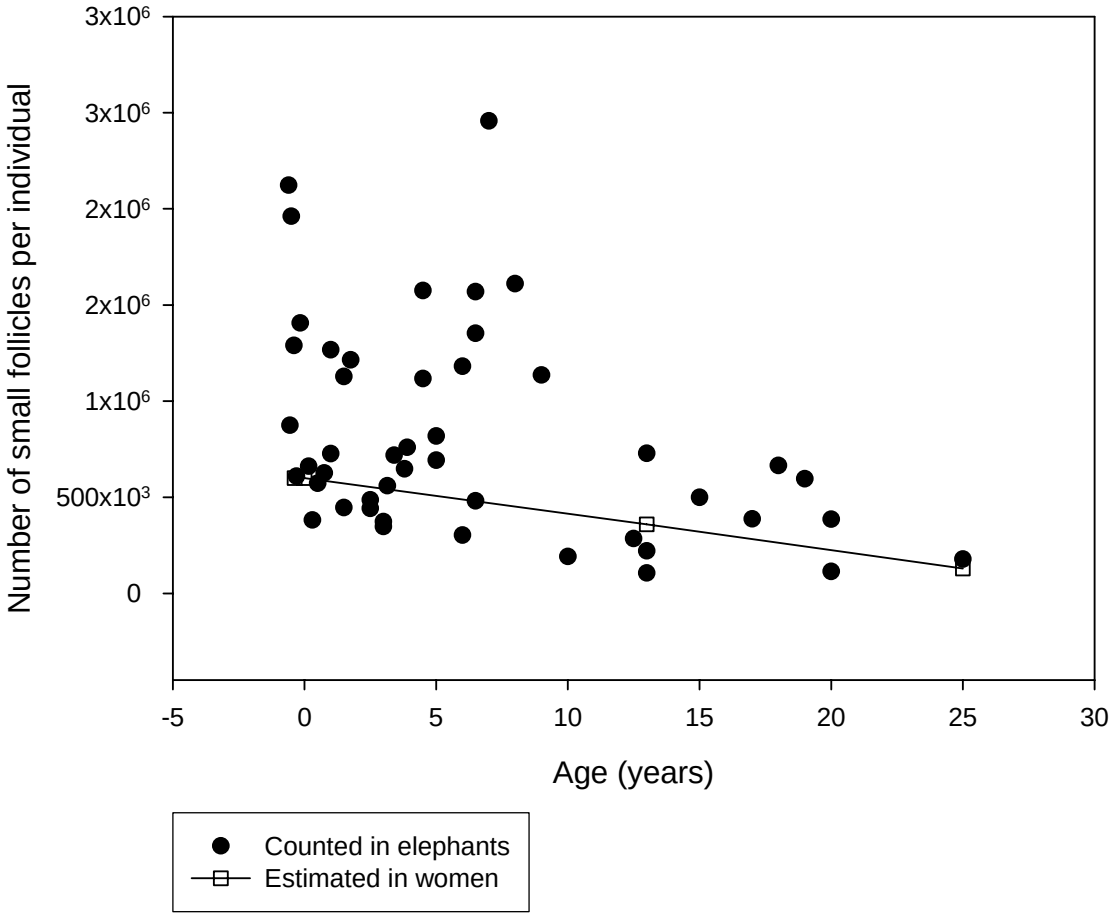


Figure 8.3 The numbers of small ovarian follicles per elephant and the average estimated per woman from approximately mid gestation to 25 years of age (Human data from Wallace and Kelsey 2010)

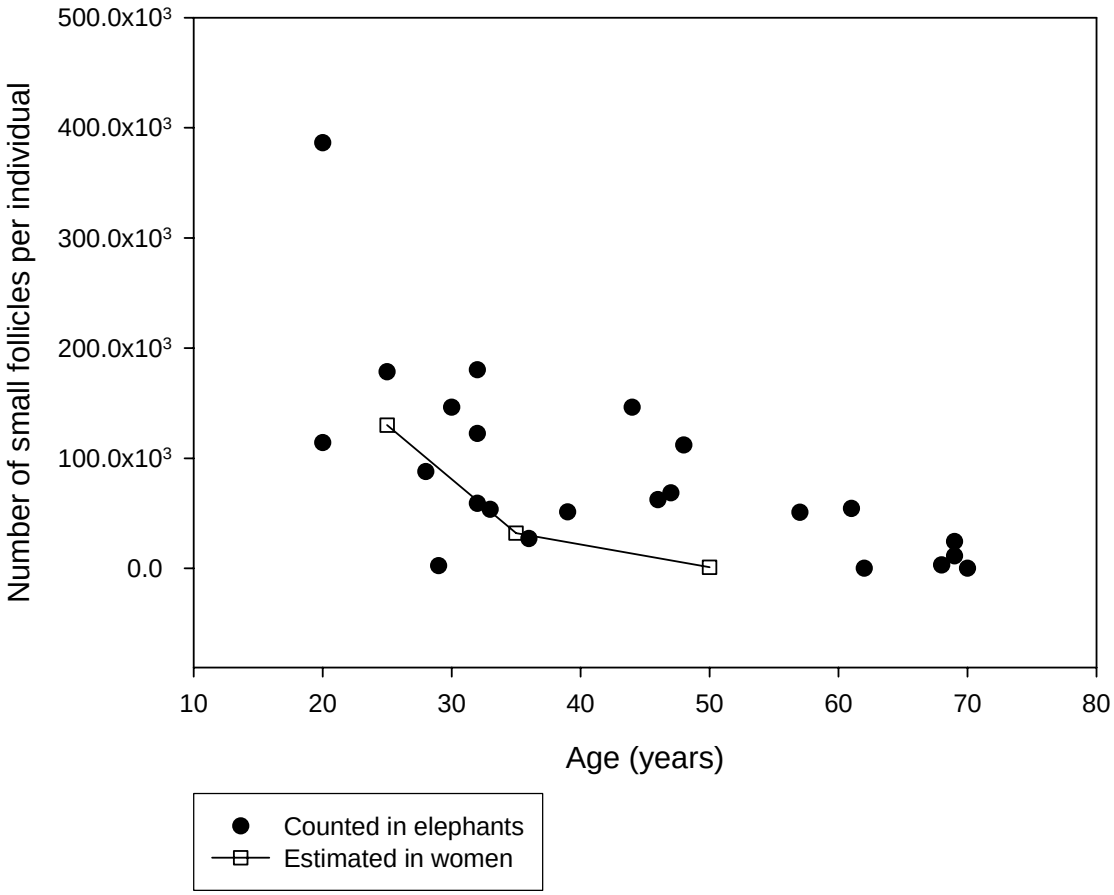


Figure 8.4 The numbers of small follicles in elephant and the average number estimated per woman from approximately 20–70 years of age (Human data from Wallace and Kelsey 2010)

8.9. Outstanding questions and possible future studies

The lack of previous research on small follicle reserves in the African elephant necessitated the broad scope of the present study in its attempt to determine the cause of any ovarian age-related reduction in the fecundity of the female elephant. In the process many questions have been generated for future studies, some of which are listed below.

- What is the cause and biological significance of the marked antral follicle development in the fetal ovaries during the second half of gestation?

- What is the role of the ovarian interstitial cells in late fetal life and early neonatal life? Does the fetus support the maintenance of gestation through steroid production by its gonadal interstitial cells in the second half of gestation?
- Is there a real increase in follicle numbers during prepubertal life? If so, how might it occur and could this be another link to the elephants aquatic past?
- Why are there such sharp declines in follicle numbers following puberty and after 25 years?
- Does the fact that the granulosa cells of the small follicles stain positively for 3β–HSD in fetal and pre-pubertal life have any biological significance?
- Is there any significance in the depletion of the EP follicle reserve by the age of 45 in wild elephant and the fact that TP follicles constitute the follicle reserve for the last 25 years of elephant life?

Chapter 9. References

Abercrombie, M. 1946 Estimation of nuclear population from microtome sections. *Anatomical Record* **94**, 236–247.

Adams, G. P., Jaiswal, R., Singh, J. & Malhi, P. 2008 Progress in understanding ovarian folicular dynamics in cattle. *Theriogenology* **69**, 72-80.

Adams, I. R. & McLaren, A. 2002 Sexually dimorphic development of mouse primordial germ cells: switching from oogenesis to spermatogenesis. *Development* **129**, 1155-1164.

Adhikari, D. & Liu, K. 2009 Molecular mechanisms underlying the activation of mammalian primordial follicles. *Endocrine Reviews* **30**, 438-464.

Aitken, R. J., Findlay, J. K., Hutt, K. J. & Kerr, J. B. 2011 Apoptosis in the germ line. *Reproduction* **141**, 139-150.

Allen, W. R. 2006 Ovulation, pregnancy, placentation and husbandry in the African elephant (Loxodonta africana). *Philosophical Transactions of the Royal Society of London. B Biological Science* **361**, 821-34.

Allen, W. R., Mathias, S. & Ford, M. 2005 Placentation in the African elephant, *Loxodonta africana*. IV. Growth and function of the fetal gonads. *Reproduction* **130**, 713-20.

Allen, W. R., Mathias, S., Wooding, F. B. & van Aarde, R. J. 2003 Placentation in the African elephant (*Loxodonta africana*): II Morphological changes in the uterus and placenta throughout gestation. *Placenta* **24**, 598-617.

Allen, W. R., Mathias, S. S., Wooding, F. B., Skidmore, J. A. & van Aarde, R. J. 2002 Placentation in the African elephant, *Loxodonta africana*. I. Endocrinological aspects. *Reproduction Supplement* **60**, 105-16.

