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.

a. Small follicles counted in the same 8 elephants by means of serial sections and stereology
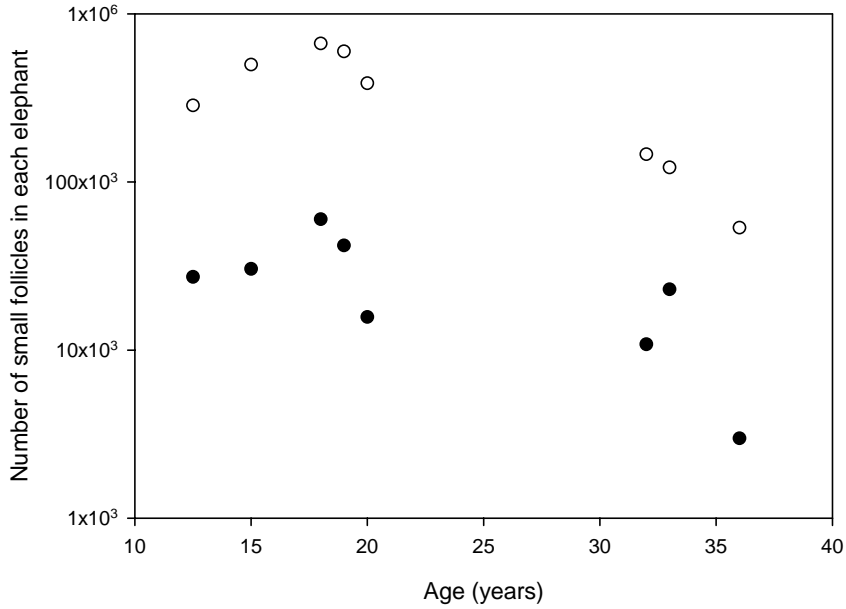
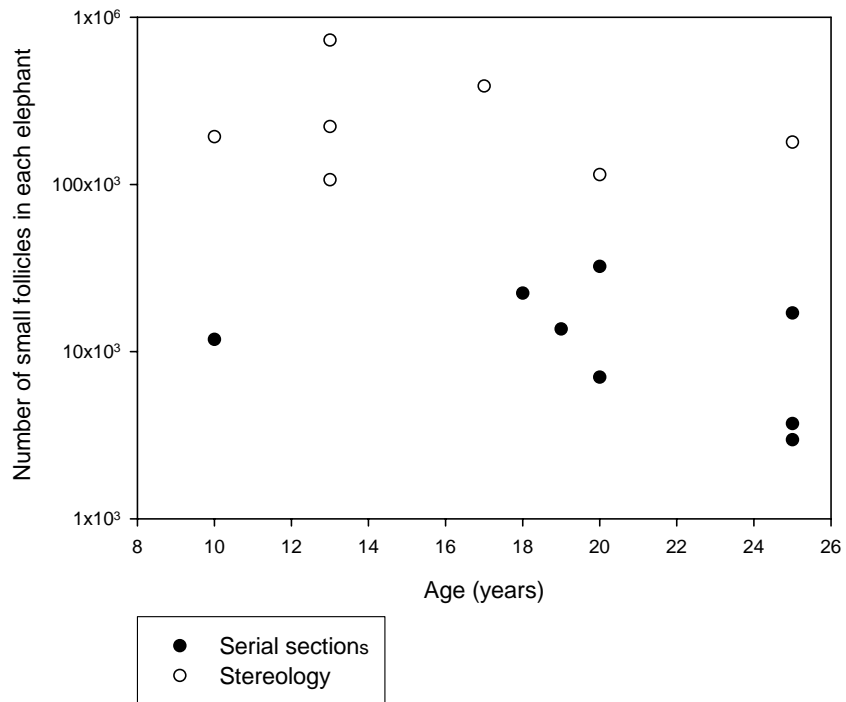**b. Different elephants used for counting small follicles with serial sections and stereology**

- ● Serial sections
- ○ Stereology

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.

