Relationship between longevity of spermatozoa after insemination and the percentage of normal embryos in brown marsupial mice (*Antechinus stuartii*)

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Summary. Twenty-six female brown marsupial mice in a laboratory colony were mated at intervals ranging from 1 to 20 days between coitus and ovulation. The numbers of corpora lutea and normal embryos were counted.

A multiple regression model examined the parabolic relationship between the proportion of normal embryos and the time from coitus to ovulation. The proportion of normal embryos increased until a mean of 9.5 days and decreased thereafter. This relationship was independent of the year of breeding and the number of corpora lutea. After survival of spermatozoa for up to 13 days in the female reproductive tract, the fertility levels of females was 88–92%. Low fertility levels after 13 days appeared to be due to a decrease in the number of spermatozoa.

Reproductive tracts from 7 females killed after insemination and examined histologically showed many spermatozoa in the isthmus of the oviduct and the uterus at 5 days *post coitum*; spermatozoa confined to the isthmus between 6 and 13 days; and few spermatozoa in the isthmus at 14 days after copulation.

A comparison between the fertility levels in the females which had been inseminated once and a further 17 females which had been inseminated 2 or 3 times suggested that spermatozoa from 2nd and 3rd inseminations can contribute spermatozoa for fertilization. In these females fertility levels did not decline with time after the first mating.

Introduction

In most eutherian mammals, spermatozoa in the female reproductive tract survive for a relatively short period of time, mostly measured in hours (Bishop, 1970). Fertilization usually occurs within 1 day or less after coitus. The most notable exception is found in some bats in which the interval between copulation and ovulation can be several months (Racey *et al.*, 1975; Racey, 1979). In a small number of other mammals, longevity of several days has been reported (Chang, 1965; Doak *et al.*, 1967; Bishop, 1970; Thibault, 1973; Ullman, 1976; Gould *et al.*, 1984). In the latter group of mammals, these are maximum times for survival of viable spermatozoa and fertilization usually occurs within a much shorter period of time.

Although less is known of longevity of spermatozoa in marsupials, it also appears to be of short duration in families with a short oestrus. Motile spermatozoa have been found in the oviduct 1–2 days after copulation in macropodids (Sharman, 1955; Tyndale-Biscoe & Rodger, 1978) and 10–12 hours after copulation in didelphids (Rodger & Bedford, 1982a). In dasyurids oestrus is often prolonged and spermatozoa in the female reproductive tract may be viable for many days. The Eastern quoll, *Dasyurus viverrinus* (Hill & O'Donoghue, 1913), had motile spermatozoa in the female tract 2 weeks after oestrus. The stripe-faced dunnart, *Sminthopsis larapinta* (= *S. macroura*), ovulated 2½ days *post coitum* (Godfrey, 1969). Fertilized eggs were obtained from the fat-tailed

dunnart, *Sminthopsis crassicaudata*, 4 days after copulation (Selwood, 1987), indicating that spermatozoa survived for at least 3 days as passage of eggs through the oviduct is usually 24 h or less in marsupials (Selwood, 1982a; Rodger & Bedford, 1982a). In brown marsupial mice (*Antechinus stuartii*), motile spermatozoa have been found in the reproductive tract 5 days *post coitum* (Woolley, 1966a) and ovulations resulting in fertilized eggs have occurred 7–9 days after copulation (Selwood, 1983).

The capacity for relatively long-term survival of spermatozoa in the female reproductive tract is important in the unusual life history of populations of *A. stuartii*. The female is monoestrous (Marlow, 1961; Woolley, 1966b) and ovulates spontaneously (Woolley, 1966b). Oestrus, ovulation and birth are each highly synchronized and occur at the same time each year within any one population in the laboratory and in the field (Wood, 1970; Selwood, 1982a, 1985; Dickman, 1985). At 3 weeks before ovulation cornified epithelial cells appear in the urine of the females and matings occur within this period (Selwood, 1980, 1982a, b, 1985). After mating spermatozoa are found in the isthmus of the oviduct (Woolley, 1966a; Selwood, 1982a; Bedford *et al.*, 1984). Females mate with a number of males in the laboratory (Woolley, 1966a; Selwood, 1980, 1982a, b, 1983) and in the field (Scott & Tan, 1985). All males die by the end of the mating period (Woolley, 1966b; Wood, 1970; Lee *et al.*, 1982). The dominant males occupy larger territories, are presumed to mate with more females and tend to die earlier than subordinate males (Braithwaite, 1979). Death of some of the males well before all the females have ovulated means that some form of storage of spermatozoa in the female reproductive tract is necessary in this species if these males are to contribute spermatozoa for fertilization.

