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Ultrasonography of wallaby prenatal development shows that the climb to the pouch begins *in utero*

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Marsupials have a functional placenta for a shorter period of time compared to that of eutherian species, and their altricial young reach the teats without any help from the mother. We have monitored the short intrauterine development of one marsupial, the tammar wallaby, with high-resolution ultrasound from reactivation of the 100-cell diapausing blastocyst to birth. The expanding blastocyst could be visualized when it had reached a diameter of 1.5 mm. From at least halfway through pregnancy, there are strong undulating movements of the endometrium that massage the expanding vesicle against the highly secretory endometrial surface. These unique movements possibly enhance exchange of uterine secretions and gases between the mother and embryo. There was a constant rate of development measured ultrasonographically from mid-gestation, regardless of when the blastocyst reactivated. Interestingly climbing movements by the fetus began *in utero* about 3 days before birth, mimicking those required to climb to the pouch.

P regnancy in marsupials is uniformly brief, ranging in length from 10.7 days in the dasyurid *Sminthopsis macroura*[1] to the potoroo, *Potorous tridactylus*, with a post-oestrus gestation length of 38 days[2] and that of the Eastern grey kangaroo, *Macropus giganteus* 36.4 ± 1.6 days[3]. The best studied marsupial is the tammar wallaby, *Macropus eugenii*, a small member of the kangaroo family of which all but one species have embryonic diapause[4,5]. Female tammar wallabies give birth in late January and come into oestrus within one hour after parturition[6]. The resulting conceptus undergoes cleavage and arrests its development at the 80–100-cell blastocyst stage when it is around 0.25 mm in diameter[7]. It remains in this state of embryonic diapause for 11 months until reactivation at the summer solstice[4]. Loss or removal of the pouch young (RPY) and its sucking stimulus during the breeding season (Jan–May) reactivates the diapausing embryo and its quiescent corpus luteum (CL). This results in a progesterone pulse at day (d) 4, 5, 6 or 7 after RPY[8] which establishes the length of post-diapause pregnancy so that they give birth 25, 26, 27, or 28 days later (mean of 26.5 days)[4,8,9]. Similarly, when pregnancy is artificially initiated by administration of exogenous progesterone, thus by-passing the CL, the duration of pregnancy is 3 to 4 days shorter than pregnancy after RPY[7].

After RPY, the blastocyst remains quiescent for 3 days until the first mitoses are observed at d4 RPY[10] and metabolic activity begins[10,11]. The blastocyst begins expansion at d8 RPY[7] and has expanded three-fold by d10 RPY[11]. The tammar blastocyst is surrounded by three distinct embryonic coats of maternal origin comprised of the zona pellucida, the mucin layer and the outermost shell coat[4,9,12–15]. The blastocyst continues to expand via uptake of uterine secretions through the shell coat[11,16]. When the embryonic vesicle has reached a diameter of ∼1– 1.5 mm, the embryonic disc forms, and by 4 mm it has the beginnings of the primitive streak on the surface of the bilaminar yolk sac[17]. The mesoderm spreads out from the embryonic plate between the trophectoderm and the endoderm from d11 to d15 RPY[17], eventually forming the vascular trilaminar yolk sac. It is only in the last third of intra-uterine development around d18 RPY that the shell coat breaks down under the influence of uterine proteases[18]. After shell coat rupture, direct embryo-maternal contact is established by close apposition of the embryonic trophectoderm to the uterine epithelium forming an active maternal-fetal exchange across the chorio-vitelline placenta[19,20]. Only one uterus becomes gravid in any one pregnancy, and uterine spontaneous contract-ility of the myometrium remains lower in the gravid uterus than in the non-gravid uterus in late-pregnancy[21,22]. There are other localized differences between the two anatomically separate uteri including marked differences in

endometrial wet weight, local hormone concentrations, the development of the sub-epithelial capillaries and in the degree of proliferation of the endometrial glands, as a result of the presence of the conceptus[4,23,24].

All our knowledge of the rate of embryonic development is derived from timed pregnancies and from sampling at known times after RPY, as well as from experimental manipulations of the time of reactivation, but serial measurements of embryonic growth in the same animals *in vivo* have not been possible previously. The aim of this study was therefore to record the development of the tammar embryo from the time of RPY through to birth *in vivo*, using high-resolution ultrasound to provide a comparative visualisation of pregnancy. This study provides the first complete ultrasonographic data from a marsupial species and the first *in vivo* record of marsupial development. Here we describe the rate of development of the reactivated embryo, the specialised movements of the endometrium and the remarkable preparation of the altricial young for the climb to the pouch.

Results

Strong endometrial movement in the first half of pregnancy. In the early pregnant females, strong endometrial motility was evident in the gravid uterus before the first ultrasonographic detection of the vesicle. Endometrial motility was also observed in both uteri of non-pregnant females, but to a lesser degree. When the expanded vesicles were first ultrasonographically detected on d14 before birth (d12–13 RPY) (Fig. 1a, 2a, b), the undulating endometrial movement caused rolling, almost gentle massaging, of the embryonic vesicles within the lumen of the highly proliferated endometrium (Supplementary video S1). The small, echo-dense, oval-shaped embryonic disc became visible by ultrasound on the surface of the yolk sac by d10 before birth (d16 RPY) (Fig. 1b, 2c–e, S1). By d9 before birth (d17 RPY) contractions of the heart were seen as a flickering motion and from the caudal end of the embryo, the allantois protruded as a very small tear-shaped sac (Fig. 1c, 2f, S1). The embryonic head and the typical cervical flexure could be discerned (Fig. 1d, 2f, S1).

