



Unique reproductive strategy in the swamp wallaby

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Reproduction in mammals requires distinct cycles of ovulation, fertilization, pregnancy, and lactation often interspersed with periods of anoestrus when breeding does not occur. Macropodids, the largest extant species of marsupials, the kangaroos and wallabies, have a very different reproductive strategy to most eutherian mammals whereby young are born at a highly altricial stage of development with the majority of development occurring over a lengthy lactation period. Furthermore, the timings of ovulation and birth in some species occurs within a very short interval of each other (sometimes hours). Female swamp wallabies have an oestrous cycle shorter than their pregnancy length and were, therefore, speculated to mate and form a new embryo before birth thereby supporting two conceptuses at different stages of pregnancy. To confirm this, we used high-resolution ultrasound to monitor reproduction in swamp wallabies during pregnancy. Here, we show that females ovulate, mate, and form a new embryo prepartum while still carrying a full-term fetus in the contralateral uterus. This embryo enters embryonic diapause until the newborn leaves the pouch 9 mo later. Thus, combined with embryonic diapause, females are continuously pregnant and lactating at the same time throughout their reproductive life, a unique reproductive strategy that completely blurs the normal staged system of reproduction in mammals.

mammal | reproduction | pregnancy | ultrasound

Aristotle was one of the first to identify discordancy in mammalian reproduction, “*The hare is an example of this. This is an animal in which superfetation occurs, for...often some of its offspring are imperfect; others of its offspring, however, it brings to birth in a perfected state.*” (1). Other than the European brown hare as described here by Aristotle, discordancy in pregnancy is rare in mammals. Reproduction in mammals usually occurs with orderly cycles of ovulation, fertilization, pregnancy, and lactation. Some mammals have evolved mechanisms to synchronize and optimize reproduction by delaying development of the embryo until environmental conditions are more favorable (e.g., embryonic diapause) or by producing new embryos prior to the birth of incumbent fetuses to optimize restricted breeding seasons (e.g., superfetation) (2–6). Female European brown hares may come into oestrus during late gestation (~3 to 4 d before birth) thereby shortening the subsequent pregnancy by up to 5 d and increasing reproductive output by 34.5% per breeding season (4).

The Macropodidae encompass 30% of all marsupial species in Australasia (7). Marsupials have two anatomically completely separate uteri that are supplied by separate ovaries, oviducts, and cervixes (7). Macropodids are polyoestrus and monovular, and a single conceptus usually develops in one of the two uteri. Conception alternates between the two uteri so that the nongravid uterus on the contralateral side becomes gravid over the next and successive oestrous and pregnancy cycles (7). All female macropodid marsupials except the swamp wallaby have an oestrous cycle longer than their pregnancy length, so females come into oestrus, ovulate, and mate within hours after birth (7). The resulting conceptus then enters a lengthy period of embryonic diapause at the blastocyst stage due to lactational or seasonal inhibition of the corpus luteum of pregnancy. This occurs in all but one of the 13 species in the genus *Macropus* (3, 8). Once the established pouch young exits the pouch, the reduced sucking

episodes fail to inhibit reactivation of the dormant blastocyst with birth about a month later. In continuously breeding macropodids, the second young attaches to a teat in the pouch adjacent to the teat and mammary gland supporting the young at foot and drinks milk of an entirely different volume and composition (9–11). This phenomenon is known as concurrent asynchronous lactation and has been described in numerous macropodids, including the swamp wallaby (7). These reproductive strategies, consisting of embryonic diapause and concurrent asynchronous lactation allow most macropodids to support young at three different stages of development at the same time, namely, an early embryo in diapause in utero, a small young attached to one of the four teats and a third young at foot supported from a larger adjacent mammary gland in the pouch (7), all controlled by the sucking stimuli of the young (12). In the tammar wallaby (*Macropus eugenii*), a seasonal breeder, loss, or experimental removal of the pouch young (RPY) and resulting sucking stimulus during the breeding season reactivates the diapausing embryo and its quiescent corpus luteum (CL). Embryo development and subsequent pregnancy length occur over such a strictly regulated time frame that the time of birth can be predicted based on the size of embryos as detected by the timing of the progesterone pulse associated with reactivation (13) and by ultrasound even as early as day 15 RPY (14). This provides a good indicator of embryonic and fetal stages in the absence of hormone analyses.