This study determines how long spermatozoa of brown marsupial mice can survive in the female reproductive tract without marked deterioration in their fertilizing ability. It also investigates, in a small number of animals, the possible causes for changes in the fertilizing ability of spermatozoa with length of survival in the female tract.

Materials and Methods

In each year just before the start of the breeding season in mid-July, a laboratory colony of brown marsupial mice (*A. stuartii*) was set up in a room maintained with the natural daylength for Melbourne. The animals were trapped in forested areas north of Melbourne near Kinglake Victoria in 1978, 1981, 1982 and 1983. On the day of collection, each female was weighed and her pouch examined to determine whether she was in her first or second breeding season (Woolley, 1973).

The animals were housed separately in cages with detachable nest boxes (Woolley, 1973) and were fed on meat and insects supplemented with dried cat food (Selwood, 1983). Water was provided *ad libitum*. A urine sample was collected in the morning of each day from each female and was examined for the large numbers of cornified epithelial cells that appear at the beginning of oestrus (Godfrey, 1969; Selwood, 1980). The fall in the numbers of epithelial cells in the urine which occurs within 24 h of ovulation in this species (Selwood, 1980, 1982b) was also recorded for each animal. After epithelial cells were present in high numbers in the urine, each of 26 females was mated once with a male from the colony at intervals ranging between 1 and 20 days before ovulation. The animals were together for 6–16 h dependent on the duration of copulation which can be as long as 12 h (Woolley, 1966a). These females were from the colonies maintained in 1981, 1982 and 1983.

Confirmation of coitus was based on observation of penetration or the detection of spermatozoa in the urine of the female on subsequent days (Selwood, 1983). The duration of storage of spermatozoa was measured in days from the day of coitus to the estimated day of ovulation, the day of coitus being taken as Day 0. A total of 12 males was used and each male mated 1–4 times.

The females were anaesthetized with ether at various times during the 27-day gestation period (Selwood, 1980). The ovaries and uteri were removed and the number of corpora lutea was counted. Each uterus was slit along the median margin and the embryos were everted into Dulbecco's phosphate-buffered saline pH 7.2 (Dulbecco & Vogt, 1954). Embryos were scored as normal and fertilized or non-viable or unfertilized based on the criteria of (1) presence or absence of spermatozoa, (2) degree of polarization of yolk, (3) nature of cytokinesis and (4) rate of development outlined for this species (Selwood, 1983). The numbers of each type of embryo were counted for each female.

The ovaries were fixed in Bouin's fluid for 24–48 h, dehydrated, embedded in paraffin wax, serially sectioned at 8 µm and stained with haematoxylin and eosin. The corpora lutea were re-counted in the serial sections following the procedure outlined by Woolley (1966a). The corpora lutea of pregnancy were post-ovulatory follicles showing various degrees of luteinization. They can be readily distinguished from the infrequent luteinized follicles, which retain an

apparently normal ovum (Woolley, 1966a). The percentage of normal embryos was calculated as the number of normal embryos divided by the number of eggs ovulated (which was assumed to be equal to the number of corpora lutea) multiplied by 100.

A multiple regression model was used to examine the relationship between the percentage of normal embryos (dependent variable) and the 3 explanatory variables: (1) length of storage of spermatozoa in days from coitus to the estimated date of ovulation, (2) year of breeding and (3) number of corpora lutea. The best model to fit the data included days and days squared and the other variables did not contribute significantly to the variance. The analysis was carried out using the Statistical Package for the Social Sciences, version X on the Vax computer at Monash University. Because only 3 of the 26 females were in their 2nd year of breeding and the remainder were in their 1st year, the age of the female was not included in the calculations. In a previous study Selwood (1983) showed that the number of corpora lutea but not the percentage of normal embryos declined significantly with the age of the female. It was not possible to compare statistically the performance of individual males at each mating because some had mated once whereas others had mated 2 or 3 times and the numbers in some categories were very low. However, the performance of individual males (based on the percentage of normal embryos) at their first mating ($N=10$) was compared with the performance of males at their second ($N=11$).