Amoroso, E. C., Bourne, G. H., Harrison, R. J., Harrison Matthews, L., Rowlands, I. W. & Sloper, J. C. 1965 Reproductive and endocrine organs of foetal, newborn and adult seals. *Journal of Zoology* **147**, 430-486.

Amoroso, E. C. & Perry, J. S. 1964 The foetal membranes and placenta of the African Elephant (Loxodonta africana). *Philosophical Transactions of the Royal Society of London B Biological Science* **248**, 1-34.

Arey, L. B. 1966 Developmental Anatomy. A textbook and laboratory manual of embryology: Saunders.

Baker, T. 1963 Quantitative and cytological study of germ cells in human ovaries. *Proceedings of the Royal Society of London B Biological Science* **158**, 417-433.

Baker, T. G. & Hunter, R. H. F. 1978 Oogenesis and follicular growth in the cow: implications for superovulation. In: Control of Reproduction in the Cow. Luxembourg: CEC publications.

Balfour, D., Dublin, H. T., Fennessy, J., Gibson, D., Niskanen, L. & Whyte, I. J. 2007 Review of options for managing the impacts of locally overabundant African elephants (ed. IUCN), pp. 80pp. Gland, Switzerland: IUCN The World Conservation Union.

Balke, J. M. E. 1988 Reproductive anatomy of three nulliparous female Asian elephants: The development of artificial breeding techniques. *Zoo Biology* **7**, 99-113.

Barber, M. R. & Fayrer-Hosken, R. A. 2000 Possible mechanisms of mammalian immunocontraception. *Journal of Reproductive Immunology* **46**, 103-124.

Barber, M. R., Lee, S. M., Steffens, W. L., Ard, M. & Fayrer-Hosken, R. A. 2001 Immunolocalization of zona pellucida antigens in the ovarian follicle of dogs, cats, horses and elephants. *Theriogenology* **55**, 1705-17.

Barnhart, H. X., Haber, M. J. & Lin, L. I. 2007 An overview on assessing agreement with continuous measurements. *Journal of Biopharmaceutical Statistics* **17**, 529-569.

Begum, S., Papaioannou, V. E. & Gosden, R. G. 2008 The oocyte population is not renewed in transplanted or irradiated adult ovaries. *Human Reproduction* **23**, 2326-2330.

Bendsen, E., Byskov, A. G., Andersen, C. & Westergaard, L. G. 2006 Number of germ cells and somatic cells in human fetal ovaries during the first weeks after sex differentiation. *Human Reproduction* **21**, 30-35.

Benirschke, K. 2007a Comparative placentation : giraffe (Giraffa camelopardalis). In Comparative Placentation :*http://placentation.ucsd.edu/homefs.html*. London.

Benirschke, K. 2007b Comparative placentation in the elephant. In Comparative Placentation :*http://placentation.ucsd.edu/homefs.html*. London.

Berne, R. M., Levy, M. N., Koeppen, B. M. & Stanton, B. A. (ed.) 2004 *Physiology*. St Louise, Missouri, USA: Mosby.

Bertchinger, H. J., Delsink, A. K., van Altena, J. J., Kirkpatrick, J. F., Kilian, H., Ganswindt, A., Slotow, R. & Castley, G. 2008 Reproductive control of elephants. In *Elephant Management. A scientific assessment for South Africa* (ed. R. J. Scholes & K. G. Mennell), pp. 257-328. Johannesburg: WITS University Press.

Bertschinger, H. J., Delsink, A. K., van Altena, J. J., Kirkpatrick, J. F., Kilian, H., Ganswindt, A., Slotow, R. & Castley, G. 2008 Reproductive control of elephants. In *Elephant Management. A scientific assessment for South Africa* (ed. R. J. Scholes & K. G. Mennell), pp. 257-328. Johannesburg: WITS University Press.

Bhavnani, B. R., Short, R. V. & Solomon, S. 1969 Formation of oestrogens by the pregnant mare. I. Metabolism of 7-3H-dehydroisoandosterone and 4-14C-androstenedione injected into the umbilical vein. *Endocrinology* **85**, 1172-1179.

Bhavnani, B. R., Short, R. V. & Solomon, S. 1971 Formation of oestrogens by the pregnant mare. II. Metabolism of 14C-acetate and 3H-cholesterol injected into the fetal circulation. *Endocrinology* **89**, 1152-1157.

Blanc, J. J., Barnes, R. F. W., Craig, G. C., Dublin, H. T., Thouless, C. R., Douglas-Hamiliton, I. & Hart, J. A. 2007 African elephant status report 2007: an update from the African Elephant Database. In *Occasional Paper of the IUCN Species Survival Commission No. 33* (ed. I. S. A. E. S. Group), pp. 276pp. IUCN, Gland, Switzerland: The World Conservation Union.

Block, E. 1951 Quantitative morphological investigations of the follicular system in women. *Acta Anatomy* **12**, (267–285).

Bloom, W. & Fawcett, D. W. 1962 *A Textbook of Histology*: Saunders.

Bokhout, B., Nabuurs, M. & deJong, M. 2005 Vasectomy of older bulls to manage elephant overpopulation in Africa: a proposal. *Pachyderm* **39**, 97-103.

Bowles, J. & Koopman, P. 2007 Retinoic acid, meiosis and germ cell fate in mammals. *Development* **134**, 3401-3411.

Braw-Tal, R. 2002 The initiation of follicle growth: the oocyte or the somatic cells? *Molecular and Cellular Endocrinology* **187**, 11–18.

Braw-Tal, R. & Rodgers, R. J. 1996 Morphological characterization of bovine primordial follicles and their environment in vivo. *Biology of Reproduction* **55**, 1003-1011.

Braw-Tal, R. & Yossefi, S. 1997 Studies in vivo and in vitro on the initiation of follicle growth in the bovine ovary. *Journal of Reproduction and Fertility* **109**, 165-171.

Brown, J. L. 2000 Reproductive endocrine monitoring of elephants: An essential tool for assisting captive management. *Zoo Biology* **19**, 347-367.

Brown, J. L., B, C. S., Bush, M., Lehnhardt, J. & Phillips, L. G. 1991 Cyclic patterns of luteinizing hormone, follicle-stimulating hormone, inhibin, and progesterone secretion in the Asian elephant (Elephas maximus). *Journal of Zoo and Wildlife Medicine* **22**, 49-57.

Brown, J. L., Olson, D., Keele, M. & W, F. E. 2004a Survey of the reproductive cyclicity status of Asian and African elephants in North America. *Zoo Biology* **23**, 309-321.

Brown, J. L., Schmitt, D. L., Bellem, A., Graham, L. H. & Lehnhardt, J. 1999 Hormone secretion in the Asian elephant (Elephas maximus): characterization of ovulatory and anovulatory luteinizing hormone surges. *Biology of Reproduction* **61**, 1294-1299.

Brown, J. L., Walker, S. L. & Moeller, T. 2004b Comparative endocrinology of cycling and non-cycling Asian (Elephas maximus) and African (Loxodonta africana) elephants. *General and Comparative Endocrinology* **136**, 360-70.

Browne, M. A., Howard, C. V. & Jolleys, G. D. 1995 Principles of stereology. In *Image analysis in histology: conventional and confocal microscopy* (ed. R. Wootton, D. R. Springall & J. M. Polak): Cambridge University Press.

Bryden, H. A. 1889 Kloof and karroo: Sport, legend and natural history in the Cape Colony, with notice of the game birds and of the present distribution of the antelopes and the larger game. London: Longmans Green.

Bukovsky, A., Caudie, M. R., Virant-Klun, I., Gupta, S. K., Dominguez, R., Svetlikova, M. & Xu, F. 2009 Immune physiology and oogenesis in fetal and adult humans, ovarian infertility, and totipotency of adult ovarian stem cells. *Birth Defects Ressearch C Embryo Today* **87**, 64-89.

Bukovsky, A., Caudle, M. R., Svetlikova, M., Wimalasena, J., Ayala, M. E. & R, D. 2005 Oogenesis in adult mammals, including humans: a review. *Endocrine Reviews* **26**, 301-316.

Burnham, M. & Gronewold, N. 2010 Water: drought spurs life-or-death struggles in Kilimanjaro's shadow. In *Greenwire; Energy and Environment Publishing*. Washington.

Buss, I. O. 1966 Observations on reproduction and breeding behavior of the African elephant. *The Journal of Wildlife Management* **30**, 375-388.

Byskov, A. G. & Nielsen, M. 2003 Ontogeny of the mammalian ovary. In *Biology and Pathology of the Oocyte* (ed. A. O. Trounson & R. G. Gosden). Cambridge: Cambridge University Press.

Byskov, A. G. & Nielsen, M. 2010 Ontogeny of the mammalian ovary. In *Biology and Pathology of the Oocyte* (ed. A. O. Trounson & R. G. Gosden). Cambridge: Cambridge University Press.

Campfire. 2007 PAC statistics 2002-2006. Harare, Zimbabwe: Campfire Association.

Carruthers, J. 2008 The elephant in South Africa: History and Distribution. In *Elephant Management. A scientific assessment for South Africa* (ed. R. J. Scholes & K. G. Mennell), pp. 23-83. Johannesburg: WITS University Press.

Caughley, G. 1983 Dynamics of large mammals and their relevance to culling. In *Management of laerge mammals in African conservation areas* (ed. R. Owen-Smith). Pretoria: HAUM Educational Publishers.

Chamaille-James, S., Fritz, H., Valeix, M., Murindagomo, F. & Clobert, J. 2008 Resource variability, aggregation and direct density dependence in an open context: the local regulation of an African elephant population. *Journal of Animal Ecology* **77**, 135-144.

