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.

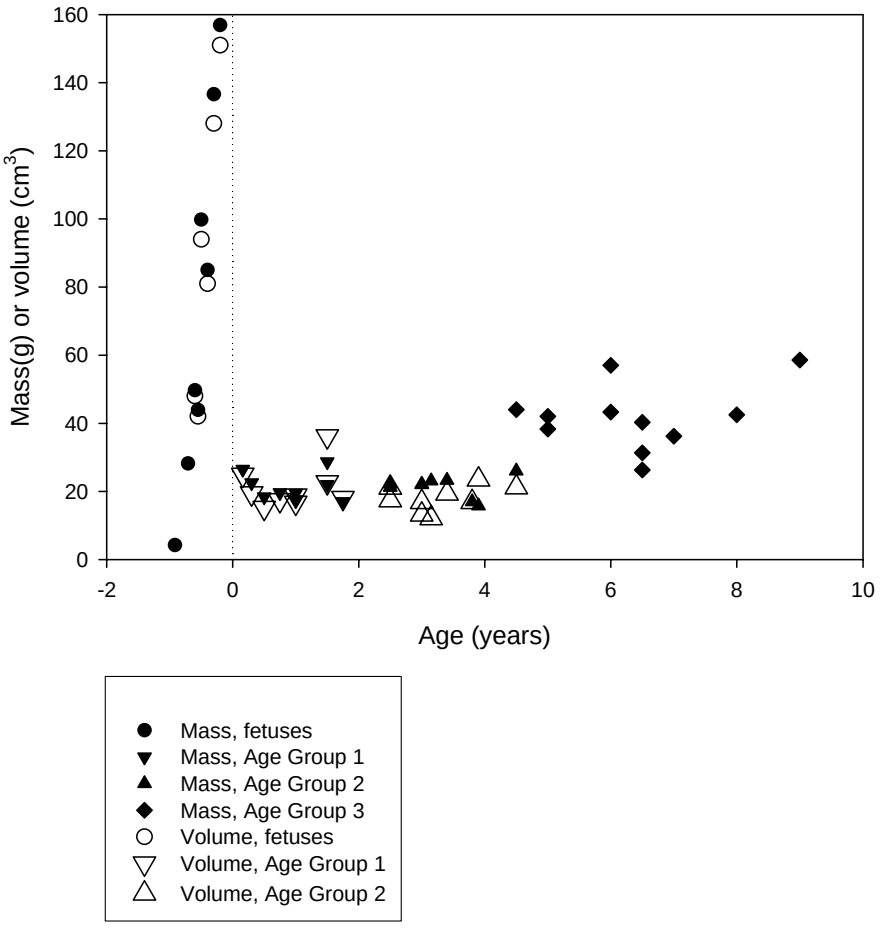

Figure 6.1 Combined weights and volumes of the ovaries of African elephant fetuses from mid-gestation onwards and of calves up to 9 years of age