The histological events during storage of spermatozoa were investigated in 5 animals which had died or were killed after mating but before or just after ovulation. Spermatozoa are known to disappear from the female tract within 24 h of fertilization (Selwood, 1982a; Bedford *et al.*, 1984) so that only those animals that were in the preovulatory phase or had ovulated within 24 h could be used for analysis. Two of these females were from colonies maintained in 1978. These animals had each mated twice, 12 and 10 days and 13 and 6 days before being killed. The other 3 females had each mated once; 5, 5 and 14 days before death. The tracts were fixed in Bouin's fluid and processed to serial sections as outlined above for the ovaries. The follicles were examined and the state of maturation of the ova was recorded. Tubal eggs were examined to see whether they were fertilized. The sections of oviduct and uterus were examined to locate spermatozoa and leucocytes in the tract. Sections from an additional 2 females killed after a single mating 1 and 14 days before were provided by Dr P. Woolley for examination.

To examine the relationship between the percentage of normal embryos and the length of storage of spermatozoa in females which had multiple matings, the results from a colony maintained in 1978 were examined. A total of 17 females, for which both sides of the reproductive tract were available was examined. The number of corpora lutea, the percentage of normal embryos, the number of matings, the number of days between the first and last mating and ovulation were recorded for each female.

Results

As shown in Table 1, the correlation matrix demonstrated that there was no significant relationship between the dependent variable, the percentage of normal embryos and the explanatory variables, the year of breeding and the number of corpora lutea. However, it showed a strong correlation between the percentage of normal embryos and the number of days between coitus and ovulation and the square of this variable (Table 2). This finding was consistent with the parabolic relationship between the percentage of normal embryos and the duration of time between coitus and ovulation (Fig. 1). The percentage of normal embryos increased with the number of days between coitus and ovulation until a mean of 9.5 days and then decreased until the 20th day.

Figure 1 also demonstrates that the relationships for individual males were the same as for the combined results for all the males. When the period between ovulation and coitus was short (≤ 5 days) or very long (≥ 14 days) the success rate for each copulation, as indicated by the percentage of normal embryos, was low. When the period between ovulation and coitus was between 6 and 13 days the success rate was generally high, and $\geq 80\%$ in all except 3 cases. Figure 1 clearly demonstrates that spermatozoa stored for as long as 13 days retained good ability for successful fertilization and that even spermatozoa stored for 16 days retained some fertilizing ability.

When the period between coitus and ovulation was short (≤ 5 days) about one-third of the abnormal embryos had failed to complete the second cleavage division and the remainder were unfertilized. When the interval between coitus and ovulation was > 5 days most of the abnormal embryos were unfertilized and spermatozoa were rarely found in the egg investments.

The performance of males at successive matings, assessed by comparing the percentage of normal embryos arising from each mating, was $67.0 \pm 36.1\%$ ($N=11$) for the first mating and $70.9 \pm 27.9\%$ ($N=10$) for the second mating. The mean percentage of normal embryos for all 26 single matings examined in this study was $70.3 \pm 30.2\%$.

Fig. 1. The relationship between the percentage of normal embryos and the number of days between coitus and ovulation after a single insemination in brown marsupial mice. On the horizontal axis, 0 is the day of ovulation. Each point represents the result for a single female and the number beside it gives the number of her male partner.

Histological examination of the reproductive tracts of the small number of females which had mated once but were killed before ovulation showed that in the specimen which had been killed 1 day after coitus, no spermatozoa were found in the oviduct. In the 2 females that had been killed 5 days after coitus, spermatozoa were found in medium to high numbers in the central lumen and in the crypts of the isthmus of the oviduct (Fig. 3), and also in considerable numbers in the uterus (Fig. 2). The eggs of all 3 females were still at the follicular primary oocyte stage with nuclei in the resting stage of meiosis I. In the 2 females that were examined 14 days *post coitum*, spermatozoa were found in very low numbers in the isthmus of 1 animal but were absent from the tract of the other animal. No obvious morphological difference was detected between spermatozoa in the uterus and in the isthmus at 5 or 14 days after copulation. Unfortunately, no female was available which had mated once and was killed 6–13 days *post coitum*. However, examination of the tract of 2 specimens which had mated twice before showed that spermatozoa were mainly confined to the oviduct. Both these specimens had just ovulated and fertilized tubal eggs were present in the ampulla. The female which had mated 10 and 12 days before death had spermatozoa in low numbers in the isthmus with many crypts being without spermatozoa and individual spermatozoa were found in the ampulla. No spermatozoa were found in the uterus. In the other female, which had mated 6 and 13 days before ovulation, large numbers of spermatozoa were present in the isthmus, small numbers in the ampulla and a few isolated spermatozoa in the uterus. Leucocytes

