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Extragonadal Sperm Reserves, Sperm-Depletion Rates, Numbers of Sperm per Mating, and Fertility With Successive Matings by Intact or Unilaterally Vasectomized Rats

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ABSTRACT: Because of the high rates of sperm production and large extragonadal sperm reserves of sexually rested male rats, mating trials are insensitive for detecting test-induced alterations in sperm production rates. Mating trials might be more sensitive if, independently of any experimental treatments under study, the number of sperm per mating was closer to the minimum requirements for normal fertility. The present study was undertaken to assess the impact of unilateral vasectomy and/or matings with up to three females in succession, for 1 hour each, on the number of sperm per mating and fertility, in comparison to corresponding values for males allowed unlimited matings with a receptive female overnight. Unilateral vasectomy did not affect sperm production, extragonadal sperm reserves, or removal of contralateral sperm during ejaculation ($P > 0.05$) but caused a 50% decrease in sperm numbers per mating. Sperm output, judged from numbers of residual extragonadal sperm in unmated and mated males, was excessive (290×10^6) during conventional overnight mating with intact males and during the first and second hours of restricted mating (105 and 184×10^6 respectively, for intact males; one-half of these amounts for unilaterally vasectomized males). In contrast, sperm output during the third successive mating was minimal (nonmeasurable) but adequate, since pregnancy rates were similar for females mated first, second, or third in succession ($P > 0.05$). Since successive matings reduce the number of sperm per mating by natural methods, this approach may enhance the sensitivity of mating tests when applied for assessing the potential effects of experimental treatments on sperm production.

Key words: Spermatogenesis, epididymis, pregnancy rates.

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Mating trials are used to confirm the safety of drugs, pesticides, food additives, and other chemicals that might cause infertility or birth defects and in other studies in which fertility is to be assessed. These are important, since the birth of normal, healthy young constitutes the only mechanism for confirming integrity of the entire reproductive process (Berndtson and Clegg, 1992). Unfortunately, they tend to be insensitive for detecting adverse effects on rates of sperm production in rats and many other laboratory species, in part because most males produce and ejaculate sperm in great excess of normal requirements (Aafjes et al, 1980; Foote et al, 1986a,b; Clegg and Zenick, unpublished data; Mably et al, 1992).

The procedures used in most mating trials with laboratory animals may contribute further to their insensitivity. Mating trials are usually conducted with sexually rested males (i.e., that have not mated for several days). During sexual rest, sperm accumulate within the extragonadal ducts so that tremendous numbers are available for the first few subsequent ejaculations. Also, conventional mating trials provide the opportunity for multiple copulations, which increase the likelihood that a female will receive an adequate or excessive number of sperm. This was demonstrated in one study by Clegg and Zenick (unpublished data). They recorded similar pregnancy rates for control vs. ethoxyethanol-treated rats, for which the number of morphologically normal epididymal sperm had been reduced by 96% when three or more copulations were allowed. In contrast, pregnancy rates differed (22 vs. 60% for treated and control animals, respectively) when mating was restricted to a single copulation. This finding is consistent with a large body of evidence demonstrating a relationship between sperm numbers and fertility associated with the artificial insemination of farm animals such as cattle (Pickett and Berndtson, 1974, 1978; Foote, 1978).

Although the single copulation approach of Clegg and Zenick (unpublished data) was successful, this may also be technically challenging. Mounting by the male rat is not always accompanied by copulation. Because copulation is very brief in rats and often follows rapid pursuit of the female by the male, it may be difficult to confirm by visual observation that ejaculation has occurred when allowing a male to mount only once. In addition, the tremendous (96%) decrease in the number of morphologi-

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cally normal cauda epididymal sperm due to ethoxyethanol treatment resulted in only a 38 percentage point difference in fertility. Therefore, an evaluation of other potential approaches for reducing the number of sperm per mating in normal males seemed appropriate.

One approach used successfully to reduce sperm output of bulls by 50% (Amann and Almquist, 1961) would be to perform unilateral vasectomy on all experimental males. However, the potential for confounding interactions with other treatments under study would need to be considered. For example, over time, vasectomized rats may develop autoantibodies against sperm (Rümke and Titus, 1970) with potential adverse consequences on sperm production or epididymal function on the contralateral side. Alternatively, physical discomfort or the stress of surgery could compromise the reproductive behavior of animals used too soon after surgery. Some investigators have reported that unilateral vasectomy does not alter daily sperm production of rats (McDonald and Scothorne, 1988) or other species (Amann and Almquist, 1961; Amann, 1962) and that the sexual behavior of vasectomized rats is equivalent to that of controls (McGlynn and Erpino, 1974). Nonetheless, the ideal time interval between vasectomy and use for a mating trial might be one providing sufficient time for healing but insufficient time for autoantibody production.

Rats copulate frequently during the first several hours of cohabitation with a receptive partner. Recognizing that sperm numbers decline with successive ejaculations (Pickett and Voss, 1973; Gebauer et al, 1974; Squires et al, 1979), we hypothesized that extragonadal sperm reserves might be reduced by allowing each male to copulate frequently with one female during a controlled period of time, thereby limiting the number of sperm available for ejaculation if that male was then placed immediately with one or more subsequent partners.