Age group (years)	Percentage of all SF that are TP	Number of SF per elephant	
		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 significantly 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).
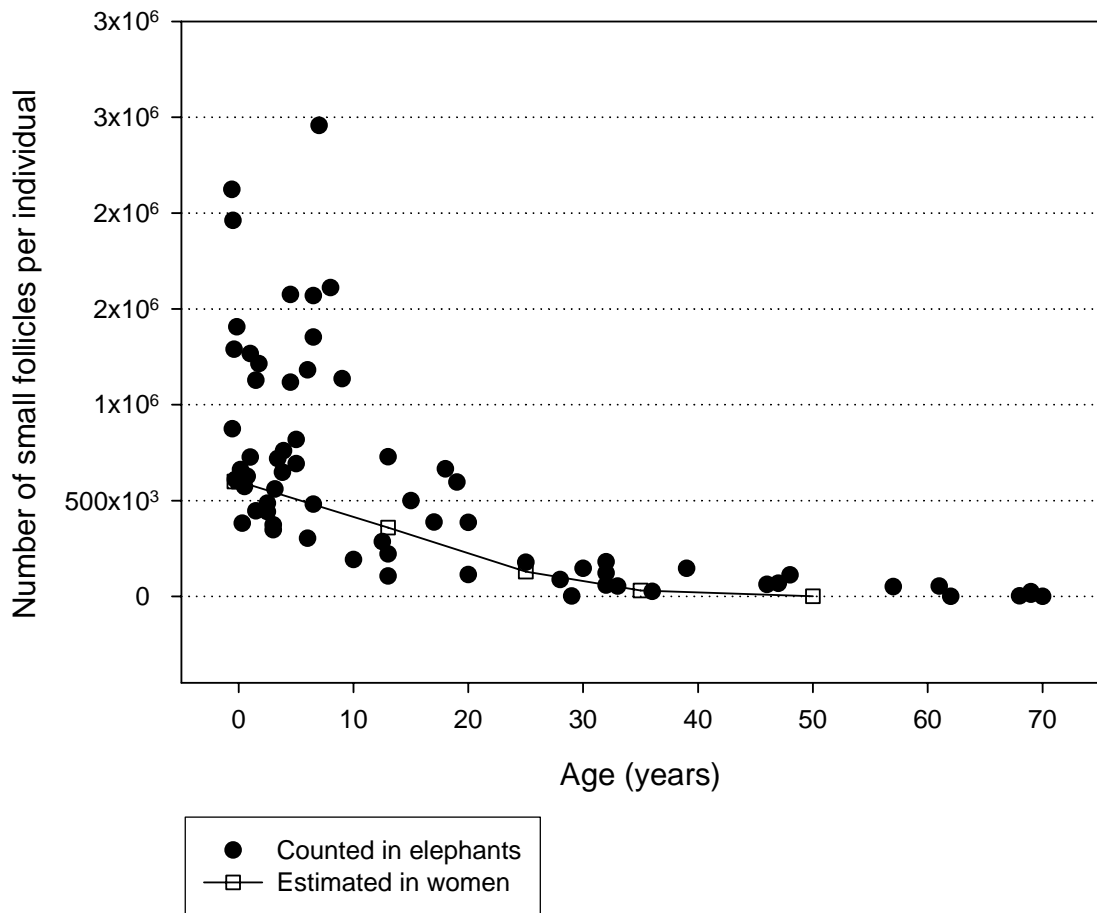
Figure 8.2 Comparison of the numbers of small ovarian follicles per elephant found in the current study and the mean number of non-growing ovarian follicles per woman, as modelled by Wallace and Kelsey (2010), from approximately mid gestation to the cessation of reproductive life

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).

