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

5.1. Introduction

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

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

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

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

5.2. Materials and methods

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

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

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

5.3. Results

5.3.1. Milestones in embryonic development

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

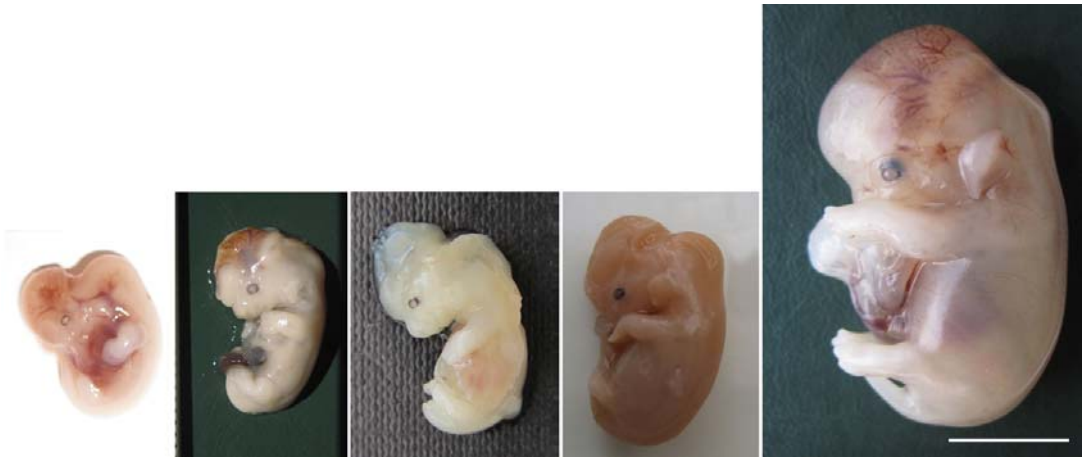

Figure 5.1 African elephant embryos aged (from left to right) 76, 81, 82, 87, and 96 days post conception (scale bar = 10mm)

