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

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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).

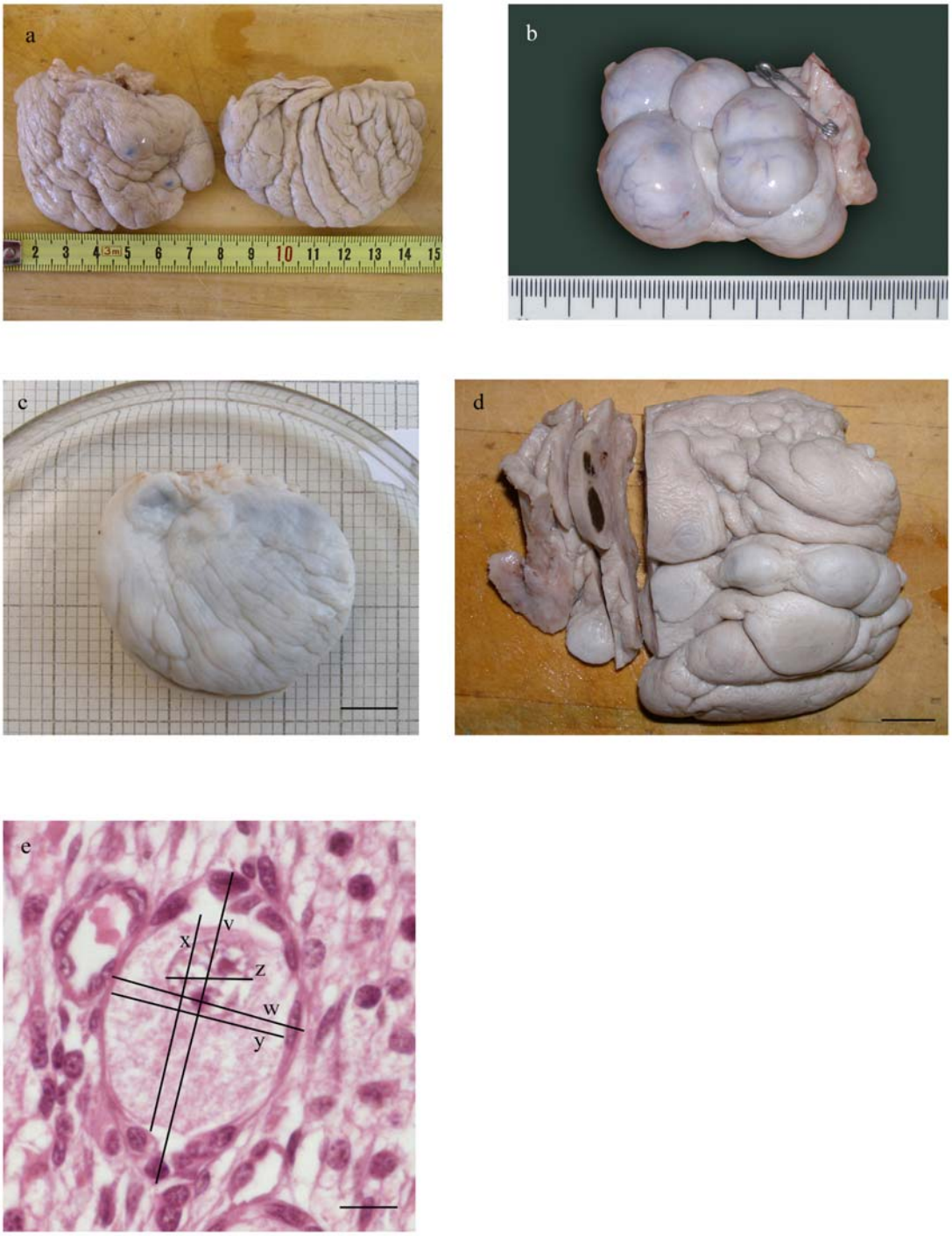


Figure 3.1 The ovaries of elephants and dimensions of measurements of small follicles

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.