Chapman, H. C. 1880 The placenta and generative apparatus of the elephant. *Journal of the Academy of the National Science Philadelphia* **8**, 431.

Charleston, J. S., Hansen, K. R., Thyer, A. C., Charleston, L. B., Gougeon, A., Siebert, J. R., Soules, M. R. & Klein, N. A. 2007 Estimating human ovarian non-growing follicle number: the application of modern stereology techniques to an old problem. *Human Reproduction* **22**, 2103-2110.

Child, G. 2004 Elephant culling in Zimbabwe. *ZimConservation Opinion.* **1**, 1-6.

Childs, A. J., Cowan, G., Kinnell, H. L., Anderson, R. A. & Saunders, P. K. 2011 Retinoic acid signalling and the control of meiotic entry in the human fetal gonad. *Plos One* **6**, e20249.

Cohen, A. A. 2004 Female post-reproductive lifespan: a general mammalian trait. *Biological Reviews* **79**, 733-750.

Cole, H. H., Hart, G. H., Lyons, W. R. & Catchpole, H. R. 1933 The development and hormonal content of fetal horse gonads. *Anatomical Record* **56**, 275-293.

Cooper, R. A., Connell, R. S. & Wellings, S. R. 1964 Placenta of the Indian Elephant, Elephas Indicus. *Science* **146**, 410-2.

Cowan, P., Duckworth, J., Cui, X., Molinia, F., Ramsey, D., Choquenot, D., Rodger, J., Mate, K., Day, D., Mahoney, T., Walmsley, A., Kirk, D., Bayliss, P., Lubitz, W. & Haller, C. 2003 Fertility control of brushtailed possums in New Zealand. In *Managing African Elephant Populations: Act or let die?* Faculty Vererinary Medicine, Utrecht University, The Netherlands.

Cox, J. E. 1975 Oestrone and equilin in the plasma of the pregnant mare. *Journal of Reproduction and Fertility Supplement* **23**, 463-468.

Coxworth, J. E. & Hawkes, K. 2010 Ovarian follicle loss in humans and mice: lessons from statistical model comparison. *Human Reproduction* **25**, 1796-1805.

Craig, G. C. 1984 Foetal mass and date of conception in African elephants: a revised formula. *South African Journal of Science* **80**, 512–516.

Cui, Y. & Yu, S. J. 1999 Ovarian morphology and follicular systems in yaks of different ages. *The Veterinary Journal* **157**, (197-205).

Cumming, D. & Jones, B. 2005 Elephants in Southern Africa: Management issues and options. Harare: WWF/SARPO Occasional Paper

de Bruin, J. P., Dorland, M., Spek, E. R., Posthuma, G., van Haaften, M., Looman, C. W. N. & te Velde, E. R. 2002 Ultrastructure of the resting ovarian follicle pool in healthy young women. *Biology of Reproduction* **66**, 1151-1160.

de Felici, M. 2010 Germ stem cells in the mammalian adult ovary: considerations by a fan of the primordial germ cells. *Molecular Human Reproduction* **16**, 632-636.

de Felici, M., Klinger, F. G., Farini, D., Scaldaferri, M. L., Iona, S. & Lobascio, M. 2005 Establishment of oocyte population in the fetal ovary: primordial germ cell proliferation and oocyte programmed cell death. *Reproductive Biomedicine Online* **10**, 182-191.

Deanesly, R. 1977 Germ cell proliferations in the fetal horse ovary. *Cell and Tissue Research* **185**, 361-371.

Debruyne, R. 2005 A case study of apparent conflict between molecular phylogenetics: the interrelationships of African elephants. *Cladistics* **27**, 31-50.

Delsink, A. 2011 www.makalaliresearch.co.za. Pretoria.

Delsink, A. K. 2006 Regulation of a small, discrete African elephant population through immunocontraception in the Makalali Conservancy, Limpopo, South Africa. *South African Journal of Science* **102**, 403 - 405.

Delsink, A. K., van Altena, J., Grobler, D., Bertschinger, H. J., Kirkpatrick, J. F. & Slotow, R. 2007 Implementing immunocontraception in free-ranging African elephants at Makalali conservancy. *Journal of the South African Veterinary Association* **78**, 25-30.

Dohoo, I., Martin, W. & Stryhn, H. 2009 *Veterinary Epidemiologic Research*. Charlottetown, Prince Edward Island, Canada: VER, Inc.

Douglas-Hamilton, I. 1972 The ecology and behaviour of the African elephant, vol. PhD, pp. 276: University of Cambridge.

Dow, T., Holaskova, I. & Brown, J. 2011 Results of the third reproductive assessment survey of North American Asian (Elephas maximus) and African (Loxodonta africana) female elephants. *Zoo Biology* **30**, 699-711.

Drews, B., Hermes, R., Goritz, F., Gray, C., Kurz, J., Lueders, I. & Hildebrandt, T. B. 2008 Early embryo development in the elephant assessed by serial ultrasound examinations. *Theriogenology* **69**, 1120 - 1128.

Driancourt, M. A. 1991 Follicular dynamics in sheep and cattle. *Theriogenology* **35**, 55-79.

Driancourt, M. A., Cahill, L. P. & Bindon, B. M. 1985 Ovarian follicular populations and preovulatory enlargement in Booroola and control Merino ewes. *Journal of Reproduction and Fertility* **73**, 93-107.

Driancourt, M. A., Paris, A., Roux, C., Mariana, J. C. & Palmer, E. 1982 Ovarian follicular populations in pony and saddle-type mares. *Reproduction Nutrition and Development* **22**, 1035-1047.

Druce, H. E., Mackey, R. L. & Slotow, R. 2011 How immunocontraception can contribute to elephant management in small, enclosed reserves: Munyawana population as a case study. *Plos One* **6**, e27952.

Eltringham, S. K. 1997 *The Illustrated Encycopedia of Elephants*. London: Salamander Books.

Erickson, B. H. 1966 Development and senescence of the post natal bovine ovary. *Journal of Animal Science* **25**.

Eshre. 2005 Fertility and Ageing. *Human Reproduction Update* **11**, 261-276.

Estes, R. D. 1992 The Safari Companion. A guide to watching African Mammals. Elephant.

Faddy, M. J. 2000 Follicle dynamics during ovarian ageing. *Molecular and Cellular Endocrinology* **163**, 43-48.

Faddy, M. J. & Gosden, R. G. 1995 A mathematical model of follicle dynamics in the human ovary. *Human Reproduction* **10**, 770-775.

Faddy, M. J. & Gosden, R. G. 1996 A model conforming the decline in follicle numbers to the age of menopause in women. *Human Reproduction* **11**, 1484-1486.

Faddy, M. J. & Gosden, R. G. 2010 Modelling the dynamics of ovarian follicle utilization throughout life. In *Biology and Pathology of the Oocyte* (ed. A. O. Trounson & R. G. Gosden): Cambridge University Press.

Faddy, M. J., Gosden, R. G., Gougeon, A., Richardson, S. J. & Nelson, J. F. 1992 Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. *Human Reproduction* **7**, 1342-1346.

Fair, T. 2003 Follicular oocyte growth and acquisition of developmental competence. *Animal Reproduction Science* **78**, 203-216.

Fair, T., Hulshof, S. C., Hyttel, P., Greve, T. & Boland, M. 1997 Oocyte ultrastructure in bovine primordial to early tertiary follicles. *Anatomical Embryology* **195**, 327-336.

Fawcett, D. W. 1997 Female Reproductive System. In *Bloom & Fawcett: Concise Histology* (ed. D. W. Fawcett & R. P. Jensh). New York: Chapman & Hall.

Fayrer-Hosken, R. A. 2008 Controlling animal populations using anti-fertility vaccines. *Reproduction in Domestic Animals* **43**, 179-185.

Fayrer-Hosken, R. A., Bertschinger, H. J., Kirkpatrick, J. F., Grobler, D., Lamberski, N., Honneyman, G. & Ulrich, T. 1999 Contraceptive potential of the porcine zona pellucida vaccine in the African elephant (Loxodonta africana). *Theriogenology* **52**, 835-46.

Findlay, J. K. 2010 Endocrine regulation of meiosis - local and systemic hormonal influences. In *Biology and Pathology of the Oocyte* (ed. A. O. Trounson & R. G. Gosden): Cambride University Press.

Findlay, J. K., Britt, K., Kerr, J. B., Donnell, L. O., Jones, M. E., Drummond, A. E. & Simpson, E. R. 2001 The road to ovulation: the role of oestrogens. *Reproduction, Fertility and Development* **13**, 543-547.

Floderus, S. 1944 Untersuchungen uber den Bau der menschlichen hypophyse mit besonderer berucksichtigung der quantitativen mikromorphologischen verhaltnisse. *Acta Pathology and Microbiology Scandinavia* **53**, 1-276.

Foggin, C. 2003 The elephant population problem in Zimbabwe: can there be an any alternative to culling. In *Managing African Elephant Populations: Act or let die?* (ed. B. Colenbrander, J. de Gooijer, R. Paling, S. Stout, T. Stout & A. T). Faculty of Veterinary Medicine, University of Utrecht, The Netherlands. .

Forabosco, A. & Sforza, C. 2007 Establishment of ovarian reserve: a quantitative morphometric study of the developing human ovary. *Fertility and Sterility* **88**, 675-683.

Forabosco, A., Sforza, C., de Pol, A., Vizzotto, L., Marzona, L. & Ferrario, V. F. 1991 Morphometric study of the human neonatal ovary. *Anatomical Record* **231**, 201-208.

