

# Gestating for 22 months: luteal development and pregnancy maintenance in elephants

Imke Lueders<sup>1,\*</sup>, Cheryl Niemuller<sup>2</sup>, Peter Rich<sup>3</sup>, Charlie Gray<sup>3</sup>,  
Robert Hermes<sup>1</sup>, Frank Goeritz<sup>1</sup> and Thomas B. Hildebrandt<sup>1</sup>

<sup>1</sup>Leibniz-Institute of Zoo- und Wildlife Research (IZW), Reproduction Management, Alfred-Kowalke-Strasse 17, 10315 Berlin, Germany

<sup>2</sup>Kingfisher International Inc. (KFI), Conservation Biology Laboratories, 165 Mostar Street, Unit 8, Stouffville, Ontario, Canada L4A 1E3

<sup>3</sup>African Lion Safari and Game Farm Ltd. (ALS), R.R.#1, Cambridge, Ontario, Canada N1R 5S2

The corpus luteum, a temporally established endocrine gland, formed on the ovary from remaining cells of the ovulated follicle, plays a key role in maintaining the early mammalian pregnancy by secreting progesterone. Despite being a monovular species, 2–12 corpora lutea (CLs) were found on the elephant ovaries during their long pregnancy lasting on average 640 days. However, the function and the formation of the additional CLs and their meaning remain unexplained. Here, we show from the example of the elephant, the close relationship between the maternally determined luteal phase length, the formation of multiple luteal structures and their progesterone secretion, the timespan of early embryonic development until implantation and maternal recognition. Through three-dimensional and Colour Flow ultrasonography of the ovaries and the uterus, we conclude that pregnant elephants maintain active CL throughout gestation that appear as main source of progestagens. Two LH peaks during the follicular phase ensure the development of a set of  $5.4 \pm 2.7$  CLs. Accessory CLs (acCLs) form prior to ovulation after the first luteinizing hormone (LH) peak, while the ovulatory CL (ovCL) forms after the second LH peak. After five to six weeks (the normal luteal phase lifespan), all existing CLs begin to regress. However, they resume growing as soon as an embryo becomes ultrasonographically apparent on day  $49 \pm 2$ . After this time, all pregnancy CLs grow significantly larger than in a non-conceptive luteal phase and are maintained until after parturition. The long luteal phase is congruent with a slow early embryonic development and luteal rescue only starts ‘last minute’, with presumed implantation of the embryo. Our findings demonstrate a highly successful reproductive solution, different from currently described mammalian models.

**Keywords:** corpus luteum; luteinized follicles; luteal rescue; embryo; *Paenungulata*

## 1. INTRODUCTION

The establishment and maintenance of pregnancy are among the most fascinating physiological events occurring in nature. Several models explain the diverse mammalian mechanisms of luteal rescue, maternal recognition of pregnancy and pregnancy maintenance [1,2]. Despite general comparability, e.g. in hormones, anatomical and functional structures, mammals exhibit an amazing variety of functional detail in comparative reproductive physiology. The elephant species (Asian elephant: *Elephas maximus*; African savannah and forest elephants: *Loxodonta africana africana* and *Loxodonta africana cyclotis*), represent a fantastic example for an exceptional reproductive anatomy and physiology.

The special reproductive features of the female elephant became known during post-mortem dissections of African elephants derived from culling procedures in Southern and East Africa during the 1950s and has continued into the 1990s. Since then, the anatomy of the reproductive tract with an 1.5 m long vestibulum (canalis urogenitalis) preceding the vagina [3,4], and ovaries showing multiple

corpora lutea (CLs) [5–8], as well as the secretion of reproduction-associated hormones have been the focus of much research. Elephants show a double luteinizing hormone (LH)-peak during the follicular phase and a 12–16 weeks long oestrous cycle [9–11]. The pregnancy lasts from 620 to 680 days, which led to research interest on placentation and embryonic development [4,12–14]. However, several findings have remained without adequate explanation. The role of accessory CLs (acCLs) in the elephant pregnancy as well as timing of implantation and maternal recognition have remained unclear [8,10].

In polyestric, spontaneously ovulating mammals, ovulation is followed by the luteal phase; a species-specific predetermined period of elevated progesterone (P4) levels secreted by the temporarily formed corpus luteum (CL). The CL-derived P4 initially aids in ensuring an adequate uterine surrounding for early embryonic development and implantation [2]. Typically, one CL develops on the mammalian ovary from each ovulated follicle, so that the number should correspond with the number of embryos. In the elephant, the luteal phase duration is between six and 10 weeks, if no conception occurs [11,15]. Elephant cows normally carry only one foetus—the incidence of twinning was calculated to be less than 1 per cent [16]—yet show up to 30 and more [17], but usually between two and 12 pregnancy CLs on their

\* Author for correspondence ([imke.lueders@gmail.com](mailto:imke.lueders@gmail.com)).

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ovaries [5–8]. Different hypotheses have been put forward over the years to account for this phenomenon [8,18,19]. However final proof is lacking, especially since in post-mortem studies only a single stage of gestation may be evaluated per female, not accounting for the ongoing ovarian process.

Initially, the mammalian CL is maintained if conception occurs, a process called luteal rescue [20]. In many species, there is a shift from CL to placentally derived P4 at a certain stage of gestation (luteoplacental-shift). However, the elephant placenta has been shown to be steroidogenically inert [10,12,21,22]. Also, no chorionic gonadotropin and no endometrial cup-like structures could be identified in the gravid elephant uterus [4], suggesting that elephants pursue a different strategy compared with the mechanism of acCL formation in the pregnant mare. There is evidence that the foetal gonads of both genera may contribute to circulatory progesterone levels of the female elephant in late gestation, because, similar as in the foetal horse, the gonads enlarge from mid gestation and stain positive for  $5\alpha$ -reduced pregnanes [23,24].