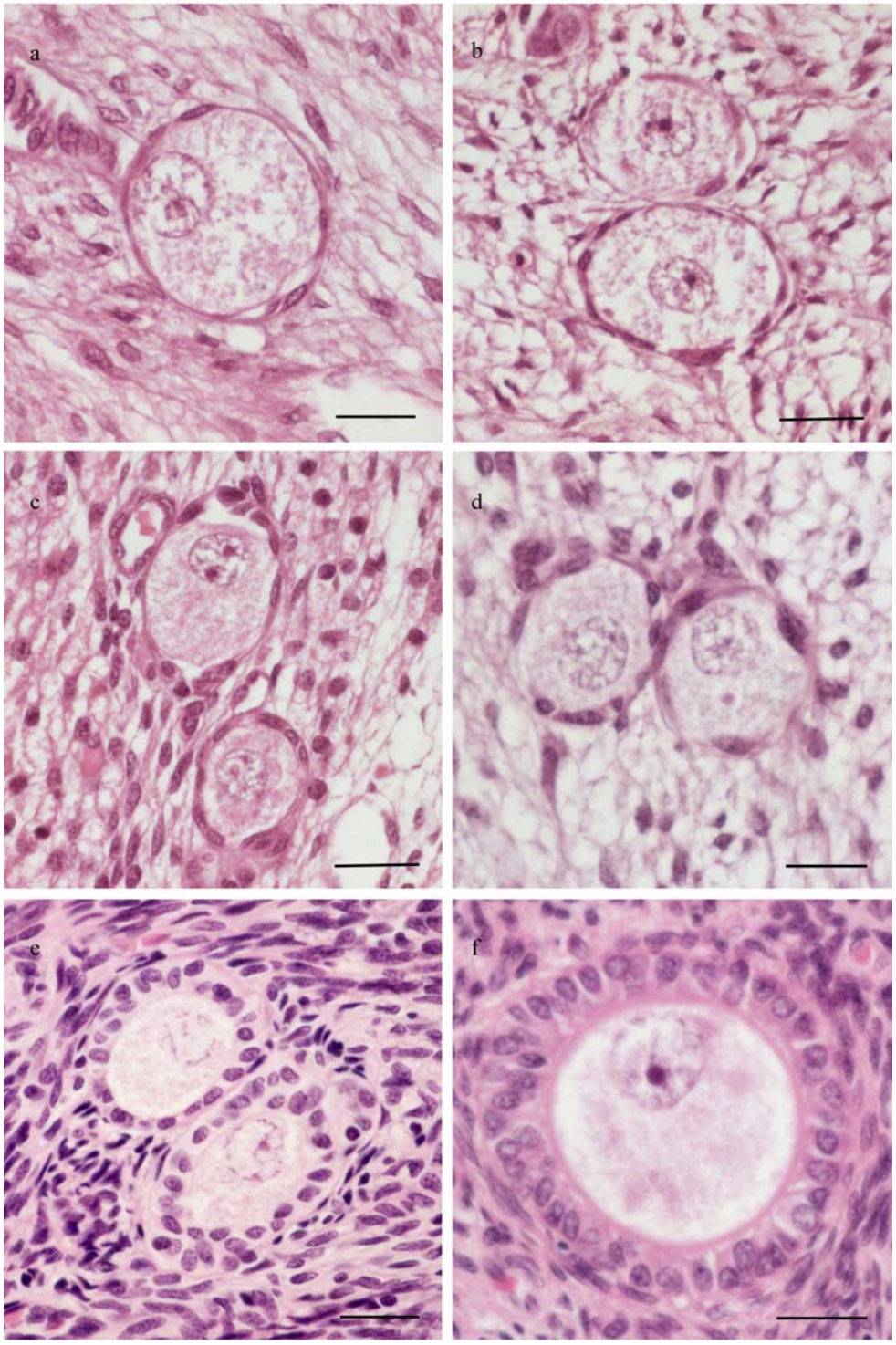


Figure 3.2 Small follicles in elephant ovaries

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

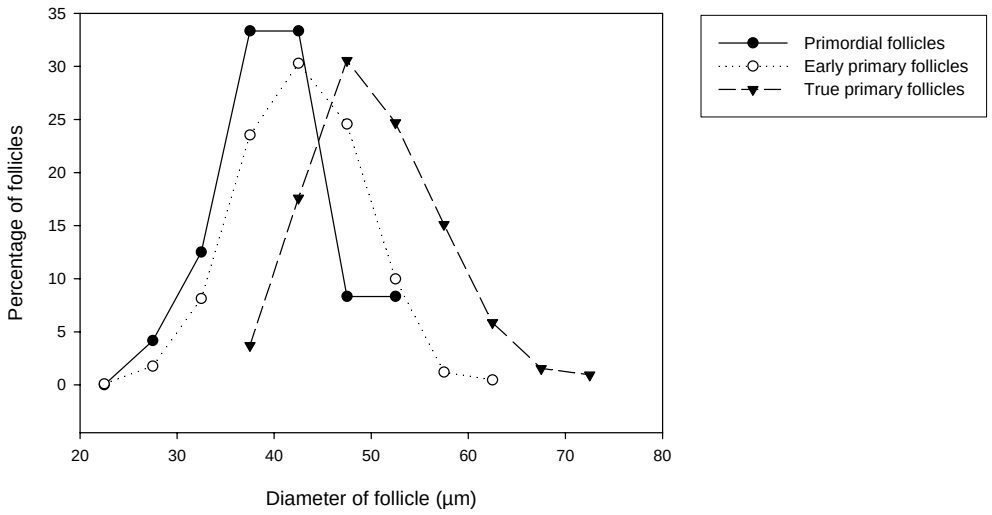
a < b < c, p < 0.001, n = 16 for each comparison

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).