Forbes, W. A. 1879 On the anatomy of the African elephant. *Proceedings of the Zoological Society of London*, 420.

Fortune, J. E. 1994 Ovarian follicular growth and development in mammals. *Biology of Reproduction* **50**, 225-232.

Fortune, J. E. 2003 The early stages of follicular development: activation of primordial follicles and growth of preantral follicles. *Animal Reproduction Science* **78**, 135-163.

Fortune, J. E., Cushman, R. A., Wahi, C. M. & Kito, S. 2000 The primordial to primary follicle transition. *Molecular and Cellular Endocrinology* **25**, 53-60.

Franchi, L. L., Mandl, A. M. & Zuckerman, S. 1962 *The ovary*: Academic Press: London.

Freeman, E. W. 2004 Examination of the Interrelationships of Behavior, Dominance Status, and Ovarian Activity in Captive Asian and African Elephants. *Zoo Biology* **23**, 431-448.

Freeman, E. W., Whyte, I. J. & Brown, E. M. 2008 Reproductive evaluation of elephants culled in Kruger National Park, South Africa between 1975 and 1995. *African Journal of Ecology* **47**, 192 - 201.

Freeman, E. W., Whyte, I. J. & Brown, E. M. 2009 Reproductive evaluation of elephants culled in Kruger National Park, South Africa between 1975 and 1995. *African Journal of Ecology* **47**, 192 - 201.

Gaeth, A. P., Short, R. V. & Renfree, M. B. 1999 The developing renal, reproductive, and respiratory systems of the African elephant suggest an aquatic ancestry. *Proceedings of the National Academy of Sciences* **96**, 5555-5558.

Garrett, W. M. & Guthrie, H. D. 1999 Expression of Bcl-2 and 3 Beta hydroxysteroid dehydrogenase protein during oocyte and follicle development in fetal and post-natal pig ovaries. *Reproduction, Fertility and Development* **11**, 463-470.

Genuth, S. M. 2004 The Endocrine System. In *Physiology* (ed. R. M. Berne, M. N. Levy, B. M. Koeppen & B. A. Stanton), pp. 950. St Louis, USA: Mosby.

Gleicher, N., Weghofer, A. & Barad, D. H. 2011 Defining ovarian reserve to better understand ovarian aging. *Reproductive Biology and Endocrinology* **9**, 1-11.

Glickman, S. E., Short, R. V. & Renfree, M. B. 2005 Sexual differentiation in three unconventional mammals: spotted hyenas, elephants and tammar wallabies. *Hormones and Behaviour* **48**, 403-17.

Gondos, B. 1978 Oogonia and oocytes in mammals. In *The Vertebrate Ovary* (ed. R. E. Jones). New York: Plenum Press.

Gondos, B. & Zamboni, L. 1969 Ovarian Development: The functional importance of germ cell interconnections. *Fertility and Sterility* **20**, 176-177.

Gonzalez-Angulo, A., Hernandex-Jauregui, P. & Martinez-Zedilo, G. 1975 Fine structure of the gonads of the horse and its functional implications. *Journal of Reproduction and Fertility Supplement* **23**, 563-567.

Gosden, R. 1995 Oocyte development through life. In *Gametes: The Oocyte* (ed. G. Yovich): Cambridge Reviews in Human Reproduction.

Gosden, R. & Lee, B. 2010 Portrait of an oocyte: Our obscure origin. *The Journal of Clinical Investigation* **120**, 973-983.

Gosden, R. G. 1987 Follicular status at menopause. *Human Reproduction* **2**, 617-621.

Gosden, R. G. 2004 Germline stem cells in the postnatal ovary: is the ovary more like a testis? *Human Reproduction Update* **10**, 193-195.

Gosden, R. G. 2005 Growth and development of mammalian oocytes. In *Clinical Embryology Workbook*: University of Leeds.

Gosden, R. G. & Telfer, E. 1987 Numbers of follicles and oocytes in mammalian ovaries and their allometric relationships. *Journal of Zoology, London* **211**, 169-175.

Gougeon, A. 1996 Regulation of ovarian follicular development in primates: Facts and hypotheses. *Endocrine Reviews* **17**, 121 – 156.

Gougeon, A. 2010 The early stages of follicular growth. In *Biology and Pathology of the Oocyte* (ed. A. O. Trounson & G. R. G). Cambridge: Cambridge University Press.

Gougeon, A. & Chainy, G. B. N. 1987 Morphometric studies of small follicles in ovaries of women at different ages. *Journal of Reproduction and Fertility* **81**, 433–442.

Gougeon, A., Ecochard, R. & Thalabard, J. C. 1994 Age-related changes of the population of human ovarian follicles: Increase in the disappearance rate of non-growing and early-growing follicles in aging women. *Biology of Reproduction* **50**, 653 – 663.

Griffin, J., Emery, B. R., Huang, I., Peterson, C. M. & Carrell, D. T. 2006 Comparative analysis of follicle morphology and oocyte diameter in four mammalian species (mouse, hamster, pig, and human). *Journal of Experimental Clinical Assisted Reproduction* **3**, 1-9.

Grobler, D. G., vanAltena, J. J., Malan, J. H. & Mackey, R. L. 2008 Elephant translocation. In *Elephant management: a scientific assessment for South Africa.* (ed. R. J. S. a. K. G. Mennell), pp. 241-256. Johannesburg: WITS University Press.

Grootenhuis, A. 2003 Contraception by ZP immunization: Organon's involvement. In *Managing African Elephant Populations: Act or let die?* Faculty Veterinary Medicine, Utrecht University, The Netherlands.

Groves, C. P. & Grubb, P. 2000 Do *Loxodonta cyclotis* and *Loxodonta africana* interbreed? *Elephant* **2**, 4-7.

Gunasena, K. T., Lakey, J. R., Villines, P. M., Bush, M., Raath, C., Critser, E. S., McGann, L. E. & Critser, J. K. 1998 Antral follicles develop in xenografted cryopreserved African elephant (*Loxodonta africana*) ovarian tissue. *Animal Reproduction Science* **53**, 265-75.

Gundersen, H. J. G. & Jensen, E. B. 1987 The efficiency of systematic sampling in stereology and its prediction. *Journal of Microscopy* **147**, 229-263.

Guraya, S. S. 1978 Recent advances in the morphology, histochemistry, biochemistry, and physiology of interstitial gland cells of mammalian ovary. *International Review of Cytology* **55**, 171-245.

Guy, P. R. 1975 The daily food intake of the African elephant, *Loxodonta africana* Blumenbach, in Rhodesia. *Arnoldia Rhodesia* **7**, 1-6.

Hanks, J. 1971 Reproductive physiology of the African elephant, vol. PhD. Cambridge: University of Cambridge.

Hanks, J. 1972 Reproduction of elephant, Loxodonta africana, in the Luangwa Valley, Zambia. *Journal of Reproduction and Fertility* **30**, 13-26.

Hanks, J. 1973 Growth and development of the ovary of the African elephant, *Loxodonta africana* Blumenbach. *The Puku* **7**, 125-131.

Hanks, J. 1979 A Struggle for Survival. The Elephant Problem: C Struik Publishers.

Hanks, J. & Short, R. V. 1972 The formation and function of the corpus luteum in the African elephant, *Loxodonta africana*. *Journal of Reproduction and Fertility* **29**, 79-89.

Hansen, K. R., Knowlton, N. S., Thver, A. C., Charleston, J. S., Soules, M. R. & Klein, N. A. 2008 A new model of reproductive aging: the decline in ovarian non-growing folicle number from birth to menopause. *Human Reproduction* **23**, 699-708.

Hay, M. F. & Allen, W. R. 1975 An ultrastructural and histochemical study of the interstitial cells in the gonads of the fetal horse. *Journal of Reproduction and Fertility* **Supplement 23**, 557-561.

Heistermann, M. 1997 Assessment of ovarian function in the African elephant (Loxodonta africana) by measurement of 5[alpha]-reduced progesterone metabolites in serum and urine. *Zoo Biology* **16**, 273-284.

Hermes, R. 2000 Ultrasonography of the estrous cycle in female African elephants (Loxodonta africana). *Zoo Biology* **19**, 369-382.

Hildebrandt, T., Drews, B., Gaeth, A. P., Goeritz, F., Hermes, R., Schmitt, D., Gray, C., Rich, P., Streich, W. J., Short, R. V. & Renfree, M. B. 2007 Foetal age determination and development in elephants. *Proceedings of The Royal Society B, Biological Sciences* **274**, 323-331.

Hildebrandt, T. B. 1997 Assessment of health and reproductive status in African elephants by transrectal ultrasonography. In *Proceedings of American Association of Zoo Veterinarians Annual Conference*, pp. 207-211. Houston, TX.

Hildebrandt, T. B., Goritz, F., Pratt, N. C., Brown, J. L., Montali, R. J. M., Schmitt, D. L. & Hermes, R. 2000 Ultrasonography of the urogenital tract in Elephants: An important tool for assessing female reproductive function. *Zoo Biology* **19**, 321-332.

Hildebrandt, T. B., Hermes, R. & Goritz, F. 2004 Management of reproduction of African and Asian elephant populations in nature reserves, semi domesticated herds and zoos. In *15th Symposium on Tropical animal health and production. Management of elephant reproduction* (ed. J. H. E. de Gooijer & R. W. Paling), pp. 28-30. Utrecht University, The Netherlands.

Hildebrandt, T. B., Lueders, I., Hermes, R., Goeritz, F. & Saragusty, J. 2011 Reproductive cycle of the elephant. *Animal Reproduction Science* **124**, 176-183.