Recent application of transrectal ultrasonography in trained elephants made observation of reproductive processes possible over time [14,19,25–27]. We showed previously that during the six-week-long follicular phase of the oestrous cycle, the acCLs are derived from luteinized unruptured follicles [26]. Luteinization of the first wave of follicles is induced by the first, anovulatory LH peak. Three weeks later, a second LH peak occurs which induces ovulation of a single-dominant follicle [26]. However, some intriguing questions remain unanswered: are there additional CLs forming during early pregnancy? Why is the oestrous cycle and gestation so much longer when compared with other large mammals, and how is the elephant able to maintain this enormously long gestation if there is no contribution of progesterone from the placenta?

Here, we address the long-standing debate on the function and formation of multiple CL during pregnancy in elephants through direct ultrasonic observation and discuss costs and benefits of the extraordinary pregnancy length.

## 2. MATERIAL AND METHODS

### (a) *Elephants*

Fifteen Asian and two African elephant females housed in zoological facilities in Canada, the USA, Britain, Germany and Australia were examined during this study between 2006 and 2011. Animals were aged between 7 and 28 years (mean age: 16.9 years). Ten Asian and the two African elephants conceived naturally while another five Asian elephants became pregnant via artificial insemination. Nine elephants were nulliparous, five had produced one calf previously while four females had given birth twice or more. In one Asian elephant, pregnancy loss occurred around day 120.

### (b) *Ultrasound*

A total of 336 transrectal ultrasound examinations were performed using previously reported approaches [13,25,26]. Five Asian elephants were ultrasonographically examined on random days during the entire pregnancy and in one case until day 459. Ultrasound was performed during non-

conceptive cycles prior to the fertile mating in five females. Here, examinations were performed once a week during the luteal phase, three times per week during the follicular phase and daily around ovulation, and in one female occasionally throughout both phases. In addition, five Asian and two African elephants were examined by ultrasound between three and 10 times during gestation. Another five Asian elephants were sonographically examined once during pregnancy.

Measurements were taken from the embryo (diameter of the embryonic vesicle (EV), length of the embryo) as well as from the largest cross-sectional diameter of the CL (height and length in the two-dimensional image). Colour Flow Doppler (CFD) or Power Doppler (PD) mode were applied during beginning and the end of gestation in four Asian elephant females to depict the blood flow within the CL and the ovaries. Ultrasounds were performed by three different operators. For consistency, images and videos were evaluated and structures measured by the same researcher.

### (c) *Hormone determination*

Elephants were housed in different countries, thus hormone determinations were performed in different laboratories by described standard methods. As in a previous study, for statistical analysis only the serum data was used from elephants in Canada ( $n = 3$ ) and Britain ( $n = 3$ ), which derived from two different enzyme immunoassays (EIA) producing comparable results [26]. The first and second LH peaks were determined from daily blood samples collected during the follicular phase in five animals (starting two weeks after the progesterone metabolite ( $P_m$ ) drop to non-luteal levels) from the ear vein into serum separator tubes (Corvac; Tyco Healthcare Group, Mansfield, MA, USA). Blood was centrifuged at 200g for 30 min at room temperature. The serum and urine samples were stored at  $-20^\circ\text{C}$  until assayed.

The previously established EIA methodologies for progesterone and its metabolites ( $P_m$ ) in elephants were applied to determine hormone concentrations from serum [28] or urine [9]. The protocols for serum  $P_m$  analysis were slightly adapted as previously published [27]. Progesterone assay sensitivity was  $0.06 \text{ ng ml}^{-1}$  at 90 per cent binding. The intra- and inter-assay coefficients of variation were less than 15 per cent. Serum LH was quantified by a double-antibody EIA [29]. Assay sensitivity was  $0.08 \text{ ng ml}^{-1}$  at 90 per cent binding. Intra- and inter-assay coefficients of variation were less than 15 per cent.

### (d) *Data analysis*

The first day of pregnancy was set as the day of the ultrasonographically observed ovulation which typically occurred the day after the second (ovulatory) LH peak [26]. Both, ovulatory LH peak and ovulation were determined for artificial insemination procedures, but not necessarily for natural mating. In case LH was not determined or ultrasound was not performed on the day of ovulation, day 1 of pregnancy was set as the day of last observed mating, as breeding usually stops with follicle rupture [26]. Not all parameters could be retrieved for all animals, depending on time and number of investigations. Furthermore, it was not possible to perform ultrasound examinations at the same day of pregnancy for all individuals. Hence, values measured in the same week of pregnancy were taken to calculate the overall mean when presented in the graphs and to perform the statistical analysis. For five females, CL measurements from a previous non-conceptive oestrous cycle were available. Statistics were performed using GRAPHPAD

PRISM v. 4.0 (GraphPad Software, La Jolla, CA, USA). Assumption for Gaussian distribution was tested using the method of Kolmogorov and Smirnov (KS). Owing to the problem of multiple measurement of the same individual, we used an additive generalized mixed model to explain variance in CL size in R (R 2.14.0, the R Foundation for Statistical Computing, Lucent Technologies, New Jersey, USA). Fixed factors were defined as time (weeks), P4 concentration ( $\text{ng ml}^{-1}$ ), number of CLs in the ovulatory ovary, as well if the elephant was pregnant and if the CL was derived from ovulation (ovCL) or not, both as factor. As a random factor, we defined the individual animal. We used only a complete dataset with 163 entries comprising data of five elephants up until week 19 of pregnancy (table 1). The significance level was set to  $\alpha = 0.05$ . Values are given as mean  $\pm$  s.d. and as mean  $\pm$  s.e. of the mean (see the electronic supplementary material) in the graphs.