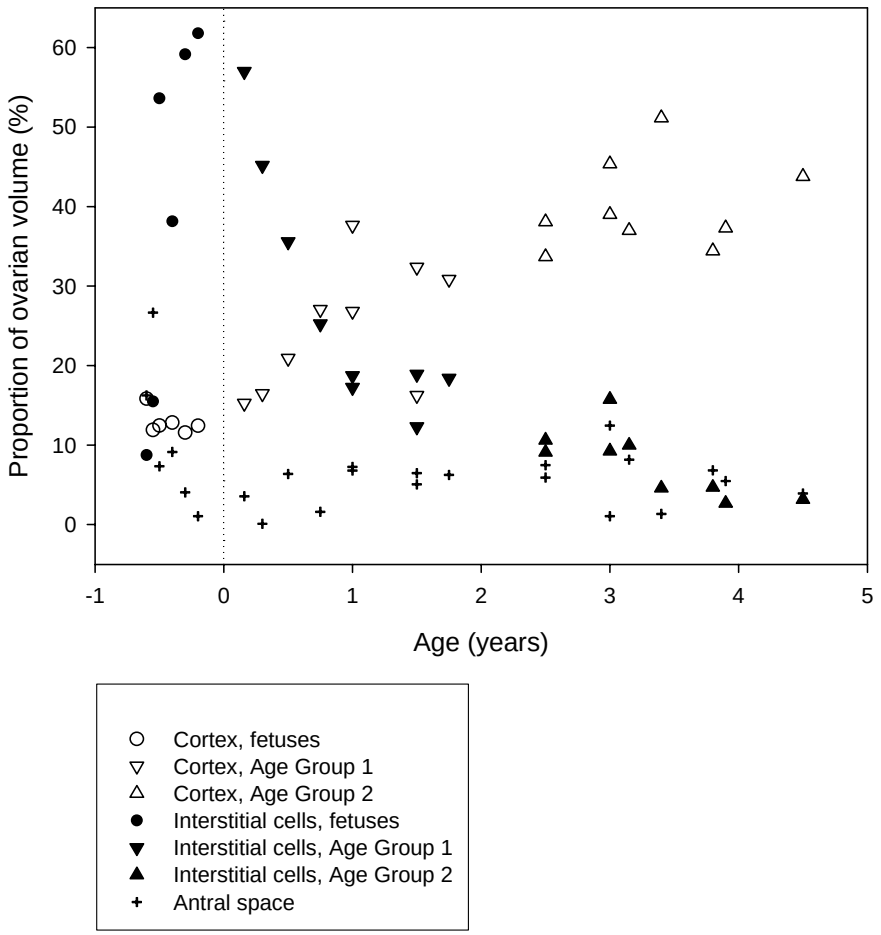

Figure 6.2 Relative contributions of cortex, interstitial cells and follicular antra to the volumes of the ovaries of elephant fetuses from mid-gestation onwards and of calves up to 4.5 years of age

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.