Hirshfield, A. N. 1989 Granulosa cell proliferation in very small follicles of cycling rats by long-term continuous triated thymidine infusion. *Biology of Reproduction* **41**, 309-316.

Hirshfield, A. N. 1991 Development of follicles in the mammalian ovary. *International Review of Cytology* **124**, 43-101.

Hirshfield, A. N. 1992 Heterogeneity of cell populations that contribute to the formation of primordial follicles in rats. *Biology of Reproduction* **47**, 466-472.

Hobson, B. M. & Boyd, I. L. 1984 gonadotrophin and progesterone concentrations in placentae of grey seals (Halichoerus grypus). *Journal of Reproduction and Fertility* **72**, 521-528.

Hodges, J. K. 1998 Endocrinology of the ovarian cycle and pregnancy in the Asian (Elephas maximus) and African (Loxodonta africana) elephant. *Animal Reproduction Science* **53**, 3-18.

Hodges, J. K., Heistermann, M., Beard, A. & van Aarde, R. J. 1997 Concentrations of progesterone and the 5 alpha-reduced progestins, 5 alpha-pregnane-3,20-dione and 3 alpha-hydroxy-5 alpha-pregnan-20-one, in luteal tissue and circulating blood and their relationship to luteal function in the African elephant, Loxodonta africana. *Biology of Reproduction* **56**, 640-6.

Hodges, J. K., van Aarde, R. J., Heistermann, M. & Hoppen, H. O. 1994 Progestin content and biosynthetic potential of the corpus luteum of the African elephant (Loxodonta africana). *Journal of Reproduction and Fertility* **102**, 163-8.

Hofmeyr, M. 2004 Translocation of elephant from the Kruger National Park to the Limpopo National Park as part of the initial development of the Greater Limpopo Transfrontier Park. In *EMOA Elephant Symposium*. Bakgatla Camp, Pilanesberg national Park, South Africa.

Hollister-Smith, J. A., Poole, J. H., Archie, A. E., Vance, E. A., Georgiadis, N. J., Moss, C. J. & Alberts, S. C. 2007 Age, musth and paternity success in wild male African elephants, Loxodonta africana. *Animal Behaviour* **74**, 287-296.

Howard, C. V. & Reed, M. G. 2005 *Unbiased Stereology*. Oxford: BIOS Scientific Publishers.

Hugh-Jones, M. E. & de Vos, V. 2002 Anthrax and wildlife. *Revue Scientifique et Technique* **21**, 359-383.

Hulshof, S. C. J., Bevers, M. M., van der Donk, H. A. & van den Hurk, R. 1992 The isolation and characterization of preantral follicles from foetal bovine ovaries. In *Proceedings of the 12th International Congress on Animal Reproduction Vol 1.*, pp. 336-338. The Hague.

Jachmann, H. 1988 Estimating age in African elephants: a revision of Laws' molar evaluation technique. *African Journal of Ecology* **26**, 51–56.

Jainudeen, M. R., Eisenberg, J. F. & Tilakeratne, N. 1971 Oestrous cycle of the asiatic elephant, Elephas maximus, in captivity. *Journal of Reproduction and Fertility* **27**, 321-8.

Jansen, R. P. S. 1995 *Bioethics and the oocyte: reproductive choice*. In Gametes. The Oocyte.

John, G. B., Gallardo, T. D., Shirdey, L. J. & Castrillon, D. H. 2008 Foxo3 is a PI3K-dependent molecular switch controlling the initiation of oocyte growth. *Developmental Biology* **321**, 197-204.

Johnson, J. 2005 Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. *Cell* **122**, 303-315.

Johnson, J., Canning, J., Kaneko, T., Pru, J. K. & Tilly, L. 2004 Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature* **428**, 145-150.

Johnson, M. H. & Everitt, B. J. 2004 Adult ovarian function. In *Essential Reproduction*. London: Blackwell Science Ltd.

Johnstone, R. A. & Cant, M. A. 2010 The evolution of menopause in cetaceans and humans: the role of demography. *Proceedings of the Royal Society Biological Sciences* **Published online**.

Junqueira, L. C. & Carneiro, J. 2005 *Basic Histology; Text and atlas*: McGraw Hill.

Kacinskis, M. A., Lucci, C. M., Lugue, M. C. & Bao, S. N. 2005 Morphometric and ultrastructural characterization of Bos indicus preantral follicles. *Animal Reproduction Science* **87**, 45-57.

Kapustin, N., Critser, J. K., Olson, D. & Malven, P. V. 1996 Nonluteal estrous cycles of 3-week duration are initiated by anovulatory luteinizing hormone peaks in African elephants. *Biology of Reproduction* **55**, 1147-54.

Kayanja, F. I. B. & Sale, J. B. 1973 The ovary of rock hyrax of the genus Procavia. *Journal of Reproduction and Fertility* **33**, 223-230.

Kellas, L. M. 1958 Ovaries of some fetal and prepubertal giraffes. *Nature* **181**, 487-488.

Keller, G. & Warrack, B. 2000 *Statistics for Management and Economics*: Duxbury Thomson Learning, California.

Kerley, G. I. H. & Landman, M. 2006 The impacts of elephants on biodiversity in the Eastern Cape Subtropical Thickets. In *South African Journal of Science*, vol. 102, pp. 395-402.

Kerr, J. B., Duckett, R., Myers, M., Britt, K. L., Mladenovska, T. & Findlay, J. K. 2006 Quantification of healthy follicles in the neonatal and adult mouse ovary: evidence for maintenance of primordial follicle supply. *Reproduction* **132**, 95 - 109.

Kerr, M. A. 1978 Reproduction of elephant in the Mana Pools National Park, Rhodesia. *Arnoldia Rhodesia* **8**, 1-11.

Kidson, A., Loskutoff, N. M., Raath, C., Wood, C. A., Williams, K. R., Schalkwyk, J. O. v., Dyche, W. K., Barry, D. M. & Bartels, P. 1995 Age- and parity-dependent differences in ovarian activity and oocyte maturity in the African Elephant (*Loxodonta Africana*). *Theriogenology* **43**, 246.

King, L. E., Douglas-Hamilton, I. & Vollrath, F. 2007 African elephants run from the sound of disturbed bees. *Current Biology* **17**.

Kingsbury, B. F. 1914 The interstitial cells of the mammalian ovary: Felis domestica. *The American Journal of Anatomy* **16**, 59-95.

Kirk, R. E. 1968 *Experimental Design: Procedures for the behavioural sciences*: Wadsworth Publishing company, Inc, California.

Kirkpatrick, J. F., Liu, I. K. & Turner, J. W. J. 1990 Remotely delivered immunocontraception in feral horses. *Wildlife Society Bulletin 18*, 326-330.

Kirkpatrick, J. F., Turner, J. W. J., Liu, I. K., Fayrer-Hosken, R. A. & Rutberg, A. T. 1997 Case studies in wildlife immunocontraception: wild and feral equids and white-tailed deer. *Reproduction, Fertility and Development* **9**, 105-110.

Konishi, I., Fujii, S., Okamura, H., Parmley, T. & Mori, T. 1986 Development of interstitial cells and ovigerous cords in the human fetal ovary: an ultrastructural study. *Journal of Anatomy* **148**, 121-135.

Krumrey, W. A. & Buss, I. 1968 Age estimation, growth and relationships between body dimensions of the female African elephant. *Journal of Mammalogy* **49**, 22–31.

Krysko, D. V., Diez-Fraile, A., Criel, G., Svistunov, A. A., Vandenabeele, P. & D'Herde, K. 2008 Life and death of female gametes during oogenesis and folliculogenesis. *Apoptosis* **13**, 1065-87.

Langman, J. 1981 *Medical Embryology*: Williams and Wilkins.

Lass, A., Silye, R., Abrams, D., Krausz, T., Hovatta, O., Margara, R. & Winston, R. M. L. 1997 Follicular density in ovarian biopsy of infertile women: a novel method to assess ovarian reserve. *Human Reproduction* **12**, 1028-1031.

Latshaw, W. K. 1987 *Veterinary developmental anatomy*: Decker Incorporated.

Laufer, N., Simon, A., Samueloff, A., Yaffe, H., Milwidsky, A. & Gielchinsky, Y. 2004 Successful spontaneous pregnancies in women older than 45 years. *Fertility and Sterility* **81**, 1-10.

Laws, R. M. 1966 Age criteria for the African elephant. *East African Wildlife Journal* **4**, 1–37.

Laws, R. M. 1967 Occurrence of placental scars in the uterus of the African elephant (*Loxodonta africana*). *Journal of Reproduction and Fertility* **14**, 445-9.

Laws, R. M. 1969 Aspects of reproduction in the African Elephant, *Loxodonta Africana*. *Journal of Reproduction and Fertility* **Supplement 6**, 193–217.

Laws, R. M., Parker, I. S. C. & Johnstone, R. C. B. 1970 Elephants and habitats in Northern Bunyoro, Uganda. *East African Wildlife Journal* **8**, 163.

Laws, R. M., Parker, I. S. C. & Johnstone, R. C. B. 1975 *Elephants and their habitats. The ecology of elephants in North Bunyoro, Uganda*: Clarendon Press. Oxford.

Lee, P. 2010 Effects of drought in Amboseli National Park, pp. Personal communication to Stansfield, F J.

Lee, P. C. 1987 Allomothering among African elephants. *Animal Behaviour* **35**, 278-291.

Lee, P. C. & Moss, C. J. 1986 Early maternal investment in male and female African elephant calves. *Behaviour, Ecology, Sociobiology* **18**, 353-361.