Shell coat rupture as an ultrasonographic marker in embryonic staging. The conceptus, which previously was spherical due to the expanded yolk sac, assumed an oval shape with irregular borders after the shell coat ruptured between d6–8 before birth (d19–20 RPY, Supplementary video S2). After break-down of the shell coat, the deep endometrial folds, which had previously surrounded the vesicle, became visible (Fig. 1d–e, S1, S2). The developing yolk sac placenta followed the contour of the endometrium. At this stage, the apposing embryonic (yolk sac placenta) and maternal (endometrium) boundaries could still be identified. Uterine movements became less vigorous after shell coat rupture.

Preparation for birth in the last third of pregnancy. Between d7–5 before birth (d20–22 RPY), the vitelline vessels, consisting of two vitelline veins and one artery, became more pronounced and blood flow was documented by colour Doppler flow (Fig. 2h, Supplementary video S3).The head and rump of the fetus were differentiated and the fore-limb buds were visualized (Fig. 2h). The allantoic sac increased in size and appeared as a fluid filled-oval shaped vesicle completely enclosed within the yolk sac cavity (Fig. 1g, S3). The sinus terminalis, which separates the bilaminar from the trilaminar portion of the yolk sac placenta, became apparent by ultrasound. As pregnancy progressed, the yolk sac placenta could not be discerned separately from the underlying endometrium, indicating that placental attachment had occurred. The first visualization of the spinal cord was recorded by d5 before birth (d22 RPY) and the fore-limb buds had formed into arms (Fig. 1h). By d4 before birth (d23 RPY) the allantois had further increased in size but was never in contact with the chorion (S3). The thin amniotic membrane, which closely enveloped the embryo, was also visible by this stage. Occasionally, random movements of the fore-arms were observed. By d3 before birth (d23 RPY) the mouth was open with the tongue protruding, ready for sucking (Fig. 1i, 2i). The functioning yolk sac placenta was characterized by the strong blood flow of its vitelline vessels (Fig. 1j, 2i). By d2–0 before birth (d25–28 RPY), the fetal head had its typical square muzzle (Fig. 1j, k, S4). The fetal arms were prominent and at the tip of the fingers, the claws were visible (Fig. 1k, l, 41). The fetus undertook repetitive climbing movements by alternate grasping actions of its forearms and hands akin to the climbing movements of the neonate (Supplementary video S4). These movements simulated those needed to climb to the pouch and increased in intensity approaching the time of birth. Similar movements also continue for some months after birth when the young grasps the mammary hairs surrounding the teat to which it becomes attached during sucking.

Constant rate of prenatal growth. The first confirmation of pregnancy by ultrasound was possible on d12–13 RPY by the visualization of a round anechoic vesicle representing the free unattached embryonic vesicle. The size of the embryonic vesicle on d14 RPY varied considerably between individuals ranging from 1.5 mm to 5.9 mm (mean 3.3 ± 0.17 mm, n = 9). On d19 and d20 RPY, the greatest length of the embryos in the same females varied from 1.6 to 8.0 mm (mean 4.9 ± 0.24 mm, n = 5) and 3.5 to 8.6 mm (mean 5.9 ± 2.2, n = 6), respectively. These measurements in later pregnancy stages revealed that smaller embryos did not catch up with the larger ones, but that the embryonic growth velocity was almost identical between individual females. If embryonic growth is plotted against dRPY, the three-day variability in gestation length is obvious, whereas if embryonic growth is plotted against observed gestation length after first visualization of the embryonic vesicle, that variability almost disappears (compare Fig. 3 and Fig. 4). These data support earlier data that the variability in gestation length in the tammar is due to the variability in blastocyst activation correlated with the timing of the progesterone pulse.

Of the eight females continuously observed from the morning of d25 RPY, seven gave birth between d25 RPY and d28 RPY. The eighth animal was alive on d27 RPY but after scanning on d29 RPY the embryo was found to be dead. This embryo died, the fluids were lost, and embryonic resorption began without any clinical signs.

The mean pregnancy length from RPY until the exact time of birth was variable within a range of 25.3 to 28.0 days (n = 7) with a mean of 26.4 ± 1.2 days. The mean crown-rump-length (CRL) and head-length (HL) of the offspring at birth was 14.6 ± 1.2 mm (n = 7) and 7.1 ± 0.6 mm (n = 7), respectively. The mean birth weight was 393 ± 13 mg (n = 5). Four male, two female and one offspring of undetermined sex were born.

There was a significant correlation between the size of the vesicle on d14 RPY and the pregnancy length of the individual females (Spearman rank correlation, n = 7, r = -0.964, p = 0.003). The size of the vesicles on d14 RPY accurately reflected the order of parturitions observed (Table 1). Hence, once reactivation of the blastocyst occurred, the rate of embryonic development and the timing of birth showed almost no variation between animals.

A set of formulae was derived that allows the exact prediction of the day of birth from a single ultrasound measurement. The remaining days until parturition can be calculated based on the first ultrasonographic detection of the embryonic vesicle.

