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**A STUDY OF THE FLUCTUATING FACTORS IN
THE SEMINIFEROUS EPITHELIAL CYCLE IN MICE
— WITH SPECIAL ATTENTION TO STATISTICAL
STUDIES ON DIURNAL FLUCTUATIONS AND ON
VARIATIONS DUE TO TESTES AND TESTIS LOCI IN
THE RELATIVE FREQUENCIES OF THE STAGES —**

Weng Kong SUNG

*Department of Veterinary Obstetrics
Faculty of Veterinary Medicine
Hokkaido University, Sapporo, Japan*

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A total of 30 male mice, DD strain, between 50~60 days old, were divided into 6 groups consisting of 5 mice each. All the mice had been reared in an almost constant temperature at between 22~24°C and they also had an almost constant daily period of natural and artificial light from about 6 am to about 6 pm. These conditions were maintained throughout the experimental period. The groups were killed at four-hour intervals over a 24-hour period. Cross sections of seminiferous tubules were examined microscopically to determine whether the diurnal fluctuations and variations due to testes within animals or due to loci within testes might exist in the relative frequencies of the stages. Identification of each stage of the cycle applied here was similar to that described previously by ROSEN-RUNGE & GIESEL. The method of testing for significant differences in the frequencies among groups of each experiment is based on discriminatory analysis and the results obtained were as follows:

- 1) A typical cellular association arrangement for each stage was found in all but a few seminiferous tubules observed here.
- 2) No obvious diurnal fluctuation in the relative frequency of the stages was found among the 6 times required for removal of testes or among the uniform periods dispersed throughout a 24-hour period.
- 3) Significant differences in the frequencies of the stages were found between testes within individuals and among 3 loci within testes ($P < 0.05$).

Analysis of the stages of the seminiferous epithelial cycle (hereafter referred to as cycle) is one of the methods effective in revealing the kinetics of spermatogenesis. The relative duration (frequency) of stages is considered to be the length of time relative to one cycle, and it is generally admitted that the duration of the different stages of the cycle is a biological constant within a species. The question is whether the constancy of the relative frequencies of the stages is consistently maintained and not influenced by certain factors.

If the stages are influenced, the following phenomena will appear: 1) a change in the absolute duration of the cycle; 2) a change in the relative length of time of each stage making up a cycle; and 3) a disorder of the cellular association and the appearance of undeveloped or degenerated germ cells. Until now no one has reported any factors influencing the absolute duration of the cycle within a species (CLERMONT, 1972). At present qualitative and quantitative research on the germ cells is in progress, and the physiological degeneration and renewal of the germ cells^{3,4,6,7,10,13,17}) or the diurnal fluctuations of the frequency of cell division^{2,12,15,16}) are questions under discussion. As to the change in the relative length of the cycle, there have been several studies published on the difference in the relative frequencies of the stages among individuals, between testes and among testis loci; however, no uniform conclusions have been reached. Very few works on the diurnal fluctuation of the relative frequencies of the stages have been reported, with the exception of the present author's fundamental studies on mice in this area. The main objectives of the present research were to examine closely the variation of the relative frequencies of the stages between testes and among testis loci and to analyse the findings statistically.

MATERIALS AND METHODS

Male DD strain mice, ranging from 50 to 60 days of age and 22 ± 0.5 g in body weight, and never employed for breeding, were used in this study. They had been reared in an almost constant temperature at between $22 \sim 24^\circ\text{C}$ and in addition had an almost constant daily period of natural and artificial light from 6 am to 6 pm. A total of 30 mice were divided at random into 6 groups consisting of 5 mice each. The groups were killed in April 1966 by cervical dislocation at four-hour intervals over a period of 24 hours from 6 pm. Immediately after killing, the testes were fixed in Bouin's solution. Successive histological sections 10μ thick cut midsagittally from each testis were prepared and stained with hematoxylin-eosin at every 20 sections, at intervals of 200μ in the testis. The cycle was divided into eight stages based on criteria described by ROOSEN-RUNGE & GIESEL. All of the round or ovoid tubules were examined. Discriminatory analysis was made on the relative frequencies of the stages. Prior to statistical analysis the percentages were transformed to arcsine.

