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Reduced Interlitter Variability in Rats Resulting from a Restricted Mating Period, and Reassessment of the "Litter Effect" [1]

J. F. HOLSON, W. J. SCOTT, D. W. GAYLOR AND J. G. WILSON
Children's Hospital Research Foundation and Department of Pediatrics, University of Cincinnati, Cincinnati, Ohio 45229, and National Center for Toxicological Research, Jefferson, Arkansas 72079

ABSTRACT Rats were mated for two or 15 hours and variability of day-12 embryos in weight, protein content, and [^{3}H]thymidine incorporation was compared in the long mating period (LMP) and short mating period (SMP) groups by a 2-level nested analysis of variance. Variability in day-20 fetal weight was similarly compared. In both groups day-12 embryonic weight was relatively more variable than day-20 fetal weight, and variability was less in SMP than LMP animals for each comparison made, although statistical significance was attained only for thymidine incorporation. "Litter effects" were noted but not of the magnitude reported by other investigators. It was concluded that inappropriate statistical methods have encouraged the belief that among-litter variability usually exceeds within-litter fetal weight variability. The teratological implications of reduced developmental variability and the "litter effect" are discussed.

For many years embryologists and teratologists have felt handicapped by inter- and intralitter variation in polytocous animals, especially rodents (Burlingame and Long, '39; Nicholas, '42; Christie, '64; Jollie, '64; Alliston and Pardee, '73). Jensh et al. ('70) and Barr ('71) found that in Wistar-derived rats near-term fetal weight variability among litters was greater than the variability within litters when expressed as mean squares. Since most investigators using pregnant rats employ an overnight mating period a 12-16-hour variation in copulation time is possible. Trying to reduce interlitter variation that may have this origin, a few investigators have used a restricted mating period (Jollie, '64; Dagg et al., '68; Degenhart et al., '68; Krowke et al., '71; Cox and Gunberg, '72; Kohler et al., '72).

Cox and Gunberg ('72) commented on the uniformity of somite counts of 11- and 12-day rat embryos in litters produced by a 2-hour mating period. Barr ('71), however, concluded that a 2-hour opposed to an overnight mating period offered no conclusive advantage in reducing variability. To judge variation he used fetal and placental weight on day 21.5 of rat gestation. Since the relative growth rate is much greater during early than late pregnancy, variation in embryonic age should be more clearly reflected by weight differences on day 12 than day 20 of gestation. Nicholas ('42) suggested that variation present during early gestation might be compensated for by the time parturition approaches, although he supplied no experimental data to support his contention. We examined interlitter variability in relation to duration of the breeding time by studying early embryos as well as near-term fetuses.

MATERIALS AND METHODS

Royalhart rats, derived from the Wistar stock, were used. A light:dark ratio of 14 hours:10 hours was used; the lights being off from 2 AM to noon. Rats for the long mating period (LMP) and short mating

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period (SMP) were housed in the same room. All females were nulliparous and of comparable ages and weights. Rats for the LMP group were bred by placing females in vaginal estrus each with a male for approximately 15 hours. Vaginal lavages were taken from LMP animals at 10-11 PM and successful mating was confirmed by the presence of sperm in the vaginal smear. Rats in the SMP group were bred by a modification of the technique described by Blandau et al., ('41). At 9 AM (7 hours into the dark cycle) females were manipulated perineally in limited lighting; instead of lordosis a characteristic ear quivering in response to perineal manipulation was chosen as the index of sexual receptivity and females exhibiting this sign were each immediately placed with a male for two hours. Three to five hours after removal from males the vaginal lavages were examined for the presence of sperm. The tightly adherent and frequently inconspicuous vaginal plug encountered soon after mating often interfered with obtaining an adequate lavage. All females were randomly chosen from stock animals. In both breeding methods pregnancy was considered to have begun at 10 AM, and the day of finding sperm was considered day 0 of pregnancy.