Between d14–d6, and d8–d0 before birth the growth of the diameter of the embryonic vesicle (EV) and the growth of the CRL of the embryo, respectively, plotted against the days before birth can be modeled by linear growth curves and are therefore good predictors of gestational age (GA) during these periods of pregnancy:

Equation (1) d14 to d6 before birth: GA [days before birth] $= 14.47 - 5.13 * EV$ [cm] (Linear regression, $R^2 = 0.96$, F = 267.9, P<0.0001).
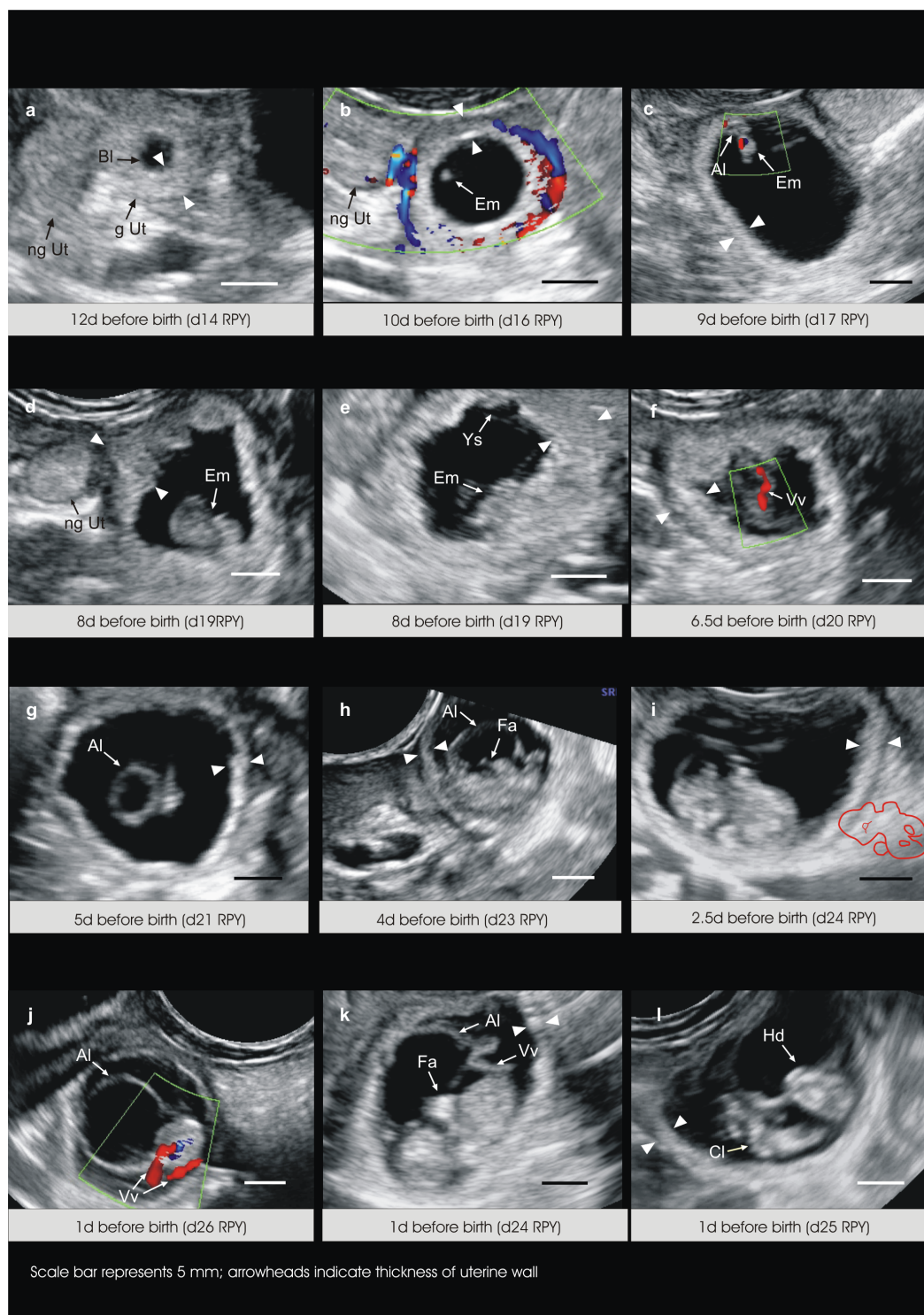
Figure 1 | Ultrasonographic markers for developmental staging. (a) Non gravid uterus (ng Ut) and gravid uterus with blastocyst (Bl). (b) Embryo (Em) on the dorsal rim of the yolk sac. The blood flow of the gravid uterus is displayed by color Doppler flow. (c) The embryo (Em) protrudes into the yolk sac cavity. The yolk sac membrane is closely apposed to shell coat and endometrium and is not visible by ultrasound. The allantois (Al) is displayed as a small tear-shaped sac and the embryonic heart is depicted by color Doppler. (d) Conceptus after shell coat rupture. The gravid uterus is visibly extended by the conceptus compared to the non gravid uterus (ng Ut). The embryo (Em) has markedly increased in size and is differentiated in head and rump.
(e) Conceptus after shell coat rupture: the endometrium displays multiple folds and the yolk sac membrane (Ys) becomes visible on the ventral part of the embryo (Em). The endometrium has gained in thickness compared to (b). The embryo (Em) displays head and rump and assumes a curled posture.