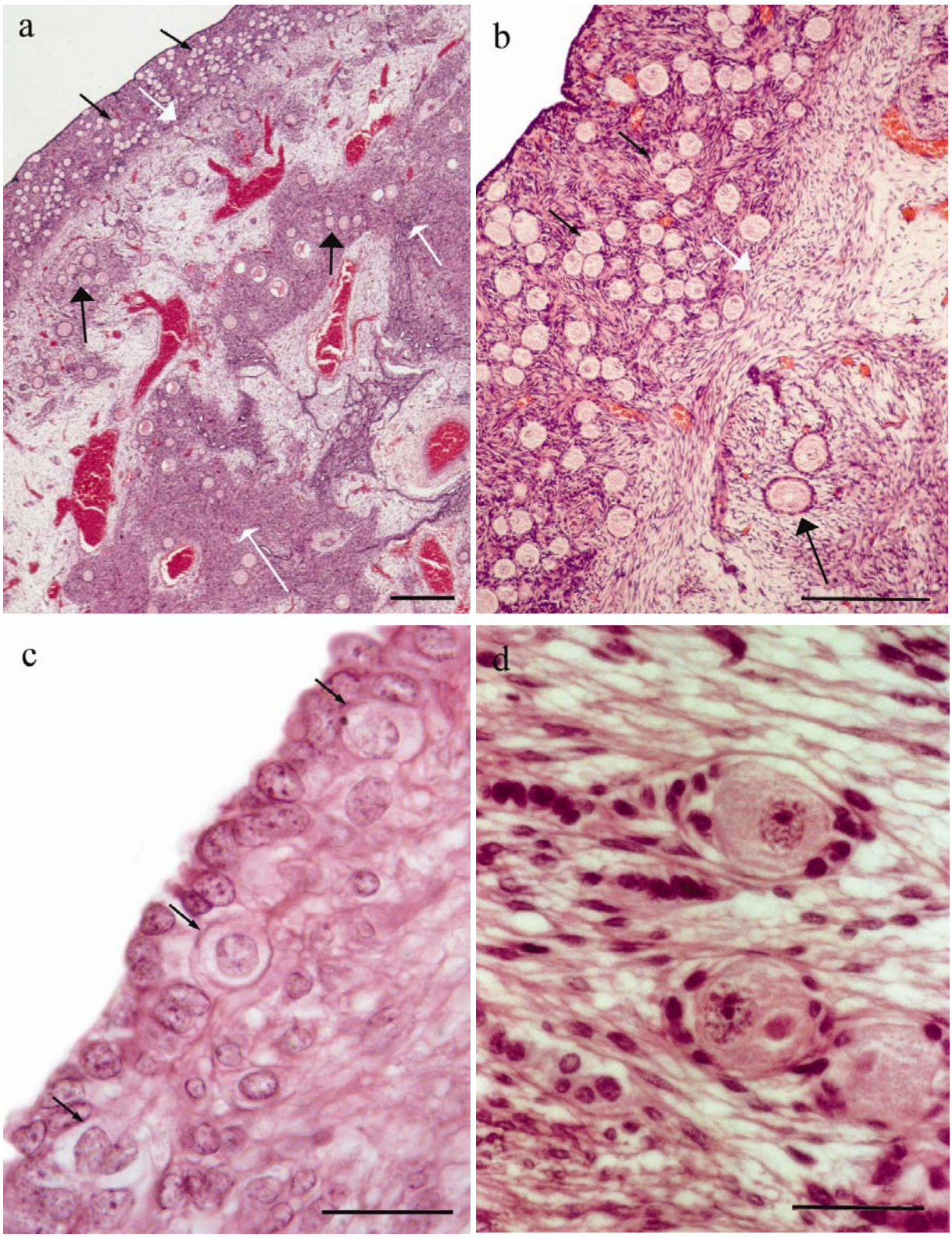

Figure 6.3 Sectioned ovary of an elephant fetus at 11 months of gestation

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).

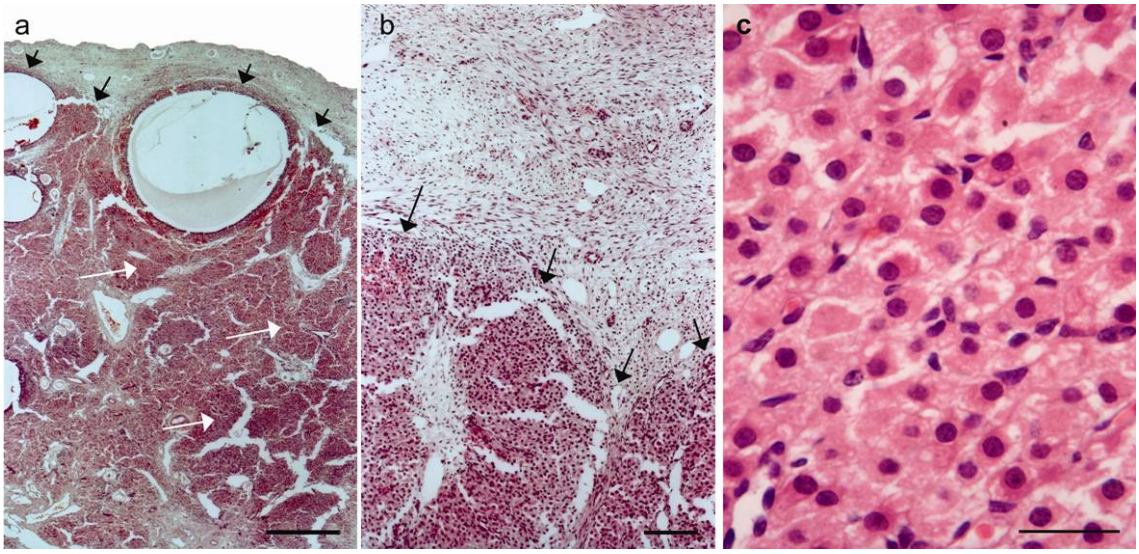

Figure 6.4 Sections of the ovaries of an elephant fetus at 13.5 months of gestation

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.

