Luteal maintenance of pregnancy in the African elephant (*Loxodonta africana*).

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Abstract

The ovaries of 8 African elephant fetuses and their mothers between 2 and 22 months of gestation, and those of 2 cycling and 2 lactating elephants, were examined grossly, histologically and immunocytochemically, with emphasis on the development and regression of the accessory corpora lutea (CL) of pregnancy and the steroidogenic capacities of these and the fetal ovaries. The results supported recent findings (Lueders *et al.* 2011) that the accessory CL form as a result of luteinisation, with and without ovulation, of medium sized follicles during the 3-week interluteal period of the oestrous cycle. They enlarge significantly and become steroidogenically active around 5 weeks of gestation, probably in response to placental lactogen (elPL) which is secreted by the implanting trophoblast of the conceptus. The large luteal cells stained strongly for 3β hydroxysteroid dehydrogenase (3βHSD) activity throughout the 22 month gestation period although they showed vacuolation and other degenerative changes in the final months of gestation coincident with hypertrophy and hyperplasia of 3βHSD-positive interstitial cells in the fetal gonads. It is proposed that the progestagens secreted by the enlarged gonads of the elephant fetus may function both to assist the maternal ovaries in supporting the pregnancy state and to induce torpor and intrauterine immobility of the rapidly growing fetus.

Introduction

As in equids (Squires and Ginther 1975), a feature of pregnancy in elephantids is the presence of multiple, large, accessory corpora lutea (CL) in the maternal ovaries (Perry, 1953; Hodges, 1998). In both genera these secondary luteal structures make their appearance at the end of the first month of gestation (Amoroso *et al*. 1948; Allen *et al*. 2002) and they appear to form both by ovulation and luteinisation of unruptured follicles (Allen, 1975; Lueders *et al*. 2011). Their advent causes a marked secondary rise in progesterone/progestagen concentrations in maternal blood (Allen, 1975; Meyer *et al*. 2004). In the pregnant mare, the accessory CLs develop equally on both maternal ovaries as a result of the LH-like biological activity of equine Chorionic Gonadotrophin (eCG) causing ovulation/luteinisation of mature Graafian follicles stimulated to grow by 10-12 day waves of pituitary FSH released throughout the physiological breeding season in both cycling and pregnant animals (Evans and Irvine, 1975; Urwin and Allen, 1982). The equine accessory CLs also secrete oestrogens (Daels *et al*. 1991) and they regress and disappear around mid-gestation when the diffuse, epitheliochorial placenta is now sufficiently well established to secrete the progestagens necessary to maintain the pregnancy state without any further contribution from the maternal ovaries (Holtan *et al*. 1979).

In the pregnant elephant, by contrast, mature follicles are rarely, if ever, seen in the maternal ovaries (Perry, 1964, 1974; Short and Buss, 1965; Laws, 1969; Hodges, 1998; Allen, 2006) and it is now proposed that the accessory CLs of pregnancy first form as a result of luteinisation of small (0.8-1.2 cm) follicles that develop in response to the first of the two peaks in serum LH concentrations which characterise the 20-22 day interluteal period of the elephant oestrous cycle (Lueders *et al*. 2010, 2011). These accessory luteal structures, seen clearly by serial transrectal

ultrasonographic examinations of the maternal ovaries (Lueders *et al*. 2011), form predominantly, but not exclusively, on the ovary ipsilateral to the uterine horn that will contain the conceptus (Allen *et al.* 2002) and they apparently remain dormant (and non-secretory) during the remainder of the interluteal and follicular phases, the release of the second ovulation-inducing LH peak (Kapustin *et al*. 1996; Brown *et al*. 1999) and the first 5-6 weeks of gestation (Lueders *et al*. 2011). They then enlarge greatly to reach diameters of 10-38mm (Hodges, 1998; Lueders *et al*. 2011) and begin to secrete appreciable quantities of 5α-dihydroprogesterone (5αDHP) and other 5α-pregnanes (Hodges *et al.* 1994, 1997), as evidenced by the sharp increase in maternal serum progestagen concentrations at this time (Meyer *et al*. 2004). Whereas eCG secreted by the fetal endometrial cups (Allen and Moor 1972) is the stimulus for secondary luteal development in the pregnant mare (Allen, 1975; Terqui and Palmer, 1979), it now seems very likely that placental lactogen (elPL) secreted by the trophoblast from around the time of implantation and commencing development of the zonary endotheliochorial placenta is the essential luteotrophic stimulus in the pregnant elephant (Yamamoto *et al*. 2011). Furthermore, the zonary elephant placenta remains steroidogenically inactive throughout gestation (Allen *et al*. 2002) and the accessory CLs also persist until birth, presumably secreting progestagens required to maintain the pregnancy state (Allen 2006).

Another common feature of pregnancy in equids and elephantids is considerable hypertrophy of the fetal gonads, both ovaries and testes, during the second half of gestation, followed by a rapid regression and shrinkage back to a typically pre-pubertal size prior to the birth of the horse foal (Cole *et al*. 1933; Hay and Allen 1975) or elephant calf (Hanks 1971; Allen *et al*. 2005). This enlargement stems primarily from hyperplasia and hypertrophy of interstitial cells in the fetal

gonads of both genera and is added to in the female elephant fetus, but not in the female horse fetus, by the growth of multiple antral follicles (Allen *et al*. 2005). In both genera the hypertrophic interstitial cells in the fetal gonads secrete steroid hormones, 5αDHP and other 5α-reduced pregnanes in the elephant (Allen *et al*. 2002) and a range of C-19 androgens, including androstenedione, dehydroepiandrosterone (DHEA), 3ß-hydroxy-5,7-pregnanedien-20-one and 3ßhydroxy-5,7 androstadien-17-one (Tait *et al*. 1983; 1985) in the mare which are subsequently aromatised to phenolic and Ring B unsaturated oestrogens by the diffuse epitheliochorial placenta (Bhavnani *et al.* 1969; 1971).