The swamp wallaby, *Wallabia bicolor*, is a medium to large sized solitary macropodid (males: 17 kg, females: 13 kg). Females reach sexual maturity at 15–18 mo of age and can live up to 16 y in captivity (15). It has a reproductive strategy similar to the majority of the macropodids in that there is a peripartum oestrus with a period of lactationally controlled embryonic diapause. However, in the swamp wallaby, oestrus occurs some days before birth instead of the postpartum oestrus typical of the Macropodids (see Fig. 1; 2). Sharman, Calaby, and Poole (2) “tentatively assumed” that the swamp wallaby had a prepartum oestrus based mainly on the observation of matings of three females before birth. However, there are no details about the timing of ovulation and conception

Significance

We have shown that female swamp wallabies ovulate, mate, and form a new embryo prepartum thereby continuously supporting conceptuses and young at different development stages before and after birth. This system is unique compared to the normal staged system of reproduction in mammals so that swamp wallabies are normally pregnant and lactating throughout their reproductive life.

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at the prepartum oestrus and no data to confirm this. Thus, the swamp wallaby appears to conceive during active pregnancy similar to the superfetation that has so far only been described for the European brown hare *Lepus europeus* in which females copulate again, 3 to 4 d before birth of the incumbent young, forming new conceptuses during an active pregnancy (3, 4). However, in contrast to *L. europeus* that has distinct breeding seasons, pregnancy and lactation in the swamp wallaby occurs concurrently and continuously throughout their reproductive life.

To determine if swamp wallabies ovulate prepartum during a prepartum oestrus and, therefore, have a secondary conception (superfetation) during an active pregnancy, we removed PY from 10 mature females to initiate reactivation of their dormant blastocysts. Previous attempts to collect blood plasma from swamp wallabies over the course of reactivated pregnancy resulted in very low birth rates of about 10% because they are highly sensitive and susceptible to the stress of handling. Thus, six animals were not scanned but were monitored for the presence of sperm in vaginal smears once per day between days 26–35 after RPY as an indicator of mating and by checking their pouches for neonatal young. The remaining four animals were monitored over the course of pregnancy using a high-resolution ultrasound technique (20 scans in total) under light anesthesia as described previously for the tammar wallaby (14). Two of these were also monitored over a subsequent pregnancy cycle.

Results and Discussion

Ninety percent (9/10) of females were carrying dormant blastocysts (in embryonic diapause) and gave birth at 30.17 ± 0.17 d after RPY. Animals in the ultrasound group were first scanned at day 15 RPY at which stage the developing embryonic vesicle was 3.6 mm in diameter (Fig. 2A and Table 1). At this time, the gravid-side ovary had a large CL (Fig. 2F; CL; diameter: 5.7 mm) while the contralateral ovary contained, at least, six small follicles (Fig. 2K; diameter: 0.5–1.0 mm). By day 19, RPY the embryonic vesicle was 10.21 mm and occupied the whole uterine lumen (Fig. 2B). At this stage, power Doppler showed a highly vascularized endometrium surrounding the vesicle (Fig. 2B) and CL of pregnancy (Fig. 2G). Follicles in the contralateral ovary at this time measured 1–1.5 mm diameter (Fig. 2L). At day 26 RPY, the conceptus had developed into a fetus with the head, limbs, and heartbeat clearly visible ultrasonographically, while the contralateral ovary contained four large follicles (2.0–3.0 mm; Fig. 2D and N). One to two days before birth (day 28 to 29), the largest follicle on the contralateral ovary had ovulated and a new highly vascularized CL was evident (CL diameter: 6.24 mm; Fig. 2O). These prepartum ovulations and new CL, which have only ever been observed after birth in all other macropodid marsupials, were observed at the same developmental stage in three animals in the ultrasound group (day 28 to 29 RPY; *SI Appendix*, Fig. S1). While we did not observe the day of ovulation ultrasonographically in the fourth animal, it did have a large preovulatory follicle on day 26 RPY (*SI Appendix*, Fig. S1). Furthermore, in the six females that were vaginally smeared from days 26–35 postpartum but not scanned by ultrasound, sperm were identified in the urogenital tract 1 to 2 d before birth but at no other time (Fig. 3L). Thus, swamp wallabies ovulate and mate 1 to 2 d before birth during pregnancy.