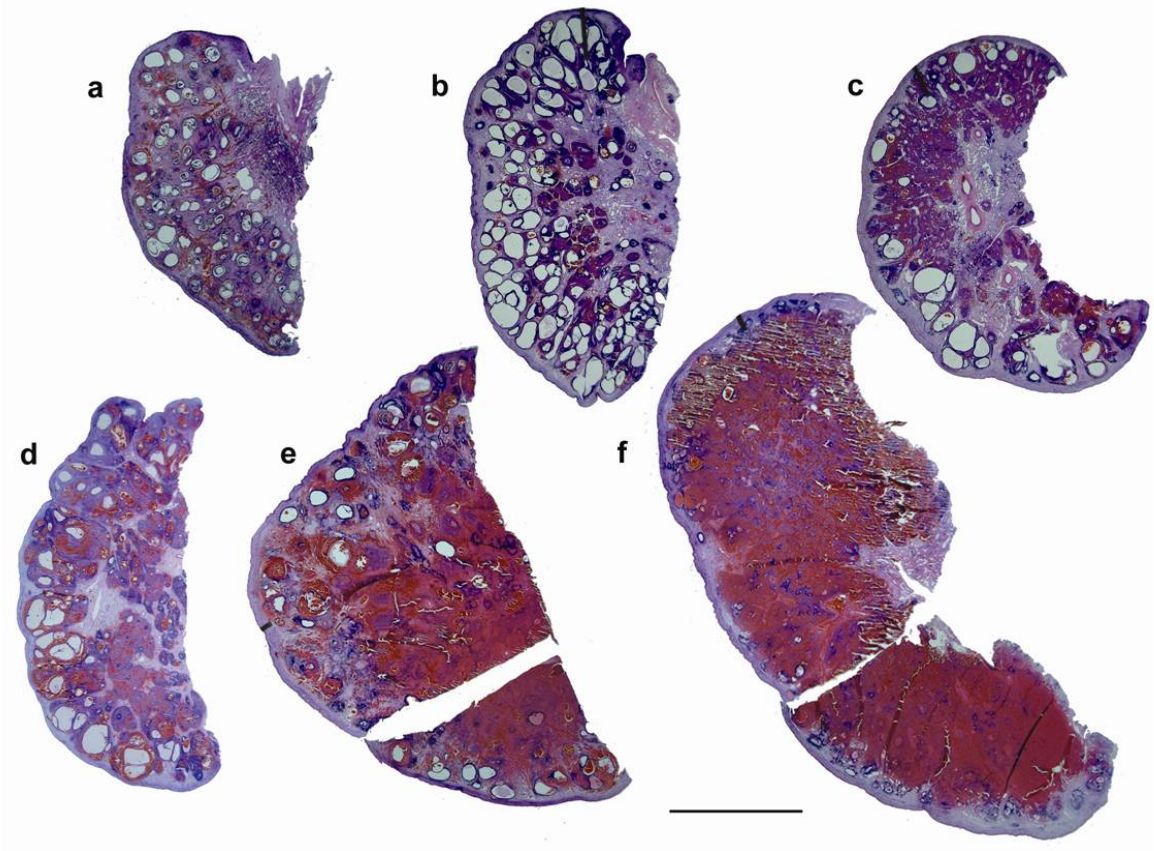

Figure 6.5 Photographs, taken above a light box, of 25 µm thick sections of fetal elephant ovaries recovered between 15 and 21 months of gestation (Scale bar = 10 mm)

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).