Pregnant females were killed at two times — between 10 and 11 AM on days 12 and 20. In the former the uterine position of each conceptus was recorded, and the embryos were removed, dissected free of their membranes, rinsed in isotonic saline, carefully blotted, and weighed individually. For measuring the rate of DNA synthesis 200 μCi/kg [^{3}H]thymidine were administered ip to some females two hours before being killed. Methods of preparing specimens and of isotopic counting were described previously (Ritter et al., '71). Embryonic protein determinations were done by the method of Lowry et al. ('51).

After the uterine positions of day-20 fetuses were recorded they were dissected free of their membranes, the umbilical cords clamped and severed, and fetuses weighed individually immediately after being blotted dry.

RESULTS

The resorption rates of the SMP and LMP groups killed at day 12 were not statistically different although the mean litter size in the former was 3.5 more than in the latter, which was a statistically significant difference ($P < 0.01$). However, there were no statistically significant correlations between litter size and the

TABLE 1

Mean day-12 rat embryonic weight per litter after short and long mating periods

Short mating period			Long mating period		
No. embryos	Embryo wt. (mg)		No. embryos	Embryo wt. (mg)	
	$\bar{x} \pm SD$	*range*		$\bar{x} \pm SD$	*range*
11	17.86 ± 3.93	9.14-22.35	12	16.03 ± 3.59	7.27-21.45
14	20.01 ± 3.27	12.51-26.04	9	13.76 ± 2.39	9.78-18.19
12	19.66 ± 2.56	13.91-22.73	12	23.37 ± 3.20	18.30-29.61
16	15.85 ± 2.07	12.18-18.89	9	19.09 ± 1.94	16.52-21.41
14	15.34 ± 1.96	12.24-18.39	12	23.99 ± 2.36	19.31-27.15
15	20.07 ± 3.43	14.68-26.14	12	20.03 ± 3.67	13.33-26.13
15	16.23[1] ± 2.07	13.33-19.16	11	21.83 ± 2.77	17.47-26.28
16	17.14 ± 3.00	11.32-20.88	13	17.09 ± 2.37	10.55-19.68
16	18.41 ± 2.82	15.08-22.98	9	15.20 ± 2.18	10.18-17.40
15	21.40 ± 2.10	17.89-25.08			
14	21.44 ± 2.02	19.21-24.53			
	$\bar{x} \pm SD$			$\bar{x} \pm SD$	
	18.45 ± 3.36			19.15 ± 4.38	

[1] Embryo 3 SD below mean omitted.

variances of the features measured, as determined by normal correlation coefficient analysis, and it is therefore probable that the differences in litter size did not affect comparisons of the variances. Data pertaining to embryonic weight, protein content, and [^{3}H]thymidine incorporation are presented in tables 1-3 and day-20 fetal weight data in table 4. The means in the tables were obtained by dividing the sum of all values in a group by the total number of embryos or fetuses in the group.

Before making the study it was decided to exclude values lying outside three standard deviations of the mean. Such values were considered to represent atypical embryos. A 2-level nested analysis of variance was computed for each variable. The form of each analysis of variance is shown in table 5. Comparisons of components of variance ($\hat{\sigma}^2_w$ or $\hat{\sigma}^2_a$) and total variances (σ'^2) were made by an approximate F test of their ratios using Satterthwaite's ('46) formula for the degrees of freedom.

There was a statistically significantly smaller ($P < 0.01$) total variance in thymidine incorporation in SMP than in LMP embryos, but the total variances in embryonic and fetal weight and protein content were not statistically significantly different

TABLE 2

Mean day-12 rat embryonic protein content per litter after short and long mating periods

Short mating period			Long mating period		
No. embryos	Embryo protein (μg)		No. embryos	Embryo protein (μg)	
	$\bar{x} \pm SD$	*range*		$\bar{x} \pm SD$	*range*
11	777 ± 151	415-935	11	665[1] ± 79	500-800
14	857[1] ± 113	655-1060	9	698 ± 111	510-855
12	873 ± 88	655-1015	12	929 ± 126	675-1150
16	726 ± 64	615-855	9	769 ± 100	560-930
15	713 ± 70	555-810	12	994 ± 78	870-1130
15	829 ± 120	625-1040	12	748 ± 137	505-915
15	620[1] ± 58	535-725	11	835 ± 123	620-990
15	776 ± 77	605-900	13	817 ± 108	525-970
14	808 ± 73	700-925	9	737 ± 100	495-810
	$\bar{x} \pm SD$			$\bar{x} \pm SD$	
	772 ± 117			806 ± 147	

[1] Embryo 3 SD below mean omitted.