Two animals scanned the day before birth (day 30) with normal full-term fetuses (Fig. 3A) did not have fetuses in their reproductive tracts or neonates in the pouch when examined on day 31 (the last possible day of pregnancy; Fig. 3B). A third animal not scanned over the peripartum period had a new pouch young when examined on day 32 RPY. We conclude that either the cleaning of the pouch and the application of ultrasound gel disturbed the natural pouch secretions required for the neonate to locate the pouch and teats (16) or they aborted due to the stress of handling and light anesthesia. However, given these fetuses were alive and moving on day 30 (the average reactivated pregnancy length in all other animals),

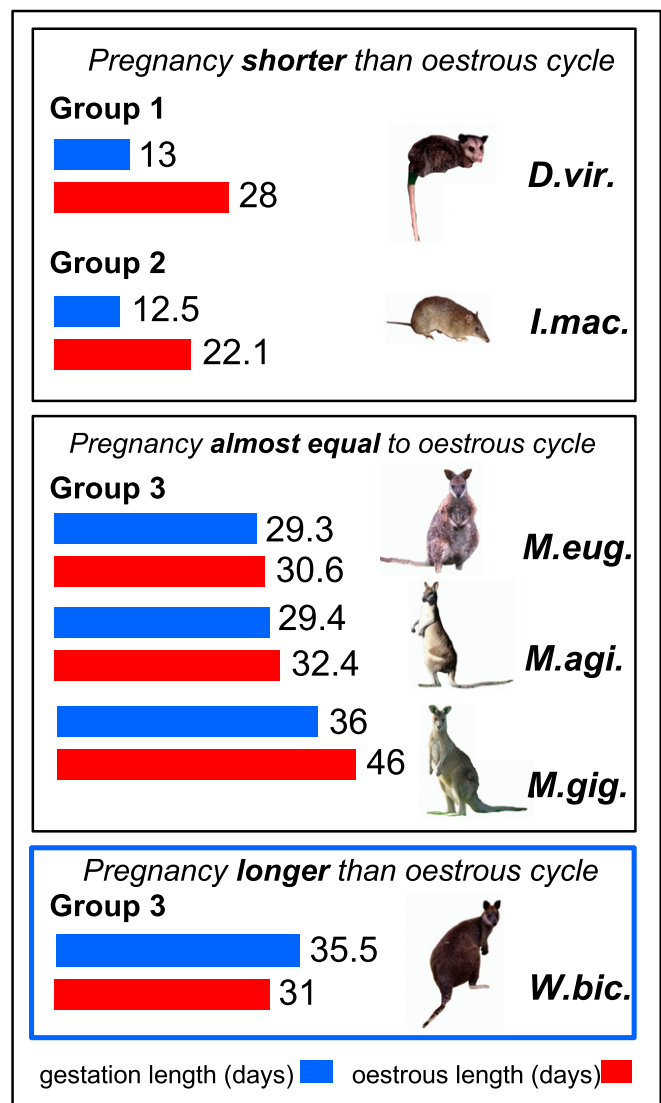


Fig. 1. Comparison of oestrous cycle and pregnancy lengths in marsupials. All marsupials other than the swamp wallaby have an oestrous cycle longer than the pregnancy length. Macropodid marsupials have some of the longest pregnancy lengths relative to the oestrous cycle of all marsupials due to their propensity for birthing larger young.

they had actually completed 97–100% of gestation. The absence of neonates allowed us to monitor the development of any prepartum conceived embryos in these two females which we had observed had ovulated 1 to 2 d before birth. This would not have been possible had the neonates survived because the sucking stimulus would have inhibited further development of the prepartum conceived embryos past the blastocyst stage (when they are undetectable ultrasonographically). In both of these animals, we detected new embryos (0.66 and 0.86 mm in diameter, respectively) in the contralateral uteri at day 41 RPY (that is, 10 d after the loss of the full term fetuses) as evidenced by their distinctive refractive conceptus shell coat and endometrial movement (Fig. 3J). Based on well-established growth charts from thousands of measurements of the developing tammar wallaby embryo (7, 17, 18), which have a very similar pregnancy duration, we estimated the age of these embryos to be day 11 to 12 postcoitum (day 10 tammar embryo: 0.5 mm, day 12 tammar embryo: 1.0 mm). These embryos would, therefore, have been conceived around the time that we observed the ovulation from the contralateral ovary and detected sperm in