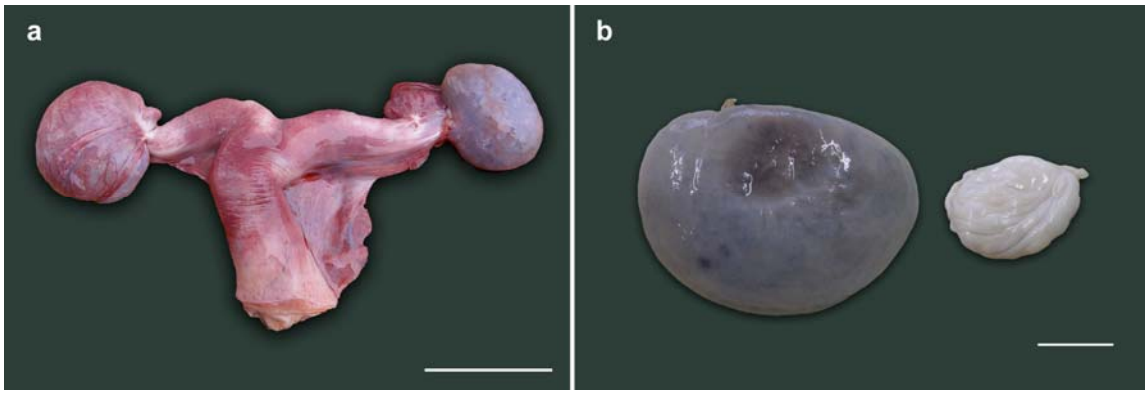

Figure 6.6 The ovaries of two late-stage African elephant fetuses

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.

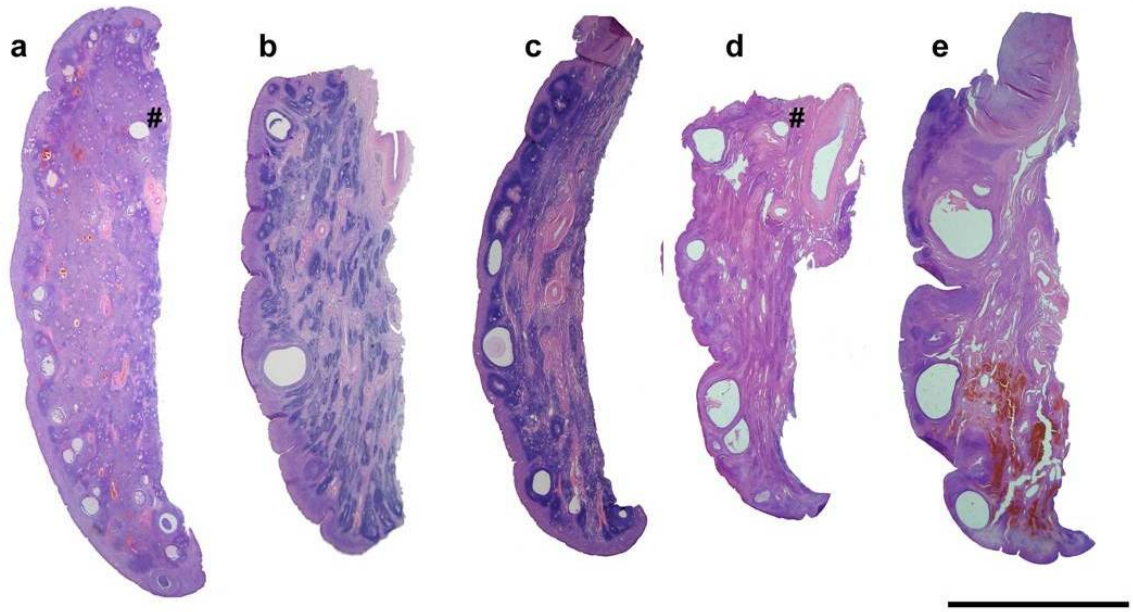

Figure 6.7 Photographs of 25 µm thick sections of the ovaries of prepubertal elephant calves (Scale bar = 10 mm)

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.