(f) The vitelline vessels (Vv) now deliver nutrients from the yolk sac placenta to the fetus. (g) The allantoic membrane (Al) is visible as a ring like structure within the yolk sac. (h) The conceptus has again increased in size and the fore-arms (Fa) are differentiated. The allantois (Al) has increased in size but does not reach the chorion. The endometrium is stretched out thin. (i) Fetus with advanced ossification. Head and rump can be clearly distinguished. The mouth is opened and the strong fore-arms are evident. (j) The allantois (Al) has considerably enlarged. The vitelline vessels (Vv) are prominent.
(k) The fetus exhibits climbing movements with its strong fore-arms (F). The vitelline vessels (Vv) and the allantois (Al) are visible. (l) The claws (Cl) on the forelimbs are evident and the head (Hd) exhibits a square muzzle. Arrowheads indicate the thickness of the uterine wall.
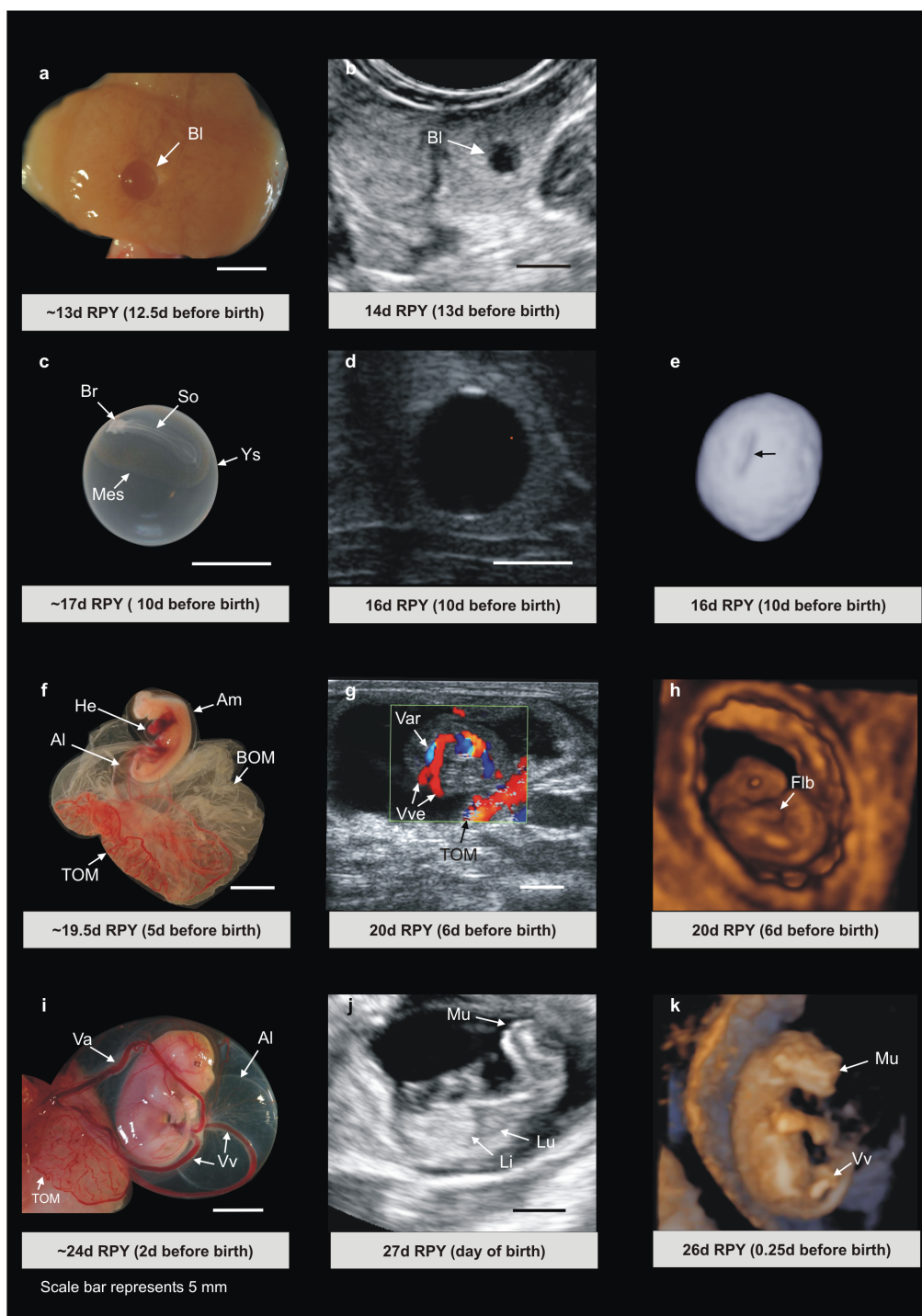
Figure 2 | Developing embryos and fetuses at different stages. Estimated gestational age of conceptuses after removal of pouch young (RPY). Age has been calculated both from days before birth and from formulas I and II in the text respectively. (a) Photograph of an unattached blastocyst (Bl) within the uterus (~d13 RPY, estimated 12.5 d before birth). (b) 2D ultrasound image of an unattached blastocyst (Bl), d14 RPY, estimated 13 d before birth). (c) Photograph of a conceptus (~d17 RPY, estimated 10 d before birth). The embryo has 17 somites (So) and the brain begins to develop. The extra-embryonic mesoderm (Mes) has started to spread over the yolk sac (Ys). (d) 2D ultrasound image of a conceptus (d16 RPY, estimated 10 d before birth). The conceptus is an anechoic vesicle within the endometrium of the uterus. (e) 3D ultrasound image of the conceptus shown in (d). The impression of the embryo into the yolk sac is visible (arrow). (f) Photograph of a fetus (~d19.5 