Some interesting questions arise from our sparse and somewhat presumptive knowledge of luteal development, function and dependence during pregnancy in the elephant. For example, do the accessory CLs found routinely in the ovaries throughout gestation really develop from luteinisation of medium sized unruptured follicles in response to the first of the two serum LH peaks during the 3-week non-luteal phase of the oestrous cycle? If so, how do they remain dormant and non-secretory during the second ovulation-inducing LH peak and the first 5 weeks of gestaton, as proposed recently by Lueders *et al.* (2011) following their ground breaking serial ultrasonographic examinations of the ovaries of cycling and pregnant elephants maintained in zoos? Also, can a temporary and highly productive endocrine gland like a CL remain active and fully functional for as long as the 22-month gestation period of the elephant?

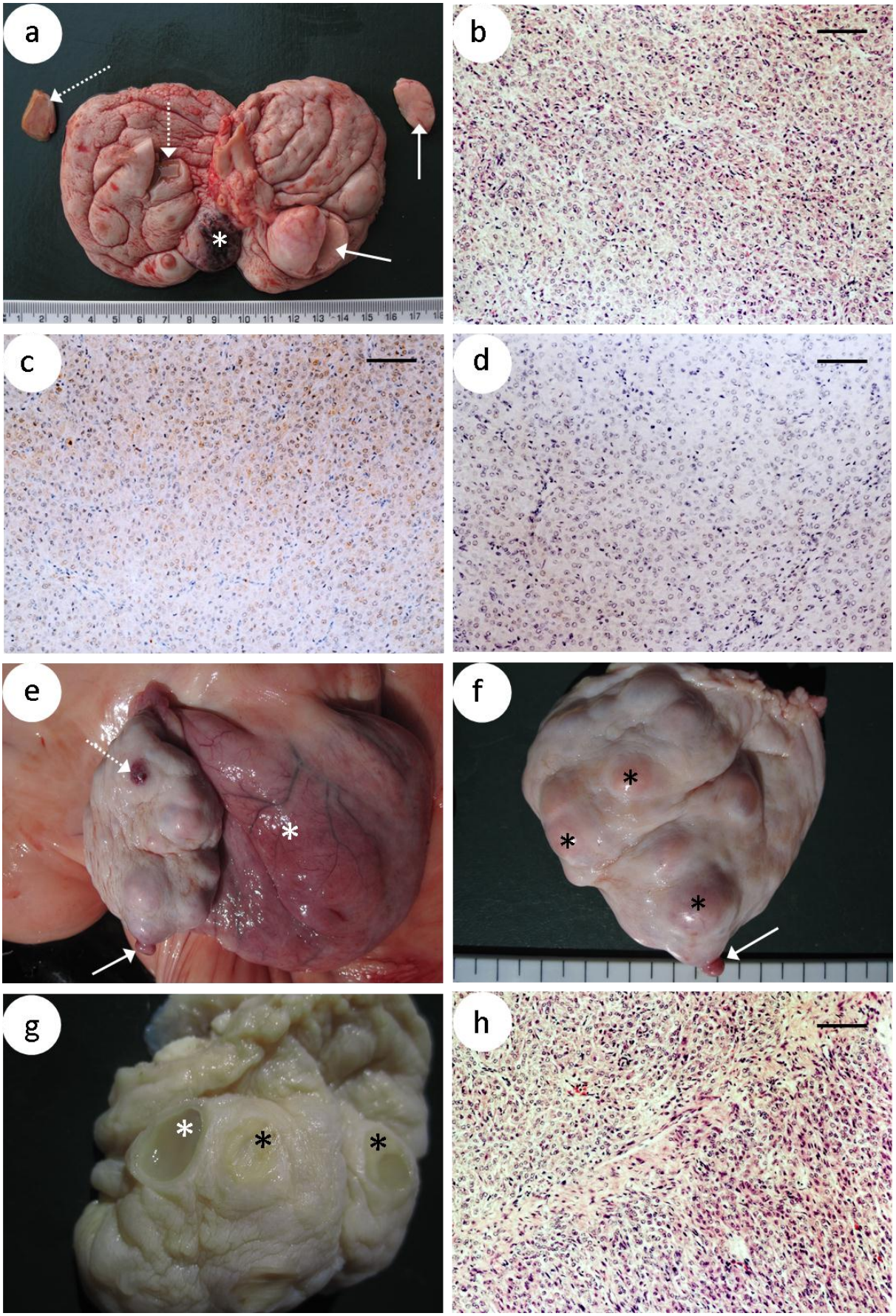
The opportunistic recovery of the uteri and ovaries from adult cycling, anoestrous and pregnant African elephant cows culled for management reasons in Zimbabwe enabled an examination of these and other aspects of luteal function during pregnancy in this species. The findings are reported in this paper.

Results

Dealing first with the two cycling females, the right ovary of the 30 y.o. semi-tamed female that had died suddenly of natural causes exhibited two CLs, one of which was composed of homogenous, creamy coloured luteal tissue indicative of a relatively young and functional organ while the luteal tissue of the second, smaller CL was more pitted and khaki-coloured and was therefore thought to indicate an ageing and regressing CL heading towards corpus nigrans (CN) status, likely representing the CL of the previous oestrous cycle (Figure1a). Histologically, the tissue of the younger CL was a dense mass of typically small luteal cells each with relatively little, pale staining cytoplasm and a round, very pale nucleus with one or more prominent nuclei (Figure 1b). Interestingly, the cytoplasm of these small luteal cells stained only faintly for 3ßHSD activity, thereby suggesting a very limited potential to synthesise progestagens (Figure 1c). A collapsed, haemorrhagic follicle (or cyst?) was also present on the right ovary (Figure 1a) and neither follicles nor CLs could be indentified in the left ovary.