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

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

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

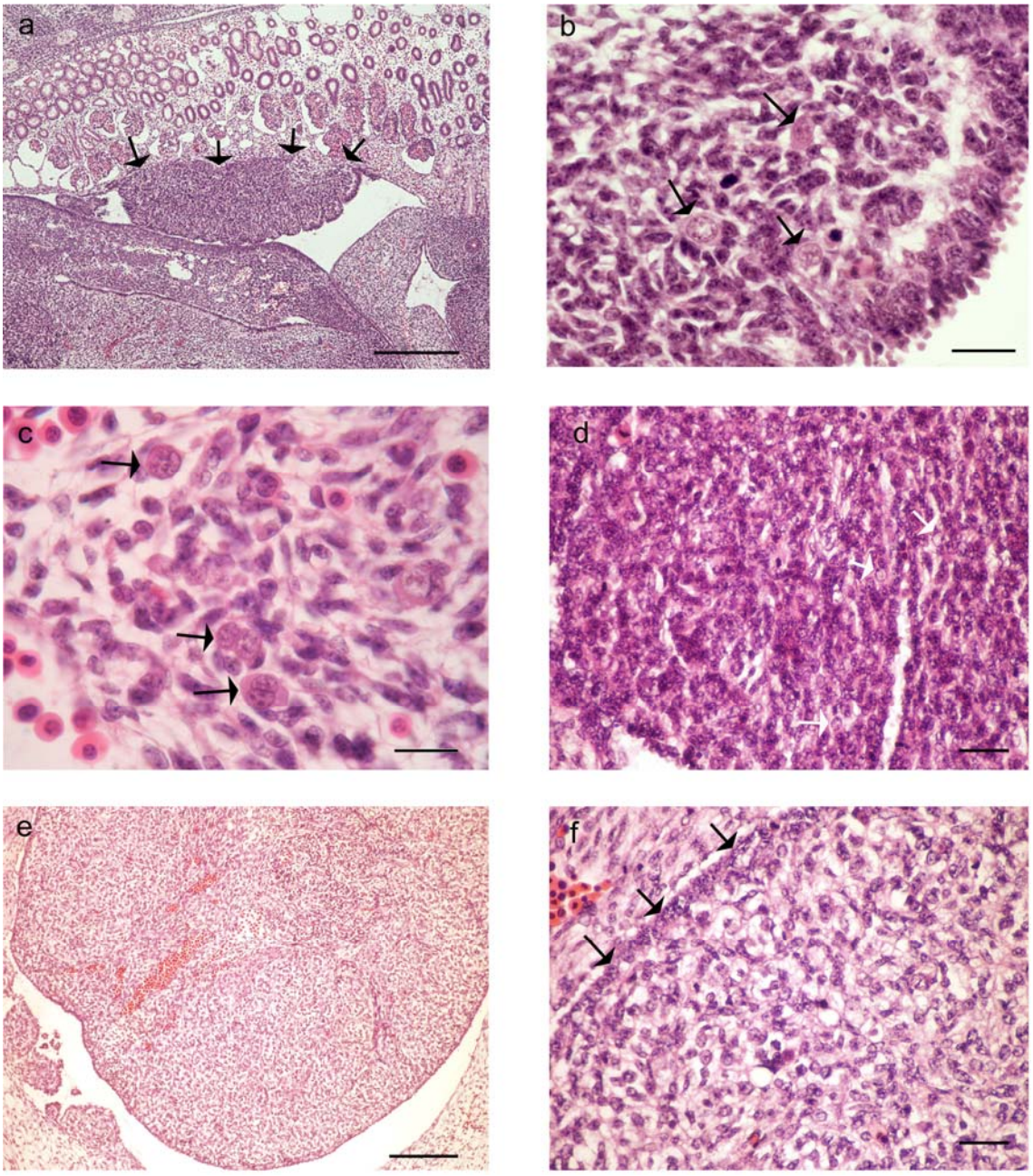

Figure 5.2 Development of the gonad of the African elephant embryo

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

Continued

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

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

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

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

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