### 3. RESULTS

Mean gestation length recorded in this study was  $647 \pm 23$  days ( $n = 16$ ). A set of  $5.4 \pm 2.7$  CLs (range: 2–11;  $n = 17$ ), including multiple acCLs and a single ovCL, which had formed originally during the previous fertile oestrous cycle, was maintained over the entire course of the pregnancy in number and size, and no additional follicular growth or CL development was observed on the ovaries of 11 females that were examined multiple times at the beginning and the end of the gestational period. There was no significant difference in the total number of CLs between cycling (mean  $\pm$  s.d.:  $4.9 \pm 1.5$  CLs, range 2–7) and pregnant females (mean  $\pm$  s.d.:  $5.5 \pm 2.9$  CLs, range: 3–11; paired  $t$ -test,  $p = 0.45$ ,  $t_{13} = 0.78$ , KS = 0.15,  $n = 14$ ). Different phases of functional structural development were distinguishable throughout the oestrous cycle and the pregnancy as described below.

#### (a) Ovarian and hormonal observations until ovulation ( $n = 5$ )

During the follicular phase, two follicular waves occurred, both ending with an LH peak. After the first LH peak, luteinization of 2–10 larger, non-ovulatory follicles (diameter: 7.0–19.0 mm) occurred on both ovaries, as seen by a distinct hyperechoic wall. In contrast to other antral, non-luteinizing follicles, as soon as signs of luteinization appeared, blood supply towards the luteal wall could be depicted when the CFD was applied. At the second LH peak, only one large follicle dominated alongside one to five subordinate follicles. The time span between the two LH peaks was between 19 and 22 days. Within 24 h after the second LH peak, ovulation occurred. The ovulatory follicle on the day of ovulation measured  $21.0 \pm 2.0$  mm. At the second LH peak, the follicles which previously luteinized without ovulation (LUF) had grown to diameters averaging  $22.9 \pm 4.0$  mm. The diameter of the luteal wall increased, while a fluid-filled centre was maintained. Based on the continuous observation of the same structures, the LUF are the source of acCLs that form beside the main CL derived from ovulation. While in an LUF the hypoechoic, fluid-filled centre is prominent, later on, the acCL had only little or no fluid content and luteal tissue clearly predominated.

Table 1. Results of the mixed model calculations testing the influence of gestation time (weeks) until week 19 on  $P_m$  concentration and number of CLs as well as the effect if the CL derived from ovulation (ovCL yes) and if it occurs during pregnancy. (In result, the ovCL is the largest, and pregnancy has a strong effect on ovCL size, but not on the number of CLs. The level of  $P_m$  increases with advance of the pregnancy.)

	mean	s.e.	d.f.	$t$ -value	$p$ -value
intercept	12.621	1.738	153	7.26	<0.001
time (weeks)	0.438	0.076	153	5.73	<0.001
$P_m$ ( $\text{ng ml}^{-1}$ )	0.404	0.110	153	3.67	<0.001
number of CLs	0.677	0.399	153	1.70	0.091
pregnancy (yes)	5.296	0.997	153	5.31	<0.001
ovCL (yes)	8.119	0.703	153	11.55	<0.001

#### (b) The luteal phase of the non-conceptive cycle

A clear ovCL was only ultrasonographically recognizable a week after ovulation. It became distinctly visible initially as a hyperechoic, irregular-shaped mass measuring  $17.1 \pm 1.5$  mm. The previously formed acCLs had also grown considerably in size at that stage and measured  $26.0 \pm 4.3$  mm. The anechoic centre remained present in most of the accessory luteal structures until week three after ovulation.  $P_m$  concentrations started rising anywhere from 1 to 3 days post ovulation.

The ovCL always became the largest of all luteal structures. The acCLs and the ovCL reached their maximum diameter after week three, and after week four post ovulation, respectively. A regression of all CLs was noticeable around week five to six and matched the  $P_m$ -level decrease. While the luteal phase ended when  $P_m$  concentration reached baseline, typically between weeks eight to 10 post ovulation, the CLs remained visible throughout the next follicular phase, although they continued to regress.

#### (c) Ovulation and early pregnancy

The development of the ovCL and acCLs as well as the  $P_m$  secretion during early pregnancy was identical to a typical luteal phase until week seven post ovulation (figure 1*a,b,d*). In week four post ovulation, no significant difference was measurable in size between the ovCL of early pregnant females ( $33.7 \pm 4.0$  mm) and their non-conceptive luteal phase ( $32.1 \pm 2.9$  mm). Similarly, the variation in mean size of the largest acCLs in week four during the cycle ( $21.2 \pm 1.2$  mm) when compared with the pregnancy ( $25.1 \pm 6.7$  mm) was insignificant (repeated measure ANOVA,  $t = 0.8860$ ,  $t = 2.137$   $p > 0.05$ ,  $n = 6$ ). The now mature CL appeared hypoechoic compared with the freshly formed luteal tissues (figure 2*a,b*). CFD ultrasonography showed a marginal vascularization with smaller colour echoes branching into the luteal stroma (figure 2*b-d*). Even small blood vessels within the CLs were clearly visible when the PD was applied and when this mode was combined with a three-dimensional scan (figure 2*d*).