The other non-pregnant elephant was particularly interesting in that its right ovary exhibited a visible 'blood spot' indicative of a very recent ovulation (Figure 1e) plus 7 small-to-medium sized (6 to 11 mm diameter) luteinising follicles/accessory CL, one of which had a clear ovulation stigma that was also relatively young in appearance (Figure 1f). The left ovary exhibited two more small luteal bodies both of which had youthful ovulation stigmata. All the luteal bodies, were composed either of already pale, homogenous luteal tissue or they still had a central core of pale follicular fluid surrounded by a variably luteinised follicle wall (Figure 1g). The ovulation 'blood spot' covered a small accumulation of clotted blood adhered to the already luteinising follicle wall. Two of the luteal bodies were

Figure 1. (a) Right ovary of a 30 y.o. cycling elephant in dioestrus. The solid white arrows indicate the pale cream-coloured wedge of homogenous luteal tissue dissected from the active CL and the dotted arrows show the khaki-coloured wedge of luteal tissue dissected from the smaller, older CL thought to be from the previous oestrous cycle. The asterisk indicates a haemorrhagic follicle or cyst. (b) Histological section of the active CL showing the tightly packed, small luteal cells intermixed with strands of fibroblasts (scale bar=150 µm). (c) Section of the same CL stained with the anti-3βHSD antibody. Only a small proportion of the luteal cells stain very lightly (scale bar=150 µm). (d) Section of the F same CL stained with a mouse monoclonal anti-herpes virus I antibody as a negative control (scale bar=150 µm). (e) Caudal edge of the right ovary of a cycling 13 y.o. elephant sitting on the external surface of the ovarial sac (asterisk). The dotted arrow indicates a very recent ovulation 'blood spot' and the solid arrow points to the recent



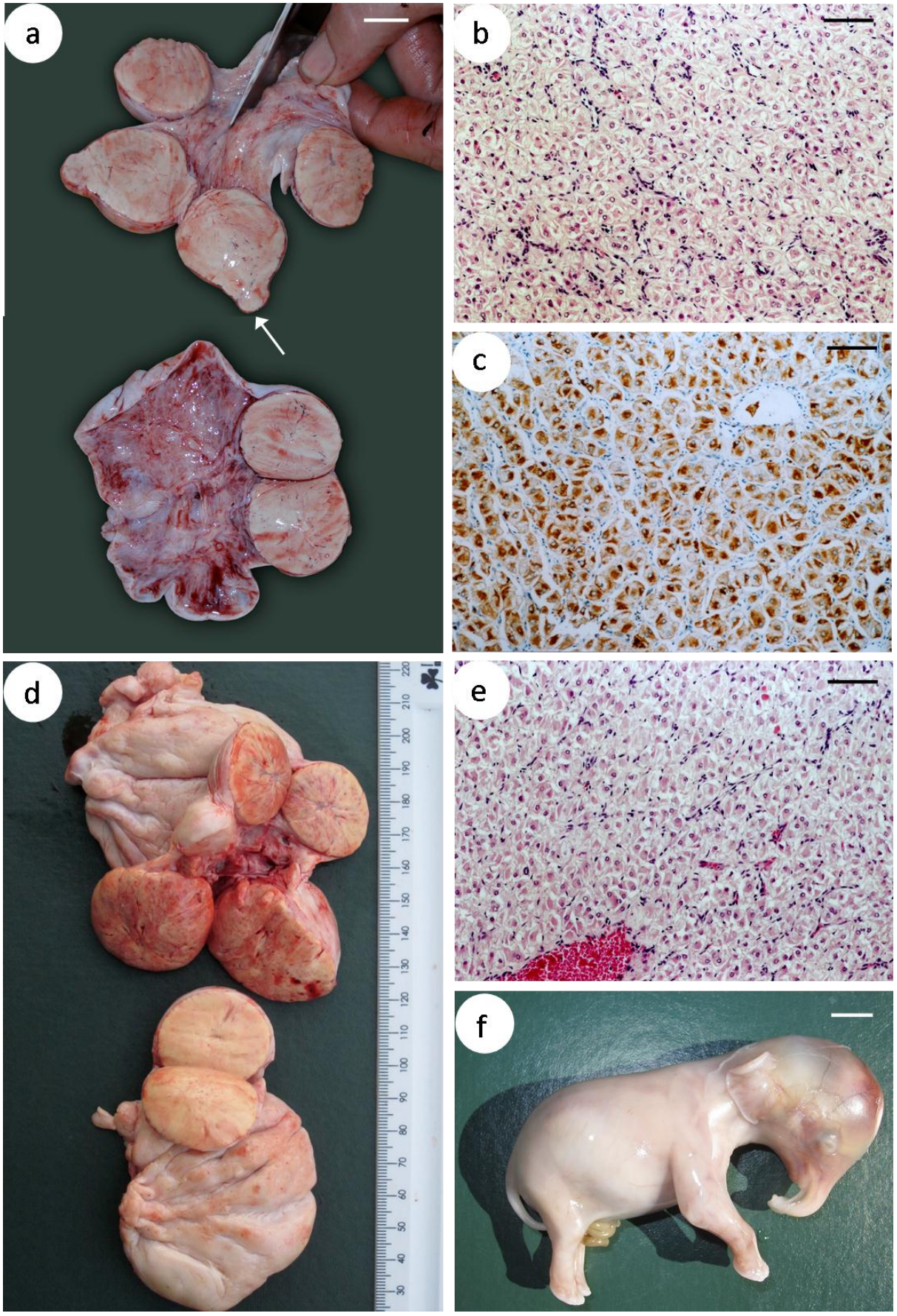
ovulation stigma of an accessory luteal body. (f) The medial surface of the same ovary in (e) with the asterisks indicating three of the seven partly or fully luteinised secondary follicles/CL in the ovary. The arrow points to the same ovulation stigma shown in (e). (g) The same ovary shown in (e) and (f), now fixed and sliced to reveal one luteinising follicle (white asterisk) and two secondary luteal bodies (black asterisks). (h) Histological section of the central accessory luteal body shown in (g). As in the other dioestrous female (b), the tissue consists of a dense mass of small luteal cells and structural elements (scale bar=150µm).

examined histologically and both showed the same accumulation of small luteal cells with pale staining nuclei and minimal cytoplasm seen in the nulliparous cycling female (Figure 1h). Thus, it seemed reasonable to conclude that this animal had probably been in oestrus recently and the 'blood spot' represented the recent, possibly fertile, ovulation resulting from the second LH peak of the interluteal period while the 9 luteal bodies, 3 of which had clear ovulation stigmata, represented the luteinised follicles described by Lueders *et al.* (2011) as developing following the first LH rise of the interluteal period that had occurred 3 weeks previously.