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

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

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

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

5.3.2. Milestones in fetal development

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

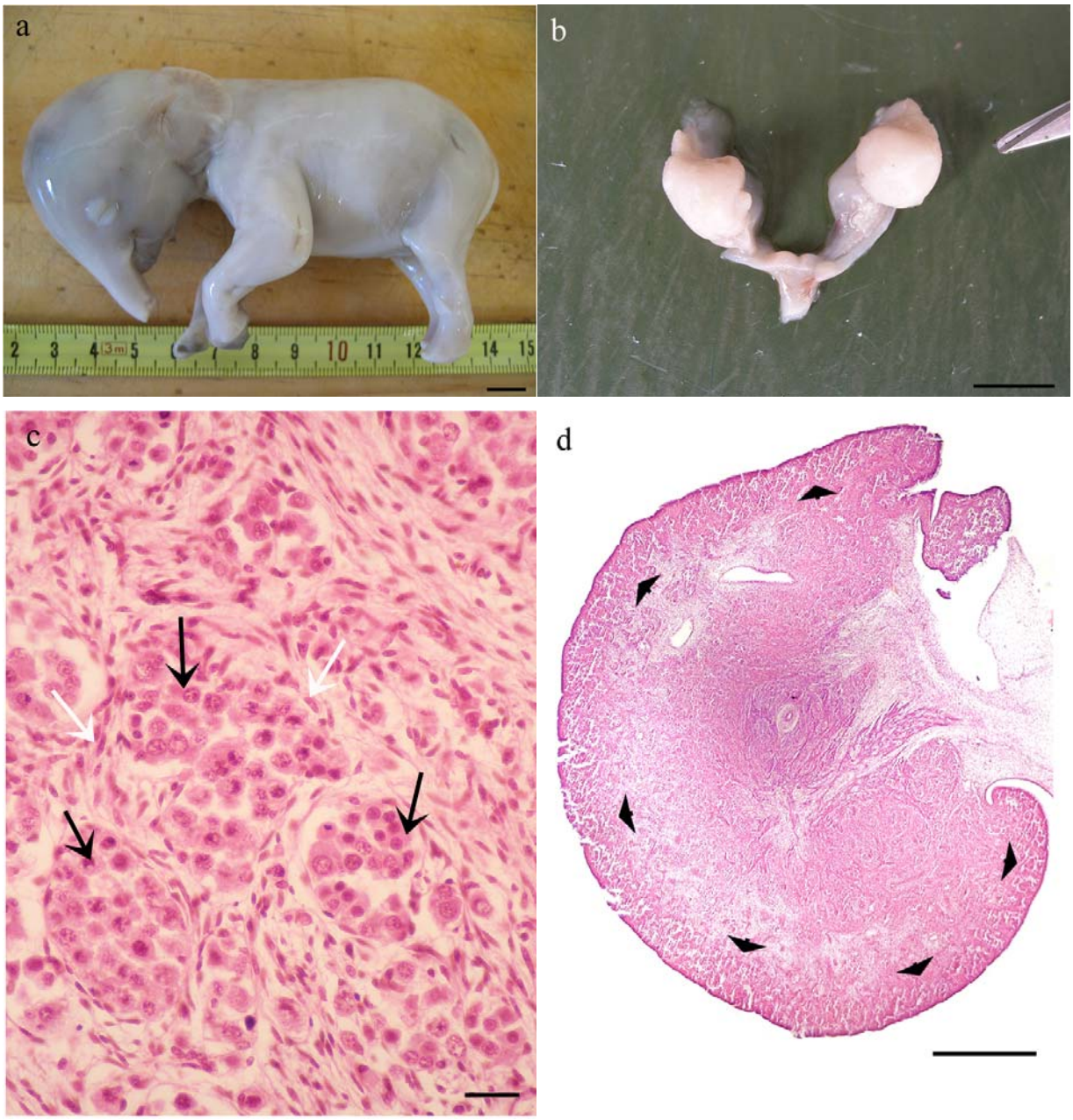

Figure 5.3 Photographs and photomicrographs of the gonads of elephant fetuses at 4.8 to 5.9 months of gestation

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

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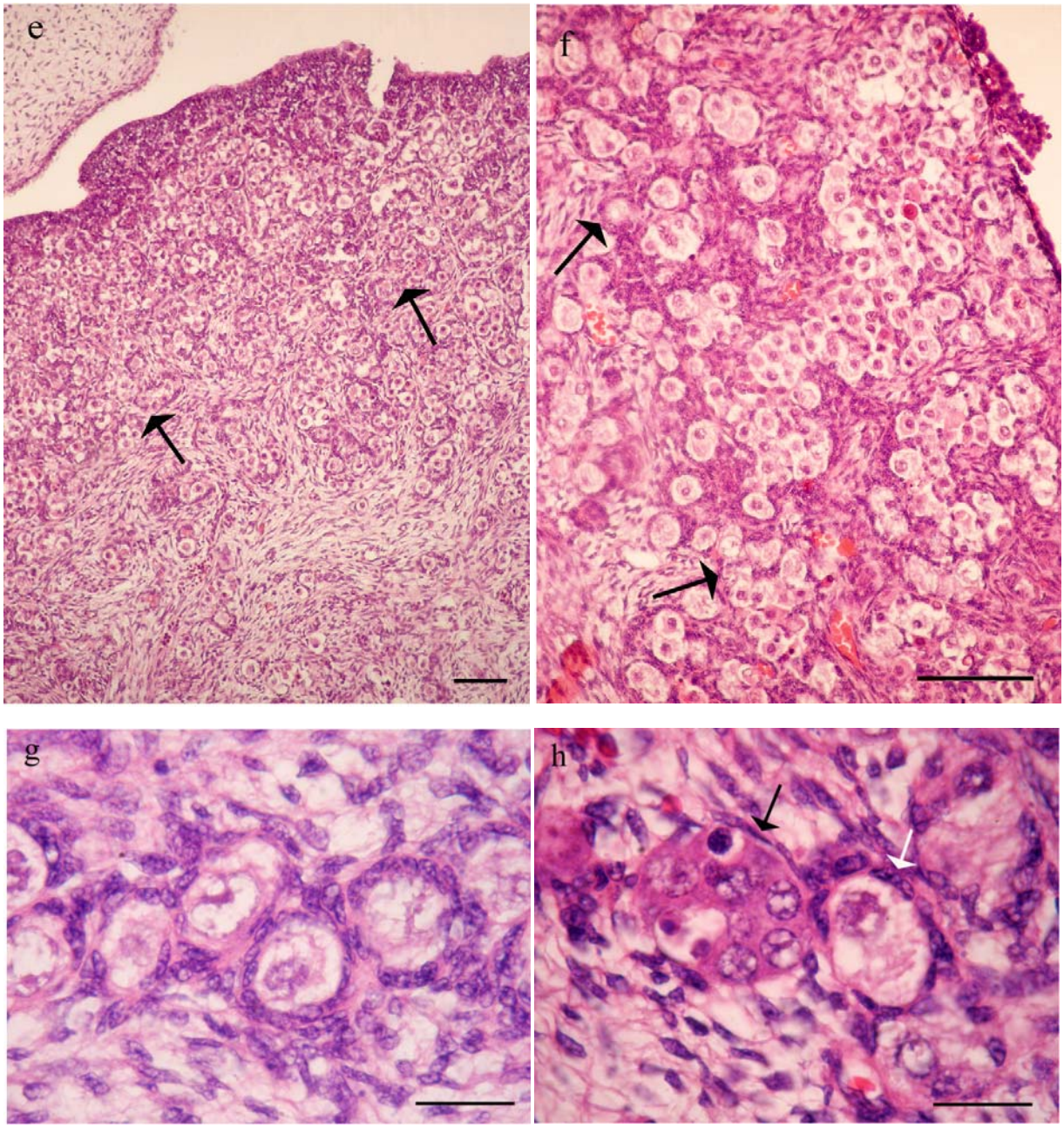