RPY, estimated 5 d before birth) with the tri- (TOM) and bilaminar (BOM) portion of the yolk sac. The heart (He) protrudes from the ventral body wall with amnion (Am) and allantois (Al) visible. (g) 2D ultrasound image of a conceptus after attachment (d19.5 RPY, estimated 6 d before birth). The vitelline veins (Vve), the vitelline artery (Var) and vascularization of the yolk sac placenta (TOM) appear by colour Doppler. The flexure of the embryo in vivo is stronger than seen after removal of the uterus (f). (h) 3D ultrasound image of a fetus. The fore-limb buds (Flb) are evident. (i) Photograph of a fetus (~d24 RPY, estimated 2 d before birth) with the vitelline veins (Vve) and vitelline artery (Var) supplying the trilaminar yolk sac portion (TOM). (k) 2D ultrasound image of a fetus (d26 RPY). The border between lung (Lu) and liver (Li) is evident and the jaws are fully ossified, forming a square muzzle (Mu). (l) 3D ultrasound reconstruction of a fetus (d26 RPY, estimated 0.25 d before birth). Note the well-developed arms and muzzle (Mu). The vitelline vessels (Vv) protrude from the caudal part of the fetus.
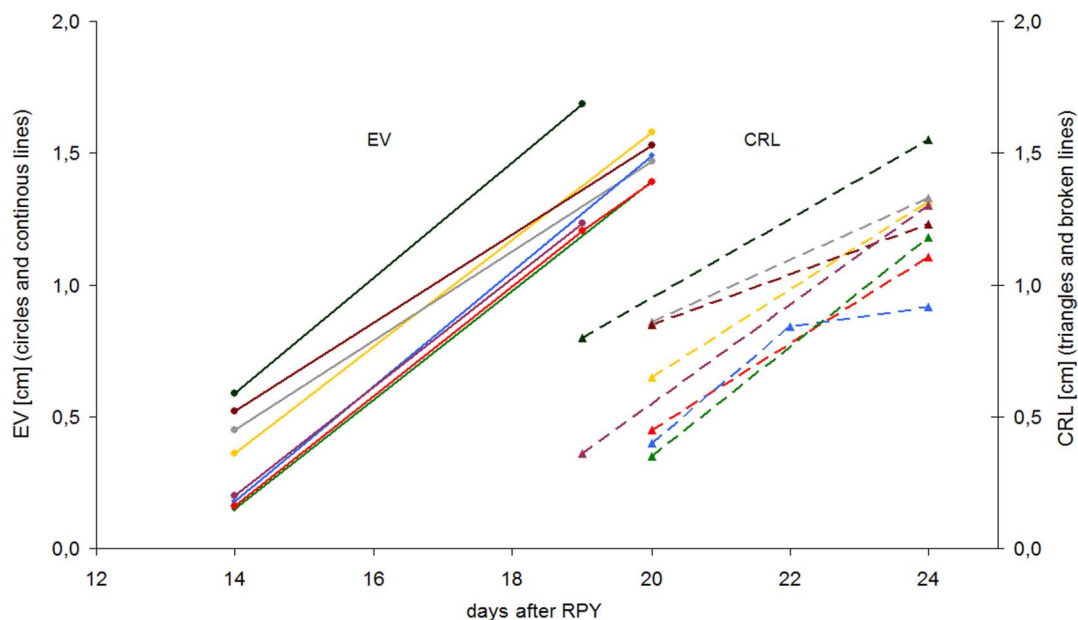
Figure 3 | Prenatal morphologic measurements relative to the days after removal of the pouch young (RPY). Measurements of the diameter of the embryonic vesicle (EV, circles) and the crown-rump-length of the embryo (CRL, triangles) at certain pregnancy days from the females where birth date was exactly known. Different individuals are represented in different colours. The lines between the measurements illustrate the "parallel" and constant velocity of prenatal growth and development (not identical with growth curves as shown in Fig. 4; RPY: removal of pouch young).