Turning to pregnancy, in the earliest stage of gestation encountered at 60 days, the left (ipsilateral to the conceptus) ovary contained 2 large CL of 42 and 38 mm diameter respectively (Figure 2a). Both consisted of creamy-coloured, homogenous luteal tissue and both showed prominent ovulation stigmata (Figure 2a). The right (contralateral) ovary contained a 40 mm diameter CL of the same appearance and with a definite, although much smaller, ovulation stigma (Figure 2a). Histologically, each CL was composed of a uniform, pavement-like arrangement of luteal cells that were now much larger, due to appreciably more cytoplasm, than the small luteal cells that constituted the cyclical CL. Small blood vessels were scattered throughout the luteal tissue along with strands of fibroblast cells constituting the structural framework (Figure 2b). These large luteal cells were uniformly stained by the anti-3ßHSD antibody but with a particularly dark area of staining surrounding the nucleus of most cells (Figure 2c).

At 5.7 months of gestation (36g fetus, Figure 2f) the right (ipsilateral) ovary similarly contained two CL (51 and 35 mm diameter) and the left a single CL of 41 mm, all composed of healthy, homogenous luteal tissue that was very slightly more yellowish in colour than those at 60 days; the large 51 mm CL in the ipsilateral ovary

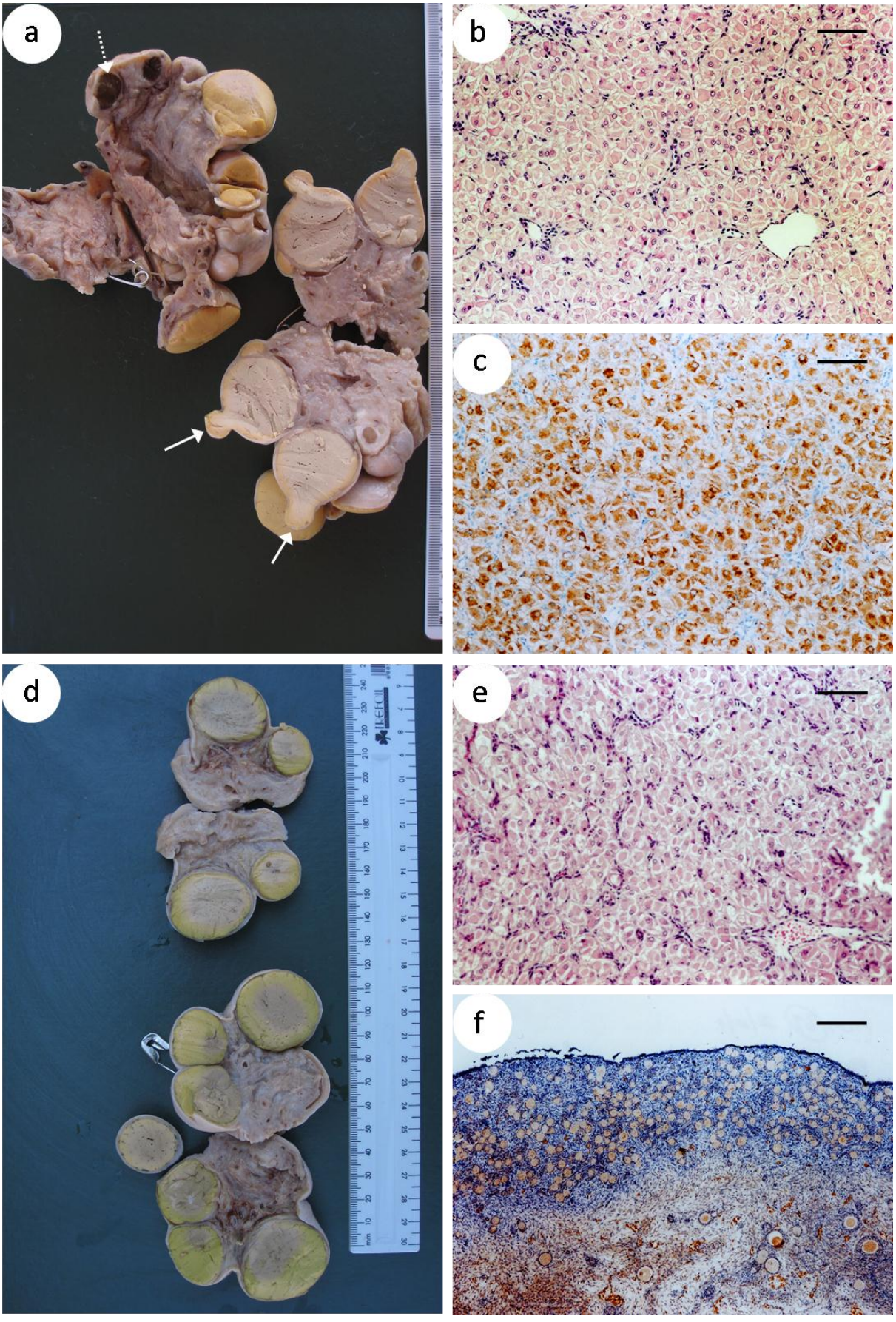
Figure 2. (a) Sectioned ovaries of a pregnant elephant at 60 days of gestation showing two large pale cream-coloured CL (42 and 38 mm diameter) in the ipsilateral ovary and one (40 mm diameter) in the contralateral ovary. The arrow points to a prominent ovulation stigma (scale bar=1 cm). (b) Histological section of the CL with the ovulation stigma in (a) showing the dense mass of now large, epithelioid luteal cells with pale staining nuclei. There are relatively few strands of supporting fibroblasts (scale bar=150 μm). (c) Section of the same CL stained with the anti-3βHSD antiserum and showing strong staining in the central region of the cytoplasm surrounding the nucleus (scale bar=150 μm). (d) Sectioned ovaries from a pregnant elephant at 5.7 months of gestation showing two large CL (51 and 35 mm diameter) in the ipsilateral right and one (41 mm diameter) in the contralateral left ovary. The luteal tissue in all three CL was homogenous and very pale yellow in colour and only one of the CL in the ipsilateral ovary showed an ovulation stigma. (e) Histological section of one CL in the ipsilateral ovary showing the typical dense mass of large, healthy looking luteal cells (scale bar=150 μm). (f) The 36 g foetus recovered from this conceptus (scale bar=1 cm).



had a prominent ovulation stigma (Figure 2d). Histologically, the tightly packed large luteal cells were very similar in appearance to those in the earlier specimen but with slight shrinkage and vacuolation of the cytoplasm of some cells (Figure 2e). These were also stained uniformly by the anti-3ßHSD antiserum and they likewise showed increased density of staining around the otherwise pale staining nucleus containing one or more prominent nucleoli.