Unilateral vasectomy and/or restricted periods of mating with successive female rats had not been examined as potential ways to reduce the number of sperm per mating closer to minimal requirements. In addition, neither the minimal number of sperm needed for normal fertility in the rat nor the rate of sperm depletion from the extragonadal ducts during successive matings had been determined. Thus, the objectives of this study were to characterize the rate of sperm depletion during successive matings and the relationship between sperm numbers during natural mating and fertility.

Materials and Methods

Animals

Sprague-Dawley rats, obtained from Charles River Laboratories, Wilmington, Massachusetts, at 59 days of age, were housed in individual stainless steel cages in accordance with the University of New Hampshire Animal Care and Use Committee guidelines. The vivarium provided a controlled environment with a 12:12 light : dark cycle (lights off at 10:00 AM, on at 10:00 PM), a temperature at 23 ± 1°C, and humidity of 50 ± 5%. Rats were maintained under low-intensity red lights (24 hours/day) throughout their residency to permit observation of copulatory activity during the mating trials. Food and water were provided *ad libitum*. Mating trials began when the animals were 105–109 days old, since daily sperm production of rats reaches maximal levels by 100 days of age (Robb et al, 1978).

Unilateral Vasectomy

Unilateral vasectomy was performed on the left side of 51 males, aged 98–102 days, 7 days prior to their scheduled mating trial. The 7-day time interval was chosen arbitrarily to allow sufficient time for healing but insufficient time for autoantibody production. Acepromazine (10 mg/ml), Ketamine (100 mg/ml), and Xylazine (20 mg/ml) were used for anesthesia and were administered intramuscularly at a dosage of 0.5–0.7 ml per kg of body weight. All surgeries were performed aseptically.

Vaginal Smears

Vaginal smears were taken from all females for several days during the rearing period to allow each animal to become accustomed to the procedure. Vaginal smears were taken again beginning 2 days prior to the mating trials and continued daily until that female was mated or no longer needed. Slides were air-dried and stained with a Hema-3 stain kit (Curtin Matheson Scientific, Inc., Houston, Texas) containing a methanol-based fixative and eosinophylic and basophylic solutions. Each slide was examined by two observers under oil immersion at 40× magnification. The observations were used for tentative identification of estrus, but females were only considered to be in estrus if receptivity was confirmed by visual observation of mating.

Mating Trials

Mating trials were conducted during the dark cycle, since rats are nocturnal breeders. One group of males was unmated (designated 0-hour group). Each male of a second group (1 hour) was presented with a receptive female and observed. The timing of the first copulation was recorded. Exactly 1 hour later, that female was returned to her cage. Males of a third group (2 hour) were allowed to mate with one female for 1 hour, as for the preceding group, after which that female was removed and replaced with another. Matings with the second female were timed for 1 hour as before. In like manner, males from a fourth group (3 hour) were allowed to mate three different females, each for 1 hour. The fifth group of males served as conventional mating-trial controls. These were presented with a receptive female and observed. Once copulation was observed, these rats remained together overnight (i.e., throughout the remainder of the dark period, approximately 10–12 hours, designated the 12-hour group). Assignment of males to one of the five mating schemes (0-, 1-, 2-, 3-, and 12-hour groups) was done randomly from within the intact and unilateral vasectomized populations. Assignment of female rats to their male partner was performed

randomly from the pool of receptive females available on any given day. If a female was placed in a cage with a male and seemed unreceptive, she was removed and replaced by another. If a male failed to copulate, perhaps due to inexperience, he was eliminated from the study. (Note: Males that failed to work were not assigned to the 0-hour group.) Immediately following their last assigned mating, males were sacrificed by carbon dioxide asphyxiation; unmated males were sacrificed at these same times. Body, vas deferens, epididymal, and testicular weights were recorded. Also, each testis, epididymis, and nonligated vas deferens was removed, frozen, and stored for subsequent quantification of daily sperm production and extragonadal sperm reserves. Mated females were sacrificed by carbon dioxide asphyxiation 14 days after mating. The uterus and ovaries were removed to allow assessment of the number of embryos, number of fetal resorption sites, and number of corpora lutea on each ovary.

Since the protocol required that unilaterally vasectomized males be used exactly 7 days postsurgery, they received preferential assignment of females. That is, on any given day, unilaterally vasectomized males that were scheduled for mating were assigned female partners first. Then, any remaining receptive females were assigned to the intact males on a female-available basis. Despite the priority of assignment to unilaterally vasectomized males, matings by intact males were well distributed within the 5-day mating period.

Quantification of Daily Sperm Production

Daily sperm production was quantified by the procedure of Amann and Lambiase (1969). For this, testes were thawed and the tunica albuginea was removed and its weight recorded. The testicular parenchyma was minced in 10 ml homogenization fluid (0.05% [v/v] Triton-X, 0.9% NaCl) and transferred into a commercial blender; the beaker was rinsed with 10 additional ml of homogenization fluid, which also was added to the blender. The sample was homogenized for 2 minutes. Each testis sample was then diluted in half with additional homogenization fluid.

Mature, elongated spermatids in the testis are resistant to homogenization and were enumerated via hemacytometry. Counts were conducted in duplicate by each of three different observers. If the duplicate counts of an individual differed by more than 10–12%, additional counts were taken. The counts over all three evaluators were averaged. Daily sperm production was calculated by dividing the number of homogenization-resistant elongated spermatids per gram or per testis by a time divisor of 6.10 days, which corresponds to the number of days of sperm production represented by these cells (Robb et al, 1978).