RESULTS

Most of the seminiferous tubules examined showed typical well-defined cellular associations, which are characteristic of the stage. Most tubules were

easily recognized in this study; those unidentifiable were eliminated from the statistical analysis. These were: 1) two or more stages found in an examined tubule; 2) the presence of the secondary spermatocytes in the cellular associations besides stage 4; 3) degeneration of germ cells; and 4) incomplete cellular association, which showed a lack in some types of germ cells. The mean relative frequencies for stage 1 through 8 are shown in table 1.

TABLE 1 *Relative frequency of the 8 stages at each time for removal of testes (%)*

	18:00		22:00		02:00		06:00		10:00		14:00	
	L	R	L	R	L	R	L	R	L	R	L	R
1	11.5	12.3	12.3	12.2	11.9	12.7	12.4	12.5	10.9	12.5	11.9	12.9
2	4.6	3.9	4.2	3.7	4.3	4.0	4.5	4.1	5.5	4.2	5.6	3.9
3	8.9	8.8	10.0	10.5	9.8	10.0	10.2	10.9	9.9	9.9	10.5	10.4
4	9.4	9.9	10.3	9.6	9.7	9.4	11.1	9.2	11.0	10.7	9.8	9.8
5	8.3	9.2	8.9	8.6	9.0	7.9	8.9	7.9	8.4	6.8	8.9	7.7
6	23.5	22.7	24.6	20.3	25.0	21.0	24.3	21.6	27.1	19.7	29.1	22.0
7	15.9	14.8	15.6	16.6	13.4	16.5	12.9	16.8	13.1	16.5	11.7	15.3
8	17.4	18.0	13.7	18.1	16.3	18.2	15.0	16.8	13.4	19.5	11.9	17.8
No. of Tubules	15119	12846	13228	11585	15367	13809	13714	15323	11229	10884	13339	14155

L: Left testes R: Right testes

I Diurnal fluctuations of the relative frequencies of the stages

The relative frequencies of the stages in 6 groups, classified according to the time of killing, were statistically analysed using the discriminatory analysis. The mean discriminant scores (mean scores), which were calculated from the relative frequencies of the stages of 5 mice in each group, are shown in table 2. The difference of the mean scores was significant statistically only at the right testes ($P < 0.05$), as shown in the same table. The difference of the mean scores of 3 groups, which consisted of two successive killing periods, was tested statistically using the χ^2 -test. The χ^2 -value was the greatest when one day was

TABLE 2 *χ^2 -value in mean score among 6 groups of times*

Testes	χ^2 -value
Left testes	$\chi^2_8 = 44.08 < \chi^2_{40}(0.05) = 56.00$
Right testes	$\chi^2_8 = 56.63 > \chi^2_4(0.05) = 56.00$
Both testes	$\chi^2_8 = 51.42 < \chi^2_{40}(0.05) = 56.00$

TABLE 3 χ^2 -value in mean score among 3 groups of times

Testes	χ^2 -value
Left testes	$\chi^2_s = 21.35 < \chi^2_{16}(0.05) = 26.30$
Right testes	$\chi^2_s = 27.66 > \chi^2_{16}(0.05) = 26.30$
Both testes	$\chi^2_s = 21.84 < \chi^2_{16}(0.05) = 26.30$

divided into 3 periods, as shown in table 3. A significant difference was found only in the right testes ($P < 0.05$).

Moreover, the difference of the mean scores of 2 groups, which consisted of three successive killing periods, that is twelve hours for each group, was tested using the F-test. The great F-values, in the left testes and in both testes, were found between the killing period of 6 pm, 10 pm, and 2 am, and period of 6 am, 10 am, and 2 pm. In the right testes, the great values were found between the period of 10 pm, 2 am, and 6 am, and period of 10 am, 2 pm, and 6 pm. The F-values between these two groups are shown in table 4; however, no significant difference was found.

The results of the statistical analysis on the diurnal fluctuations are shown in table 5. Even though the difference of the mean score of two groups was significant in the right testes, the significant difference on the diurnal fluctuations in the relative frequencies of the stages could not be estimated.