At the next stage of gestation examined (7.2 months, 463g fetus), the left and right ovaries each contained 3 significant CL of 35, 32 and 29 mm diameter on the left (ipsilateral) ovary the first two of which showed large ovulation stigmata and 34, 22 and 21 mm on the right (contralateral) two of which showed stigmata (Figure 3a). Histologically, the tightly packed large luteal cells still appeared normal and healthy with perhaps a slight increase in shrinkage and vacuolation of the cytoplasm (Figure 3b) and a basically similar pattern of staining for 3ßHSD (Figure 3c). Four months later in mid-pregnancy (7 kg fetus, 11.2 months gestation) the left (ipsilateral) ovary contained 4 sizeable CL (28 – 43 mm diameter) and the right ovary two (24 and 36 mm diameter). Only one CL in the ipsilateral ovary showed a small ovulation stigma (Figure 3d). The luteal tissue was now becoming slightly pale brown or tan in colour but, histologically, the majority of large luteal cells still appeared structurally intact and functional (Figure 3e) and they still stained uniformly for 3ßHSD activity. The ovaries of this 11 month female fetus had not yet begun to hypertrophy (0.42 and 0.45g) and, when sections of them were stained with the anti-3ßHSD antibody, they showed patchy staining of groups of the still small and fibroblast-like interstitial cells in the medulla (Figure 3f). Interestingly, the cytoplasm of the oocytes and the partly haemolysed red blood cells in the vessels appeared to also take up the chromagen non-specifically. The cortex of the fetal ovary was tightly packed with small primary

Figure 3. (a) Sectioned ovaries (fixed) from a pregnant elephant at 7.2 months of gestation. Each ovary contains three significant CL (21–35 mm diameter). The solid white arrows indicate two prominent ovulation stigmata and the dotted arrow indicates a CN from a previous oestrous cycle or pregnancy. (b) Histological section of one CL in (a). The tightly packed large luteal cells still appear healthy and (c) they stain strongly for 3βHSD activity particularly around the nucleus (both scale bars=150 μm). (d) Sectioned ovaries of a pregnant elephant at 11.2 months of gestation carrying a 7 kg foetus. The lower (ipsilateral) left ovary contains four CL (28–43 mm diameter) and the upper (contralateral) right ovary two CL (24 and 36 mm diameter). The homogenous luteal tissue now has a very pale brown/khaki colouration but, (e) histologically, the large, tightly packed luteal cells still appear healthy and functional (scale bar=150 m). (f) Section of the ovary of the 11.2 month female foetus stained with the anti-3βHSD antibody and showing the tightly packed small primary follicles in the cortex with a few enlarging follicles that have migrated into the medulla. The interstitial cells in the medulla are still small and fibroblast like and they stain only patchily for 3βHSD activity at this stage (scale bar=240μm).

