Differential transport of spermatozoa into the two sides of the genital tract of a monovular marsupial, the tammar wallaby (*Macropus eugenii*)

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Summary. Ovulation in the tammar wallaby alternates between the ovaries. The genital duct of each side enters the median vaginal culs-de-sac separately. Post-partum oestrus occurred 0·4 days after birth and ovulation 1 day later. After a single copulation spermatozoa were found in both cervical canals at 0·5 h and extended to the oviduct on the non-parturient side only by 8 h. Very few spermatozoa were found in sections of the post-partum uterus or its associated oviduct at any time. Spermatozoa were recovered by flushing from both sides but the numbers were 2–20 times greater in the non-parturient than in the post-partum side: the greatest difference occurred in the cervical canals 2–5 h after copulation. In females which had undergone a previous infertile cycle, spermatozoa were abundant in both cervixes and both uteri. It is concluded that the differential distribution of spermatozoa in post-partum animals was (1) due to failure of transport in the recently pregnant side of the tract, rather than attraction of spermatozoa to the ovulation side, and (2) established at the cervix which, on the ovulation side, provides a reservoir of spermatozoa for 24 h after copulation.

Introduction

Various regions of the female tract of vertebrates have been shown to act both as barriers to sperm migration and as reservoirs for spermatozoa. Barriers are thought to remove defective spermatozoa and to reduce the number of spermatozoa reaching the site of fertilization, thus preventing polyspermy (for reviews see Thibault, 1973; Hunter, 1975). Reservoirs on the other hand maintain a viable population of spermatozoa until ovulation, perhaps within hours, as in most mammals (Thibault, 1973), days as in birds (Kamar & Hafez, 1975) or months as in bats and some reptiles (Thibault, 1973; Saint-Girons, 1975; Racey, Suzuki & Medway, 1975). The uterotubal junction and cervix are the most common barrier/reservoir regions in mammals but any or several region(s) can be utilized as sperm reservoirs (Thibault, 1973).

In monovular animals it might be expected that transport of spermatozoa to the non-ovulating side would be reduced in favour of the ovulating side, but such differential transport has not been reported for any eutherian species.

Marsupials at oestrus have much enlarged anterior vaginal canals which become distended with fluid composed of semen and vaginal secretions (Sharman, 1954; Hughes & Rodger, 1971) and it has been suggested that they may act as a sperm reservoir. Wood Jones (1944) believed that sperm storage for some weeks or months might account for delayed pregnancy in kangaroos but Sharman (1955a, b) disproved this and showed that spermatozoa disappeared from the genital tract of the quokka (*Setonix brachyurus*) and tammar wallaby (*Macropus eugenii*) within 10 days of oestrus. In these monovular animals, oestrus frequently occurs a few days after parturition and Tyndale-Biscoe (1963) observed that quokka spermatozoa were abundant in the non-pregnant uterus associated with the imminent ovulation and absent from the parturient uterus. This unusual pattern of distribution was subsequently observed in tammar wallabies shot during the breeding season on Kangaroo Island, South Australia, and led us to undertake a study of sperm transport and distribution in this macropod marsupial. As in all other marsupials, the two uteri of the tammar open into the median vaginal culs-de-sac through entirely separate cervical canals (Sharman, 1954) so that the observed distribu-

tion of spermatozoa could be due to differential uptake by each cervix or to subsequent differential survival.

Materials and Methods

Tammar wallabies were shot on Kangaroo Island, South Australia during January 1970 (Series 2), or were taken from a breeding colony of Kangaroo Island stock maintained in Canberra. The experiments with captive animals (Series 1 and 3) were carried out on females with pouch young during the main breeding season in March and April. The reproductive cycle was reactivated by removing the pouch young (RPY) and the females were checked for parturition each day, or more frequently from Day 25 after RPY. After giving birth females were placed with a male and observed for 1 h. If copulation did not occur they were removed for a few hours. This procedure was repeated several times but if copulation still failed to occur the pair were left together for some hours and then checked for signs of copulation. When copulation was observed directly or indirectly the female was isolated from males until autopsy.

The rate of sperm movement and the fate of spermatozoa after copulation can be studied by fixing the female tract and counting spermatozoa in serial sections of the tract or by separately flushing the parts of the tract and counting the recovered spermatozoa. Thibault (1973) very strongly favours the former approach but Robinson (1975) contends that the methods give comparable results if carefully used. We used both methods in this study.