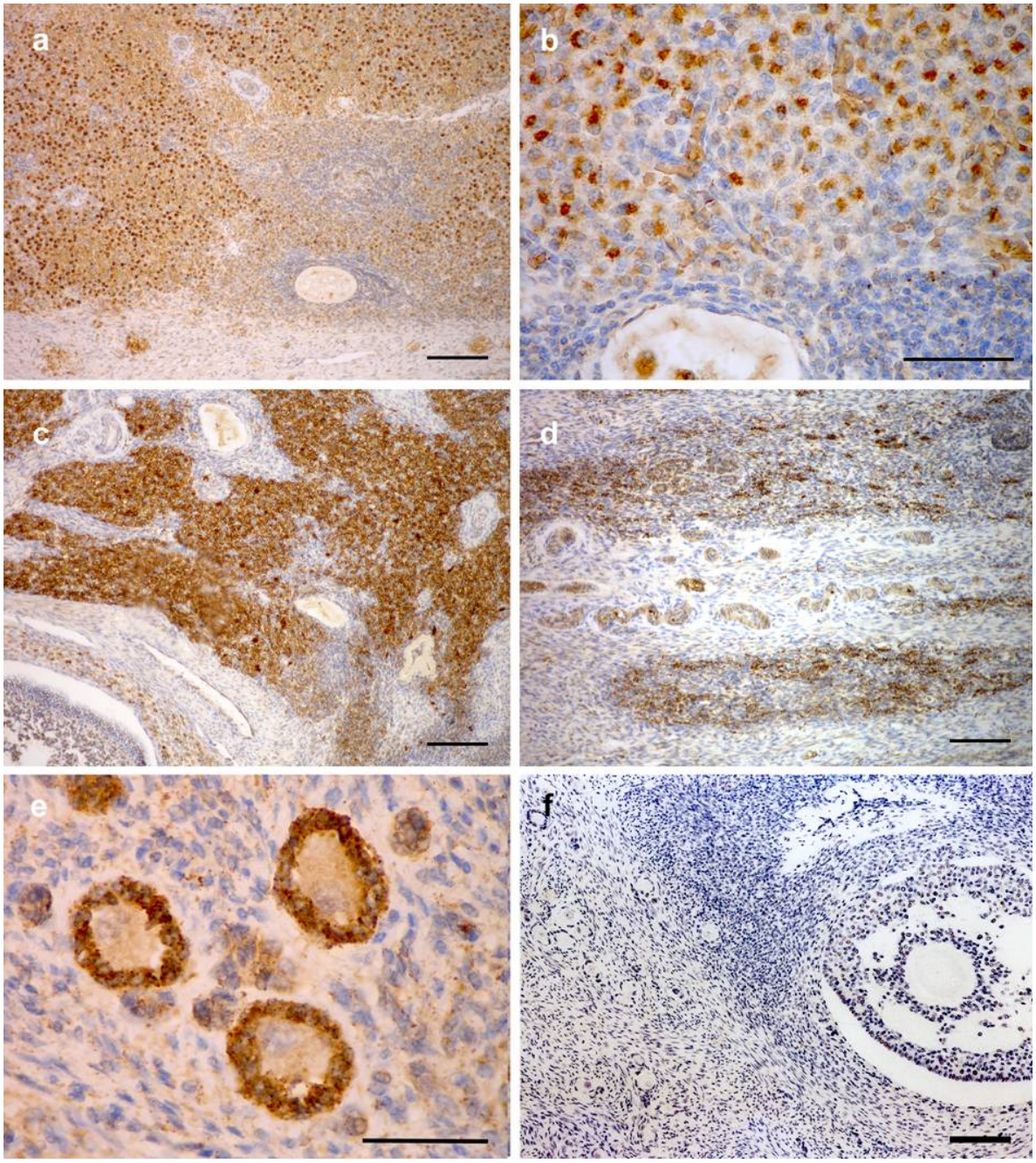

Figure 6.8 Sections of the ovaries of elephant fetuses and calves stained immunocytochemically with an anti-3β–HSD antibody

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).

