

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

Chapter 1.	General Introduction	1
1.1.	Classification of elephants	1
1.2.	A brief overview of studies related to reproductive processes in the elephant....	3
1.3.	The gross and microscopic anatomy and physiological functions of the elephant ovary and its follicles	5
1.3.1.	Research pertaining to fetal, neonatal and prepubertal ovaries	5
1.3.2.	Ovarian structures during different phases of the reproductive cycle of adult elephants	7
1.4.	Further anatomy and physiology of the elephant.....	15
1.4.1.	Anatomy of the female reproductive system	15
1.4.2.	Puberty	19
1.4.3.	Fertility.....	19
1.4.4.	Fetal size	22
1.4.5.	Placentation.....	23
1.4.6.	Lactation	25
1.4.7.	Ovarian ageing in elephants.....	25
1.4.8.	Elephant age classification.....	25
1.5.	Follicle development in mammals	26
1.5.1.	Origin of the ovarian reserve	26
1.5.2.	The ovarian reserve and follicle activation.....	35
1.5.3.	Continued follicle growth to pre-ovulatory size	42
1.5.4.	Follicle classification	43

1.5.5.	Small follicles in elephant ovaries	46
1.6.	Background to the over-abundance of elephants in Zimbabwe.....	47
1.6.1.	Elephant numbers in Zimbabwe	47
1.6.2.	Attempts to counteract over-abundance	51
1.7.	Objectives of the study	56
Chapter 2.	Materials and methods	57
2.1.	Source of specimens	57
2.2.	Collection of specimens.....	57
2.2.1.	Ovary and lower jaw collection.....	57
2.2.2.	Body measurements.....	58
2.2.3.	Further data collection	59
2.3.	Estimating the age of elephants	60
2.4.	Histology and stereology	63
2.4.1.	Establishment of the protocol	63
2.4.2.	Histology.....	64
2.4.3.	Stereological examination.....	68
2.4.4.	Sampling and bias.....	69
Chapter 3.	Follicle morphology in the ovary of the African elephant and the composition of the ovarian reserve.....	78
3.1.	Introduction.....	78
3.2.	Materials and methods	79
3.2.1.	Statistical Analysis.....	83
3.3.	Results.....	83

3.4.	Discussion	89
3.4.1.	Follicle classification	90
3.4.2.	The ovarian reserve	92
3.5.	Conclusions	94
Chapter 4.	The distribution of small preantral follicles within the ovaries of prepubertal African elephants (<i>Loxodonta africana</i>)	95
4.1.	Introduction	95
4.2.	Materials and methods	96
4.2.1.	Specimens and stereology	96
4.2.2.	Statistical analyses	97
4.3.	Results	99
4.4.	Discussion	105
Chapter 5.	Development of the germinal ridge and ovary in the African elephant (<i>Loxodonta africana</i>)	108
5.1.	Introduction	108
5.2.	Materials and methods	110
5.3.	Results	111
5.3.1.	Milestones in embryonic development	111
5.3.2.	Milestones in fetal development	116
5.3.3.	Germ cell counts	122
5.4.	Discussion	125
5.4.1.	Developmental stage and age of the embryos	125
5.4.2.	Morphology of the gonadal ridge and developing ovary	126
5.4.3.	Germ cell counts	129

Chapter 6. Growth and development of the ovary and small follicle pool from mid fetal life to pre-puberty in the African elephant (<i>Loxodonta africana</i>)	132
6.1. Introduction.....	132
6.2. Materials and methods	133
6.2.1. Tissue recovery	133
6.2.2. Tissue preparation.....	133
6.2.3. Immunohistochemical staining.....	133
6.2.4. Histological and stereological examinations	134
6.2.5. Classification of prepubertal calves into 3 physiological groups	134
6.3. Results.....	135
6.3.1. Fetal ovarian morphology (11–20 months of gestation).....	135
6.3.2. Prepubertal calf ovarian morphology	142
6.3.3. Immunohistochemical staining of interstitial cells	143
6.3.4. Ovarian weight.....	146
6.3.5. Follicle number	146
6.4. Discussion.....	149
6.4.1. Late fetal and prepubertal ovarian morphology.....	149
6.4.2. Interstitial cells.....	151
6.4.3. Follicle numbers	153
6.5. Conclusion	157
Chapter 7. The progression of small follicle reserves in wild African elephants (<i>Loxodonta Africana</i>) from puberty to reproductive senescence.....	158
7.1. Introduction.....	158
7.2. Materials and methods	159