In Series 1 and 2 the whole urogenital tract was removed, and weighed. Each half with its associated ovary was then pinned to aluminium foil and fixed whole in Bodian's fluid. After fixation each half was photographed and cut transversely into 5 mm lengths beginning at the os uterus. Sections cut at 10 μ m were taken from the posterior end of each length and stained with haematoxylin and eosin. The sections were searched and scored for presence or absence of spermatozoa. In Series 3 an attempt was made to obtain total counts of spermatozoa in the cervix, uterus and oviduct of each side. The seminal coagulum was removed from the vaginal complex and the internal surface was irrigated with Krebs-Ringer-phosphate (Umbreit, Burris & Stauffer, 1972). The oviducts were flushed from the uterotubal junction by using a 26-gauge needle and 2 ml Krebs-Ringer-phosphate. The uterus was then flushed twice with 2 ml Krebs-Ringer-phosphate by means of an 18-gauge needle inserted into the lumen through the cervical canal. Then, by putting the same needle through the uterotubal junction so that it touched the inner end of the cervical canal, the cervix was flushed. Subsequent histological examination of the tracts disclosed some spermatozoa in folds of the cervix of only one tammar (No. 15), very few spermatozoa in the uterine glands and none in the lumina so we conclude that the flushing technique was efficient and the estimates of total spermatozoa recovered are close to the true totals in the tracts.

The flushings were first searched under a binocular microscope for eggs and early embryos and then centrifuged at 870 g. The supernatant was discarded and the pellet resuspended in a known volume (100–200 μ l) of Krebs-Ringer-phosphate. The number of spermatozoa recovered from the various regions of the tract was estimated by counting the spermatozoa in each suspension in an Improved Neubauer Bright-Line haemocytometer. Depending upon the concentration of spermatozoa in the suspensions, the spermatozoa were counted in the five 'red cell' areas (volume 0.02 mm³), the five 'white cell' areas (volume 0.5 mm³) or over the entire ruled area (volume 0.9 mm³). Counts were carried out in duplicate on both grids of a haemocytometer. Every care was taken in the handling of the material and in cleaning the haemocytometers between counts. A fresh disposable Pasteur pipette was used for each pipetting operation. All centrifuge tubes and vials were soaked in detergent, rinsed in tap water and distilled water and then oven dried before use.

The flushed tracts were fixed by immersion in Bouin's solution for 48 h, washed in several changes of 70% alcohol and stored in 70% alcohol. Wax blocks were prepared and 7–10 μ m sections cut. Sections were stained with haematoxylin and eosin so that tissue structures and spermatozoa could be identified or for histochemistry with 0.1% Alcian blue, pH 2.5 (Pearse, 1961); periodic acid-Schiff (PAS) with diastase control (Pearse, 1961); methylene blue, pH 8.1–2.2 (Pearse, 1961); or 0.25% toluidine blue, pH 4.4 (Drury & Wallington, 1967).

Results

Relationship of events around the time of birth

Birth, post-partum oestrus, ovulation and tubal transport of the egg occur in that order but differences in the time relationships in individual animals led us to relate all the events back to the start of the reproductive cycle that culminated in birth. The observations made on 19 animals during this period are summarized in Table 1. The mean time of birth after RPY was in agreement with that of 27.5 ± 0.7 (s.d.) days given by Berger (1970). Two young weighed immediately after birth were 0.363 and 0.382 g while two others at 8 and 16 h after birth and attachment weighed 0.418 and 0.492 g respectively.

Table 1. Distribution of birth, oestrus and ovulation after removal of the pouch young (RPY) from female tammar wallabies (Series 1 and 3)

	Day after RPY							Mean $\pm$ s.d.	(N)
	-25	-26	-27	-28	-29	-30	-31		
Birth	0	4	2	5	4	1	0	27.6 ± 1.2	(16)
Copulation	0	0	4	4	4	0	1*	28.0 ± 0.9	(13)
Graafian follicle	—	2	2	2	4	2	0	29.0 ± 1.5	(17)
New corpus luteum	0	0	0	0	1	1	3		
Egg in oviduct	0	0	0	0	1	0	0	29	
Egg in uterus	0	0	0	0	0	1	3	30.7	

* Non-pregnant cycle.