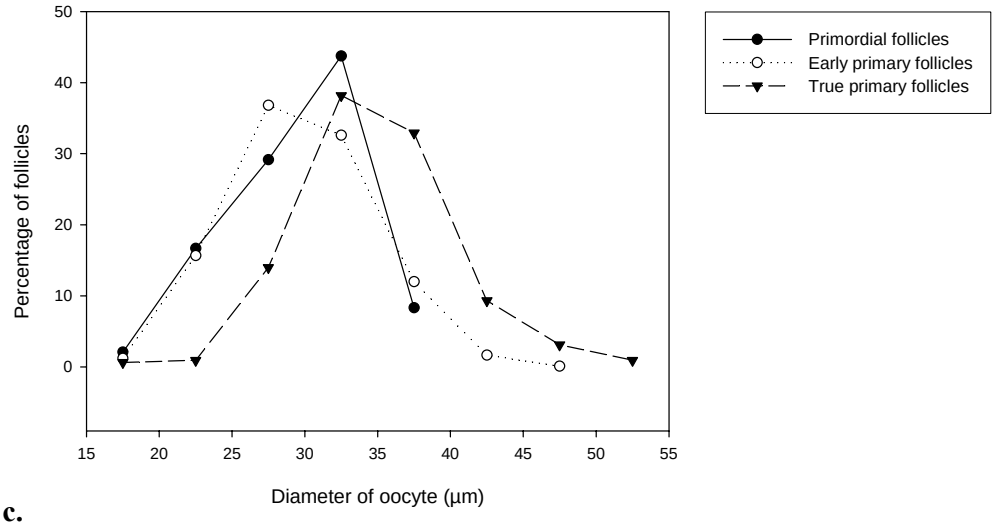
Seven elephants had one or more CL in their ovaries and 9 had none. The median age of elephants with CL was 20 (19–25) years, which was similar to the median age of 18 (16–25) years in the elephant without any CL (P = 0.56). Nevertheless, the 7 elephants with CL in their ovaries had 5 920 (853–10 287) 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.

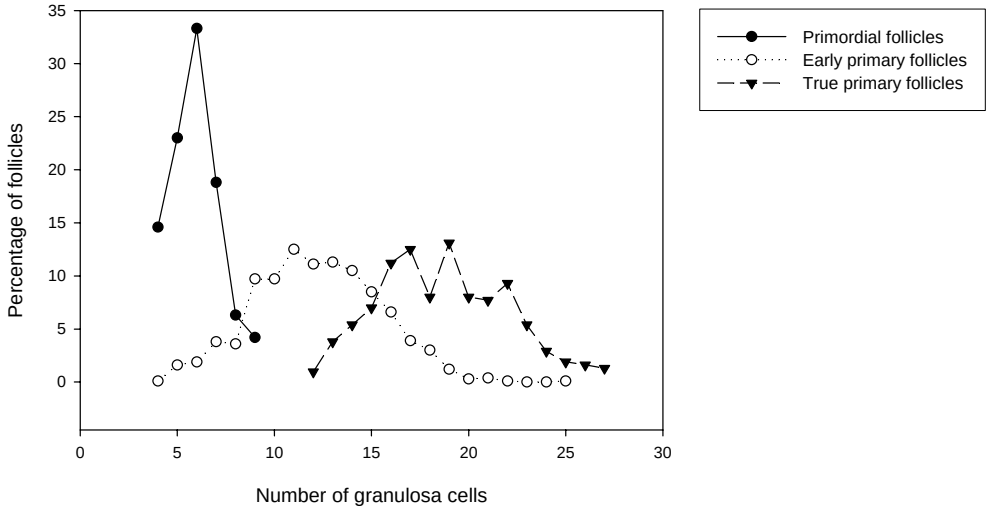


Figure 3.3 Comparison of follicle diameter, nuclear diameter and numbers of granulosa cells of small ovarian follicles in the African elephant

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.