Table 1. Developmental milestones in the gravid and nongravid sides of the reproductive tract of the swamp wallaby (*W. bicolor*)

Days after RPY	Gravid-side ovary	Gravid-side uterus	Non-gravid-side ovary	Non-gravid-side uterus	Number of scans and animals
15	Active CL	Vesicle	Six small foll	Quiescent	1 ^a
19	Active CL	Vesicle	Large dominant foll	Quiescent	1 ^b
23	Active CL	Fetus	Large dominant foll	Quiescent	2 ^{b,c}
25 to 26	Active CL	Fetus	Graafian foll/Ov	Secretory	2 ^{a,b}
28 to 29	Active CL	Full-term fetus	New CL	Embryo	3 ^{a,b,c}
30	Luteolytic CL	Birth/loss	Active CL	Conceptus	2 ^{c,d}
31	CA	Quiescent	Active CL	Conceptus	2 ^{c,d}
32	CA	Quiescent	Active CL	Conceptus	2 ^{c,d}
35	CA	Quiescent	Active CL	Conceptus	1 ^c
37	CA	Quiescent	Active CL	Conceptus	1 ^d
38	CA	Quiescent	Active CL	Conceptus	1 ^c
41	CA	Quiescent	Active CL	d11 to 12 embryo	2 ^{c,d}

Superscript letters denote different animals.

RPY: removal of pouch young; CL: corpus luteum; CA: corpus albicans; foll: follicle; Ov: ovulation; d: day.

their reproductive tracts (day 28 to 29 RPY). Furthermore, the new CL of pregnancy supporting the newly gravid uterus showed strong Doppler coloration, something that would not be present in a quiescent or regressing CL (Fig. 3K). Thus, these embryos were conceived before birth of the fetuses from the contralateral uterus 2 d later. To confirm that these ultrasound images showed bona fide

conceptuses, we checked for new pouch young 2 wk after their expected birth date, and both females had pouch young of ~14 to 15 d postpartum as determined by PY head length charts (15). These pouch young could only be the result of the immediate development of the day 11 to 12 conceptuses observed by ultrasound in their contralateral uteri.