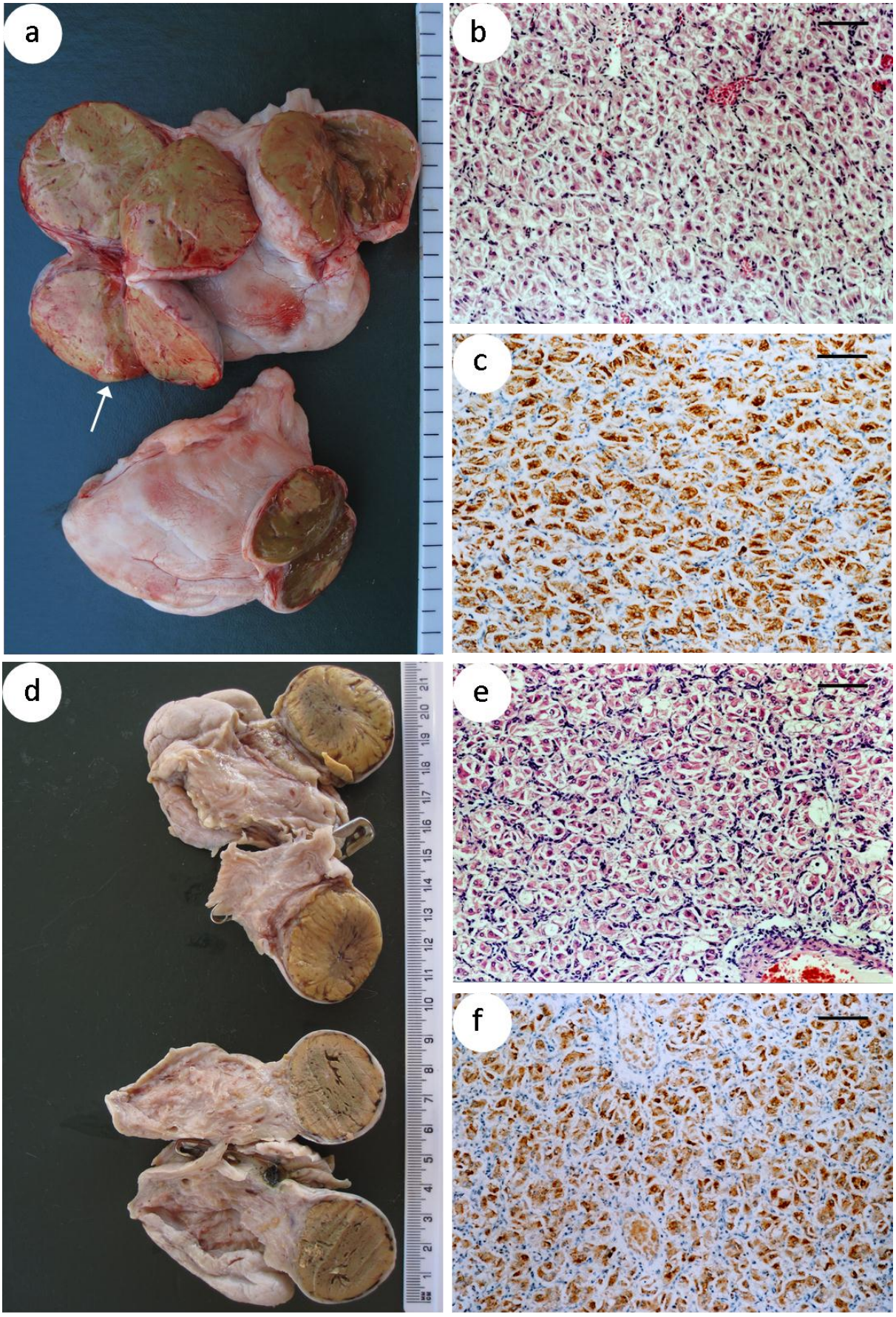


follicles, a few of which had migrated into the medulla and were just beginning to enlarge (Figure 3f).

Towards the end of pregnancy (17.5 months, 51.5 kg fetus) the ipsilateral (right) ovary contained 3 large CL (31 – 37 mm diameter), the stroma of which, although basically still homogenous, was considerably more yellow/khaki in colour while the single, smaller CL in the left (contralateral) ovary (25 mm diameter) was even more khaki coloured (Figure 4a). Histologically, the large luteal cells were showing definite signs of degeneration, including pronounced shrinkage of the cytoplasm away from the cell wall leading to the formation of vacuoles that encircled the entire perimeter of most cells, with smaller, isolated vacuoles within the persisting cytoplasm (Figure 4b). This cytoplasm shrinkage was accompanied by darker, more granular staining of the centrally located persisting cytoplasm with the anti-3ßHSD antiserum (Figure 4c). In addition, the structural strands of fibroblasts were more prominent and now appeared to be separating the aging luteal cells into smaller and more isolated cell pockets (Figures 4b & c).

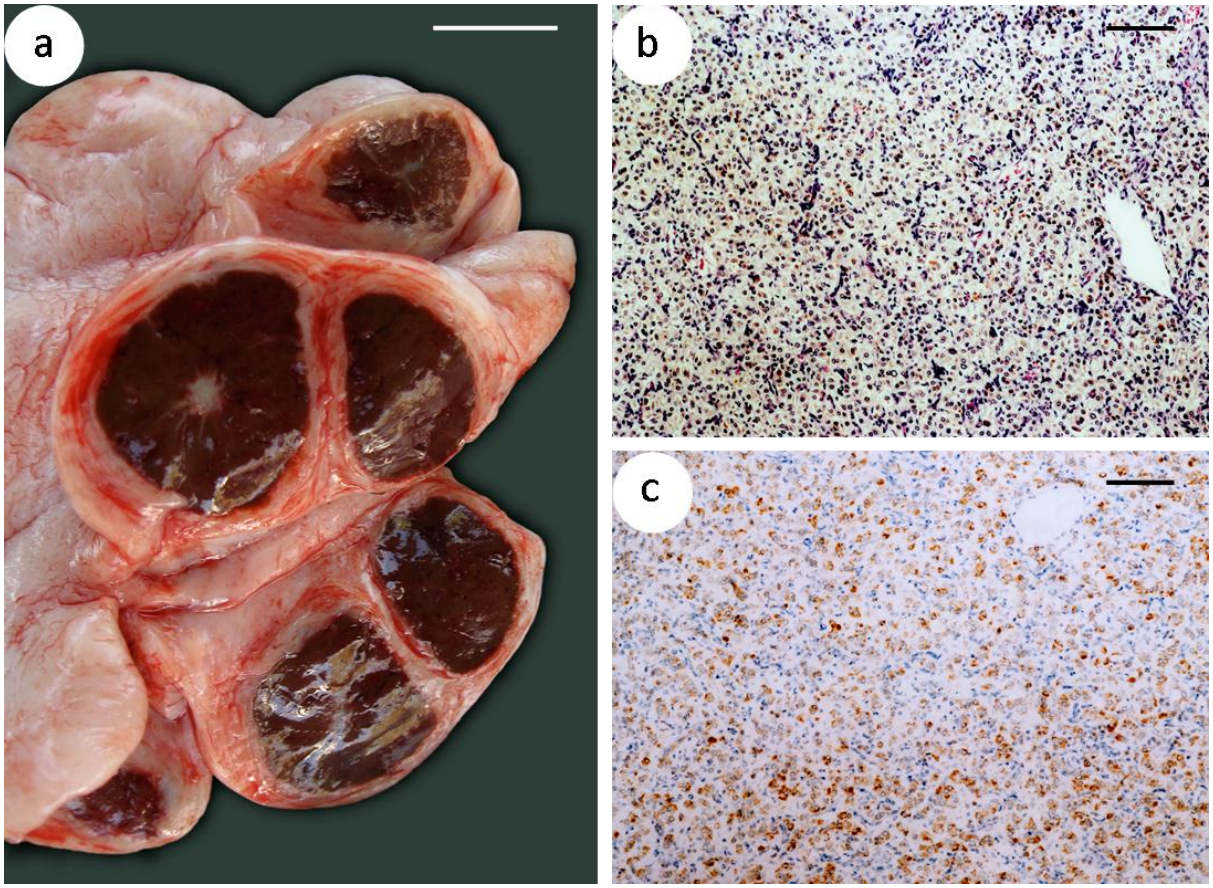
Just prior to parturition (139kg fetus, 22 months gestation) the two large CL, one in each ovary (42 and 36 mm diameter, both with stigmata), were now quite markedly orange/khaki in colour and the luteal tissue was noticeably less smooth and homogenous, with prominent cracks and fissures radiating out from the central core (Figure 4d). Histologically, cytoplasmic shrinkage and vacuolation of the luteal cells was very advanced and the structural strands of fibroblasts even more prominent and numerous (Figure 4e). Overall, staining with anti-3ßHSD serum was markedly reduced and now very concentrated to the small volume of cytoplasm present in each luteal cell (Figure 4f).