TABLE 4 F-value in mean score between 2 groups of times

Testes	F-value
Left testes	$F_s = 1.25 < F_{21}^s(0.05) = 2.42$
Right testes	$F_s = 1.64 < F_{21}^s(0.05) = 2.42$
Both testes	$F_s = 1.23 < F_{51}^s(0.05) = 2.13$

TABLE 5 Difference in mean score for each group of time

Group	Left testes	Right testes	Both testes
6	NS	*	NS
3	NS	*	NS
2	NS	NS	NS

NS: no significant difference ($P < 0.05$)

* : significant level $P < 0.05$

II The differences of the mean scores between the testes and among the loci of the testes

The mean scores between the left and right testes of 30 mice were compared, disregarding the time of killing. The F-test showed that the difference of the mean scores between the testes was very significant [$F_s = 3.45 > F_{15}^8(0.01) = 2.13$]. In addition, the differences of the mean scores among the three loci of the testes—the apical pole, the equatorial zone, and the caudal pole—were tested; the mean relative frequencies of the stages in each locus are shown in table 6. The χ^2 -test among 6 groups (two testes \times three loci) showed significant differences [$\chi_s^2 = 142.81 > \chi_{40}^2(0.01) = 6.360$]. Moreover, the comparison among 3 groups (three loci in both testes) also showed a very significant difference [$\chi_s^2 = 55.86 > \chi_{16}^2(0.01) = 32.00$].

From these statistical analyses the relative frequencies of the stages are very significant between the testes and among the loci of testes.

TABLE 6 *Mean relative frequencies of the 8 stages in 3 loci of testis (%)*

Testes	Locus	Stage							
		1	2	3	4	5	6	7	8
Left testes	Apical pole	12.0	4.4	9.7	9.9	9.0	25.6	14.6	13.6
	Equat. zone	11.7	5.0	10.0	10.2	8.6	25.6	13.2	14.9
	Caudal pole	11.4	4.6	9.2	10.8	8.9	24.6	13.0	16.6
Right testes	Apical pole	12.4	3.8	10.6	9.7	7.8	21.2	16.5	17.2
	Equat. zone	12.3	4.1	9.8	9.7	8.1	21.3	16.0	18.2
	Caudal pole	12.8	3.7	9.4	10.1	7.6	21.0	15.2	19.3
Both testes	Apical pole	12.2	4.1	10.1	9.8	8.4	23.4	15.5	15.4
	Equat. zone	12.0	4.5	9.9	9.9	8.4	23.4	14.6	16.5
	Caudal pole	12.1	4.1	9.3	10.4	8.2	22.8	14.1	17.6

DISCUSSION

In spite of several irregularities of cell composition found in a few seminiferous tubules in mice as well as in many other mammals, each typical cellular association is considered as a single stages of the cycle.

From the data of the previous report (SUNG), it has been shown that the relative frequency of each stage is relatively constant either among the 3 days or among the 3 times required for removal of the testes. Another experiment on the diurnal fluctuation in the relative frequency, therefore, has been designed

only for a given 24-hour period in this study. The result that no statistically significant difference in the relative frequency was found among the 6 times or among uniform periods dispersed throughout a 24-hour period confirmed preliminary data on diurnal fluctuation reported previously.

No hasty conclusion should be made that there are no diurnal fluctuations in the progressive rate of each stage. It is still unknown whether the diurnal fluctuation may exist in the progressive rate of the cycle, and if so, whether the appearance of the fluctuation may be ubiquitous in all stages or may be limited to some given stages. If the former case is true, it may be suggested that an increase or a decrease in the relative frequency of each stage will run exactly parallel to the fluctuation of the progressive rate of each stage. Consequently, it may be impossible to find out a diurnal fluctuation or any other cyclic variations of the progressive rate, even though it surely exists, by analyzing the relative frequency of the stage. To answer these questions, the fluctuation in the rate of seminiferous epithelial cycle must be clarified.