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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.
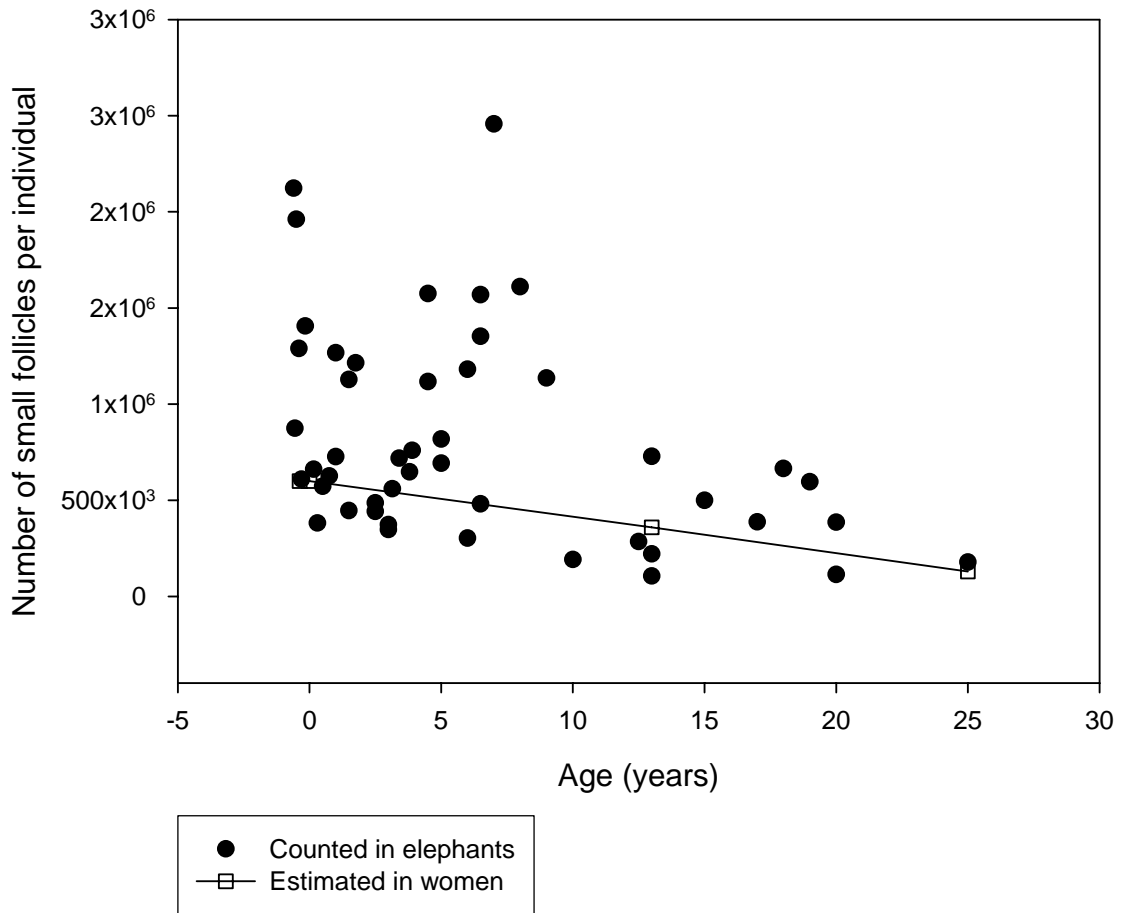
Figure 8.3 The numbers of small ovarian follicles per elephant and the average estimated per woman from approximately mid gestation to 25 years of age (Human data from Wallace and Kelsey 2010)
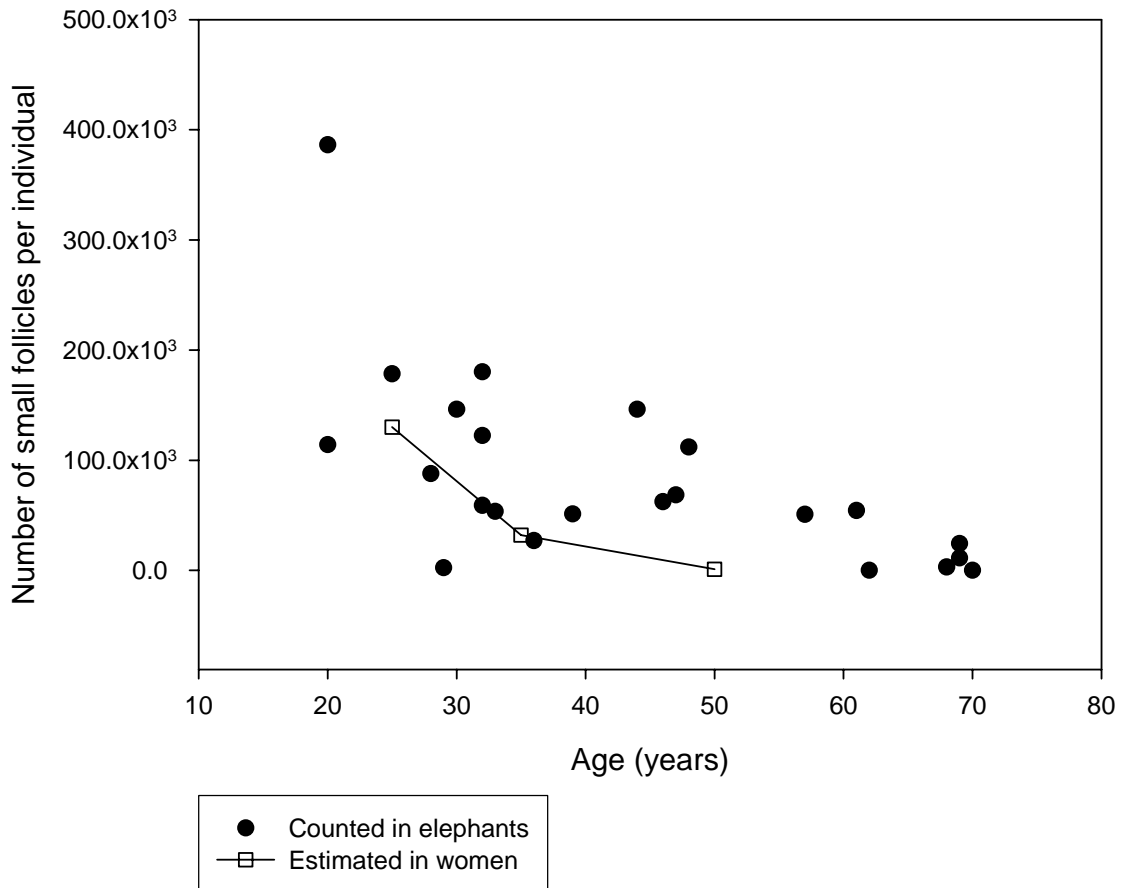
Figure 8.4 The numbers of small follicles in elephant and the average number estimated per woman from approximately 20–70 years of age (Human data from Wallace and Kelsey 2010)

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?