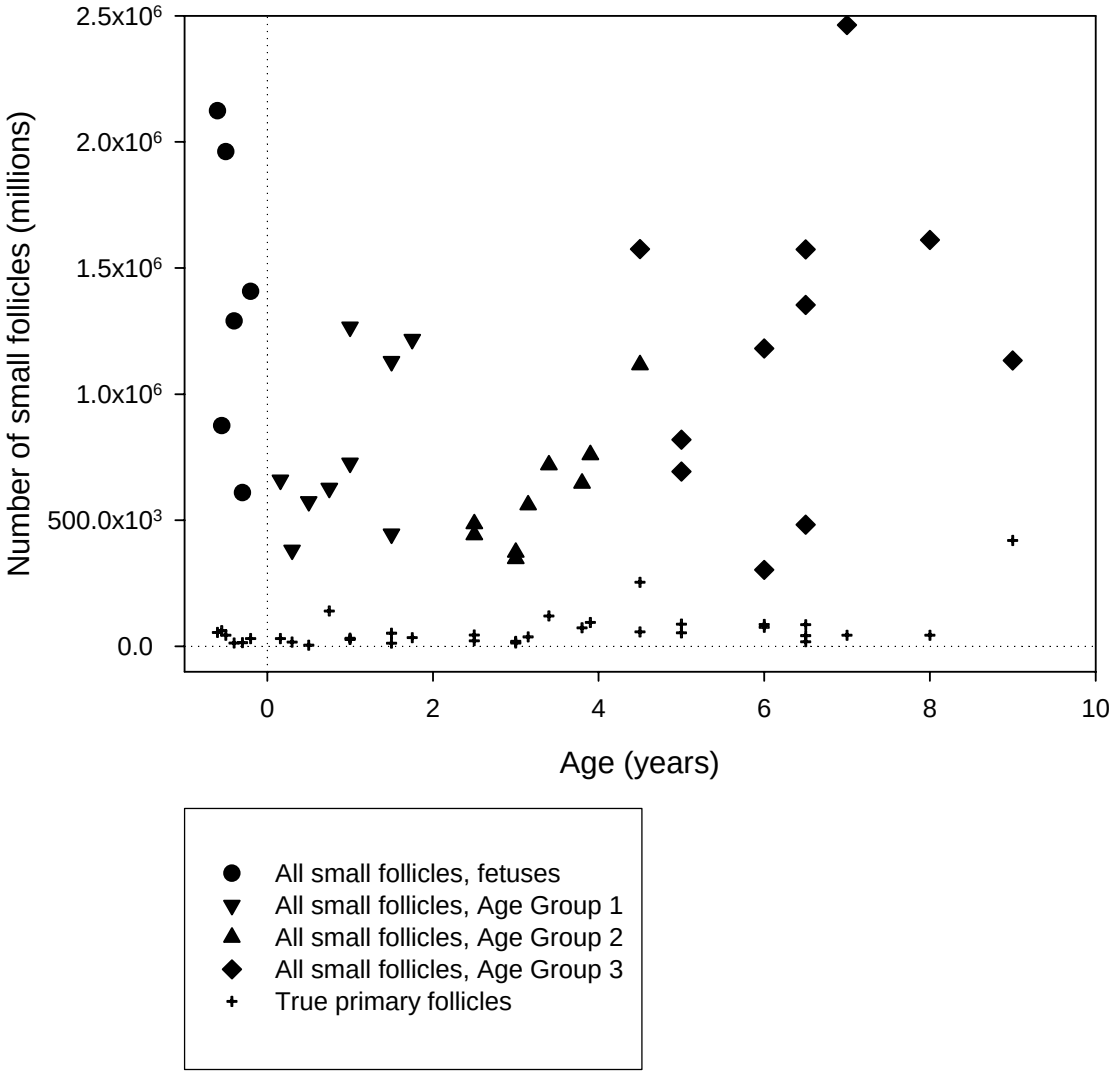

Figure 6.9 The number of small follicles in the ovaries of elephant fetuses and prepubertal calves in relation to age