Figure 5.3 (continued)

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

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

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

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

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

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

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

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

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

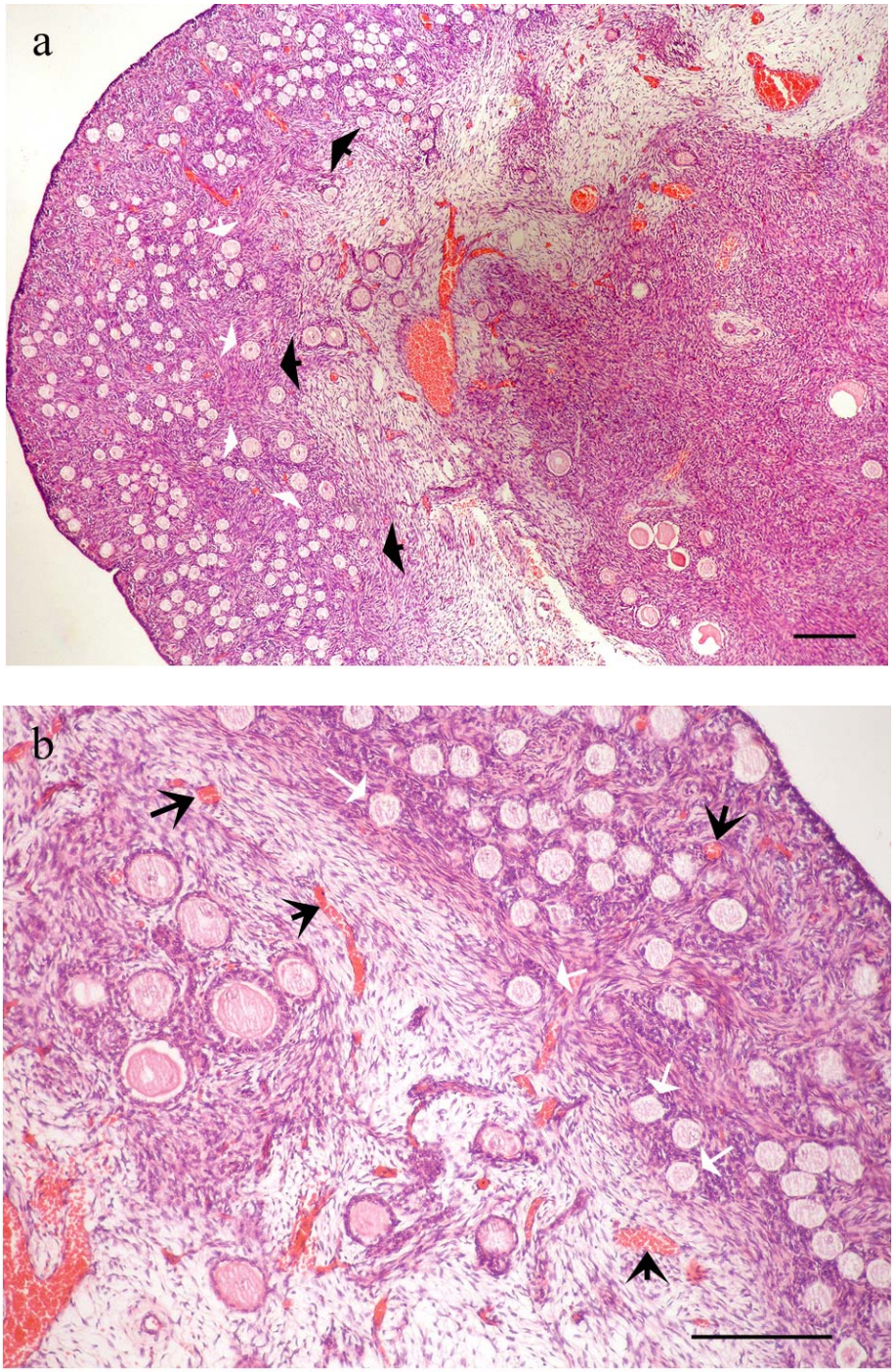

Figure 5.4 Ovarian sections from a mid-term (11.2 month) African elephant fetus

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

5.3.3. Germ cell counts

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

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

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

[a] Percentage in cortex:percentage in medulla

Figure 5.5 Elephant fetuses of different ages

Continued

Figure 5.5 (continued)

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

5.4. Discussion

5.4.1. Developmental stage and age of the embryos

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

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

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

5.4.2. Morphology of the gonadal ridge and developing ovary

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

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

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

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

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

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

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

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

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

5.4.3. Germ cell counts

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

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

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

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

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

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

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

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

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

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