TABLE 3

Mean day-12 rat embryonic [^{3}H]thymidine incorporation per litter after short and long mating periods

Short mating period			Long mating period		
No. embryos	cpm/mg embryo		No. embryos	cpm/mg embryo	
	$\bar{x} \pm SD$	*range*		$\bar{x} \pm SD$	*range*
11	288 ± 20	248-317	12	327 ± 28	281-385
16	300 ± 16	274-325	12	278 ± 20	255-327
15	286 ± 26	256-363	9	302 ± 22	272-344
15	308[1] ± 22	269-348	12	285 ± 13	271-311
15	324 ± 28	272-366	12	209 ± 25	137-248
14	307 ± 19	280-342	13	323 ± 20	287-353
			9	333 ± 28	283-381
	$\bar{x} \pm SD$			$\bar{x} \pm SD$	
	303 ± 25			293 ± 46	

[1] Embryo 3 SD below mean omitted.

TABLE 4

Mean day-20 fetal weight per litter after short and long mating periods

Short mating period			Long mating period		
No. fetuses	Fetal wt. (g)		No. fetuses	Fetal wt. (g)	
	$\bar{x} \pm SD$	*range*		$\bar{x} \pm SD$	*range*
12	3.49 ± 0.259	3.09-3.74	13	3.34 ± 0.298	2.94-3.92
16	3.66 ± 0.212	3.21-4.04	15	3.14 ± 0.277	2.54-3.66
11	3.46 ± 0.278	3.01-3.97	12	3.60 ± 0.139	3.44-3.91
12	3.37 ± 0.236	3.05-3.80	13	3.34 ± 0.240	2.85-3.80
15	3.18 ± 0.135	2.98-3.45	14	3.43 ± 0.150	3.13-3.81
14	3.45 ± 0.207	3.08-3.70	14	2.89 ± 0.178	2.53-3.17
11	3.49 ± 0.167	3.23-3.76	13	3.43 ± 0.316	2.89-3.92
	$\bar{x} \pm SD$			$\bar{x} \pm SD$	
	3.44 ± 0.253			3.30 ± 0.316	

TABLE 5

Form of 2-level nested analysis of variance

Source of variation	Degrees of freedom	Mean square	Expected mean square
Among litters	L-1	A	$\hat{\sigma}^2_w + K\hat{\sigma}^2_a$
Within litters	N-L	W	$\hat{\sigma}^2_w$
Total	N-1		

Let a_i represent the deviation of the average of the ith litter mean from the overall mean, with a variance among litters of σ^2_a. The deviation of the jth embryo or fetus from the ith litter mean is denoted by w_{ij} with a variance within litter of σ^2_w. In selecting an embryo or fetus at random from the experiment its deviation from the mean is the sum of these two components $(a_j + w_{ij})$ which has a total variance of $\sigma^2 = \sigma^2_a + \sigma^2_w$. This total variance is estimated from the analysis of variance by $\hat{\sigma}^2 = [(K-1)W+A]/K$ with approximate degrees of freedom, *f*, given by Satterthwaite's ('46) formula, $f \sim [(K-1)W+A]^2 / [[(K-1)W]^2/(N-L) + A^2/(L-1)]$. Estimates of σ^2_w, σ^2_a, and σ^2 are given in table 6.

L, number of litters.

N, number of animals (embryos or fetuses).

N_i, number of animals (embryos or fetuses) in the ith litter.

$K, \frac{1}{L-1}\left[N - \frac{\Sigma N_i^2}{N}\right]$

σ^2_w, within-litter variance.

σ^2_a, among-litters variance.