Lee, P. C., Sayialel, S., Lindsay, W. K. & Moss, C. J. 2012 African elephant age determination from teeth: validation from known individuals. *African Journal of Ecology* **50**, 9-20.

Lemieux, A. M. & Clarke, R. V. 2009 The international ban on ivory sales and its effects on elephant poaching in Africa. *British Journal of Criminology* **49**, 451-471.

Li, J., Kawamura, K., Cheng, Y., Liu, S., Klein, C., Liu, S., Duan, E. & Hsueh, A. J. W. 2010 Activation of dormant ovarian follicles to generate mature eggs. *PNAS* **107**, 10280-10284.

Lindeque, M., Skinner, J. D. & Millar, R. P. 1986 Adrenal and gonadal contribution to circulating androgens in spotted hyaenas (*Crocuta crocuta*) as revealed by LHRH, hCG and ACTH stimulation. *Journal of Reproduction and Fertility* **78**, 211-217.

Lindsey, P. A., Roulet, P. A. & Romanach, S. S. 2007 Economic and conservation significance of the trophy hunting industry in sub-Saharan Africa. *Biological Conservation* **134**, 455-469.

Lintern-Moore, S. & Moore, G. P. M. 1979 The initiation of follicle and oocyte growth in the mouse ovary. *Biology of Reproduction* **20**, 773-778.

Lintern-Moore, S., Peters, H. & Moore, G. P. M. 1974 Follicular development in the infant ovary. *Journal of Reproduction and Fertility* **39**, 53-64.

Lotter, H. P. P., Henley, M., Fakir, S. & Pickover, M. 2008 Ethical considerations in elephant management. In *Elephant Management. A scientific assessment for South Africa* (ed. R. J. Scholes & K. G. Mennell), pp. 406-445. Johannesburg: WITS University Press.

Lueders, I. 2010 Are luteinizing follicles a pre-condition for ovulatory follicle deviation? In *International Elephant Foundation Symposium 2010*. Pretoria, South Africa.

Lueders, I., C, N., Gray, C., Rich, P. & Hildebrandt, T. B. 2010 Luteogenesis during the estrous cycle in Asian elephants (Elephas maximus). *Reproduction* **140**, 777-786.

Lueders, I., Niemuller, C., Rich, P., Gray, C., Hermes, R., Goeritz, F. & Hildebrandt, T. B. 2012 Gestating for 22 months: luteal development and pregnancy maintenance in elephants. *Proceedings of the Royal Society B Biological Sciences* **Published online 20 June 2012**.

Lueders, I., Taya, K., Watanabe, G., Yamamoto, Y., Yamamoto, T., Kaewmanee, S., Niemuller, C., Gray, C., Streich, W. J. & Hildebrandt, T. B. 2011 Role of the double luteinizing hormone peak, luteinizing follicles, and the secretion of inhibin for dominant

follicle selection in Asian Elephants (Elephas maximus). *Biology of Reproduction* **85**, 714-720.

Lundy, T., Smith, P., O'Connell, A., Hudson, N. L. & McNatty, K. P. 1999 Populations of granulosa cells in small follicles of the sheep ovary. *Journal of Reproduction and Fertility* **115**, 251-262.

Mamsen, L. S., Lutterodt, M. C., Andersen, E. W., Byskov, A. G. & Y, A. C. 2011 Germ cell numbers in human embryonic and fetal gonads during the first two trimesters of pregnancy: analysis of six published studies. *Human reproduction* **26**, 2140-2145.

Marais, J. & Hadaway, D. 2006 *Great Tuskers of Africa*. Johannesburg: Penguin Books.

McComb, K., Moss, C., Durant, S. M., Baker, L. & Sayialel, S. 2001 Matriarchs as repositories of social knowledge in African elephants. *Science* **292**, 491-4.

McGeady, T. A., Quinn, P. J., FitzPatrick, E. S. & Ryan, M. T. 2006 Male and Female Reproductive Systems. In *Veterinary Embryology*: Blackwell Publishing.

McLaren, A. 2001 Mammalian germ cells: birth, sex, and immorality. *Cell structure and function* **26**, 119-122.

McLaren, A. 2003 Primordial germ cells in the mouse. *Developmental Biology* **262**, 1-15.

McLaughlin, E. A. & McIver, S. C. 2009 Awakening the oocyte: controlling primordial follicle development. *Reproduction* **137**, 1-11.

McNatty, K. P., Fidler, A. E., Juengel, J. L., Quirke, L. D., Smith, P. R., Heath, D. A., Lundy, T., O'Connell, A. & Tisdall, D. J. 2000 Growth and paracrine factors regulating follicular formation and cellular function. *Molecular and Cellular Endocrinology* **163**, 11-20.

Meredith, M. 2001 *Africa's Elephant. A Biography*. London: Hodder and Stoughton.

Meredith, S., Dudenhoeffer, G. & Jackson, K. 1999 Single-section counting error when distinguishing between primordial and early primary follicles in sections of rat ovary of different thickness. *Journal of Reproduction and Fertility* **117**, 339–343.

Meredith, S., Dudenhoeffer, G. & Jackson, K. 2000 Classification of small type B/C follicles as primordial follicles in mature rats. *Journal of Reproduction and Fertility* **119**, 43–48.

Meyer, J. M., Walker, S. L., Freeman, E. W., Steinetz, B. G. & Brown, J. L. 2004 Species and fetal gender effects on the endocrinology of pregnancy in elephants. *General and Comparative Endocrinology* **138**, 263-70.

Miller, P. B. 1999 Morphometric analysis of primordial follicle number in Pigtailed Monkey ovaries: Symmetry and relationship with age. *Biology of Reproduction* **61**, 553-556.

Miller, P. B., Charleston, J. S., Battaglia, D. E., Klein, N. A. & Soules, M. R. 1997 An accurate, simple Method for unbiased determination of primordial follicle number in the primate ovary. *Biology of Reproduction* **56**, 909-915.

Mizroch, S. A. 1981 Analyses of some biological parameters in the Antarctic fin whale. *International Whaling Commission* **31**, 425-434.

Mori, H. & Matsumoto, K. 1970 On the histogenesis of the ovarian interstitial gland in rabbits I. Primary interstitial gland. *American Journal of Anatomy* **129**, 289-306.

Moss, C. 1996 *Getting to know a population*. Studying Elephants: Handbook 7 of African Wildlife Foundation.

Moss, C. 2001 The demography of an African elephant (*Loxodonta africana*) population in Amboseli, Kenya. *Journal of Zoology London* **255**, 145-156.

Moss, C. & Poole, J. H. 1983 Relationships and social structure of African elephants. In *Primate social relationships: an integrated approach* (ed. R. E. Hinde): Oxford: Blackwell Scientific.

Moss, C. J., Croze, H. & Lee, P. C. 2011 The Amboseli Elephants: A long-term perspective on a long-lived mammal.

Moss, C. J. & Lee, P. C. 2011 Female reproductive strategies: individual life histories. In *The Amboseli Elephants: A long-term perspective on a long-lived mammal* (ed. C. J. Moss, H. Croze & L. P. C).

Mounton, P. R. 2002 Principles and practices of unbiased stereology. Baltimore: The John Hopkins University Press.

Myers, M., Britt, K. L., Wreford, N. G. M., Ebling, F. J. P. & Kerr, J. B. 2004 Methods for quantifying follicular numbers within the mouse ovary *Reproduction* **127**, 569-580.

Nduku, W. K. 1991 Notes on culling of elephant (Press requests). pp. 3. Harare, Zimbabwe.: Department of National Park and Wildlife Management.

Nelson, A., Bidwell, P. & Sillero-Zubiri, C. 2003 A review of human-elephant conflict management strategies (ed. P. W. Initiative), pp. 25 pages. Oxford University: Wildlife Conservation Research Unit.

Neuville, H. 1937 Recherches comparatives sur l'organe femelle des elephants, avec remarques sur les formations dites hymenales. *Annals Scientifica Naturelle (Zoology)* **10e** 245.

Nichols, S. M., Bavister, B. D., Brenner, C. A., Didier, P. J., Harrison, R. M. & Kubisch, H. M. 2005 Ovarian senescence in the rhesus monkey (Macaca mulatta). *Human Reproduction* **20**, 79–83.

Odor, D. L. 1960 Electron microscopic studies on ovarian oocytes and unferilized tubal ova in the rat. *Journal of Biophysics, Biochemistry and Cytology* **7**, 567-591.

Ogle, T. F., Braach, H. H. & Buss, I. O. 1973 Fine structure and progesterone concentration in the corpus luteum of the African elephant. *Anatomical Record* **175**, 707-23.

Oktay, K. 1995 Proliferating cell nuclear antigen marks the initiation of follicular growth in the rat. *Biology of Reproduction* **53**, 295–301.

Oktem, O. & Oktay, K. 2008 The ovary: anatomy and function throughout human life. *Annals of the New York Academy of Science* **1127**, 1-9.

Oktem, O. & Urman, B. 2010 Understanding follicle growth in vivo. *Human Reproduction* **25**, 2944-2954.

Osborn, F. V. & Parker, G. E. 2003 Towards an integrated approach for reducing the conflict between elephants and people: a review of current research. *Oryx* **37**, 80-84.

Packer, C., Tatar, M. & Collins, A. 1998 Reproductive cessation in female mammals. *Nature* **392**, 807-811.

Pashen, R. L. & Allen, W. R. 1979 The role of fetal gonads and placenta in steroid production, maintenance of pregnancy and parturition in the mare. *Journal of Reproduction and Fertility Supplement* **27**, 499-509.