Figure 4. (a) Sectioned ovaries from a pregnant elephant at 17.5 months of gestation (57.5 kg foetus). The ipsilateral right ovary contains three large CL (31–37 mm diameter), only one of which has an ovulation stigma (solid arrow). The contralateral left ovary has a single accessory CL (25 mm diameter). The luteal tissue now has a definite yellowish/khaki colouration and, histologically, (b) the still tightly packed large luteal cells are showing vacuolation and shrinkage of the cytoplasm away from the cell wall (scale bar=150 µm). (c) Luteal cell degeneration is reflected in a more patchy staining with the anti-3βHSD antibody although activity is still concentrated around the nucleus in the reduced volume of cytoplasm (scale bar=150 µm). (d) Sectioned ovaries from a near-term pregnant elephant carrying a 139 kg foetus at 22 months of gestation. Each ovary contains a single large CL (38 and 36 mm diameter) which is now noticeably orange/khaki in colour and the luteal tissue of which is less smooth and homogenous in appearance. (e) Histologically the luteal cells are showing signs of advanced degeneration with widespread, often confluent vacuolation of the cytoplasm



although the nuclei still appear intact. There is a noticeable increase in the amount of structural fibroblast tissue appearing to surround and compartmentalise groups of luteal cells (scale bar=150µm). (f) Mirroring the histological degeneration of the luteal cells, staining with the anti-3βHSD antibody is patchy and less intense, although still concentrated around, and often including, the cell nuclei (scale bar=150µm).

Figure 5. (a) Sectioned ovary from an anoestrous lactating female elephant at 7 months postpartum showing the cut surfaces of three dark brown corpora nigrans (CN) as remnants of the accessory CL of the previous pregnancy (scale bar=1 cm). (b) Section of one CN from this animal showing a dense mixture of fibroblasts and very shrunken luteal cells (scale bar=150 µm). (c) A section of the same CN stained with the anti-3βHSD antibody. Surprisingly, the much-reduced cytoplasm of the shrunken luteal cells still stains positively (scale bar=150 µm).



Small brown CN were present in the ovaries of the two anoestrous lactating females, calculated on the basis of the weight and height at the shoulder of their calves (Laws, 1966; Krumrey and Buss, 1968; Whyte 1996; Shrader *et al.* 2006) to be 7 and 18 months post-partum respectively (Figure 5a). Histologically, these consisted of an approximately equal mixture of fibroblasts and former luteal cells that had shrunk dramatically to become even smaller than those that comprised the cyclical CL, but still retained their small, pale-staining, spherical nuclei (Figure 5b). And, curiously, these very shrunken and degenerate-looking luteal cells still showed some patchy staining with the anti-3ßHSD antiserum (Figure 5c).

Discussion

The origin and time of formation of the large accessory CLs that develop in the ovaries of pregnant elephants has aroused considerable speculation over many years (Short, 1966; Laws, 1969; Smith *et al.* 1969; Hanks and Short, 1972; Smith and Buss, 1975; Hodges *et al.* 1994, 1997; Hodges 1998 and others), the more so since significant follicular growth is not seen at any stage of gestation in the elephant ovary (Allen 2006; Lueders *et al*. 2011). However, the findings in the present study lend strong circumstantial support to the novel proposal by Lueders *et al.* (2011) that these accessory luteal structures form initially by luteinisation of relatively small (5-15 mm diameter) follicles shortly after the first of the two pronounced serum LH peaks which characterise the 3-week inter-luteal period in the elephant, but with the notable exception that, clearly as shown by our demonstration of ovulation stigmata on many of the accessory CL, some of these follicles can actually ovulate before luteinisation commences. In others, particularly the smaller follicles situated deep in the ovarian stroma, luteinisation without ovulation occurs as proposed by Lueders *et al.* (2011). .

It is not currently understood why, if ovulation occurs at the first LH peak and mating behaviour involving young bulls is observed (Moss, 1983), that pregnancy does not ensue. Ovulation is the culmination of a complex series of events leading to oocyte maturation and its release into the ovarial sac, it is therefore highly likely that these ovulated oocytes are potentially fertile. Blockage to pregnancy therefore may be behavioural in that mating, despite the enthusiastic attention of young bulls, does not take place as females are not fully receptive (Poole *et al* 2011), or it may be related to the preparation of the reproductive tract. Mature bulls with mating experience are capable of differentiating between females following the first LH peak and those at fertile oestrous. This signalling from the female, as in the Asian elephant, is probably through pheromone production (Rasmussen and Schulte, 1998) which in turn may be influenced by a hormone stimulus, suggested to be oestrogens as in other mammals (Lueders et al, 2011), which concurrently prepares the fallopian tubes for conception and the uterus for sperm transport and later implantation.

The likelihood that these accessory CL remain dormant in terms of steroid production during at least the remainder of the luteal period, and probably also the first 5-6 weeks of pregnancy, is supported by the present findings of a lack of 3ßHSD activity in the luteal cells and those of Meyer *et al*. (2004) of continuing basal levels of progestagen in the peripheral blood of female elephants between the two LH peaks of the interluteal period and only a moderate rise in plasma progestagen concentrations after the second LH peak which may reasonably be assumed stems from the primary or gestational CL that develops from the mature (2.0 – 2.2 cm) follicle which has ovulated normally in response to the second LH peak. And then, at the end of the first 5-6 weeks of gestation, and presumably in response to the luteotrophic action of the commencing elPL secretion by the implanting trophoblast

(Yamamoto *et al*. 2011), the "dormant accessory CL" are stimulated into action, their small luteal cells hypertrophy to form the typically large luteal cells of a mature CL and they then begin to secrete the much larger quantities of progestagens that give rise to the marked, secondary increase in serum progestagen concentrations measured by Meyer *et al*. (2004). All these pieces of the jigsaw begin to fall into place well, but the mechanism which enables the accessory CL to lie doggo and remain inactive until stimulated by elPL, but in the face of the second pituitary LH release of the interluteal period and the maturation, ovulation, luteinisation and commencing progesterone secretion of the primary (fertile) CL, remains a mystery.