Like in a non-gravid luteal phase,  $P_m$  concentrations as well as all luteal structures started declining significantly after week five (figure 1*a,b,d*). In week seven, CL diameters (mean diameter of all CLs:  $22.4 \pm 5.3$  mm) were significantly smaller than in week four of pregnancy ( $25.1 \pm 6.6$  mm; paired  $t$ -test,  $p = 0.0185$ ,  $t_4 = 3.836$ ;



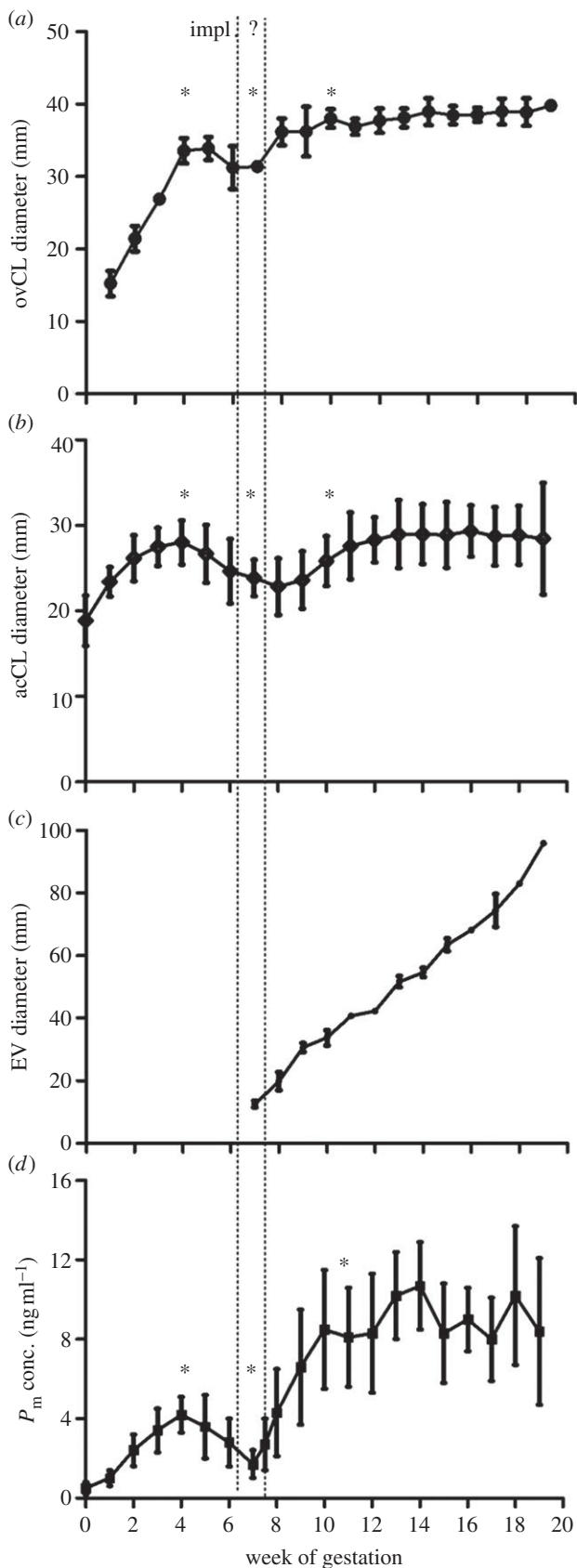


Figure 1. (Caption opposite.)

KS = 0.26,  $p > 0.05$ ,  $n = 5$ ). This matched the onset of luteolysis in a non-gravid luteal phase and was reflected by the  $P_m$  concentrations (figure 1d). The  $P_m$  levels also reached a transient minimum of  $1.7 \pm 0.7 \text{ ng ml}^{-1}$  during week seven of pregnancy. However, in contrast

Figure 1. (Opposite.) Weekly measurements depicted as mean  $\pm$  s.e.m. for (a) ovulatory CL (ovCL) diameter, (b) diameter of the largest accessory CL (acCL), (c) embryonic vesicle (EV) diameter, and (d) progesterone ( $P_m$ ) concentration during the first 19 weeks of gestation. Note the different growth curves for ovCL and acCL, the parallel decline in  $P_m$  concentrations and CL diameter from week five to seven or eight, as well as the second growth phase of CLs and increase of  $P_m$  simultaneously with ultrasonic appearance of the EV around week seven, which presumably equals implantation (impl). While the ovCL is relatively preserved in size across individuals (ovulatory follicle reaches 20 mm), the largest acCL (depicted representatively for development of all acCL) may vary strongly in diameter, depending on the size of the luteinized unruptured follicle it derived from. (a,b,d) show measurements derived from the same five Asian elephants, (c) shows EV measurements from four out of the five Asian elephants from above as well as of three additional Asian and two African elephants. ov, ovulation. Asterisks (\*) denote the significant points in time during early gestation for CL diameter and  $P_m$  concentration changes.

to a normal, non-gravid luteal phase, concentrations did not continue to fall, but increased again during the following week with significant higher levels attained from week 11 onwards (figure 1d). During temporary hormone depression in week seven, the serum  $P_m$  concentration was significantly lower than during week 11 ( $8.1 \pm 5.5 \text{ ng ml}^{-1}$ ; paired  $t$ -test,  $p = 0.0315$ ,  $t_4 = 3.246$ ; KS = 0.17,  $p > 0.05$ ,  $n = 5$ ).