Table 1. Fertility of brown marsupial mice, each mated once during three breeding periods

No. of days between coitus and ovulation	Year	Female no.	No. of CL	% of normal embryos
1	1982	13	15	53
4	1982	3	17	35
5	1982	14	11	64
5	1982	15	9	78
6	1982	16	10	100
6	1983	7	10	80
6	1981	5	12	92
7	1982	31	10	90
7	1981	8	14	86
8	1982	1	11	91
8	1982	4	9	78
8	1982	21	9	89
9	1983	2	9	100
9	1981	6	10	60
9	1981	4	11	73
10	1982	7	10	90
10	1981	9	14	100
12	1982	28	12	92
13	1982	9	8	88
13	1982	25	13	92
13	1983	12	11	91
14	1982	6	10	0
14	1982	22	9	56
14	1983	13	12	17
16	1982	23	9	33
20	1982	29	10	0

Table 2. Analysis of variance for the multiple regression model, the dependent variable being percentage of normal embryos and the explanatory variables being days (between coitus and ovulation) and days squared (between coitus and ovulation)

Source	Sum of squares	Degrees of freedom	Mean square	F value	Significance
Additive model	1.15771	2	0.57886	11.89653	$P = 0.0003$
Days	0.70806	1	0.70806	15.4292	$P = 0.0007$
Days ²	0.44965	1	0.44965	9.2416	$P = 0.0058$
Residual total	1.11912	23	0.04866		

were found in the connective tissue and associated blood vessels of the female tract and appeared to increase in number with increasing time after insemination.

In the 17 females which had multiple matings and which were killed during pregnancy in 1978, the number of corpora lutea, the percentage of normal embryos and the number of days between ovulation and the first and last mating were recorded. Table 3 shows that, in contrast to the results

Fig. 2. Spermatozoa (arrows) in the uterine lumen of a female brown marsupial mouse which had been inseminated 5 days before death. No spermatozoa were found in the uterine lumen of females mated more than 14 days before ovulation. The bar = 50 μ m.

Fig. 3. The isthmus of the oviduct of the same specimen as in Fig. 2. Spermatozoa (arrows) are found in the crypts and lumen of this region. At 14 days after copulation spermatozoa were infrequent or absent from the oviduct.

Table 3. Fertility of female brown marsupial mice, each mated more than once, during one breeding season (1978)

No. of days between ovulation and:		No. of female	No. of CL	No. of matings	% Normal embryos
First coitus	Last coitus				
9	7	18	12	2	100
10	2	14	10	3	70
10	4	1	11	2	72
11	3	2	12	3	75
11	3	4	11	3	93
11	4	8	12	3	93
11	4	11	8	2	90
12	4	5	13	3	78
12	7	6	11	2	33
13	11	20	9	3	88
14	4	9	13	2	30
14	10	17	12	2	56
15	11	7	8	2	40
16	6	16	14	3	72
17	9	13	11	2	72
19	11	10	13	2	69
21	11	19	14	3	93

in females that had mated once, in females that had mated more than once no obvious relationship existed between the percentage of normal embryos and the number of days between ovulation and the first or last mating. Fertility levels were high (>80%) in animals in which the first copulation had occurred 9, 11, 13 or 21 days before ovulation. Similarly, no relationship appeared to exist between the percentage of normal embryos and the number of days between the last copulation and ovulation. Except for Female 6/78, fertility levels of <70% were restricted to females in which both inseminations occurred a relatively long time before ovulation (Females 7/78, 17/78) or the first and second insemination occurred 14 and 4 days respectively before ovulation (Female 9/78).