Quantification of Extragonadal Sperm Reserves

The heads of extragonadal sperm are resistant to homogenization, permitting quantification by the same method used to estimate daily sperm production. For this, each epididymis was separated into two segments, the head plus body and the tail (Robb et al, 1978). Sperm numbers were quantified in both segments. Homogenates of epididymal samples were diluted with additional homogenization fluid if necessary to provide sperm concentrations suitable for counting. Since few sperm were expected in the vas deferens (Robb et al, 1978), only 15 ml of homogenization fluid was used in processing that tissue.

Statistical Analysis

This experiment consisted of a 2 × 5 factorial design with two male status categories (intact and unilateral vasectomy) and five mating groups (0, 1, 2, 3, and 12 hour). Data were analyzed using Systat and were subjected to one-way and two-way analysis of variance followed by Tukey's test for statistical significance with unequal replication (Steel and Torrie, 1960). One unilaterally vasectomized male assigned to the 1-hour mating scheme group copulated only three times, after which he remained sexually inactive. Upon evaluation of his tissues, extragonadal sperm reserve values were extremely high; thus, data were eliminated for this one animal. Pregnancy rates were analyzed using SAS and were subjected to Fisher's exact test followed by a modified version of Tukey's test for proportions (Zar, 1984). Since a lack of statistically significant treatment effects could be due to the true absence of an effect or to limited experimental power and sensitivity, the power and sensitivity of experiments for detecting treatment effects was determined by the methods of Berndtson (1991) or of Casagrande, Pike, and Smith (as described by Zar, 1984).

Results

Body and Tissue Weights

Body weights of unilaterally vasectomized males averaged 6% less ($P < 0.01$, Table 1) than those of intact males (478.1 vs. 448.9 g. respectively), probably due to surgeries performed 7 days earlier, but no detrimental effect on the reproductive capacity of unilaterally vasectomized males (other than interference with sperm transit on the ipsilateral side) was evident from any of the other evaluations. For example, the weights of the testes, testicular parenchyma, right epididymides, and vas deferentia did not differ ($P > 0.05$) among the intact and the unilaterally vasectomized males (Table 1). The weights of the head and body of the left epididymis of intact vs. unilaterally vasectomized males also did not differ ($P > 0.05$). The weight of the tail of the left epididymis and total left epididymal weight were greater ($P < 0.01$) for unilaterally vasectomized vs. intact males, as were the paired epididymal weights. These differences can be attributed to vasectomy on the left side causing sperm accumulation followed by an increase in epididymal weight rather than to any unexpected surgically induced malfunction.

Daily Sperm Production

There was no significant difference ($P > 0.05$) in daily sperm production/g or daily sperm production/testis of intact vs. unilaterally vasectomized rats (Table 2). Similarly, there was no interaction of male status (i.e., intact or unilaterally vasectomized) by side (i.e., left or right) and for data pooled across intact and unilaterally vasectomized groups; the daily sperm production of left and right testes

Table 1. *Body and reproductive organ weights (mean ± SEM)*

Item	Intact (n = 57)	Unilateral vasectomy (n = 51)	P
Body weight (g)	478.1 ± 6.57	448.9 ± 6.20	<0.01
Testes (g)			
Left	1.66 ± 0.02	1.66 ± 0.03	>0.05*
Right	1.67 ± 0.02	1.65 ± 0.03	>0.05*
Paired	3.34 ± 0.04	3.31 ± 0.05	>0.05*
Parenchyma (g)			
Left	1.56 ± 0.02	1.54 ± 0.03	>0.05*
Right	1.56 ± 0.02	1.54 ± 0.03	>0.05*
Paired	3.12 ± 0.04	3.07 ± 0.05	>0.05*
Left epididymis (g)			
Head and body	0.30 ± 0.004	0.30 ± 0.007	>0.05*
Tail	0.29 ± 0.006	0.51 ± 0.014	<0.01
Total	0.59 ± 0.007	0.81 ± 0.017	<0.01
Right epididymis (g)			
Head and body	0.30 ± 0.004	0.29 ± 0.004	>0.05*
Tail	0.30 ± 0.006	0.28 ± 0.006	>0.05*
Total	0.60 ± 0.008	0.57 ± 0.009	>0.05*
Paired epididymal			
Weights (g)	1.18 ± 0.02	1.38 ± 0.02	<0.01
Vas deferens (g)			
Left	0.10 ± 0.002	—	>0.05*
Right	0.10 ± 0.002	0.10 ± 0.002	>0.05*

* The experiment provided at least a 90% chance for the detection of a 10% treatment response, as determined by the method of Berndtson (1991).

did not differ ($P > 0.05$). Accordingly, unilateral vasectomy had no effect on daily sperm production of the ipsilateral or contralateral testis.