7.2.1.	Animals	159
7.2.2.	Collection and processing of specimens	160
7.2.3.	Estimation of the age of <i>corpora nigra</i> (CN)	160
7.2.4.	Statistical analysis	161
7.3.	Results	162
7.3.1.	The relationship between the number of small follicles and age	162
7.3.2.	The relationship between the type of small follicles and age	164
7.3.3.	The relationship between reproductive status or tusklessness and the number of small follicles	166
7.3.4.	Reproductive status of the old elephant	166
7.4.	Discussion	168
7.4.1.	The follicle reserve after puberty	168
7.4.2.	The change in follicle numbers around puberty	169
7.4.3.	A switch in type of small follicle constituting the reserve	170
7.4.4.	The value of the current study with respect to understanding infertility of Zoo elephants	171
7.4.5.	The relationship between tusklessness and the follicular reserve	173
7.4.6.	Reproductive senescence	173
7.5.	Conclusion	174
Chapter 8.	General Discussion	176
8.1.	Main findings on the ontogeny of the follicular reserve in the African elephants	176
8.2.	Scope of inference from the salient findings	177

8.3.	Follicle counting methods, stereology versus serial sectioning.....	178
8.4.	The distribution of small follicles in the ovary of the African elephant.....	180
8.5.	Insights gained in the type of follicles constituting the follicle reserve in the African elephant.....	180
8.6.	Insights gained in the cessation of reproductive life.....	183
8.7.	The value of the current study with respect to understanding and improving strategies for contraception of African elephants	185
8.8.	Does the longevity of reproductive life in the elephant offer insights into reproductive senescence in humans, or <i>vice versa</i> ?.....	187
8.9.	Outstanding questions and possible future studies	192
Chapter 9.	References.....	194

List of tables

Table 1.1 Ovarian structures in an elephant shot immediately after mating during oestrus; data from Short (1966)	12
Table 1.2 Numbers of corpora lutea reported in the ovaries of pregnant elephants	14
Table 1.3 Weights of luteal tissue reported in the ovaries of pregnant elephants	14
Table 1.4 Range in luteal size reported in pregnant and cycling elephants	15
Table 1.5 Age of puberty in African elephants	19
Table 1.6 Published data on intercalving intervals in African elephants	20
Table 1.7 Studies reporting on fertility in old African elephants	21
Table 1.8 Reproductive status of elephants older than 50 years culled in Kruger National Park, South Africa during 1975–1995. Data from Freeman <i>et al.</i> (2008).	22
Table 1.9 Age classifications of African elephants based on Sykes (1971) and Laws (1969)	26
Table 1.10 Chronology of events during the differentiation of the mammalian gonad in various species (Days post conception)	28
Table 1.11 Numbers of primordial follicles in the ovary of mammals at varying ages (or number of oocytes during gestation)	32
Table 1.12 Small follicle classification in mammals	37
Table 1.13 Small follicle classification (SF)	39
Table 1.14 Diameter of follicles in the resting pool in various mammalian species	40
Table 1.15 Diameters of various parts of primary follicles (μm)	41
Table 1.16 Diameter of various parts of preantral follicles (μm)	45
Table 1.17 Estimated elephant numbers in Zimbabwe	48

Table 2.1 Hierarchy of experimental variability (Howard & Reed 2005)	70
Table 2.2 Types of probes used during stereological studies	71
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 <i>corpora lutea</i> (CL) in their ovaries.	84
Table 3.2 Numbers of small follicles (SF) in the ovaries of 16 African elephants.	85
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.	87
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	88
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.	89
Table 4.1 Mean (\pm 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 (<i>Loxodonta africana</i>) calves aged 2 months to 4.5 years	101
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	102
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	103
Table 5.1 Mass, crown-rump length (CRL) and estimated ages of the 5 elephant embryos	112
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	115