The constancy of the relative frequencies of the various stages among loci within testes, between testes within animals, or among animals within species has been discussed with regard to other mammals. In the case of the bull, AMANN concluded that the frequency is constant for normal animals within the species. In contrast, in the same species, a significant difference has been found in the frequency among individuals by KRAMER and among individuals and between testes by TIBA. Similarly, in contrast to a report on rats by LEBLOND & CLERMONT or HOCHEREAU, ROOSEN-RUNGE & GIESEL observed a large amount of variation in the frequency among individuals. Moreover, observations on the ram (ORTAVANT), the rabbit (SWIERSTRA et al.), and the boar (SWIERSTRA) support the view that the frequencies of the stages is specific in species and that within a species the frequencies are relatively constant among animals. In view of differences in species, it is difficult to make direct comparison and to discuss the results obtained in those reports. However, in the bull and the rat, contradictory conclusions have been found. If a significant difference can be found in the relative frequency among the loci within testes, it would be unable to determine precisely the representative frequency of a testis by observing a small number of tubules in a given locus of a testis. In other words, as for comparing the difference in the frequency between testes within animals, it should be examined by increasing the number of tubules classified per locus or by increasing the number of loci per testis. The frequencies would be easily affected by the above-mentioned factors in the species which possess a relatively large volume of testes and strongly coiled tubules, the bull or boar for examples.

On the other hand, the conclusions deduced from the observations will vary

properly with the method of statistical analysis. Regarding the test for the difference of the frequency among groups, the data have usually been analysed according to the χ^2 -test or the analysis of variance. However, for these methods, the level of each factor must be independent from each other.

The relative frequencies of the various stages obtained in this study were estimated by classifying all of the cross-sectioned tubules appearing in each serial section of a whole testis. The data were analysed by means of the discriminatory analysis in which the frequencies of the various stages could be considered as 8 dependent variables.

In many clinical cases a diagnosis or a prognosis was made for the case of infertility in male animals, which usually depended on information from examining a small piece of testicular biopsy. For clinical examination, it is practically difficult to collect many biopsies from all over the testis. However, in evaluating the conditions of disease, it will be necessary to take into consideration that the data obtained may be influenced by the loci within testes or by the testes within animals.

Although an attempt was made in this study to clarify whether there are any internal factors affecting the rate of spermatogenesis, "the cycle" or "the stage" is no more than a part of the dynamic histological phenomena of spermatogenesis. In order to make further biological explanations for the results obtained here, further experiments also should be carried out for clarifying the relationship between the phenomena and the mechanisms that regulate the synchronous development and coordinate evolution of the germ cells.

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EXPLANATION OF PLATES

PLATE I

Figures 1-8 are photomicrographs of partial cross sections of mouse seminiferous tubules representative of stages 1 through 8. hematoxylin-eosin stained. ×500.