Equation (2) d8 to 0 before birth: GA [days before birth] $= 10.58 - 6.15 * CRL$ [cm] (Linear regression, $R^2 = 0.92$, $F = 157.9$, $P < 0.0001$).

If the embryo itself is already visible, then the length of the embryo, or rather CRL, can be used to calculate the gestational age (days before birth). If the embryo is not yet detectable, the diameter of the EV is a good predictor of gestational age (days before birth).

After plotting all of the data for EV size in a diagram it is obvious that a linear growth curve is only a rough estimate for a short period of gestation. In later stages the embryonic cavity does not grow anymore, although the embryo itself still increases in size (Fig. 4).

To model the growth of the mean diameter of the embryonic cavity over the whole gestation period a sigmoidal growth curve was modeled:

Equation (3) EV [cm] $= 0.025 + 1.60/(1 + ((\text{age [days before birth]} + 15)/10.30)^{\wedge}7.49)$ (Non-linear regression, $R^2 = 0.97$).
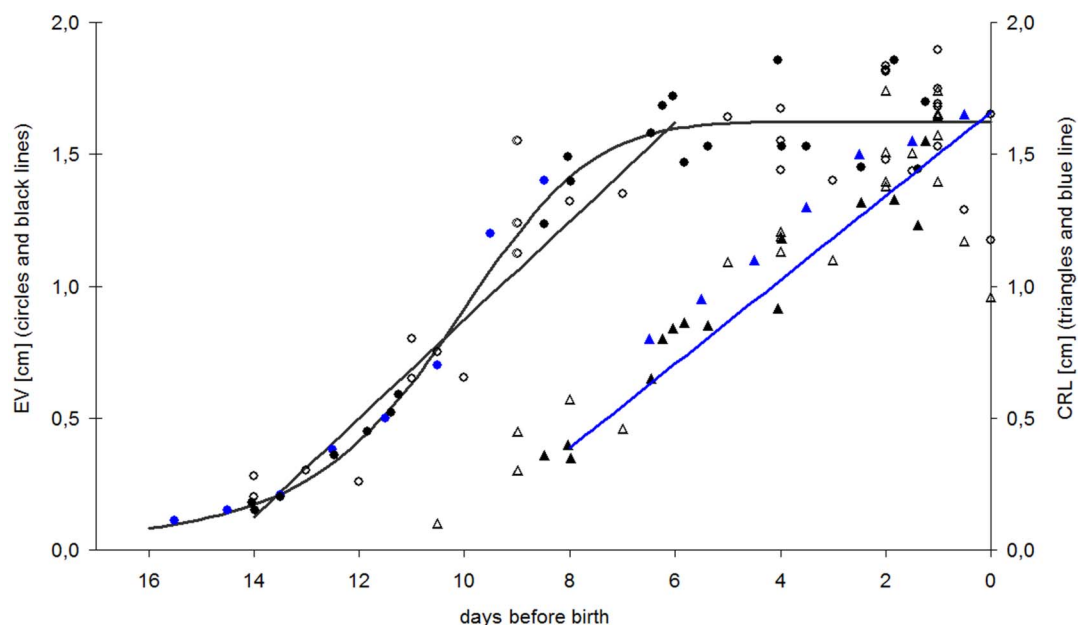

Figure 4 | Embryonic growth relative to the days before birth. Prenatal measurements of the diameter of the embryonic vesicle (EV, circles) and crown-rump-length (CRL, triangles) from all 20 females examined (black closed marks: exact birth time known including the measurements displayed in Fig. 3, black open marks: animals where birth date was only roughly known by date) and from post mortem examination of pregnant wallabies (blue marks[4]) combined. Linear (straight black line) and sigmoidal (curved black line) growth model for EV and linear growth model for CRL (blue line) according to the equations (I), (III) and (II) in the text, respectively.

Table 1 | Pregnancy data measured by ultrasound and initiated by removal of the incumbent pouch young (RPY)

Animal #	EV [cm] at d14 RPY	Predicted birth order	Observed birth order	Birth date and time	Observed gestation length [dRPY*]
1	0.15	8	6	17.3.11 9:30 h	28.0
2	0.16	7		18.3.11 0:00 h	died *in utero*
3	0.18	6	7	17.3.11 11:00 h	28.0
4	0.20	5	5	16.3.11 22:00 h	27.5
5	0.36	4	4	15.3.11 21:00 h	26.5
6	0.45	3	3	15.3.11 6:00 h	25.38
7	0.52	2	2	14.3.11 19:07 h	25.4
8	0.59	1	1	14.3.11 16:02 h	25.2
Mean	0.33				26.4
SD	0.18				1.20

*all pouch young were removed on the same day (17.2.11, 10:00 h).
EV: diameter of embryonic vesicle; CRL: crown-rump length; HL: head length; SD: standard deviation.

Factors influencing activation of blastocyst. There was no significant correlation between the body weight (used as a measure of body condition) of the females and the diameter of the blastocyst on d14 RPY (Spearman rank correlation, n = 8, r = 0.417, p = 0.299). However, the age of the incumbent pouch young at the time of RPY may have had an influence on the time of activation of the blastocyst and the subsequent development of the next embryo. The calculated age of the pouch young at the time of RPY was within the range of 9.5 to 22 days (Table 2) as calculated from established growth curves [4]. There was a significant positive correlation between the size of the embryonic vesicle on d14 RPY and the age of the pouch young at the time of RPY (Spearman rank correlation, n = 8, r = 0.766, p = 0.037). Thus, mothers that carried larger pouch young had blastocysts that had already entered diapause at the time of RPY. The embryonic vesicle at d14 RPY of these mothers was larger than vesicles resulting from removal of young that were only d8–9 post partum and whose blastocysts had not yet entered diapause.

Discussion

All marsupials have short pregnancies relative to most eutherians, and give birth to highly altricial young that must complete the journey to the teat unaided by the mother. Our data, which, to our knowledge, represent the first series of ultrasonographic images during pregnancy in the Marsupialia, demonstrate that growth and development unfolds in the tammar wallaby according to a strict program that starts when the diapausing blastocyst receives the signal to reactivate, and ends with the tiny fetus training for its climb to the pouch.

Table 2 | Size and sex of pouch young at removal

Animal #	HL [mm]	Sex	Estimated age [days]
1	10.9	F	9.5
2	11.2	F	10.0
3	13.1	M	15.5
4	10.8	F	10.0
5	14.1	M	18.0
6	11.9	M	12.5
7	15.6	F	22.0
8	13.8	M	17.0

The data confirm earlier observations that the 3 day variation in gestation length observed after RPY in the tammar wallaby is linked to the timing of reactivation of the corpus luteum of pregnancy, its subsequent progesterone production, onset of uterine secretory activity and embryo reactivation[4,7–9]. After the progesterone pulse synchronises reactivation, the rate of development remains remarkably constant. The rate of development proceeded at a predetermined velocity that was the same for every embryo, confirming that the variation of the embryonic development at a certain day RPY is due to the different time of reactivation of the blastocyst in each individual female. Neonatal weights were comparable to those previously recorded[9] so the handling during ultrasonography had no effect on fetal development.