Copulation occurred at an average of 0.4 days after birth. In two animals (Tammars 1 and 2) copulation did not occur at 14 h but did so at 15 and 16 h while in Tammar 3 it had occurred at 10 h but not at 8 h. The onset of oestrus therefore appears to be quite sudden, 10–16 h after birth. The duration of oestrus was not determined in this study because the females were isolated after the first copulation, but it is usually not more than 12 h. The six observed copulations lasted 8.3 ± 4.8 (s.d.) min.

The time of ovulation in the ovary contralateral to that associated with the previous pregnancy was determined from 15 autopsies. The earliest new corpus luteum (CL) after RPY was seen at 28 days 6 h (No. 19), and the latest observed Graafian follicle was at 29 days 8 h (No. 4); the mid-point for all animals was 29 days or 1 day after the onset of oestrus and about 1.4 days after birth. Only one oviductal egg was recovered and was from No. 19 with the earliest CL; a zygote recovered at 29 days 4 h after RPY (No. 26) was an early cleavage stage and was already in the uterus (Table 1). These results agree closely with the observations of Sharman (1955a) on the quokka.

Distribution of spermatozoa

Series 1. By 40 min after a single copulation the anterior vaginal canals and the median vaginal culs-de-sac were distended with semen and the whole tract weighed 75 g, compared to 39 g at 24 h (Text-fig. 1 and Pl. 1, Fig. 1). Spermatozoa were abundant at each cervical orifice (Pl. 1, Fig. 4) and extended throughout the 15 mm-long cervical canals but none was found in either uterus or oviduct (Text-fig. 1a). The same distribution of spermatozoa was observed in females at 1 and 2 h after copulation, but at 4 h spermatozoa were distributed throughout the lumen and glands of the non-parturient uterus; at 8 h spermatozoa were found in the lower segment of the oviduct on this side only. No spermatozoa were found in the uterus or oviduct of the parturient side of any animal up to 8 h *p.c.* At 4 and 8 h, spermatozoa were absent from the posterior end of the cervix or were being phagocytosed there. At 24 h *p.c.* spermatozoa were abundant in the lumen and glands of the non-parturient uterus (Pl. 1, Fig. 2) and a few were within 10 mm of the fimbria on this side. In the parturient uterus of the same animal (Tammar 6) very few spermatozoa were found in the posterior part of

Text-fig. 1. Distribution of spermatozoa in the two halves of the female genital tract of tammar wallabies at increasing time after copulation. (a) Presence of spermatozoa along each tract from the os cervix represented by open columns on the post-partum side and stippled columns on the non-parturient side. Open circles indicate the preovulatory Graafian follicle and closed circles the new corpus luteum of ovulation. (b) Weight of whole genital tract, including contents (▲), and of new born young (△) of females represented in (a) above.

the uterus (Pl. 1, Fig. 3) but none in the oviduct. None of the 6 animals had ovulated, although each had a Graafian follicle in the ovary on the non-parturient side.

Series 2. In these 8 females from Kangaroo Island copulation was not observed but had occurred about 1–4 days before autopsy. Females in post-partum oestrus were commonly seen being followed by a queue of up to 8 males and the urogenital tracts of the females examined were much heavier than those of the captive animals, which had been permitted to copulate only once (Text-fig. 1b). The two heaviest tracts weighed 125 g and 110 g and each contained 80 ml of partly coagulated seminal fluid, which had presumably been contributed by several males. Six of the 8 animals had given birth and the weight of the young and the weight of the urogenital tracts were inversely correlated and provide a means of placing the animals in a sequence (Text-fig. 1b). The young of the female with the heaviest tract (Tammar 7) weighed 0.489 g, which is within the range for the 1st day after birth, and the female had not yet ovulated. This specimen, therefore, is equivalent to Tammar 6 of Series 1 while the other females were at later stages after oestrus and all had ovulated.

The differential distribution of spermatozoa is more pronounced in this series; no spermatozoa were found in any part of the parturient uteri and oviducts of 5 of the 8 animals, and only a very few were found in the other 3 animals (Text-fig. 1a). Two of these 3 animals (Tammars 8 and 12) had undergone an infertile cycle before oestrus. However, spermatozoa were found in the non-pregnant side of all 8 animals, variously distributed in the cervix, uterus or oviduct. A 2-cell fertilized egg was present in the anterior end of the uterus of Tammar 14.