Extragonadal Sperm Numbers

For intact, unmated (0 hour) male rats, the total numbers and distribution of extragonadal sperm were similar ($P > 0.05$) within the left vs. right sides (Table 3). In addition, the corresponding numbers of sperm were similar ($P > 0.05$) between the right (nonvasectomized) side of unilaterally vasectomized rats vs. the right side for intact controls. Since the experiment provided moderate power and sensitivity for detecting potential treatment effects on most of these end points (except sperm number within the vas deferens, see Table 3), it appears reasonable to conclude that unilateral vasectomy did not alter the total numbers of extragonadal sperm or the distribution of these sperm within the contralateral extragonadal ducts. In contrast, sperm numbers and their distribution were altered in the ipsilateral epididymis of unmated unilaterally vasectomized animals; sperm numbers were reduced slightly in the head and body and increased in the tail and in total (Table 3). The decreases in sperm number in the head and body are difficult to explain since daily sperm production was not altered. However, the decrease was quite small (114.5 vs. 118.8 × 10^6 for unilaterally vasectomized and intact males, respectively) and of no obvious significance relative to the objectives of this study. Also, this difference was not statistically significant when sperm numbers in the head and body of the epididymis were analyzed using data for all mating schemes. Indeed, the number of sperm in the head and body of either the left or right side was not affected by mating scheme nor was there a male status by mating scheme interaction (Table 4).

Sperm accumulated in the left tail of the epididymis (i.e., ipsilateral to the vasectomy); sperm numbers averaged 470.4 × 10^6 compared to 324.1 × 10^6 for all nonligated epididymides of unmated (0 hour) males (Table 5). Thus, 146 × 10^6 sperm accumulated in the tail during 7 days after vasectomy, averaging approximately 21 × 10^6/testis/day. The daily sperm production estimated from testicular homogenates averaged 38 × 10^6/testis/day. The reason for the disparity between the estimated daily sperm production and the actual accumulation of sperm within the epididymis is not apparent.

The left and right sides of intact males contained similar ($P > 0.05$) numbers of cauda epididymal sperm, and sperm numbers were similar for the right tail of the epididymis of intact rats vs. those for the right (nonligated) side of unilaterally vasectomized rats (Table 5). However, the main effect of mating scheme was significant ($P < 0.05$) for both the right and nonligated left cauda epididymides. These findings, and the absence of a male status by mating scheme interaction for left (nonvasectomized) cauda sperm numbers, indicate that sperm removal in re-

Table 2. *Daily sperm production (DSP) (×10^6) of intact and unilaterally vasectomized (UV) rats (mean ± SEM)*

Male status	n	DSP/g			DSP/testis		
		Left	Right	Mean*	Left	Right	Mean*
Intact	57	23.9 ± 0.56	24.5 ± 0.60	24.2 ± 0.41	37.1 ± 0.99	38.2 ± 0.98	37.6 ± 0.69
UV	51	24.6 ± 1.09	25.6 ± 0.73	25.1 ± 0.66	37.1 ± 1.27	39.2 ± 1.09	38.2 ± 0.84
Mean†		24.2 ± 0.59	25.0 ± 0.47		37.1 ± 0.79	38.6 ± 0.72	

* Means for intact and UV males did not differ ($P > 0.05$) within these experiments, which were determined by the method of Berndtson (1991) to have provided at least a 90% chance for the detection of a 10% treatment response.

† Mean for intact and UV combined.

Table 3. *Extragonadal sperm numbers (EGR, $\times 10^6$) in unmated (0 hour) intact and unilaterally vasectomized (UV) males (mean ± SEM)**

Status and side	Epididymis†			Vas deferens	Total EGR
	Head and body	Tail	Total		
Intact (n = 14)					
Left	118.8 ± 3.1[b]	321.0 ± 13.6[d]	439.9 ± 15.7[d]	3.6 ± 1.0	443.5 ± 15.9
Right	118.9 ± 3.4	327.6 ± 18.9	446.5 ± 19.7	4.3 ± 0.9	450.9 ± 19.7
UV (n = 10)					
Left	114.5 ± 24.9[c]	485.0 ± 20.5[e]	599.5 ± 32.7[e]	—	
Right	119.9 ± 4.1	323.5 ± 8.9	443.4 ± 8.3	5.9 ± 2.0	449.3 ± 9.3

* The total number of extragonadal sperm and their distribution among each segment of the extragonadal ducts was similar ($P > 0.05$) between the left vs. right sides of intact males and between the right (nonligated) sides of intact vs. UV males. From the method of Berndtson (1991), it was estimated that the present experiment provided a 90% chance of detecting 25, 30, 25, >100, and 25% changes in sperm numbers within the epididymal head and body, the epididymal tail, the total epididymis, the vas deferens, and the entire extragonadal ducts, respectively.

† [b,c] Means for the left sides of intact vs. unilaterally vasectomized rats within the same column that bear a different superscript differ at $P < 0.05$. [d,e] Means for the left sides of intact vs. unilaterally vasectomized rats within the same column that bear a different superscript differ at $P < 0.01$.

sponse to mating was similar for intact males and for nonligated ducts of unilaterally vasectomized rats. This, in turn, would suggest that ejaculatory behavior was not altered by unilateral vasectomy. Because results were similar for all nonligated epididymides, these data were pooled for the analysis of the main effect of mating scheme on residual sperm numbers. Sperm numbers in the tail of the epididymis were reduced, to a point, with increased mating activity (Table 5). Sperm numbers for the 2-, 3-, and 12-hour mating schemes did not differ ($P > 0.05$), indicating that within the first 2 hours of mating, virtually all of the sperm available for ejaculation were removed from the extragonadal ducts.