TABLE 6

Estimates of variance components in SMP and LMP groups for protein content, embryonic weight, [^{3}H] thymidine incorporation, and fetal weight

	$\hat{\sigma}^2_w$	$\hat{\sigma}^2_a$	$\hat{\sigma}^2$
Protein (μg^2)			
SMP	6,587	6,804	13,391
LMP	10,910	8,814	19,724
Embryo wt. (mg^2)			
SMP	5.79	5.55	11.34
LMP	7.61	9.11	16.72
Thymidine (CPM^2)			
SMP	368	231	599
LMP	522	1,828	2,350
Fetal wt. (g^2)			
SMP	0.039	0.016	0.055
LMP	0.045	0.048	0.094

(table 7). However, in all instances the estimated $\hat{\sigma}^2$ for SMP animals was numerically less than for LMP animals. It was calculated that approximately 50 litters would be required to show statistically significantly reduced variances in SMP animals if results similar to those in the present study were to prevail.

The $\hat{\sigma}^2_{SMP}/\hat{\sigma}^2_{LMP}$ ratios in table 7 provide estimates of the proportion of SMP animals that would be required to give the same precision as LMP animals (Anderson and Bancroft, '52). For example, in measuring protein content the same precision would be expected from using only 68% as many SMP litters as LMP litters.

We were interested in comparing the relative magnitude of the litter component of variance, $\hat{\sigma}^2_a$, with the within-litter component of variance, $\hat{\sigma}^2_w$, because previous workers (Jensh et al., '70; Barr, '71) had used the F ratio of the mean squares which is a comparison of $(\hat{\sigma}^2_w + K\hat{\sigma}^2_a)$ with $\hat{\sigma}^2_w$, and

TABLE 7

Ratios of σ^2 for SMP to LMP animals

	$\hat{\sigma}^2_{SMP}/\hat{\sigma}^2_{LMP}$	Significance levels
Protein	0.68	$P < 0.20$
Embryo wt.	0.68	$P < 0.20$
Thymidine	0.25	$P < 0.01$
Fetal wt.	0.59	$P < 0.10$

which because of K may overemphasize the "litter effect." The component of variance among litters was significantly reduced ($P < 0.05$) in the SMP group as regards thymidine incorporation (table 6). For each variable measured the among- and within-litter variances were smaller, but not significantly so, in the SMP groups than in LMP groups. Comparison of among- with within-litter variances for SMP and LMP groups did not reveal a consistent pattern. By definition there is a statistically significant "litter effect" when the among-litter mean squares are significantly larger than the within-litter mean squares, i.e., the among-litter component of variance is significantly larger than 0. Therefore these data indicated a trend toward "litter effects."

Dividing the standard deviation ($\hat{\sigma}$) by the mean and multiplying by 100% gives a standardized percent value (coefficient of variation) which can be used to compare the extent of dispersion about two means that are not equivalent (tables 1, 4). These percentages for SMP day-12 and -20 weights are 18 and 7%, respectively, and for LMP day-12 and -20 weights, 21 and 9%, respectively. If one accepts weight as an indicator of development, these data demonstrate a striking decrease in the relative magnitude of variance as gestation progresses, as hypothesized by Nicholas ('42). A similar trend was noted by Otis and Brent ('54) who reported that developmental variance was less apparent after than prior to day 12 of gestation in mice.

DISCUSSION

An understanding of the factors contributing to intra- and interlitter variability in polytocous species is important for two primary reasons. First, intralitter variation in embryonic development may be responsible to some extent for the variability of responses (death, various degrees of malformation, normal) frequently observed (Alliston and Pardee, '73; Wilson, '73). If the spectrum of defects produced in a litter exposed to a teratogen is due to or influenced by intralitter variation in developmental age or stage, studies attempting to elucidate mechanisms of teratogenesis will have to take such factors into account. Second, the source and magnitude of variation may greatly influence statistical treatment of teratological data. Recently 3 editorials appeared in this journal presenting reasons for choosing the fetus or the litter as the experimental unit (Becker, '74; Kalter, '74; Staples and Haseman, '74). During the last several years the litter has more and more frequently been taken as the experimental unit, owing largely to the influence exerted by toxicologists and statisticians (Weil, '70; Healy, '72), and perhaps to some extent to inferences made by Jensh et al., ('70) and Barr ('71). Haseman and Hogan ('75) recently presented strong reasons for using the litter unit.