Dynamics of sperm transport

Series 3. Of the 12 tammars examined (Table 2) 9 were post-partum and 3 had undergone an infertile cycle before copulation. Spermatozoa were found in all 8 animals examined up to 24 h *p.c.* (Table 2), at 30 and 48 h *p.c.* only a few spermatozoa were recovered in the uteri, and in the animals

Table 2. The numbers ($\times 10^3$) of spermatozoa recovered by flushing the tracts (CA, side of ovary with corpus albicans; F, side of ovary with Graafian follicle or new corpus luteum) of female tammars after copulation

Tammar no.	Hours <i>post coitum</i>	Anterior vaginal canal	Cervices		Uteri		Oviducts		Before (—) or after (+) ovulation
			CA	F	CA	F	CA	F	
Post-partum females									
15	2-5	357.0	14.7	301.8	24.1	32.6	0.2	6.0	—
16	6	64.9	95.6	138.4	6.0	130.0	0	1.1	—
17	6	7.8	157.6	21.6	3.6	23.1	0	0	—
18	12	1.1	0.1	2.7	0	2.4	0	0	—
19	7-22	1.6	(20.8	71.2)*			0.4	4.4	+
20	24	0.5	9.0	11.9	3.6	26.7	0.2	2.4	—
21	48	0.3	(0.05	0)*			0	0	+
22	72-84	5.6	0	0	0	0	0	0	+
23	90	0	0	0	0	0	0	0	+
Non-pregnant females									
24	3-16	230.0	87.3	34.7	309.0	408.0	1.7	0	—
25	6	0	18.4	47.4	58.4	76.4	0.6	0.8	—
26	30	0	0	0	0	1.3	0	0	+

*Combined flushings from cervix and uterus.

examined later than this none was found. In the 6 post-partum animals examined up to 24 h *p.c.* there were marked differences in the number of spermatozoa in the two sides (Table 2). In the non-parturient or ovulation side there was a very large cervical population at 2–5 h *p.c.*, fewer spermatozoa between 12 and 24 h and none at 48 h *p.c.* The uterine and oviductal populations on this side remained fairly stable until 24 h *p.c.* and then rapidly disappeared. The cervical population in 5 out of 6 animals was lower in the parturient side than in the opposite cervix and, although the uterine population at 2–5 h *p.c.* was similar to that in the ovulation side, it was much lower by 6 h *p.c.* and no spermatozoon remained by 48 h *p.c.* The oviductal population in this side remained at 10% or less of that in the ovulation side up to 24 h *p.c.*

In 2 of the 3 non-pregnant animals (Tammars 24 and 25) very large numbers of spermatozoa were recovered from both cervixes and both uteri and the differences between the two sides were small. Except for a few spermatozoa in the uterus of the ovulation side no spermatozoa were recovered from Tamar 26 examined at 30 h *p.c.*

The vaginal complex and its contents

In animals that had mated the anterior part of the vaginal complex contained a rubbery mass similar in appearance to coagulated ejaculates from male tammars (Rodger & White, 1975) and consisted mainly of prostatic bodies, as described by Rodger & Hughes (1973) and Rodger & White (1975). The coagula did not appear to contain large numbers of spermatozoa, even in the earliest sample collected (2–5 h *p.c.*), and most of the spermatozoa present were at the surface of the mass (Pl. 1, Fig. 4) or in groups within it. However, in Tammars 15, 16, 17 and 24 large numbers of spermatozoa were washed from the surface of the epithelial lining of the vagina after removal of the coagulated plugs (Table 2). The only other vaginal complex to contain significant numbers of free spermatozoa (5600) was that of Tamar 22 in which the coagulum had degraded to a watery suspension.

Vaginal secretions

The posterior portion of the median vaginal culs-de-sac is the only region of the vaginal complex of the tammar which is lined by a columnar secretory epithelium; the remainder is lined by stratified epithelium. The secretory cells of the epithelium in Tammars 15, 19 and 22 contained numerous granules which reacted positively in several histochemical tests for mucopolysaccharides. These

granules were most concentrated in the apical cytoplasm. Material with the same staining properties was found on the luminal surface of the secretory epithelium and free in the lumen. Although the secretory cells of all 3 tammar studied were active, the secretory activity was greater in the two animals (15 and 19) which had copulated more recently and in these the whole cytoplasm of the epithelial cells was often filled with secretory granules.