Twenty-two months is indeed a long time for any temporary endocrine gland like a CL to remain active and maintain its full secretory function so it is perhaps not surprising that they should show clear histological signs of progressive cell degeneration and loss of steroid synthetic capacity during the second half of gestation. This seems to accord well with the definite decline in, and flattening of, plasma progesterone profiles during the later stages of pregnancy in the elephant (Hodges *et al*. 1997; Meyer *et al.* 2004) and it seems reasonable to propose that any shortfall in progestagen production that may be occasioned by this later-stage decline in luteal progestagen secretion rate is met by an equivalent increase in the capacity of the fetal gonads, either ovaries or testes, to secrete greater amounts of progestagens as the proportion of steroidogenically active interstitial cells increases. Furthermore, the marked increase in general vascularity seen in the fetal gonads with advancing gestation (F Stansfield & WR Allen 2012, *unpublished observations*) may function usefully to facilitate the flow of progestagens from the gonads towards the maternal blood supply via the now highly vascularised endotheliochorial placenta (Wooding *et al*. 2005).

Returning finally to the original comparison between the hormone secreting capacities of the horse and elephant fetoplacental units, bilateral gonadectomy of the horse fetus in late gestation ablated oestrogen production by the placenta which, in turn, reduced vascular development at the placental interface to lessen placental exchange and cause significant deprivation of fetal development (Pashen and Allen 1979). While equivalent surgical intervention to ablate fetal gonadal secretion of progestagens is not feasible in the elephant, it is nevertheless interesting to speculate what effects upon fetal development and continuation of the pregnancy state might occur were it to be possible. Clearly, oestrogens are not the important vasculogenic factor involved in placental exchange in the elephant that they are in the horse and it is difficult to conceive that progestagens, of luteal or fetal origin, would have the same action. It is much more likely from previous studies in other domestic animal species, especially ruminants (Buttle and Forsyth, 1976; Martal *et al.* 1997; Watkins and Reddy, 1980; Wooding and Beckers, 1987; Takahashi, 2006) that the large quantities of elPL secreted by the elephant trophoblast directly onto the endothelium of the adjacent maternal vessels in the elephant endotheliochorial placenta (Allen *et al.* 2003; Wooding *et al.* 2005) would act in concert with vasculoendothelial growth factor (VEGF) to stimulate the degree of vasculogenesis required to keep pace with the rate of placental development needed to drive fetal growth. If so, a more likely role for the increasing amounts of fetal gonadal progestagens secreted in late gestation in the elephant would be, in addition to maintaining cervical closure and myometrial quiescence, to induce torpor and general inactivity in the rapidly enlarging fetus. 5αDHP has potent anaesthetic properties (Holzbauer, 1974; Gyermek and Soyka, 1975; Mok *et al.* 1991; Pearson Murphy *et al.* 2001) and since the combined weight of the fetus and placenta approaches 140kg towards term (Laws 1966), a hyperactive

near-term fetus cramped, due to the nature of its zonary placenta, into just half of the uterus suspended in the abdominal cavity, would clearly be undesirable and could lead to all manner of malpresentations and dystocias at parturition. Thus, perhaps the intrauterine safety and destiny of the elephant fetus, at least in later gestation, is ensured by the tranquilising properties of the products of its own enlarged and hyperactive gonads.

Materials and methods

Animals and tissues

The uterus and ovaries, and occasionally the fetal ovaries, were recovered within 3 h of death by free bullet from a total of 10 female elephants aged 8.5 to 38 years (Laws, 1966), culled as whole family groups under government licence for management reasons to preserve habitat in the Savé Valley Conservancy (SVC) in southern Zimbabwe; one animal was a tamed female aged 30 years that died suddenly. One or both authors were present at the culling operations and they dissected, sectioned and photographed the maternal and fetal ovaries in the field before taking pieces of CL or CN from the maternal ovaries and pieces of fetal ovary (or the whole embryo) for fixation in >10 vols neutral buffered formalin which was replaced entirely after 1 h. The elephant cows, both pregnant and non-pregnant, were aged on the basis of eruption and wear of the molar teeth in the lower jaw as described by Laws (1966) and Lee *et al.* (2011). The stage of gestation in the pregnant animals was estimated on the basis of embryonic/fetal weight using the formulae devised by Hildebrandt *et al*. (2007) for fetuses weighing < 2 kg and Craig (1984) for heavier fetuses.

Histology and immunocytochemistry

For normal histology, pieces (approximately 2 cm^3) of each maternal CL and fetal ovary were embedded in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin (H & E).

For immunostaining, a mouse monoclonal antibody raised against recombinant-derived 3ß hydroxysteroid dehydrogenase (3ßHSD) of human origin (3ß-HSD[37-2]:sc 100466; Santa Cruz Biotechnology, California, USA) was used at a dilution of 1:100. The 5μm paraffin embedded sections were placed in a 56°C oven overnight to de-wax them. They were then immersed in a pre-heated (65°C) bath of high pH antigen unmasking solution (Dako PT link; Dako UK Ltd., Ely, Cambs, UK) and heated to 97°C for 20 mins. After cooling, the slides were rinsed in neutral buffer and transferred to a Dako Plus Autostainer (Dako UK Ltd) where a computer controlled indirect staining method was performed. Incubations of the optimally diluted primary and secondary antibodies were for 30 mins. The secondary antibody, blocking reagents, buffers, substrate, chromagen and nuclear stain were all Envision FLEX reagents (Dako UK Ltd.) optimised for use on the Autostainer Plus. After staining the slides were removed from the machine, dehydrated, cleared and mounted in DPX.

The H&E-stained and 3ßHSD-stained slides were examined using an Olympus Laborlux AH-3 microscope (Olympus, Tokyo, Japan) and photographed using the microscope's built-in camera.

Declaration of Interests

The authors declare there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported in this paper.

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