The existence of a pregnancy was confirmed through a small, circumscribed anechoic spot (fluid) within the uterine horn ipsilateral to the ovulatory ovary. In two females, a small amount of fluid accumulation (0.9 and 4.8 mm) became visible on day 38 and day 42, respectively (figure 2e). A clear EV was not evident before week seven of pregnancy (figure 1c). Typically, the EV appeared between days 48 and 52 (mean  $49.4 \pm 1.7$ ,  $n = 5$ ) and measured  $12.7 \pm 1.7 \text{ mm}$  within the uterine lumen (figure 2f).

In contrast to a non-conceptive cycle, the decrease in  $P_m$  level and regression of the CL size ended after week seven or eight of pregnancy and all structures resumed growing. Consequently,  $P_m$  rose again and reached concentrations on average higher than during the luteal phase (figures 1 and 3). With more than 11 weeks of gestation, the ovCL measured  $39.9 \pm 3.3 \text{ mm}$  and the mean of all acCLs measured  $25.6 \pm 5.5 \text{ mm}$  ( $n = 17$ ). The pregnancy ovCL was now significantly larger than the cyclical ovCL at its maximum dimension during week four of the luteal phase (pregnancy ovCL:  $38.5 \pm 2.0 \text{ mm}$  versus. cycle ovCL:  $32.1 \pm 3.0 \text{ mm}$ ; repeated measure ANOVA,  $t_5 = 3.489$ ,  $p < 0.05$ ,  $n = 6$ ). Also compared with week four of the pregnancy ( $33.7 \pm 4.0 \text{ mm}$ ), which represents the time of the first maximum diameter, the ovCL was significantly larger in week 11 ( $t_5 = 4.344$ ,  $p < 0.05$ ). Similarly, the biggest acCLs of week 11 ( $28.4 \pm 5.0 \text{ mm}$ ) of the pregnancy was significantly larger than the same structure in week four of the oestrus cycle ( $21.2 \pm 1.2 \text{ mm}$ ;  $t_5 = 3.596$ ,  $p < 0.05$ ). This shows a significant gain in size of all CLs, most likely associated with establishment of pregnancy.

The mixed model analysis confirmed the observations (table 1): all significant effects were positive. As expected, the fact that the measured CL was an ovCL had the strongest influence on CL size, because ovCL grow larger than

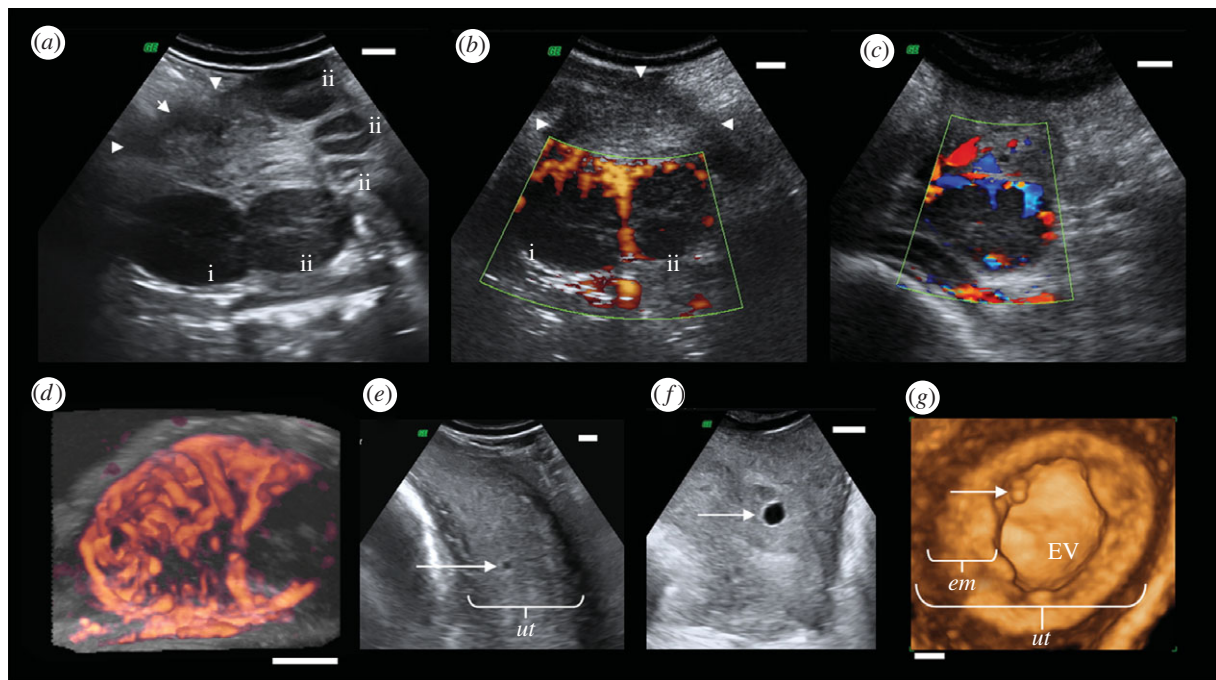


Figure 2. Ultrasonographic images from the ovary (*a–d*) and the uterus (*e–f*) of pregnant elephants. (*a*) Ovulatory ovary showing multiple hypoechoic corpora lutea (CLs), (i), ovulatory CL, (ii), acCLs, arrow heads, ovarian border (day 162); (*b*) Power Doppler (PD) mode depicting minimal blood streams of two CL ((i) ovCL, (ii) acCL, arrow, ovarian boarder) of the same ovary as in (*a*); (*c*) Colour Flow Doppler applied on the ovCL shortly before parturition (day 603); (*d*) three-dimensional PD ultrasound image (glass mode) depicting the vascularization of the ovulatory CL on day 74; (*e*) first ultrasonographic appearance of a conceptus (arrow) as a tiny fluid accumulation within the uterine horn (*ut*) on day 42; (*f*) clear EV with typical hyperechoic enhancement lines on day 55; (*g*) three-dimensional ultrasound image looking into the uterine horn (*ut*) and the EV revealing the embryo (arrow) close to the endometrium (*em*) on day 72.