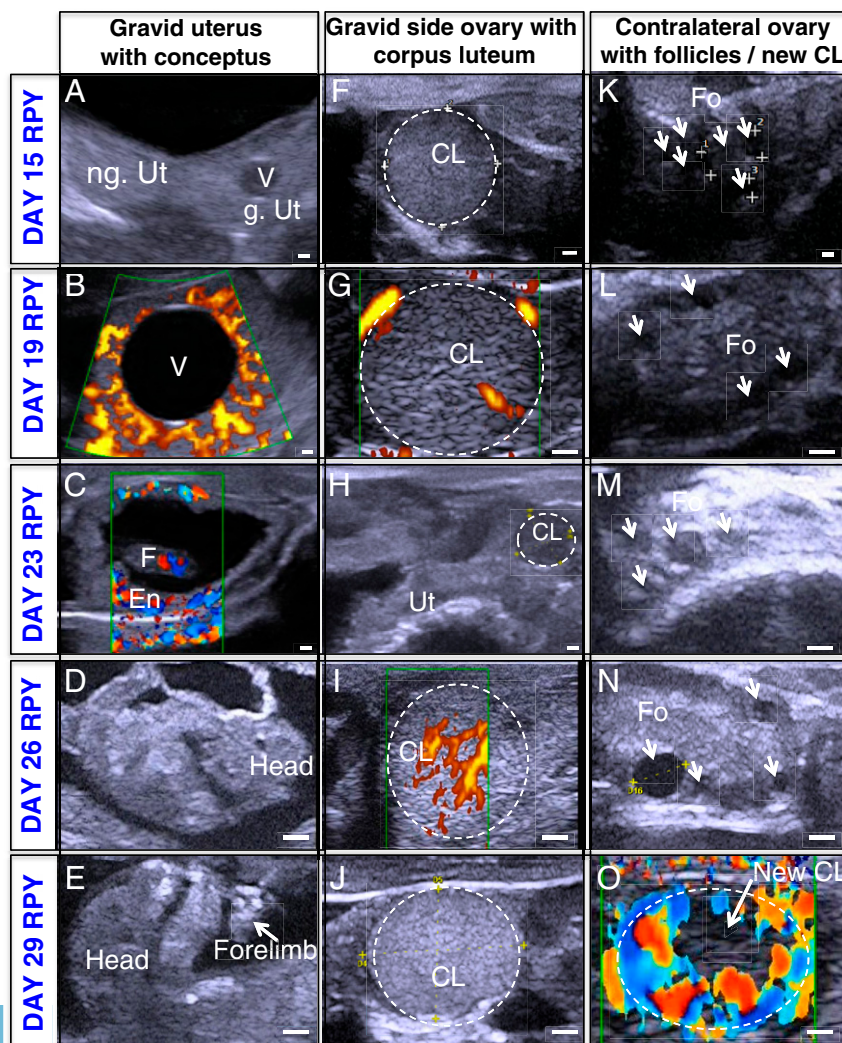


Fig. 2. High-resolution ultrasound of key developmental events supporting concurrent conception and pregnancy in the swampy wallaby. *A*, *F*, and *K* show the embryo at day 15 RPY, the supporting CL of pregnancy, and contralateral ovary with follicles, respectively. By day 19 RPY the embryo fills the whole uterine lumen (*B*) and power doppler analysis shows the highly vascularized endometrium around the embryo. The gravid side ovary (*G*) is unchanged, while the follicles in the contralateral ovary are slightly larger (*L*). At day 23 RPY the fetus, placenta, and heart beat can be visualized by ultrasound (*C*); the gravid side ovary is largely unchanged (*H*), while a dominant follicle in the contralateral ovary has started to emerge (*M*). By day 26 RPY the head, eyes, and limbs are discernible (*D*); the gravid side ovary is very active (*I*), while a dominant follicle has developed in the contralateral ovary (*N*). At day 29 RPY, shortly before birth, the fully developed fetus (*E*), supporting ovary with CL (*J*), and contralateral ovary with new CL (*O*) can be seen. Dotted lines indicate approximate dimensions of the gravid-side CL. White arrows indicate follicles. (Scale bars: 1.0 mm.)

These results clearly demonstrate that the swamp wallaby conceives a new embryo before birth and that their normal reproductive strategy involves concurrent pregnancy of two conceptuses of different ages (Fig. 4; 7). It is not clear why the swamp wallaby ovulates and mates prepartum compared to all other macropodids. It is unlikely to be related to any gain in developmental time of the embryo (1–3 d) because this “benefit” would be lost when the embryo enters its lengthy period of diapause.

Prepartum oestrus potentially poses unique endocrine questions for mammals due to the conflicting actions of progesterone and estrogen in the female reproductive tract. However, given that, at least, one other mammal, the European brown hare, also has a prepartum oestrus and superfetation, this must not be an impediment to successful pregnancy in these species (3). Additionally, changes in progesterone and estrogen do not have any effect on parturition in the best studied marsupial, the tammar wallaby (*M. eugenii*). Plasma progesterone, secreted by the CL of

pregnancy, is elevated until the time of birth and dramatically falls immediately after birth (19), but progesterone withdrawal is not essential for birth. There is a short peak of plasma estradiol 8–10 h after birth secreted by the dominant ovarian follicle associated with the postpartum ovulation and mating (Hinds and Tyndale-Biscoe 1982; Shaw and Renfree, 1984) and a luteinizing hormone pulse at about the same time, but neither are essential for parturition (19–24). The pituitary is not necessary for successful embryo reactivation, pregnancy, or parturition in the tammar because hypophysectomy eliminates the prolactin inhibition of the CL of pregnancy resulting in normal embryo reactivation, pregnancy, and birth (25). All marsupials have anatomically separate ovaries, uteri, and cervixes with separate vascular circulations (26), so a prepartum rise in estradiol from the contralateral ovary is unlikely to affect birth of the fetus on the contralateral side.