The correlation of the age of the pouch young with size of the embryonic vesicle at d14 RPY was not entirely unexpected. Because the actual day of birth of the young removed was not known, previously established growth curves for postnatal development were used[4]. While the growth curves provide a reasonable estimate of age, there is considerable (up to ± 2 days) inaccuracy of young less than 20 days, so the estimated 9 day young may well have been only 7 days old. Since the blastocyst does not enter into diapause until d8–9 of post-natal life of its sibling in the pouch[4], it is entirely possible that the blastocysts of these younger animals had not yet entered diapause, and so were at an earlier stage of development. The duration of pregnancy from oestrus to birth is 2–3 days longer than the time from RPY to birth, and this is due to the time taken to reach the blastocyst stage and enter diapause[4,25].

There was very little variation in the rate of development between individuals once the embryonic vesicle could be observed by ultrasound. In fact, development was so precise we were able to predict the order of birth (Fig. 3, Tab. 1). The precise developmental program observed in the tammar wallaby contrasts strongly with precocial eutherian species in which pregnancy length and fetal size at birth vary considerably[26]. However, even a one to two day variation in the time of reactivation to birth in a 26-day pregnancy is a significant variation in this marsupial. For many eutherian species, fetal size at birth is correlated with maternal size. However, the same relationship only exists in marsupials during lactation, when the young reach an equivalent developmental stage[27].

Before attachment, the embryo is still covered by its embryonic coats with the shell coat being the outermost layer, so that the contents of the uterine secretions must be absorbed through the shell

coat[16]. The shell is of maternal origin and is laid down at the utero-tubal junction and in the uterus[13–15,28]. It typically consists of three layers when viewed under the electron microscope[9]. During the unattached phase, there were very strong endometrial waves that rolled the vesicle around within the uterine lumen. However, there is no evidence for endometrial musculature. It is possible that these movements are due to the pulsation of blood moving through the highly vascularised endometrium. This latter suggestion is supported by the Doppler images in the video files (Figures 1, 2 and S1). In the tammar, these movements may have several functions. First, the rolling of the embryo might be essential for the circular deposition of additional shell coat material as the vesicle expands. Second, rolling of the growing embryo may enhance the transfer of nutrients from the uterine secretions across the yolk sac to the developing embryo. Third, it may increase gaseous exchange to the rapidly dividing cells.

The horse embryo also migrates around the uterus as a result of uterine movements for an extended period during early pregnancy (d6–15 post ovulation) and is even shuttled from one uterine horn to the other before the chorionic girdle forms[29]. Interestingly, just as in the tammar, the horse embryo is surrounded by a distinct embryonic coat known as the capsule. The capsule replaces the zona pellucida and envelops the conceptus between the second and third weeks of pregnancy and thickens as the embryo develops[30]. It inhibits early attachment so the horse conceptus lies free in the uterine cavity for around 15 days, similar to the free phase of the tammar conceptus[31]. In the horse, the embryo induces local myometrial activity by secretion of $PGF2\alpha$ and $PGE2\alpha$[32] and contractility diminishes after the embryo leaves a certain area of the uterine lumen[33]. However, this is not the case in the tammar in which the endometrial, not myometrial, movements were also observed in non-pregnant females and in the non-gravid uteri of pregnant females. The inherent undulating movement of the endometrium in the tammar is unusual given that progesterone has an inhibitory influence on myometrial contractility[34–36].

Attachment of the placenta of the embryo in the tammar as seen by ultrasound only occurs after the vesicle has expanded and shell coat rupture has occurred. At this size, it stretches the uterine wall to an extent that the endometrium may be unable to undulate effectively, although uterine movement did continue to a limited extent after this time. After attachment and shell coat rupture on d18–19 RPY there is rapid embryonic and fetal growth and organogenesis[37]. With shell coat rupture, the spherical conceptus is free to assume an irregular shape, and the yolk sac membrane that had been closely apposed to the shell coat becomes visible by ultrasound to form a close interdigitation with the uterine endometrium[38].

Perhaps the most surprising finding was the preparative climbing movements observed by ultrasound in the late-stage tammar fetus from three days before birth. This is clearly an essential behavior required by all marsupials for post-natal survival. While similarities can be drawn to well characterized eutherian uterine movements such as sucking (humans, apes) or swimming (dolphins, T.B. Hildebrandt, unpublished data), or the thrusting of limbs as seen in the horse, the climbing movements of the tammar are unique in that they are so pronounced and occur at such a highly altricial stage of development. These movements occur solely in the fore-limbs, since the hind–limbs are barely formed at birth, and result from a heterochronic asynchrony of fore- and hind-limb development[39]. The neuromuscular connections must develop early to allow this movement.

In conclusion, this ultrasonographic study has highlighted several unique aspects of marsupial pregnancy including the constant velocity of post-reactivation embryonic development in the tammar, the active mechanical massaging of the embryo by the endometrium before a close placental attachment is achieved, and finally the preparative climbing movements of the wallaby fetus that begin less than three days before birth. These findings emphasize the remarkable adaptations of the altricial wallaby young to survive the journey from the uterus to the pouch and successfully attach to a teat from which it will gain all its nourishment for the next nine months.

Methods

Ethics statement. All experimentation was approved by the University of Melbourne Institutional Animal Ethics Committees and conformed to the National Health and Medical Research Council of Australia (2004) guidelines.

Animals. Tammar wallabies (*Macropus eugenii*) of Kangaroo Island origin were maintained in open grassy outdoor enclosures in a breeding colony in Melbourne, Victoria. Their diet was supplemented with lucerne cubes, oats and vegetables. Drinking water was provided ad libitum.