Table 5.3 Ovarian volumes (mm ³) of elephant fetuses	119
Table 5.4 Numbers and distributions of oogonia and small and growing follicles in the ovaries of elephant fetuses	123
Table 5.5 Number of small follicles in the reserves of different mammalian species	131
Table 6.1 The median (95% confidence interval) combined number of small follicles in both ovarian cortices of African elephant fetuses and prepubertal calves	148
Table 7.1 The number of small follicles (SF) per elephant of different age groups	162
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	164
Table 7.3 True primary follicles (TP) as a percentage of total small follicles (SF) in the ovaries of elephants of different age groups	166
Table 7.4 Comparative data of <i>corpora nigra</i> (CN) of known age in African elephants	168
Table 8.1 Approximation of the small follicle component of the ovarian reserve throughout life in the African elephant.	182
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	183

List of figures

Figure 1.1 The African elephant	2
Figure 1.2 Hormonal changes in the peripheral blood of female elephants during the oestrous cycle and pregnancy (diagrammatic)	9
Figure 1.3 A schematic representation of the reproductive organs of the female African elephant	17
Figure 1.4 The uterus and ovaries of the African elephant	18
Figure 1.5 The placenta of the African elephant	24
Figure 1.6 Similar morphology of the ovarian cortex in the domestic cow and the African elephant	34
Figure 1.7 Damage to trees caused by elephants in Savé Valley Conservancy and Mana Pools National Park, Zimbabwe	50
Figure 2.1 The ageing of elephants is achieved by examining the progression of molar teeth through the mandible	61
Figure 2.2 Sources of variation and error, according to (Mounton 2002)	69
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	73
Figure 2.4 Stereology	75
Figure 3.1 The ovaries of elephants and dimensions of measurements of small follicles	80
Figure 3.2 Small follicles in elephant ovaries	82
Figure 3.3 Comparison of follicle diameter, nuclear diameter and numbers of granulosa cells of small ovarian follicles in the African elephant	86

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	105
Figure 5.1 African elephant embryos aged (from left to right) 76, 81, 82, 87, and 96 days post conception (scale bar = 10mm)	111
Figure 5.2 Development of the gonad of the African elephant embryo	113
Figure 5.3 Photographs and photomicrographs of the gonads of elephant fetuses at 4.8 to 5.9 months of gestation	117
Figure 5.4 Ovarian sections from a mid-term (11.2 month) African elephant fetus	121
Figure 5.5 Elephant fetuses of different ages	124
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	135
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	136
Figure 6.3 Sectioned ovary of an elephant fetus at 11 months of gestation	138
Figure 6.4 Sections of the ovaries of an elephant fetus at 13.5 months of gestation	139
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)	140
Figure 6.6 The ovaries of two late-stage African elephant fetuses	142
Figure 6.7 Photographs of 25 µm thick sections of the ovaries of prepubertal elephant calves (Scale bar = 10 mm)	143
Figure 6.8 Sections of the ovaries of elephant fetuses and calves stained immunocytochemically with an anti-3β-HSD antibody	145

Figure 6.9 The number of small follicles in the ovaries of elephant fetuses and prepubertal calves in relation to age	147
Figure 6.10 Cortical volumes of fetal and prepubertal ovaries of African elephants	149
Figure 7.1 Ovaries from a pregnant and a non-pregnant African elephant cow	160
Figure 7.2 The total number of small ovarian follicles (SF) in elephant of different ages	163
Figure 7.3 The numbers of early primary (EP)- and true primary (TP) follicles in the ovaries of African elephants of different ages	165
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	167
Figure 8.1 The numbers of SF in elephant ovaries counted by serial sectioning and stereology	179
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	189
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)	191
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)	192

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
3 β -HSD	3 β -hydroxysteroid dehydrogenase
5 α -DHP	5 α -dihydroprogesterone
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
eIPL	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	Phosphatidylinositol 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
Σ	Sum of
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 4½–9 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.