While all marsupials apart from the swamp wallaby have an oestrous cycle longer than the gestation length, macropodid

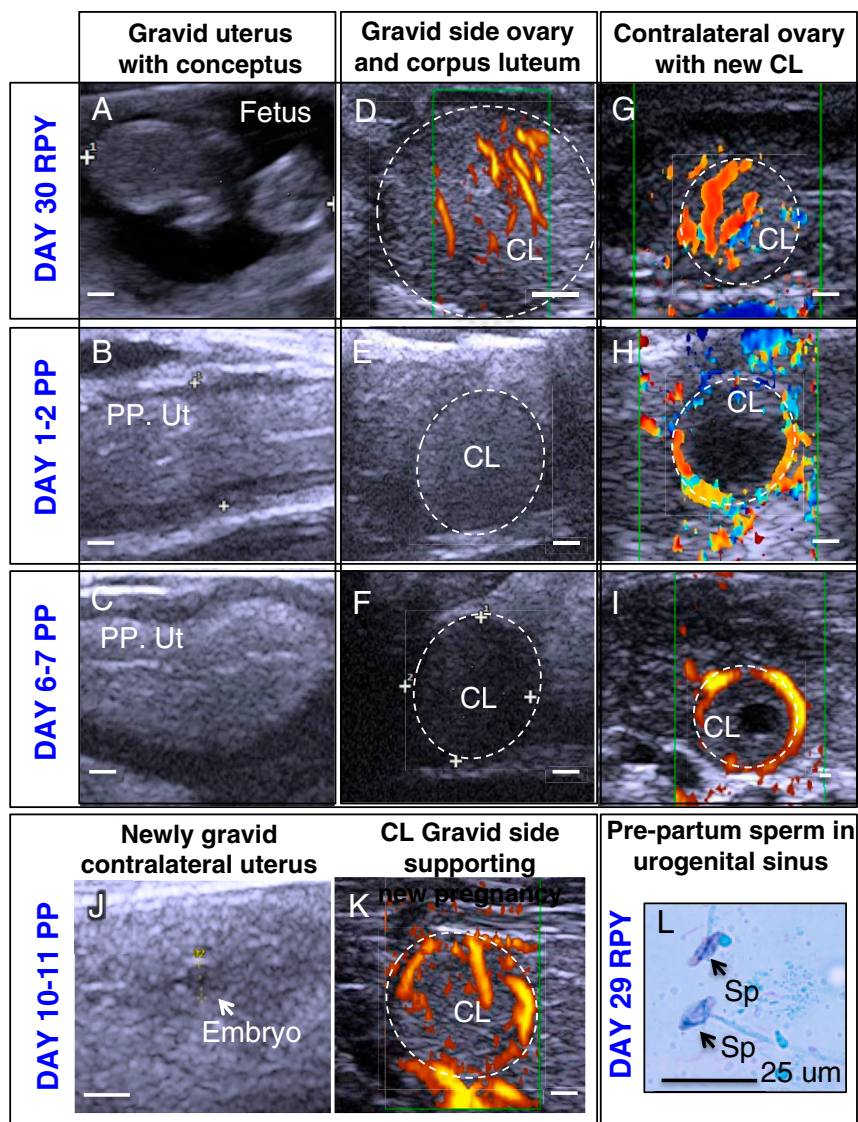


Fig. 3. High-resolution ultrasound of key developmental events supporting concurrent conception and pregnancy in the swampy wallaby. *A*, *D*, and *G* show the incumbent fetus at day 30 RPY prior to birth, the CL on the gravid-side supporting this pregnancy, and the new contralateral CL, respectively. At 1 to 2 d postpartum (PP) the empty uterus from the recent pregnancy can be seen (*B*) alongside the old CL associated with the recent pregnancy (*E*) as well as the new CL on the contralateral side (*H*). A similar set of images can be seen left to right in *C*, *F*, and *I* at days 6 to 7 PP. By days 10 to 11 PP, the new embryo can be seen in the contralateral uterus (*J*) still supported by the new contralateral CL (*K*). The size of this embryo means it could only have been conceived prepartum. Sperm detected in the urogenital sinus by vaginal smear at day 29 RPY (*L*). (Scale bars: 1.0 mm except for *L*.)