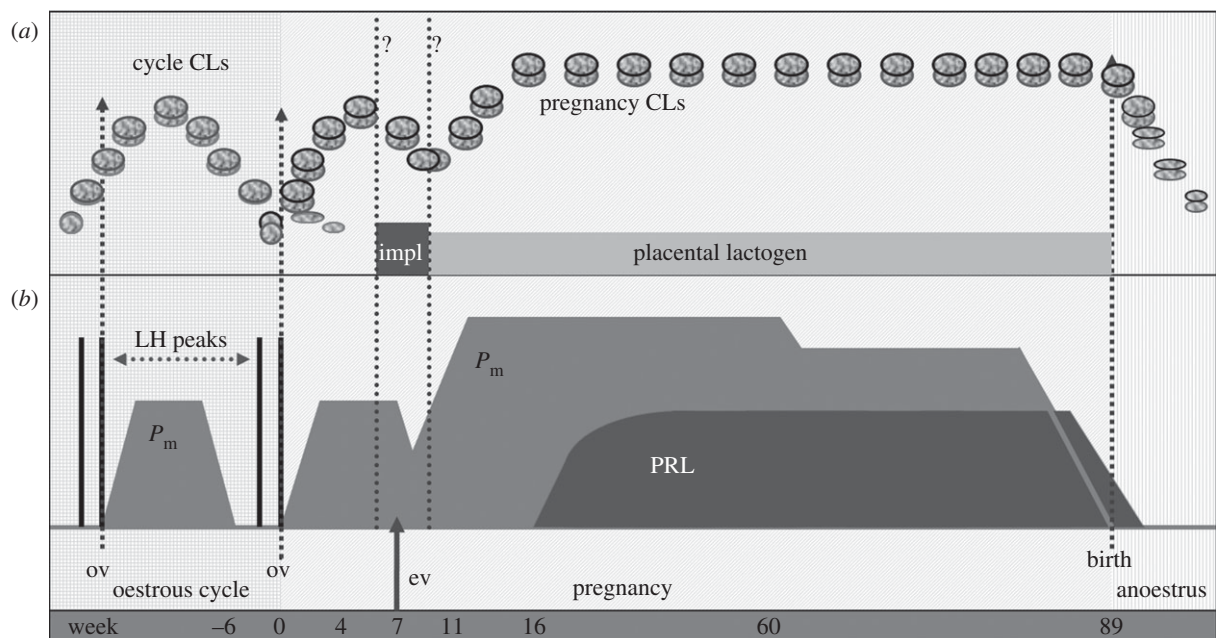


Figure 3. Schematic of (*a*) the growth behaviour of CL during the oestrous cycle (cycle CLs) and the pregnancy (pregnancy CLs) and (*b*) hormone secretion during the pregnancy and a previous oestrous cycle in relation to ovulation (*ov*), presumed implantation period (*impl.*) and placental lactogen production by the trophoblast.  $P_m$ , progesterone metabolites; PRL, prolactin; EV, embryonic vesicle becomes ultrasonographically visible for the first time.

acCLs. Also in line with expectation was that pregnancy had a strong positive effect on CL size. Compared with those factors, time and  $P_4$  concentration had a lower, but nevertheless also highly significant effect on CL size. This is in accordance with our observations of

increased CL size and  $P_m$  output from week 11 of pregnancy. The number of CLs in the ovulatory ovary showed no significant effect on CL sizes, suggesting that higher numbers of CLs do not compensate for smaller CL diameters.



**(d) The course of pregnancy and parturition**

Beyond day 200 post ovulation, the uterus descended deep into the abdominal cavity and it was no longer possible to visualize the foetus through transrectal ultrasound on a regular base. However, the ovaries remained accessible during the entire pregnancy. After week 11 and after reaching their final diameter, all CLs on both ovaries were maintained at the same number and size until parturition (figure 3). Apart from two exceptions, the ovulatory (*ov* ovary) ovary contained more CLs, which were significantly larger in size (mean CL diameter *ov* ovary:  $31.3 \pm 7.2$  mm; *contralateral* ovary (*ctrla* ovary):  $21.6 \pm 11.0$ ; paired *t*-test,  $p = 0.0025$ ,  $t_{16} = 3.573$ ; KS = 0.18,  $p > 0.05$ ,  $n = 17$ ). Between two to seven CLs (mean:  $3.4 \pm 1.7$ ) were present on the *ov* ovary, including the ovCL. The *ctrla* ovary contained either none or between one to six acCLs (mean:  $2.0 \pm 1.7$ ). The application of the CFD mode revealed a constant blood supply of all CLs until the day before parturition in two animals (figure 2c). In four females, where daily  $P_m$  measurement took place around the expected time of parturition,  $P_m$  dropped to nadir 1–3 days prior to giving birth. A noticeable diameter decrease was not seen before day 5 after parturition in two females. Thereafter, all CLs regressed slowly within two to three months.