The total number of sperm within nonligated extragonadal ducts is presented in Table 6. Again, data have been excluded for the left (vasectomized) side of unilaterally vasectomized males. Numbers of sperm within the left extragonadal ducts of intact males were affected by mating scheme ($P < 0.05$); sperm numbers declined ($P < 0.05$) during the first 2 hours of mating but remained similar ($P > 0.05$) thereafter. Sperm numbers within the right extragonadal ducts did not differ ($P > 0.05$) due to male status, and there was no male status by mating scheme interaction ($P > 0.05$). However, sperm numbers averaged over the right side of intact and unilaterally vasectomized males declined ($P < 0.05$) within the first 2 hours of mating, as they did within the left side of intact males.

Since the numbers and distributions of sperm were similar within the left vs. right extragonadal ducts of intact males, and since the corresponding sperm numbers were similar for the right (nonvasectomized) extragonadal ducts of unilaterally vasectomized vs. intact males, data have been pooled across all nonvasectomized ducts of unilaterally vasectomized and intact males for presentation in Table 7. As illustrated previously (Tables 5 and 6), sperm numbers declined in the extragonadal ducts during the first 2 hours of mating, from 447.7 × 10^6 in sexually rested males to 395.2 × 10^6 after 1 hour of mating

Table 4. *Number of sperm ($\times 10^6$) in the head and body of the left or right epididymis (mean ± SEM)* of intact and unilaterally vasectomized (UV) rats*

Mating scheme	Left†			Right‡		
	Intact	UV	Mean	Intact	UV	Mean
0	118.8 ± 3.1	114.5 ± 24.9	117.0 ± 10.2	118.9 ± 3.4	119.9 ± 4.1	119.3 ± 2.5
1	129.7 ± 4.7	99.7 ± 11.7	117.2 ± 6.3	129.1 ± 5.8	120.9 ± 4.3	125.7 ± 3.9
2	118.1 ± 4.2	106.2 ± 7.3	111.8 ± 4.4	116.3 ± 4.5	118.8 ± 6.6	117.6 ± 4.0
3	123.6 ± 4.3	98.9 ± 10.0	110.7 ± 6.1	164.9 ± 45.6§	116.6 ± 4.1	139.6 ± 21.9
12	116.0 ± 3.0	117.1 ± 5.9	116.5 ± 3.2	116.1 ± 3.5	126.1 ± 5.3	121.1 ± 3.3
Mean	121.7 ± 1.9[a]	107.1 ± 5.9[b]		128.6 ± 8.2	120.4 ± 2.2	

* Numbers of males per treatment group were as follows: intact males in 0-, 1-, 2-, 3-, and 12-hour schemes = 14, 14, 9, 10, and 10, and corresponding values for UV males were 10, 10, 10, 11, and 10, respectively.

† Numbers of sperm in the left head were not affected ($P > 0.05$) by mating scheme or mating scheme by male status. The experiment provided approximately 90% power for detecting a 15% change due to treatment, based on the power and sensitivity estimation method of Berndtson (1991).

‡ Numbers of sperm in the right head were not affected ($P > 0.05$) by male status, mating scheme, or male status by mating scheme. The experiment provided approximately 90% power for detecting a 15% change due to treatment based on the power and sensitivity estimation method of Berndtson (1991). [a,b] Means within the same row which bear different superscripts differ ($P < 0.05$).

§ One rat that appeared normal in all other respects had exceptionally high numbers of sperm in the head of the epididymis (573.6 × 10^6). The mean with data for that rat excluded equaled 119.5 × 10^6.

Table 5. *Numbers ($\times 10^6$) of sperm in the tail of the left or right epididymis (mean ± SEM)*† of intact and unilaterally vasectomized (UV) rats*

Mating scheme	Left		Right‡		Residual sperm‖
	Intact	UV§	Intact	UV	
0	321.0 ± 13.6	485.0 ± 20.5	327.6 ± 18.9	323.5 ± 8.9	324.1 ± 8.7[a]
1	261.9 ± 20.0	454.1 ± 44.5	277.5 ± 19.0	243.3 ± 25.8	262.8 ± 12.1[b]
2	170.6 ± 27.9	418.2 ± 61.6	169.6 ± 26.7	202.6 ± 32.8	181.7 ± 16.7[c]
3	190.3 ± 28.5	448.6 ± 74.9	207.7 ± 32.6	160.6 ± 21.2	185.4 ± 15.7[c]
12	191.5 ± 32.3	504.3 ± 40.4	190.3 ± 31.6	159.2 ± 16.2	180.3 ± 15.7[c]
Mean		470.4 ± 23.2			

* Numbers of males per treatment group were as follows: intact males in 0-, 1-, 2-, 3-, and 12-hour schemes = 14, 14, 9, 10, and 10, and corresponding values for UV males were 10, 10, 10, 11, and 10, respectively.

† For intact males, numbers of sperm in the tail of the epididymis did not differ ($P > 0.05$) between the left vs. right sides, and there was no side by mating scheme interaction ($P > 0.05$). It was estimated (Berndtson, 1991) that the experiment provided 90% power for detection of a 25% change due to treatment.

‡ There was no significant effect ($P > 0.05$) of unilateral vasectomy and no mating scheme by male status (i.e., intact vs. UV) interaction on sperm number in the right tail of the epididymis. It was estimated (Berndtson, 1991) that the experiment provided 90% power for detection of a 30% change due to treatment.

§ Vasectomy was performed on the left vas deferens.