Paterson, M., Koothan, P. T., Morris, K. D., O'Byrne, K. T., Braude, P., William, A. & Aitken, R. J. 1992 Analysis of the contraceptive potential of antibodies against native and deglycosylated prcine ZP3 in vivo and in vitro. *Biology of Reproduction* **46**, 523-534.

Paterson, M., Wilson, M. R., van Duin, M. & Aitken, R. J. 1996 Evaluation of zona pellucida antigens as potential candidates for immunocontraception. *Journal of Reproduction and Fertility* **Supplement 50**, 175-182.

Patterson, A. M. & Dun, R. C. 1898 The genito-urinary organs of the female Indian elephant. *Journal of Anatomy and Physiology* **32**, 582.

Pepling, M. E. 2006 From primordial germ cell to primordial follicle: mammalian female germ cell development. *Genesis* **44**, 622-632.

Pepling, M. E. 2012 Follicular assembly: mechanisms of action. *Reproduction* **143**.

Pepling, M. E., de Cuevas, M. & Spradling, A. C. 1999 Germline cysts: a conserved phase of germ cell development? *Trends in Cellular Biology* **9**, 257-262.

Pepling, M. E. & Spradling, A. C. 2001 Mouse ovarian germ cell systs undergo programmed breakdown to form primoridal follicles. *Developmental Biology* **234**, 339-351.

Perdok, A. A., de Boer, W. F. & Stout, T. A. F. 2007 Prospects for managing African elephant population growth by immunocontraception: a review. *Pachyderm* **42**, 97-107.

Perrault, C. 1734 Description anatomique d'un elephant. *Memmoirs of the Academy of Science, Paris* **3**, 91.

Perry, J. S. 1953 The reproduction of the African elephant, Loxodonta africana. Philosophical Transactions of the Royal Society of London B Biological Science **237**, 93-149.

Perry, J. S. 1964 The structure and development of the reproductive organs of the female African elephant. *Philosophical Transaction of the Royal Society of London, Series B: Biological Sciences* **248**, 35–51.

Perry, J. S. 1974 Implantation, foetal membranes and early placentation of the African elephant, *Loxodonta africana*. *Philosophical Transactions of the Royal Society of London B Biological Science* **269**, 109-35.

Peters, H. 1969 The development of the mouse ovary from birth to maturity. *Acta Endocrinology* **62**, 98-916.

Peters, H. & Levy, E. 1963 Effect of irradiation in infancy on the fertility of female mice. *Radiat Res* **18**, 421-428.

Peters, H. & McNatty, K. P. 1980 *The Ovary, a correlation of structure and function in mammals*: University of California Press.

Picton, H., Briggs, D. & Gosden, R. 1998 The molecular basis of oocyte growth and development. *Molecular and Cellular Endocrinology* **145**, 27-37.

Picton, H. M. 2001 Activation of follicle development: the primordial follicle. *Theriogenology* **55**, 1193-1210.

Poirot, C. & al, e. 2002 Human ovarian tissue cryopreservation: indications and feasibility. *Human Reproduction* **17**, 1447-1452.

Poole, J., H & Granli, P. 2011 Signals, gestures and behavior of African Elephants. In *The Amboseli Elephants. A long term perspective on a long-lived mammal* (ed. C. Moss, H. Croze & P. C. Lee), pp. 109-124. Chicago and London: The University of Chicago Press.

Poole, J. H. 1989 Mate guarding, reproductive success and female choice in African elephants. *Anim. Behav.* **37**, 842-849.

Poole, J. H., Kasman, L. H., Ramsay, E. C. & Lasley, B. L. 1984 Musth and urinary testosterone concentrations in the African elephant (Loxodonta africana). *Journal of Reproduction and Fertility* **70**, 255-260.

Poole, J. H., Lee, P. C., Jniraini, N. & Moss, C. J. 2011 Longevity, competition and musth: A long-term perspective on male reproductive strategies. In *The Amboseli Elephants. A long term perspective on a long-lived mammal*, pp. 272-290. Chicago and London: The University of Chicago Press.

Proctor, C. M., Freeman, E. W. & Brown, J. L. 2010 Results of a second survey to assess the reproductive status of female Asian and African elephants in North America. *Zoo Biology* **29**, 127-139.

Qu, J., Godin, P. A., Nisole, M. & Donnez, J. 2000 Distribution and epidermal growth factor receptor expression of primordial follicles in human ovarian tissue before and aftr cryopreservation. *Human Reproduction* **15**, 302-310.

Quirke, L. D., Juengel, J. L., Tisdall, D. J., Lun, S., Heath, D. A. & McNatty, K. P. 2001 Ontogeny of steroidogenesis in the fetal sheep gonad. *Biology of Reproduction* **65**, 216-228.

Raeside, J. I. & Liptrap, R. M. 1975 Patterns of urinary oestrogen excretion in individual pregnant mares. *Journal of Reproduction and Fertility Supplement* **23**, 469-475.

Reddy, P., Zheng, W. & Liu, K. 2010 Mechanisms maintaining the dormancy and survival of mammalian primordial follicles. *Trends in Endocrionology and Metabolism* **21**, 96-103.

Reynaud, K. & Driancourt, M. A. 2000 Oocyte attrition. *Molecular and Cellular Endocrinology* **163**, 101-108.

Richardson, S. J., Senikas, V. & Nelson, J. F. 1987 Follicular depletion during the menopausal transition: evidence for accelerated loss and ultimate exhaustion. *Journal of Clinical Endocrinology and Metabolism* **65**, 1231-1237.

Roca, A. L., Georgiadis, B. & J, O. B. S. 2005 Cytonuclear genomic dissociation in African elephant species. *Nature Genetics* **37**, 96-100.

Rodgers, R. J. & Irving-Rodgers, H. F. 2010 The morphological classification of bovine ovarian follicles. *Reproduction* **139**, 309-318.

Rosa, P. & Joubert, P. 2009 Wildlife exploitation in Sub-Saharan Africa: an enterpreneurship analysis. In *http://www.swinburne.edu.au/lib/ir/onlineconferences* /agse2009/000041.pdf (ed. AGSE), pp. 1013-1028.

Russe, I. 1983 Oogenesis in cattle and sheep. *Anatomy* **24**, 77-92.

Russe, I. & Sinowatz, F. 1991 *Lehrbuch der Embryologie der Haustiere*. Berlin, Hamburg.

Sawyer, H. R., Smith, P., Heath, D. A., Juengel, J. L., Wakefield, S. J. & McNatty, K. P. 2002 Formation of ovarian follicles during fetal development in sheep. *Biology of Reproduction* **66**, 1134-1150.

Schatten, H. & Constantinescu, G. M. 2007 *Comparative Reproductive Biology*: John Wiley and Sons.

Schmidt, K. L. T. 2003 Density and distribution of primordial follicles in single pieces of cortex from 21 patients and in individual pieces of cortex from three entire human ovaries. *Human Reproduction* **18**, 1158-1164.

Scholes, R. J., Carruthers, J., Van Aarde, R., Kerley, G. I. H., Twine, W., Grobler, D. G., Bertschinger, H., Grant, C. C., Slotow, R., Lotter, H. H. P., Blignaut, J., Hopkinson, L. and Biggs, H. C. . 2007 *Summary for Policymakers: assessment of South African elephant management.* Johannesburg: Witwatersrand University Press.

Seth-Smith, A. M. D. & Parker, I. S. C. 1967 A record of twin foetuses in the African Elephant. *African Journal of Ecology* **5**, 167.

Sharara, F. I. 2004 Assessment of ovarian reserve. Is there still a role for ovarian biopsy? First do no harm! *Human Reproduction* **19**, 470-471.

Sherry, B. Y. 1975 Reproduction of elephant in Gonarezhou, South-Eastern Rhodesia. *Arnoldia Rhodesia* **29**, 7–13.

Short, R. 1966 Oestrous behaviour, ovulation and the formation of the corpus luteum in the African elephant. *East African Wildlife Journal* **4**, 56–68.

Short, R. V. 1969 Notes on the teeth oand ovaries of an African elephant (Loxodonta Africana) of known age. *Journal of Zoology, London* **158**, 421–425.

Short, R. V. & Buss, I. O. 1965 Biochemical and histological observations on the corpora lutea of the African Elephant, *Loxodonta Africana*. *Journal of Reproduction and Fertility* **9**, 61-7.

Sikes, S. K. 1966 The African elephant, Loxodonta africana: a field method for the estimation of age. *Journal of Zoology London* **150**, 279-295.

Sikes, S. K. 1971 *The Natural History of the African elephant*. The Natural History of the African Elephant. London: Weidenfeld & Nicolson.

Sinowatz, F., Amselgruber, W., Topfer-Petersen, E., Totzauer, I., Calvete, J. & Plendl, J. 1995 Immunocytochemical characterization of porcine zona pellucida during folicular development. *Anatomical Embryology* **191**, 41-46.

Skinner, J. D. & Smithers, R. H. N. 1990 *The Mammals of the Southern African Subregion*: Cambridge University Press.

Slotow, R., Garai, M. E., Reilly, B., Page, B. and Carr, R. D. . 2005 Population dynamics of elephants re-introduced to small fenced reserved in South Africa. *South African Journal of Wildlife Research* **35**, 23-32.

Slotow, R. a. v. D., G. 2001 Role of delinquent young "orphan" male elephants in high mortality of white rhinocerous in Pilanesberg National Park, South Africa. *Koedoe* **44**, 85-94.

Smith, J. G., Hanks, J. & Short, R. V. 1969 Biochemical observations on the corpora lutea of the African elephant Loxodonta africana. *Journal of Reproduction and Fertility* **20**, 111-117.