**4. DISCUSSION**

Known extra-ovarian sources for progesterone during the mammalian pregnancy are the placenta or the gonads of the foetus. Because no placental contribution was detectable in elephants, it has been speculated in the past that additional CLs form during the early elephant pregnancy [17,25], or that foetal ovaries or testicles, which enlarge during the second half of gestation may contribute to peripheral progesterone concentration [23,24]. Although, contribution of the latter cannot be categorically excluded and is possible in late gestation, we could not observe an additional CL formation after ovulation. Instead, all acCLs were formed prior to ovulation in response to the first, anovulatory LH peak during the follicular phase of the oestrous cycle (figure 3). A single ovCL formed from the rupture of the dominant follicle after the second, ovulatory LH peak. While the ovulatory follicle usually does not exceed 20.0–23.0 mm, which is about the same size as in women or cattle (as reviewed by Evans [30]), the resulting ovCL is twice the size in elephants. The set of 1–11 acCLs and the additional ovCLs was maintained for the entire gestational period. The maintenance of number, diameter and blood supply of all luteal structures suggests their functionality throughout the entire gestational period of on average 640 days. Ovarian blood flow has been shown to be correlated to luteal progesterone secretion in cows, ewes and pigs [31]. Similarly, the constant blood supply that we could visualize via CFD sonography may be employed as a marker for luteal activity in elephants. Because steroidogenic activity of all pregnancy CLs has been reported previously [22,32] and onset of structural regression was only seen after parturition, the CLs are likely to be the main source of  $P_m$  in elephants. A recent examination of ovaries obtained from pregnant African elephant females supports the idea of active CL throughout the pregnancy [24]. Although

degeneration of large luteal cells was observed in late compared with earlier pregnancy stages, they still stained positive for  $3\beta$  hydroxysteroid dehydrogenase (an enzyme important for the biosynthesis of progesterone from pregnenolon) in CL even in near term pregnancy. However, compared with the first half of gestation, the staining was more patchy and not as uniform, implying reduced luteal secretory capacity [24]. This may be reflected in flattening serum progesterone concentrations during the second half of gestation as denoted in other studies [32,33].

Our observations on the oestrous cycle prior to conception agree with previous findings [26] and show that it is impossible to distinguish between a non-conceptive luteal phase and an early pregnancy during the first six to seven weeks post ovulation by means of ovarian ultrasonography or  $P_m$  concentration (figures 1 and 3). A normal pattern of a (non-fertile) luteal phase CL development occurs until the embryo becomes visible in the uterus around day 48–50 of gestation. Although onset of structural CL regression and decreasing  $P_m$  levels were noticed after week five, this decline was only temporary and by week 11 all CLs reached larger diameters and  $P_m$  concentrations rose higher than non-pregnancy levels. The transient functional luteolysis ( $P_m$  level decline), resembling a normal non-conceptive luteal phase, has been noted previously in early pregnancy of elephants [33], but does not appear in other domestic species [20]. In the pregnant mare, progesterone fluctuation is a result of the secretory shift from ovulatory CL to newly forming secondary CLs (see [34] and electronic supplementary material, file S1). However, we clearly show that in elephants, acCLs form already during the follicular phase of the oestrous cycle and that in contrast to many other species, antral follicle formation ceases after ovulation. Interestingly, even though LUF (acCLs) become distinct already prior to ovulation in elephants, measurable  $P_m$  concentration only occurs after the second LH peak. It was speculated that the luteal cells of acCLs only start secreting  $P_m$  in pregnant elephants after five to six weeks [24] or after induction by the second LH peak [10,19]. The observation of a rapid  $P_m$  increase after the second LH peak despite the slow ovCL formation [26] may support the latter theory. The LUF rather seems to secrete inhibin in order to enable selection of a single-dominant follicle [27]. We observed that the set of all CLs grew to a significantly larger size after ultrasonic recognition of the embryo and was maintained until after parturition. Therefore, it is likely the gain in size of pregnancy CLs beginning in week 9–11 which explains the sudden increase of  $P_m$  concentrations [33] as well as the fact that pregnancy  $P_m$  levels are on average higher than luteal phase levels [22].

It remains remarkable that final rescue of luteal structures occurs so late in elephants, even after a measureable onset of structural and functional luteolysis (figures 1 and 3). In most domestic species, this signal for luteal rescue occurs between day 10 and 15 post ovulation, prior to any onset of luteolysis [20,35]. It appears that in elephants, the maternal organism gives just enough time for the early embryo to induce luteal rescue. Taking our observations together with previous findings [13,14], we speculate that implantation takes place not earlier than 40–45 days after ovulation.

One wonders why the elephant developed this almost risky 'last minute' strategy of luteal rescue? Maternal investment into offspring is enormous for elephant mothers, with pregnancy lasting 22 months, a lactational anoestrus of up to 2 years and a resulting inter-birth interval of 3–7 years [36]. In respect to these long periods, it is even more relevant for the elephant to ensure that it invests in a healthy conceptus. Thus, only with full establishment of a maternofetal interface (attachment to the endometrium) the pregnancy may be maintained. Different from other species, where a free embryo may signal for maternal recognition, we propose, that only with the attachment of the hatched, healthy and viable blastocyst a signal is transmitted that stops further CL regression and induces additional diameter growth. A recent study supports this idea: elephant placental lactogen (ePL) was suggested to be this luteotrophic agent [37]. It appeared only with the attachment of the embryo to the endometrium. The increased  $P_m$  concentration and CL size, observed in the present study after week seven or eight of gestation, coincides with the ultrasonographic appearance of the EV (figure 1). Prolactin and placental lactogen are known to exert luteotropic actions in several species [38]. Because ePL was located in the trophoblast in African elephants through virtually all stages of pregnancy [37], ultimately, ePL may trigger enhanced luteal  $P_m$  output as well as maintenance of all CLs from implantation onwards through the course of the pregnancy. Prolactin appears in the circulation after four to six months of pregnancy (see [33,39]; figure 3; electronic supplementary material, file S1). Even though PRL may not be responsible for initiating luteal rescue in the elephant, as it appears too late, it may still be an important factor for amplifying and sustaining progesterone output from luteal cells throughout gestation [39]. Speculations on a possible pregnancy gonadotropin-like agent could not yet been proven either in Asian or in African elephants [10,12,21], highlighting again the difference in acCL development between the horse and the elephant (see the electronic supplementary material, file S1).