‖ Mean for all nonligated epididymides (i.e., left and right side of intact and right side of UV). [a,b,c] Means within the same column that bear a different superscript differ at $P < 0.05$.

and to 303.4 × 10^6 after 2 hours of mating. Sperm numbers for the 2-, 3-, and 12-hour groups were not different ($P > 0.05$), indicating that most extragonadal sperm that were available for ejaculation were removed within the first 2 hours of activity.

From the extragonadal sperm numbers summarized in Table 7, it was possible to estimate the approximate average numbers of sperm ejaculated during the various mating schemes. With conventional overnight mating, intact and unilaterally vasectomized males ejaculated an average of 290 million (i.e., 447.7 × 10^6 sperm in extragonadal ducts of sexually rested males minus 302.1 × 10^6 sperm remaining after mating, ×2 [for left plus right sides]) and 145 million sperm, respectively. Where mating was restricted to 1 hour, the average sperm output of intact and unilaterally vasectomized males was approximately 105 and 52 million during the first hour and 184 and 92 million during the second hour, respectively. It is obvious that both intact and unilaterally vasectomized males allowed to mate at will either overnight or during up to two consecutive 1-hour periods ejaculate a tremendous number of sperm (52–290 million). In contrast, because the extragonadal sperm reserves after 3 hours of mating did not differ ($P > 0.05$) from those after either 2 hours or overnight mating (and since the actual value of 325.5 × 10^6 exceeded that of 303.4 and 302.1 × 10^6 for these other groups, Table 7), it was not possible to estimate the average numbers of sperm ejaculated during the third hour of mating. However, the data do establish quite clearly that the number of sperm ejaculated during the third successive hour of mating must be quite limited.

Fertility results are shown in Table 8. There was no effect of mating scheme on pregnancy rates when analyzed by Fisher's exact test, but differences were found when mating scheme by male status means were examined by a modified version of Tukey's test for proportions

Table 6. *Total sperm numbers (mean ± SEM, $\times 10^6$) within the nonligated ducts of intact and unilaterally vasectomized (UV) rats*†*

Mating scheme	Left side‡	Right side§		
		Intact	UV	Mean
0	443.5 ± 15.9[a]	450.9 ± 19.7	449.3 ± 9.3	450.2 ± 11.9[d]
1	396.0 ± 21.5[a,b]	412.5 ± 22.3	369.8 ± 27.7	394.7 ± 17.6[d,e]
2	292.6 ± 31.7[c]	290.1 ± 30.1	325.1 ± 37.3	308.5 ± 23.9[f]
3	320.8 ± 31.7[b,c]	378.4 ± 56.6	281.8 ± 23.4	327.8 ± 30.7[e,f]
12	310.1 ± 30.7[b,c]	308.9 ± 32.5	287.1 ± 18.3	298.0 ± 18.3[f]

* Numbers of males per treatment group were as follows: intact males in 0-, 1-, 2-, 3-, and 12-hour schemes = 14, 14, 9, 10, and 10, and corresponding values for UV males were 10, 10, 10, 11, and 10, respectively.

† Numbers of sperm in the epididymis and vas deferens.

‡ Intact males only. [a,b,c] Means within the same column that bear a different superscript differ at $P < 0.05$.

§ There was no effect of male status (intact vs. UV) and no status by mating scheme interaction on the numbers of sperm in the right extragonadal ducts ($P > 0.05$). The experiment provided 90% power for detecting an approximate 25% treatment response, as judged by the method of Berndtson (1991). [d,e,f] Means within the same column that bear a different superscript differ at $P < 0.05$.

Table 7. *Numbers ($\times 10^6$) of residual sperm (per one side) in the extragonadal ducts of sexually rested or mated male rats (mean ± SEM)**

Mating scheme	Epididymides			Vas deferens	Total†
	Head and body	Tail	Total		
0	119.1 ± 1.9	324.1 ± 8.7	443.4 ± 9.3	4.5 ± 0.7	447.7 ± 9.4[a]
1	127.2 ± 3.0	262.8 ± 12.1	389.9 ± 13.5	5.2 ± 0.6	395.2 ± 13.5[a]
2	117.8 ± 3.0	181.7 ± 16.7	299.5 ± 18.6	3.9 ± 0.5	303.4 ± 18.9[b]
3	134.4 ± 14.8	185.4 ± 15.7	319.8 ± 22.6	5.8 ± 0.0	325.5 ± 22.9[b]
12	119.4 ± 2.4	180.3 ± 15.7	300.0 ± 15.6	2.4 ± 0.4	302.1 ± 15.6[b]

* Means for all nonligated extragonadal ducts (i.e., left and right sides of intact and right side of unilaterally vasectomized males) of 24, 24, 19, 21, and 20 males in the 0-, 1-, 2-, 3-, and 12-hour mating schemes, respectively. The experiment was estimated (Berndtson, 1991) to have provided 95% power for the detection of 10, 15, and 15% changes in sperm numbers in the head and body, tail, and total epididymis, respectively, but only 80% power for detecting a 70% treatment response in sperm numbers within the vas deferens.

† [a,b] Means within the same column that do not bear a similar superscript differ ($P < 0.05$).

(Zar, 1984). Pregnancy rates were similar ($P > 0.05$) for all groups for which mating was restricted to 1 hour (i.e., for the 1-, 2-, and 3-hour mating groups with intact and unilaterally vasectomized males), but the pregnancy rates of 90 and 100% for intact and unilaterally vasectomized males allowed to mate overnight were greater than those of 57.6 and 61.3% for those females mated during the first hour of breeding to intact and unilaterally vasectomized males, respectively.