Smith, N. S. & Buss, I. O. 1975 Formation, function, and persistence of the corpora lutea of the African elephant (*Loxodonta africana*). *Journal of Mammalogy* **56**, 30-43.

Spicer, L. J. & Echternkamp, S. E. 1986 Ovarian follicular growth, function and turn over in cattle: a review. *Journal of Animal Science* **62**, 428-451.

Spinage, C. 1997 *Elephants* Poyser Natural History. London: Academic Press.

Stansfield, F. J. 2006 Ovarian reserve in wild African elephant. In *School of Medicine*, vol. MSc. MSc Clinical Embryology: University of Leeds.

Stansfield, F. J. & Allen, W. R. 2012 Luteal maintenance of pregnancy in the African elephant (*Loxodonta africana*). *Reproduction* **143**, 845-854.

Stansfield, F. J., Nöthling, J. O. & Ansari, T. 2011a The distribution of small preantral follicles within the ovaries of prepubertal African elephants (Loxodonta africana). *Animal Reproduction Science* **129**, 96-103.

Stansfield, F. J., Picton, H. M. & Nöthling, J. O. 2011b Early primary-rather than primordial follicles constitute the main follicular reserve in the African elephant (Loxodonta africana). *Animal Reproduction Science* **123**, 112-118.

Steenkamp, G., Ferreira, S. M. & Bester, M. N. 2007 Tusklessness and tusk fractures in free-ranging African savanna elephants (Loxodonta africana). *Journal of the South African Veterinary Association* **78**, 75-80.

Stout, T. A. E. & Colenbrander, B. 2004 Contraception as a tool for limiting elephant population growth; the possible pitfalls of various approaches. In *15th Symposium on Tropical animal health and production. Management of elephant reproduction* (ed. J. H. E. de Gooijer & R. W. Paling), pp. 7-10. Utrecht University, The Netherlands.

Sukumar, R. 2003 The Living Elephants. Evolutionary ecology, behavior, and conservation. NewYork: Oxford University Press.

Sweeney, L. J. 1998 *Basic Concepts in Embryology*. New York: The McGraw-Hill Companies Health Professions Division.

Tait, A. D., Santikaren, L. C. & Allen, W. R. 1983 Identification of 3-beta-hydroxy-5,7 androstadien-17-one as endogenous steroids in the foetal horse gonad. *Journal of Endocrinology* **99**, 87-92.

Tam, P. P. & Snow, M. H. 1981 Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos. *Journal of Embryology and Experimental Morphology* **64**, 133-147.

Taya, K. 2007 Secretion of gonadal inhibin and steroid hormones in the elephant. In *Managing the health and reproduction of elephant populations in Asia*, pp. 106-113. Kasetsart University, Bangkok, Thailand: EU-Asia Link Project Symposium.

te Velde, E. R. & Pearson, P. L. 2002 The variability of female reproductive ageing. *Human Reproduction Update* **8**, 141-154.

Teerds, K. J. & Dorrington, J. H. 1993 Immunohistochemical localization of 2B-Hydroxysteroid dehydrogenase in the rat ovary during follicular development and atresia. *Biology of Reproduction* **49-**, 989-996.

Telfer, E. E. & McLaughlin, M. 2007 Natural history of the mammalian oocyte. *Reproductive Biomedicine Online* **15**, 288-295.

Tietze, C. 1957 Reproductive span and rate of reproduction among Hutterite women. *Fertility and Sterility* **8**, 89-97.

Tilly, J. L. 2001 Commuting the death sentence: how oocytes strive to survive. *Nature Reviews Molecular Cell Biology* **2**, 838-848.

Tilly, J. L. & Johnson, J. 2007 Recent arguments against germ cell renewal in the adult human ovary: is an absence of marker gene expression really acceptable evidence of an absence of oogenesis? *Cell Cycle* **6**, 879-883.

Tilly, J. L. & Telfer, E. E. 2009 Purification of germline stem cells from adult mammalian ovaries: a step closer towards control of the female biological clock? *Molecular Human Reproduction* **15**, 393-398.

van den Hurk, R., Bevers, M. M. & Beckers, J. F. 1997 In vivo and in vitro development of preantral follicles. *Theriogenology* **47**, 73-82.

van den Hurk, R. & Zhao, J. 2005 Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. *Theriogenology* **63**, 1717-1751.

van Noord, P. S. H., Dubas, J. S., Dorland, M., Boersma, H. & te Velde, E. 1997 Age at natural menopause in a population-based screening cohort: the role of menarch, fecundity, and lifestyle factors. *Fertility and Sterility* **68**, 95-102.

Van Wezel, I. L. & Rodgers, R. J. 1996 Morphological characterization of bovine primordial follicles and their environment *in vivo*. *Biology of Reproduction* **55**, 1003–1011.

Viljoen, J. J., Ganswindt, A., Du Toit, J. T. and Langbauer, W. R. 2008 Translocation stress and faecal glucocorticoid metabolite levels in free-ranging African savannah elephants. *South African Journal of Wildlife Research* **38**, 146-152.

Wallace, W. H. B. & Kelsey, T. W. 2010 Human ovarian reserve from conception to the menopause. *Plos One* **5**, e8772.

Wandji, S. A., Srsen, V., Eppig, J. J. & Fortune, J. E. 1996 Initiation In Vitro of growth of bovine primordial follicles. *Biology of Reproduction* **55**, 942-948.

Wandji, S. A., Srsen, V., Nathanielsz, P. W., Eppig, J. J. & Fortune, J. E. 1997 Initiation of growth of baboon primordial follicles in vitro. *Human Reproduction* **12**, 1993-2001.

Ward, E. J., Parsons, K., Holmes, E. E., Balcomb III, K. C. & Ford, J. K. B. 2009 The role of menopause and reproductive senescence in a long-lived social mammal. *Frontiers in zoology* **6**.

Wassarman, P. M. & Josefowicz, W. J. 1978 Oocyte development in the mouse: an ultrastructural comparison of oocytes isolated at various stages of growth and meiotic competence. *Journal of Morphology* **156**, 209-235.

Watson, P. F. & D'Souza, F. D. 1975 Detection of oestrus in the african elephant (*Loxodonta africana*). *Theriogenology* **4**, 203-9.

Westergaard, C. G., Byskov, A. G. & Andersen, C. Y. 2007 Morphometric characteristics of the primordial to primary follicle transition in the human ovary in relation to age. *Human Reproduction* **22**, 2225-22231.

White, Y. A. R., Woods, D. C., Kakai, Y., Ishihara, O., Seki, H. & Tilly, J. L. 2012 Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. *Nature Medicine* **18**, 413-421.

Whyte, I. J. 1996 Collecting data from dead elephants. In *Studying Elephants* (ed. K. Kangwana).

Whyte, I. J. 2001 Conservation management of the Kruger National Park elephant population. Pretoria: Pretoria.

Whyte, I. J. 2002 Headaches and heartaches: the elephant managemement dilemma. In *Environmental Ethics: What really matters, what really works* (ed. D. Schmidtz & E. Willott), pp. 293-305. Oxford: Oxford University Press.

Williamson, B. R. 1976 Reproduction in female African elephant in the Whankie National Park, Rhodesia. *South African Journal of Wildlife Research* **6**, 89–93.

Witschi, E. 1948 Migration of the germ cells of human embryos from the yolk sac to the primitive gonadal folds. *Contributions to Embryology* **32**, 67-80.

Wood, J. W. 1989 Fecundity and natural fertility in Humans. *Oxford Reviews Reproductive Biology* **11**, 61-109.

Wooding, F. B., Stewart, F., Mathias, S. & Allen, W. R. 2005 Placentation in the African elephant, *Loxodonta africana*: III. Ultrastructural and functional features of the placenta. *Placenta* **26**, 449-70.

Wright, C. S., Hovatta, O., Margara, R., Trew, G., Winston, R. M. L., Franks, S. & Hardy, K. 1999 Effects of follicle stimulating hormone and serum substitution on the in-vitro growth of human ovarian follicles. *Human Reproduction* **14**, 1555–1562.

www.stereology.info.com. 2010 Optical disector vs. unbiased brick.

Yamamoto, T., Yamamoto, Y., Taya, K., Watanabe, G., Stansfield, F. J. & Allen, W. R. 2011 Placentation in the African elephant (Loxodonta africana) V. The trophoblast secretes placental lactogen. *Placenta* **32**, 506-510.

Young, J. M. 2010 Theca: the forgotten cell of the ovarian follicle. *Reproduction* **140**, 489-504.

Zamboni, L. 1974 Fine morphology of the follicle wall and follicle cell-oocyte association. *Biology of Reproduction* **10**, 125-149.

Zamboni, L. 1982 The contribution of the mesonephros to the development of the sheep fetal testis. *Developmental Dynamics* **165**, 339-356.

Zamboni, L., Bezard, J. & Mauleon, P. 1979 The role of the mesonephros in the development of the sheep fetal ovary. *Annals of Biology, Biochemistry and Biophysics* **19**, 1153-1178.

Zamboni, L. & Upadhyay, S. 1983 Germ cell differentiation in mouse adrenal glands. *Journal of Experimental Zoology* **228**, 178-193.

Zou, K., Yuan, Z., Yang, Z., Luo, H., Sun, K., Zhou, L., Xiang, J., Shi, L., Yu, Q., Zhang, Y., Hou, R. & Wu, J. 2009 Production of offspring from a germline stem cell line derived from neonatal ovaries. *Nature Cell Biology* **11**, 631-636.

Zuckerman, S. 1951 The number of oocytes in the mature ovary. *Recent Progress in Hormone Research* **6**, 63-108.