In general, statisticians have preferred using the litter as the unit of comparison because it more closely approximates the classically designated experimental unit and since treatment is given to the pregnant female. Because it is not usually known to what extent factors within individuals of a given litter influence individual responses it is difficult to oppose analysis-by-the-litter. Additionally, tendencies to construct dose-response curves for teratogenic agents have augmented use of the litter unit. In an exceptionally large teratology study where cleft-palate incidence, fetal-weight reduction, and resorption rate were analyzed in a dose-response fashion, it was found that very poor fitting of data to probit and regression lines occurred when the fetus was used as the experimental unit instead of the litter (Nelson, '74, personal communication). The

perplexity of the problem is compounded by the paucity of knowledge of teratogenic mechanisms and by the fact that the real answer is probably a compromise between the litter and the fetus, depending on the nature of the teratogen and its interaction with unidentified factors.

Studies by Jensh et al. ('70) and Barr ('71) in which mean squares of fetal and placental weight were compared among and within rat litters indicated to these authors that there was a consistent "litter effect." They suggested that it was more important to increase the number of litters used than rely on large numbers of fetuses.

We also found "litter effects." However, there was no trend indicating a significantly larger among- than within-litter variance. Therefore, we cannot subscribe to the view that existence of the "litter effect," in itself, necessitates placing emphasis on litter number rather than number of fetuses. In the present study the within-litter variance was not statistically significantly different from among-litter variance for day-20 fetal weights in the SMP group. Actually the within-litter variance was numerically larger than the among-litter variance for the SMP group. If these differences were real they might be attributed to the effect of the reduced mating period. Close similarity between among- and within-litter variances occurred in the LMP group. For thymidine incorporation in day-12 embryos, however, there was a significantly larger ($P < 0.05$) litter effect in the LMP than SMP group. The very large difference in among-litter variability for the SMP and LMP groups substantiates the effectiveness of the reduced mating period in decreasing among-litter variability.

Other variables probably did not reach generally accepted significance levels because the number of animals used was too small. Testing for significant differences between variances or components of variances requires a much larger number of experimental units than comparisons between mean values. Jensh et al. ('70) demonstrated that an increase in the number of litters increased the level of significance obtained in comparisons between variances. Alternatively, the larger litter sizes in the SMP groups (day-12 embryos) may have caused larger among-litter variance in the SMP group thereby masking any significant reduction of among-litter variance due to the shortened mating period. Jensh et al. ('70) reported that among-litter variance of fetal weights increased as litter size increased.

Jensh et al. ('70) and Barr ('71) obtained mean squares among litters that were significantly larger than mean squares within litters, thereby indicating "litter effects." They did not, however, obtain separate estimates of the within- and among-litter components of variance to determine their relative effects. The simple F ratio test used by these authors overemphasized the differences among litters because the litter mean squares contains the within-litter components of variance; but more importantly, because the among-litter component of variance is multiplied by K in the F ratio, which is the approximate mean litter size. In our analysis of variance not only were mean squares compared but each was estimated by $\hat{\sigma}^2_w + K\hat{\sigma}^2_a$ for among litters and $\hat{\sigma}^2_w$ for within litters and then the components of variance were tested for significant differences.

Barr ('71) suggested that fetal weights in an LMP group indicated that 18 of the 20 animals used conceived within a relatively short span of time, and then concluded that the shorter mating period offered no advantage in reducing fetal weight variability.

We also believe that much of the variability in fetal weights can be attributed to a relatively few females in the group. All rats in a colony are not synchronized as to the stage of their estrus cycle, and even if one selected animals with closely similar proportions of cell types in vaginal lavages there is no doubt that a few animals would still deviate significantly from the group in time of reaching peak receptivity to the male. The elimination of variant litters, which in the present study

appears to have been accomplished in the SMP group, would be desirable for many experimental studies.

Undoubtedly other factors also influence conception time and its variability, e.g., sperm transit time, ovulation time, etc., all of which contribute to the "litter effect." Postconception events such as implantation may also contribute to this phenomenon. It is doubtful that a reduced mating period affects such factors. Although a shortened mating period reduced among-litter variability the remaining among- and within-litter variability was striking. Studies directed at reducing still further such variability are desirable.

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