The secretory products were PAS-positive and this reaction was unaffected by pretreatment with diastase to remove reactive glycogen. The material was stained by alcian blue and reacted meta-chromatically with toluidine and methylene blues. These histochemical characteristics are identical to those of the mucus secreted in large quantities (3–4 ml) by the anterior vagina and culs-de-sac of the brush-tailed possum between oestrus and ovulation (Hughes & Rodger, 1971), and suggest the presence of acidic mucopolysaccharides. However, methylene blue staining was greatly reduced below pH 4.2 and almost completely removed at pH 2.2 and this indicates that the polysaccharide is probably not highly sulphated.

Discussion

Despite the very large volume of semen in the vaginal canals after copulation, the cervical canals in the tammar are the main reservoirs of spermatozoa and appear also to act as a selective barrier to the further advance of spermatozoa through the uteri and oviducts. The relatively stable sperm numbers, especially in the non-parturient uterus and oviduct for 24 h, are perhaps maintained by a continuous supply from the cervix, where sperm numbers declined markedly during the same period. The maintenance of this cervical pool appears to depend on a special intimacy between the spermatozoa and the cervical epithelium or its secretions, as has been described in many vertebrate species (Thibault, 1973). In cattle, goats and sheep the association of spermatozoa with cervical mucus and the cervical epithelium is such that flushing only removes a small proportion of the total cervical spermatozoa (Mattner, 1966; Mattner & Braden, 1969).

The rate of transport of spermatozoa to the oviduct in the tammar is similar to that in eutherian mammals; some are carried into the oviduct within 6 h *p.c.* and the subsequent population is replenished from a cervical reservoir for up to 24 h *p.c.* when ovulation occurs. However, the differential transport of spermatozoa up the two sides of the tract of a monovular species has not previously been reported.

In all three series of tammar studied the distribution of spermatozoa in the parturient side was markedly less extensive than that in the other side which was associated with the Graafian follicle or newly formed corpus luteum. In Series 1 and 2, spermatozoa were almost wholly absent from the uterus and oviduct of the parturient side and in Series 3 the flushing method disclosed so few spermatozoa in the oviducts on this side that the probability of a spermatozoon being found in a 10 μ m section would be less than 0.1. The difference in the results obtained by the two methods is therefore explicable. However, the way in which this difference in distribution of spermatozoa to the two sides is achieved is less easy to explain.

EXPLANATION OF PLATE 1

Fig. 1. Ventral view of the genital tract of a female tammar wallaby (No. 21) 48 h after a single copulation to illustrate the enlarged anterior vaginal canals (AVC). LV = lateral vagina; PPU = post-partum uterus; NPU = non-parturient uterus; CA = corpus albicans; CL = corpus luteum.

Fig. 2. Section of the endometrium of the non-parturient uterus of a female tammar (No. 6) 24 h after copulation showing spermatozoa (Sp) in the lumina of the glands and uterus.

Fig. 3. Section of the endometrium of the post-partum uterus of Tammar 6 (see Fig. 2). Note the absence of spermatozoa in the lumina and glands.

Fig. 4. Section of the vaginal epithelium of the cervical os and adjacent seminal coagulum (C) from a female (No. 1) 0.5 h after a single copulation. Note the concentration of spermatozoa (Sp) at the epithelium.

PLATE I

It might be supposed that in Series 1 insufficient spermatozoa were provided by a single insemination to supply both sides, but in the series collected on Kangaroo Island the females were probably each inseminated by several males and yet they had the same unequal distribution of spermatozoa as did the experimental animals. Within 1 h after copulation the semen deposited in the anterior end of the urogenital sinus had reached the anterior vaginal canals, probably by contractions of the lateral vaginae (Hartman, 1924; Hughes & Rodger, 1971). Here the spermatozoa leave the semen, possibly before it coagulates, and become concentrated near the vaginal surface of the cervical os (Pl. I, Fig. 4), probably by their own motility. They then migrate or are carried into the cervical canals, predominantly into the non-parturient side, which suggests that the primary selection is being exercised here rather than in the body of the uterus. Differences in myometrial activity are thus ruled out, but the fluid content of the post-partum uterus may disperse down the cervical canal and inhibit sperm ascent, because in the non-pregnant females there was little difference between sperm numbers in the two cervical canals or in the two uteri. The secretions of the parturient uterus have not yet been investigated but our results suggest that they are the most likely factors influencing the differential distribution of spermatozoa in the genital tract of the tammar.

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