Discussion

Close synchronization of the onset of oestrus, ovulation (Selwood, 1985) and the death of all males by the end of the mating period in the field (Woolley, 1966b; Wood, 1970; Lee *et al.*, 1982) are characteristic of the life history of populations of brown marsupial mice. Because some males will die before their mates have ovulated, storage of spermatozoa in the female tract is also a characteristic of the life history. Previous studies have shown that storage of spermatozoa occurs in the isthmus of the oviduct in the female reproductive tract (Woolley, 1966a; Selwood, 1982a) and this study defines the extent of that storage. High levels of fertility were found 13 days after a single insemination, giving percentages of normal embryos of 88%, 91% and 92% (Table 1). These females had 9, 10 and 9 teats in the pouch respectively so each of these matings would provide enough pouch young to fill or almost fill the pouch. With storage of spermatozoa longer than 13 days, fertility levels decline dramatically. None of the single matings that occurred 14–20 days before ovulation would provide sufficient pouch young to occupy most or all of the teats in the pouch. All or nearly all of the teats in the pouch are occupied by pouch young in field animals (Woolley, 1966b; Wood, 1970). The lower levels of fertility after matings 14–20 days before ovulation were due to eggs not being fertilized rather than failures in early embryonic development and this is consistent with findings in other mammals (Bishop, 1970). Two facts suggest that fertilization failure at this time appeared to be due to falling numbers of spermatozoa in the female tract. Firstly, for most of the unfertilized eggs no spermatozoa were found in the egg investments and only a few spermatozoa were found in the investments of fertilized eggs. Secondly, in the 2 females which were killed 14 days *post coitum* the number of spermatozoa in the isthmus of the oviduct was very low. The increase in number of leucocytes with increase in time after copulation possibly plays a role in this reduction in sperm number as they do in bats (Krutzsch *et al.*, 1982). The relatively low fertility levels up to 5 days *post coitum* were attributed partly to fertilization failure and partly to failure during early cleavage especially during the second cleavage division. Embryonic failure in a number of polytocous marsupials has been linked to similar factors (Hartman, 1919; Hill, 1910; Lyne & Hollis, 1976; Selwood, 1980, 1983; Hughes, 1982). Histological examination of the female reproductive tract 1–5 days after copulation suggests that fertilization failure was possibly due to low numbers of spermatozoa in the oviduct. Spermatozoa appear to take a relatively long time (>1 day) to reach the isthmus of the oviduct, the storage site (Woolley, 1969; Selwood, 1982a). The numerous spermatozoa found in the uterine lumen 5 days *post coitum* may not be viable and still motile and might never have reached the oviduct. It is also possible that the relatively high fertilization or developmental failure 1–5 days *post coitum* was because spermatozoa need to spend a minimum amount of time (several days or so) in the female tract, probably in the isthmus of the oviduct, for capacitation to be completed. However, it is not known whether marsupial spermatozoa undergo capacitation in the female tract. Rodger & Bedford (1982b) have shown that the paired spermatozoa of the American opossum separate during passage through the oviduct before fertilization.

The parabolic nature of the relationship between the percentage of normal embryos *in utero* and the length of time between coitus and ovulation suggests that, when females mate once, matings

that occur in the middle of the period of rut (or 6–13 days before ovulation) yield a higher percentage of normal embryos. Scott & Tan (1985) have shown that, in field populations of *A. stuartii*, males mate with a number of different females, sometimes more than once. Mating activity is more intense in the early half of oestrus but some individual males have more matings late in oestrus. Because of the difficulty of establishing paternity of offspring (Scott & Tan, 1985) when females have mated a number of times, it has not been possible to determine which insemination contributed spermatozoa for fertilization. However, fertility levels do not decline after 13 days *post coitum* in females which have multiple inseminations. This suggests that when multiple inseminations occur spermatozoa from the 2nd or 3rd mating may contribute spermatozoa for fertilization. It is not known whether litter composition is related to the order of mating as has been demonstrated in some mammals and other animals (Dewsbury, 1982).

This study has a number of implications for laboratory rearing and field populations of *A. stuartii*. For breeding colonies maintained in the laboratory, a higher yield of embryos and pouch young will be obtained if matings are confined to the middle of oestrus. The low fertility levels produced by single inseminations at either end of the oestrous period and the contribution of fertilizing spermatozoa by each of several inseminations suggest that, in the field, the males which mate with as many females as possible, and possibly as often as possible, within the middle period of oestrus will make the greatest contribution to the next generation.

The animals for this study were obtained under permit numbers 428–1978, 81–110, 82–32 and 82–180, Fisheries and Wildlife Division, Ministry for Conservation Victoria. The Melbourne Metropolitan Board of Works kindly gave permission to trap in the Wallaby Creek Catchment Area near Kinglake Victoria and the co-operation of the staff at the catchment area is gratefully acknowledged. We thank Dr P. Woolley, Department of Zoology, La Trobe University, for the opportunity to examine some of the slide material from her thesis; Mrs I. Douglas for technical assistance; Ms B. Hage and Dr T. S. Selwood of the Department of Social and Preventative Medicine, Monash Medical School for statistical analysis; and Dr T. S. Selwood for constructive criticism of the manuscript. The project was supported by grants to L.S. from the Australian Research Grants Scheme and the Joyce W. Vickery Scientific Research Fund, Linnean Society of NSW.

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Received 1 July 1986