Among female rats that became pregnant, there was no difference ($P > 0.05$) in litter size due to male status (i.e., unilateral vasectomy vs. intact), mating scheme, or their interaction (Table 8). Thus, females receiving fewer sperm had litters of a size similar to those for females receiving larger numbers of sperm. As for pregnancy rate and for other measures of fertility discussed subsequently, most measures of fertility are associated with large inherent variability that can make confirmation of actual treatment effects by statistical significance quite difficult. However, the similarity of the actual observed means (Table 8) provides little reason to suspect that fertility was affected by the present treatments.

To remove ovulation rate as a variable in fertility rates, the number of fetuses per corpus luteum (CL) was assessed in pregnant females (Table 8). Male status, mating scheme, and the male status by mating scheme interaction were without an effect ($P > 0.05$) on the fetus/CL ratio. The overall mating scheme means for the number of fetuses/CL ranged from 0.63 to 0.67.

Fetal resorption rates in pregnant females (Table 8) were not affected by treatment. Male status (i.e., intact vs. unilaterally vasectomized), mating scheme, and the male status by mating scheme interaction were without an effect ($P > 0.05$). The overall mating scheme means for the number of resorptions ranged from 0.80 to 1.20.

Discussion

To our knowledge, this represents the first report characterizing the rate of sperm depletion in male rats during successive, natural mating periods and the first study to examine the relationship between sperm numbers and fertility during natural mating. For the purpose of extending

Table 8. *Fertility of female rats mated to intact or unilaterally vasectomized (UV) males*

Item	Male status	Mating scheme*			
		1	2	3	12
Pregnancy rate (%)	Intact	57.6 (19/33)[a]	68.4 (13/19)[a,b]	80.0 (8/10)[a,b]	90.0 (9/10)[b]
	UV	61.3 (19/31)[a]	76.2 (16/21)[a,b]	63.6 (7/11)[a,b]	100.0 (10/10)[b]
Litter size†	Intact	14.6 ± 0.33 (19)	13.3 ± 0.62 (13)	13.9 ± 1.23 (8)	13.2 ± 1.75 (9)‡
	UV	13.2 ± 0.72 (19)	14.5 ± 0.45 (16)	12.0 ± 1.25 (7)	14.7 ± 0.50 (10)
Fetuses/corpus luteum†	Intact	0.72 ± 0.03 (19)	0.65 ± 0.05 (13)	0.65 ± 0.07 (8)	0.69 ± 0.09 (9)
	UV	0.58 ± 0.04 (19)	0.69 ± 0.04 (16)	0.60 ± 0.07 (7)	0.63 ± 0.04 (10)
Fetal resorptions†	Intact	0.90 ± 0.22 (19)	0.93 ± 0.22 (13)	0.63 ± 0.32 (8)	0.78 ± 0.36 (9)
	UV	1.50 ± 0.50 (19)	0.81 ± 0.19 (16)	1.00 ± 0.38 (7)	1.40 ± 0.31 (10)

* [a,b] Means that do not bear a similar superscript differ ($P < 0.05$).

† Per pregnant females only (number of pregnant females is given within parentheses). It was estimated that the present study provided ~80–90% power for detecting 50% changes in litter size or the number of fetuses/corpus luteum and extremely limited power and sensitivity for detecting changes in the number of fetal resorptions.

‡ Includes one female with no viable fetuses but one resorption.

the present observations to future mating trials, it is important to note that the rates of daily sperm production found herein are similar to those reported in the literature (Amann, 1970, 1981, 1982, 1986; Robb et al, 1978; Working, 1988). The normalcy of our male rats was also supported by our finding that ejaculation reduced sperm number primarily within the tail rather than the head and body of the epididymis, as seen by others for the rat (Robb et al, 1978) and other species (Hale and Almquist, 1960; Lambiase and Amann, 1969; Amann, 1981). Regarding our female population, the 90% pregnancy rate for animals allowed to mate overnight was consistent with the typical 80–90% range reported in the literature (Khera et al, 1984; Thompson et al, 1984; Working et al, 1985; Dostal et al, 1988; Linder et al, 1988; Velez et al, 1988; Clegg and Zenick, unpublished data). Finally, neither male status, mating scheme, nor their interaction had an effect on litter size (Table 8). Thus, females receiving fewer sperm had litters of a size similar to those for females receiving larger numbers of sperm. Setchell et al (1988) reported that litter size in normal female rats was decreased when they were mated to subfertile males. However, our results are consistent with those of Robaire et al (1984) who reported that >90% decreases in epididymal sperm numbers caused by testosterone administration were without any effect on fertilizing potential or litter size. The average litter sizes of 12.0–14.7 in the present study (Table 8) are similar to those reported in the literature (John et al, 1983; Khera et al, 1984; Robaire et al, 1984; Thompson et al, 1984; Working et al, 1985; Velez et al, 1988). Based on the forgoing comparisons, the rats used in the present investigation appear quite representative of the larger population of sexually mature individuals used in most other reproductive studies.