To systematically study prenatal development in the tammar wallaby *in vivo* by ultrasound, pouch young were removed from 20 females within one hour on the same day to initiate the development of the diapausing blastocyst. Ultrasonographic markers for developmental staging were defined and compared with previously reported findings in embryos and fetuses of corresponding age[4,7,9,17,19,39–43]. Pregnancy length was determined from the day after RPY to the observed birth. The time between embryonic vesicle expansion, as seen by ultrasound, was recorded in relation to the time of observed births.

Reactivation of the diapausing blastocyst (and subsequent active pregnancy). After capture female wallabies were checked for pouch young. Pouch young were then sexed and head length taken with vernier callipers to determine their age from published growth tables[4]. Pouch young were removed (day 0) during the breeding season in February 2011 from their mother's teat by gentle traction. All females were adults in lactational quiescence and presumed to have blastocysts in diapause. The mean weight of the adult female tammars was 5.0 ± 0.1 kg (n = 20). The tammar young that were removed from the teat were killed with pentobarbitone sodium (60 mg/ml to effect) and used in other ethically approved experiments. The estimated ages of the removed pouch young (RPY) was from 9 to 22 days (Table 1).

Ultrasound examination. After RPY, 19 of the 20 females included in the study were diagnosed to be pregnant by ultrasound as those reactivated pregnancies proceeded. Ultrasound was performed using a mobile laptop-sized ultrasound unit (Voluson "i", GE, Austria). Several reasons hinder the procedure of ultrasound in wallabies making it more difficult than in other mammalian species. The reproductive tract of the female tammar wallaby is located mostly in the pelvic cavity. Furthermore, there are two long bones - the epipubic bones - extending from the pelvis cranially supporting the pouch and thigh muscles. Shaving of the abdominal region, as usually performed in other species, is not appropriate as the newborn offspring needs the fur coat to crawl into the pouch. For all of these reasons ultrasound was performed with a small slender transducer placed on the hairless pouch skin through the opening of the pouch (about 2×2 cm). We used a long convex array (5–9 MHz) with minimal contact surface originally developed for vaginal scans in women.

Ultrasound examination was performed by physical examination without sedation when possible. However, light sedation induced by intramuscular injection of Zoletil (12.5 mg Tiletamine and 12.5 mg Zolazepam) was used in 48/78 examinations to minimise movements that would interfere with the ultrasound. Sedation, and even full anaesthesia, does not interfere with pregnancy[40,41]. All animals were lightly sedated at least once over the course of pregnancy. Animals were still responsive but moved infrequently during light sedation. For ultrasound without sedation the animals were kept in a hessian sack and held in a restraint box. This is standard procedure for examination of tammar wallabies and has no effects on pregnancy[4,41]. For the ultrasound procedure a small amount of ultrasound gel was used to increase coupling of the probe to the surface area. The scan involved the evaluation of the entire reproductive tract including the three vaginae, the two separate uteri and the two ovaries (Supplementary Figure S1). A total of 116 ultrasound examinations were done. Examination was performed on a daily basis from d4 RPY until parturition in two randomly chosen animals. On d14, 20 and 24 RPY, 20 females were examined one at a time.

Birth watch. To determine the time of birth precisely, eight of the females were constantly observed over 24-hours starting from the morning of d25 RPY. All females were identified by tape labeled with the respective ear tag numbers on the tail. Animals were placed in a specially constructed "birth watch cage" with acrylic side and front walls. The birth watch cages were elevated to eye level to facilitate observations. Furthermore, one male was also kept in the birth watch cage with each group to indicate the onset of parturition by showing increased interest in the respective female. Starting at midnight at d24 RPY, the animals were observed continuously for signs of birth. Imminent birth was recognized by typical behavior such as adopting the birth posture and licking of the fur as previously described[42]. Immediately after birth, but before attachment to the teat, the neonatal young were removed and weighed and their head lengths and crown-rump-length measured with a vernier calliper, then replaced close to the teats where they attached themselves within 2 min. Continuous observations ceased at midnight at d27 RPY when all females, except for one had given birth. The one pregnant female that did not give birth was checked by ultrasound and the fetus was found dead within the uterus at d29 RPY.

Post-mortem samples. Comparison photos of post-mortem embryos and fetuses at equivalent RPY stages were from wild shot animals on Kangaroo Island, South Australia, taken under permits from the South Australian Department of Sustainability and Environment. The length of pregnancy from RPY to birth averages 26.5 ± 0.4 days[42]. Therefore, we have given the average RPY stage for these samples based on head length measurements, weight, developmental stage and comparison with previously published growth curves[4].

Statistical analysis. Statistical calculations were performed using SPSS ver. 18.0 statistical software (SPSS). Results are quoted as mean $\pm$ s.d, and probabilities are for two-tailed tests. Due to low sample sizes correlations were calculated using the nonparametric Spearman-rank-correlation. To establish a relationship between gestational age and the morphological parameters EV and CRL, linear regression was performed. For the sigmoidal EV model, non-linear regression was performed with estimated starting parameters.

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Author contributions

The author(s) have made the following declarations about their contributions: Conceived the study and designed the experiments: M.B.R., T.B.H., C.A.H., G.S. and B.D. Conducted the ultrasonography: B.D., T.B.H. and K.R. Performed the experiments: all authors. Analysed the data: B.D. K.R. M.B.R. and B.R.M. Wrote the paper: B.D., M.B.R., B.R.M. and K.R. All authors edited and approved the manuscript.

Additional information

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