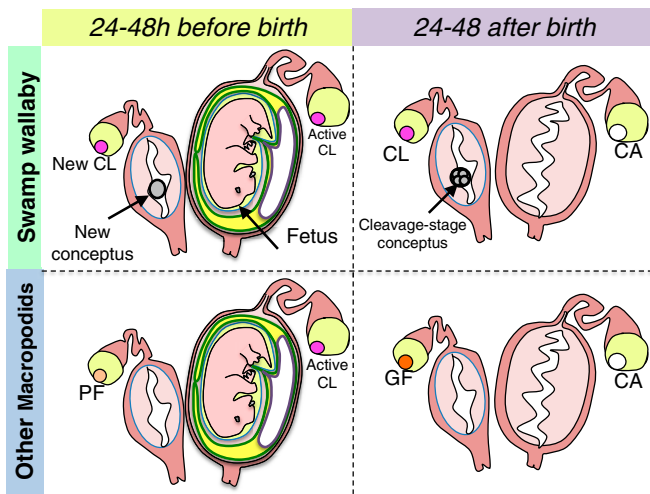


Fig. 4. Diagram of reproductive events surrounding pregnancy and birth in the swamp wallaby. The swamp wallaby ovulates and mates prepartum forming a new CL on the contralateral ovary and embryo prior to birth of the late stage fetus. If the incumbent fetus is the second or subsequent pregnancy for the animal, it will more than likely have a semidependent young still sucking occasionally from one of four mammary glands in the pouch. After birth, the new pouch young attaches to an adjacent nipple and mammary gland to that supporting the semidependent young at foot. Thus, under normal conditions, swamp wallabies are continuously pregnant and lactating with conceptuses and pouch young at different stages, respectively.

marsupials have the longest gestation periods of all marsupials and give birth to the most developed young. Eutherian pregnancies greatly exceed the length of the oestrous cycle, so during mammalian evolution, there has been selection pressure to extend the duration of pregnancy. Among marsupials, gestation in most macropodids encompasses almost the whole of the oestrous cycle. The swamp wallaby takes this one step further with its prepartum oestrus allowing marsupial gestation length to exceed the oestrous cycle length. Thus, swamp wallabies exhibit superfetation (conceptuses at different

stages of development) and asynchronous concurrent lactation (pouch young at different stages of lactation) such that under normal conditions females may be pregnant and lactating continuously.

Materials and Methods

Animals. Captive swamp wallabies were sourced from wild locations in Victoria and held at our facility under permit from the Victorian Department of Environment, Land, Water and Planning. Male and female animals were freely distributed within a fenced area of regenerated bush land approximately 5 hectares in area with access to water and lucerne cubes ad libitum. This work was completed with approval from The University of Melbourne Institutional Animal Ethics Committee and in accordance with the National Health and Medical Research Council guidelines (27).

Ultrasound Examinations. To minimize stress caused by repetitive capture and handling efforts, animals in the ultrasound group ($n = 4$) were caught and scanned five times (20 examinations in total) on average from day 13 RPY to day 20 PP with the majority of examinations clustered around the prepartum and postpartum periods (days 28–32 RPY). Not all animals could be caught and checked at each time point because they showed signs of heat stress, such as licking paws and arms or heavy panting, and no more than four animals could be monitored simultaneously at this intensity of scanning and handling. Animals were caught in large (diameter: 1.0 m) hand-held nets and transferred promptly to large hessian sacks until sedation and ultrasound could be administered as described previously (14). Examinations were conducted during this study using a portable digital ultrasound unit (MyLab One Vet, Esaote Europe B.V. Maastricht, The Netherlands, Serial No.: 9445042). The system was equipped with a high frequency linear probe (24 MHz, SL3116 VET) and used the MyLab One Vet default software for visualization and measurement of images. All measurements are given as the mean \pm the SE unless stated otherwise.

Vaginal Smears. A second separate group of animals ($n = 6$) was monitored from day 26 RPY to day 35 RPY for births and detection of mating from examination of vaginal smears. After catching, the urogenital sinus was exposed and flushed with 1 mL of sterile water using a plastic Pasteur pipette. The flushings were then smeared onto a glass slide and allowed to dry then stained with Shorr's stain for 30 s, counterstained with hemotoxylin 5–10 s, coverslipped, and examined under a light microscope.

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