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] ($P<0.05$)

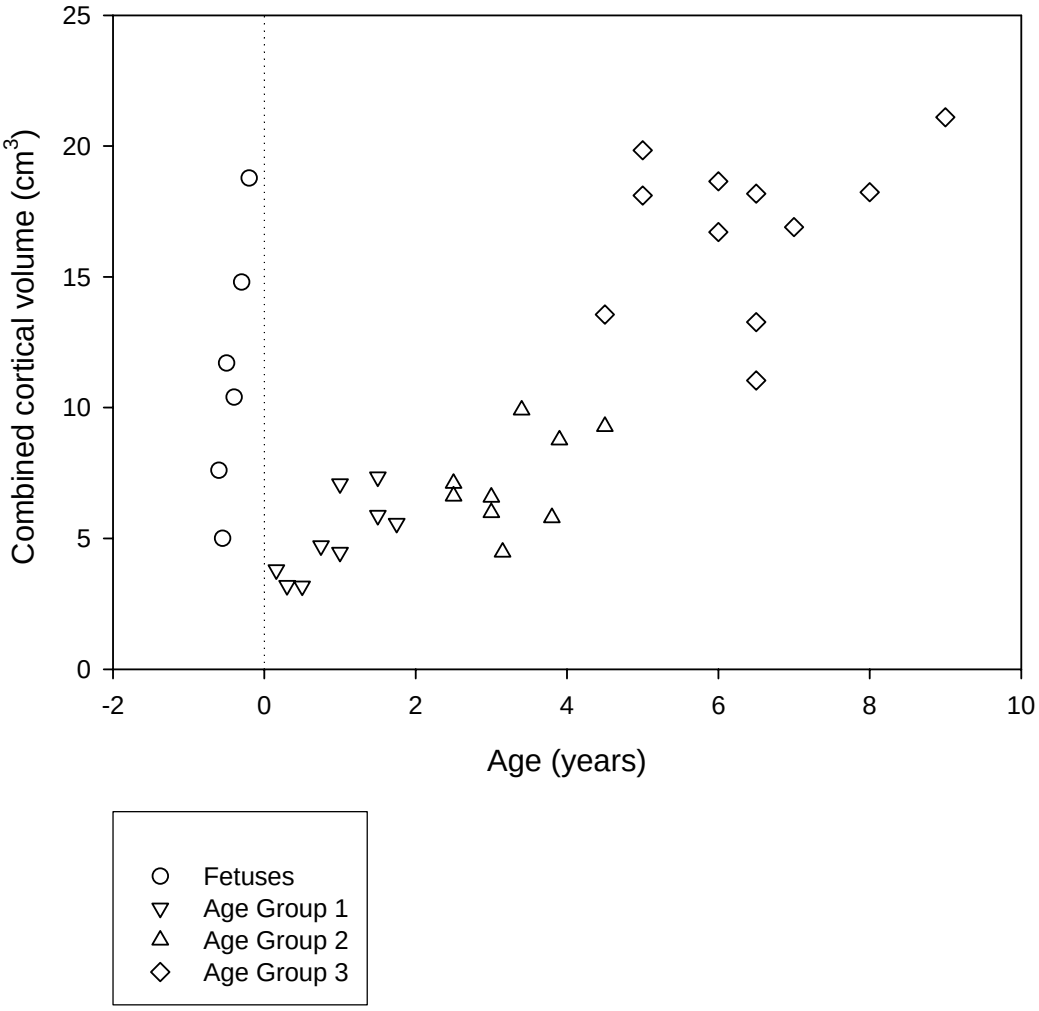

Figure 6.10 Cortical volumes of fetal and prepubertal ovaries of African elephants

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.