Although we were unable to establish the minimal number of sperm required for normal fertility, the sperm output of normal males during conventional mating tests exceeds this threshold by many orders of magnitude. It is not surprising, therefore, that fertility often is unaffected by treatments that induce severe depressions in spermatogenesis. In that regard, it was interesting to note that pregnancy rates were higher for animals mated overnight than during the first restricted 1-hour period (Table 8). It is unlikely that this was attributable to high sperm numbers. First, sperm output during the first hour of mating exceeded that for several other groups for which fertility did not differ from that for the overnight controls (Table 7). Second, if sperm number was a limiting factor, it would be unlikely that reductions from the 145–290 $\times$ 10^6 range (for females mated overnight) to the 52–105 $\times$ 10^6 range (for the 1-hour mating groups) would produce a significant reduction in fertility, while subsequent reductions in sperm number per mating to nonmeasurable levels (for females in the 3-hour groups) would restore fertility to the levels for conventional overnight controls. Perhaps frequent copulation during cohabitation overnight enhances sperm transport in the female, resulting in higher pregnancy rates. Alternatively, these findings (Table 8) may reflect the large, inherent variability in pregnancy rates and the associated difficulty in distinguishing differences in fertility that are due to treatment from those that might have arisen by chance, as discussed elsewhere in detail (Berndtson et al, 1997).

The present finding that male rats ejaculated more sperm during the second hour than during the first hour of mating was surprising, since a progressive decrease in sperm numbers in each successive ejaculate taken after sexual rest was expected from evidence with other species (Amann, 1962; Freund, 1963; Desjardins et al, 1968; Gebauer et al, 1974; Squires et al, 1979). This unexpected observation may reflect the acquisition of sexual experience during the first hour of cohabitation. Alternatively, it could simply reflect the inherent variability in reproductive characteristics, as cited above.

The present results establish that short-term unilateral vasectomy provides a viable approach for reducing the sperm output of male rats to one-half of normal and that sperm number per mating can be reduced dramatically and naturally by a successive mating approach. The benefits of a successive mating approach with intact or unilaterally vasectomized males await confirmation through actual application. Nonetheless, since normal male rats ejaculate nearly all available sperm during the first 2 hours of cohabitation, females mated immediately thereafter can be expected to receive an adequate, yet very small, number of sperm in comparison to those assigned to conventional overnight matings. By reducing sperm numbers per mating closer to the minimal requirement, the successive mating approach should reduce the magnitude of any added treatment-induced impairment of spermatogenesis needed to induce an actual reduction in fertility. It might also induce larger, more readily detectable changes in fertility (Berndtson et al, 1997) than would result with a more conventional approach.

Total sperm number is not the only factor bearing on the fertility of mated females. For example, a treatment could alter the willingness or ability of the male to mate or might induce changes in the quality of the spermatozoa, leading to fertilization failure, early embryonic loss, etc. Although observations of mating were necessary to control the length of cohabitation, they also allow assessment of adverse agent effects on reproductive behavior. Also, each reduction in the total number of sperm per mated female would reduce proportionately the number of competent spermatozoa received (i.e., those that are viable, morphologically and genetically normal, etc.). This could enhance, and certainly would not diminish, the chances that adverse treatment effects on spermatozoal

integrity would be manifested in reduced fertility. Second, the successive mating approach might yield greater insight into the probable severity of agent effects than more conventional protocols. The numbers of sperm per mating are quite high during the first two 1-hour mating periods but very low thereafter. Thus, a treatment that reduced the fertility only of those females mated third in succession would tend to indicate an effect of less severity than if the same treatment also reduced fertility of females mated first or second in succession.

While considering potential merits of the successive mating approach, it should be noted that one may perform artificial insemination with a standard number of spermatozoa in some species. This may be an excellent approach for some studies, but its appropriateness depends, in part, on the objectives of the investigator. For example, when sperm number/mating is held constant for all treatment groups, any resulting differences in fertility would need to be attributed to qualitative differences in the sperm rather than to sperm production per se. Our interest in a natural mating approach was based on: 1) the extensive utilization and acceptance of conventional mating trials, 2) an expectation that mating trials will continue to be used because of the valuable information on congenital defects, embryonic mortality, etc. only they can currently provide, and 3) our specific interest in rendering fertility more sensitive and responsive to changes in sperm production.

Among the disadvantages of the successive mating approach is that the visual observation needed for timed matings is relatively labor intensive. Although some males mated very quickly upon presentation with a receptive female, many responded quite slowly. Also, several males (20/128) were eliminated from the study because they failed to copulate when presented with females of confirmed receptivity (females that were receptive when placed with other males). We suspect that this problem might be overcome in future studies, at least in part, by the use of experienced rather than inexperienced males. Also, because our protocol specified that unilaterally vasectomized males would be used for mating exactly 7 days postsurgery and that all males would be used when 105–109 days of age, it was necessary for us to acquire and maintain more females than were actually used to ensure that sufficient numbers would be receptive when needed. Less stringent requirements may be acceptable for many future studies.

The findings that conventional mating protocols are insensitive and that sperm output is reduced after unilateral vasectomy and during successive matings are not unique. However, the problem of mating-trial insensitivity had largely been ignored, and well-established physiological principles characterizing the relationship of sperm output to frequency of copulation had not been exploited to improve the value of natural mating trials. The present study identifies one promising approach, with intact or unilaterally vasectomized males, that may serve as an effective alternative by which to render future mating trials more sensitive and reliable than their conventional counterparts.

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