Similar to previous studies [12,18,26,27], the current investigations showed that there were usually significantly more CLs on the ovary of ovulation with a significantly greater diameter compared with the contralateral ovary. Interestingly, close relatives, the sirenians, seem to also possess additional CL. Dugongs (*Dugong dugon*) and manatees (*Trichechus inunguis*) are uniparous, but show the same pattern of multiple CLs clustering on the ovary of the gravid uterine horn [40,41]. Since sirenians also show a zonary, endotheliochorial placenta very similar to elephants [42], it is possible that the function and the formation of acCLs are the same. Reproductive processes usually appear relatively conserved across a genus and the special reproductive features found in elephants and sirenians may consolidate their close relationship. However, LH concentrations have not yet been measured either in the dugong or in the manatee. Another recognized close relative of the elephant, the hyracoidea, also show a zonary placenta and multiple CL. Hyracoidea may give birth to up to four young [43] and the number of conceptus match the number of CLs [44]. Thus, CLs form from ovulated follicles rather than from LUF. Regarding lifestyle and body size, in contrast to the larger manatee/dugong and the elephant, the hyrax may afford to raise more than one young. All three Orders, sirenians, hyracoidea

and proboscidea, form together the Clade of *Paenungulata* [45]. The *Paenungulata* are assigned to the Superorder of *Afrotheria*, which also include mammals such as tenreks, aardvark and elephant shrews. The former isolation of Africa from Eurasia probably gave rise to these mammals [46]. Although not obvious, from the molecular evidence, it can be anticipated that there are shared morphological characteristics that link together this rather strange assortment of mammals [46]. Some ancient reproductive features, such as intra-abdominal testicles, are still preserved in the recent species and suggest that the connection may be found in the reproduction physiology. Another similarity in *Paenungulata* is the relatively long pregnancy. It lasts about 12 months in sirenians [40] and up to eight months in the small hyracoidea [43]. Yet, the reason for the extraordinary length of the entire pregnancy in elephants needs to be determined. An important factor in the slow development of reproductive functional structures is the large body size of the elephant. Metabolic rates decrease with body mass [47,48], thus parameters such as cell division, rates of DNA substitution and molecular evolution are also reduced in larger animals [49]. Similarly, there is a positive allometric relationship between body size and pregnancy length for most mammals [50]. However, this may not be the main reason, because in much heavier whales, shorter pregnancies than in the elephant may be found [50]. In addition, the Hyracoidea represent a remarkable exception with their comparatively long pregnancy for such a small mammal. Other factors may be more relevant for the pregnancy length. The placenta may be a limiting factor for foetal growth, because the placental hilus, measured in near term elephant pregnancies, is relatively narrow. While the total placental band may be 150.0 cm in length and as wide as 35.0 cm, the hilus, as the only connection between endometrium and conceptus, is only 2.0–4.0 cm wide [4]. Supply of the foetus may be restricted particularly in later gestation.

Elephants are long-lived mammals, living up to 65 years in the wild [51], and slow embryonic development has been shown to be an adaptation to delay ageing [52,53]. Thus, a prolonged intrauterine development in the elephant might be advantageous for its longer lifespan. From all possible factors, central nervous system development may play the most important role. Elephants are classified at the same intelligence level as great apes and dolphins [54] and also show self-recognition [55]. Neonate brain size and pregnancy length in mammals were shown to be strongly related [56]. New born elephants show an advanced level of brain and cognitive capacity, which is important to recognize the complex social structure of the herd from the first day in order to survive. Furthermore, the use of the dextrous trunk, a sophisticated organ with a unique muscle structure and sensory innervation [57], requires advanced development. Very young elephants are capable of grabbing, stopping and pushing objects with their trunks [58], implicating an established cross-linking of motory and sensory neurons at birth. Therefore, a longer intrauterine development may enhance competency and subsequently reduce neonatal mortality. Thus, the elephant is an interesting example of the links between body size and brain size in neonates and adults with respect to gestation period, basal metabolic rate and lifespan [59].

In summary, to ensure pregnancy maintenance over a period of 22 months, elephants have evolved an unusual

reproductive strategy:  $P_m$  secretion appears to be secured and supported throughout the entire gestation by forming acCLs in each oestrous cycle. The multitude of luteal structures, which enlarge only after foetomaternal interaction (implantation), allow the elephant to invest only into a viable embryo and probably to abandon an extra-ovarian progesterone source. With this distinct reproductive pattern, elephants add an interesting, yet effective model for luteal capacity building and pregnancy maintenance compared with other described species. This special reproductive strategy makes the elephant enormously successful, despite investing 4–6 years, a large proportion of the reproductive lifespan, into a single calf.

The Canadian elephant keeping facility is a registered research institution under the Animals for Research Act of the Ontario Ministry of Agriculture, Food and Rural Affairs. All other examinations were conducted in the frame of routine reproductive monitoring and pregnancy health checks as recommended by the European Endangered Species Programme for elephant breeding facilities. All examinations were conducted in accord with accepted and legally mandated Canadian standards of humane animal care.

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