Fig. 1 stage 1

Fig. 2 stage 2

Fig. 3 stage 3

Fig. 4 stage 4

Fig. 5 stage 5

Fig. 6 stage 6

Fig. 7 stage 7

Fig. 8 stage 8

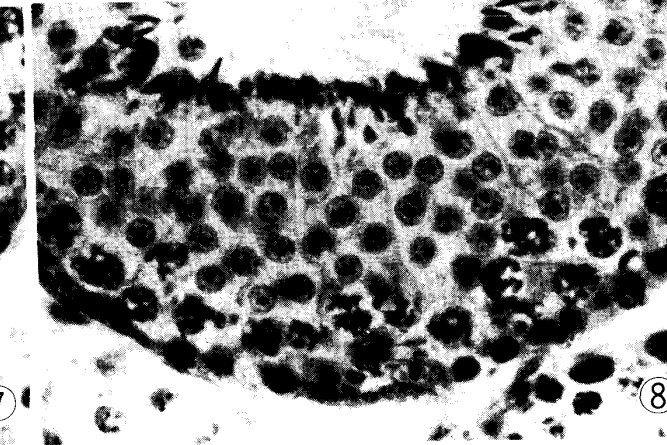
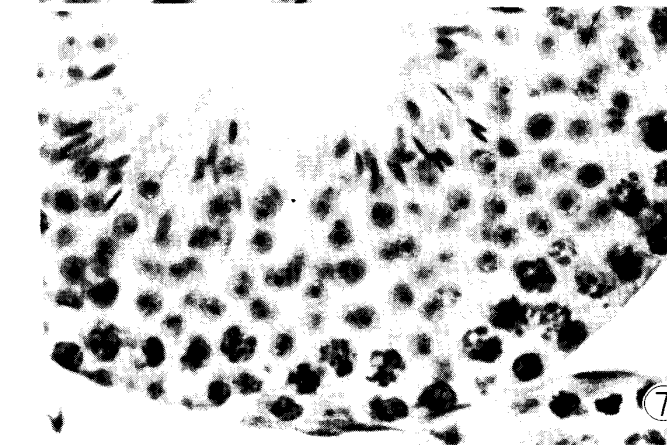
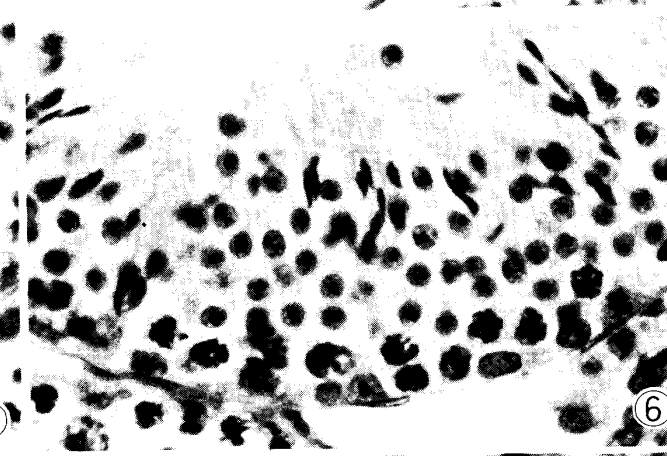
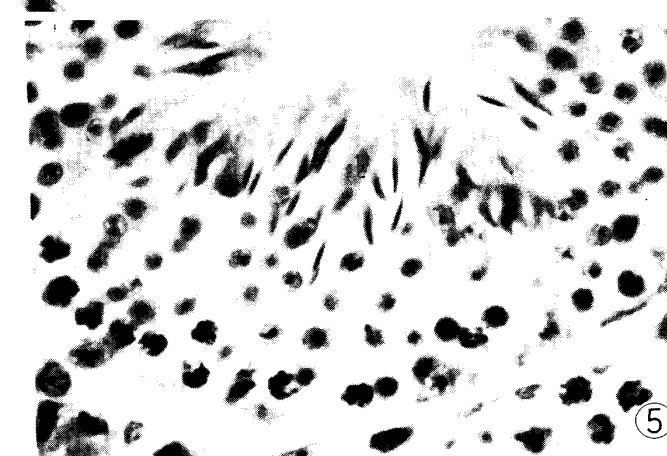
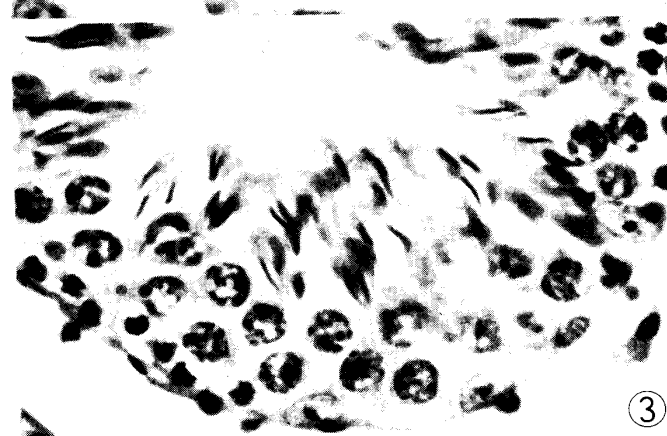
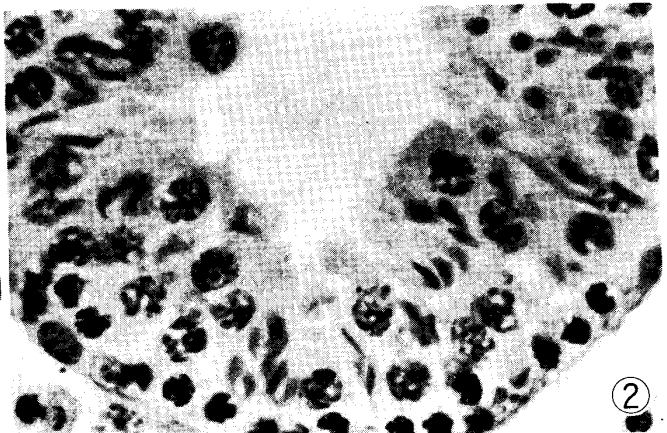
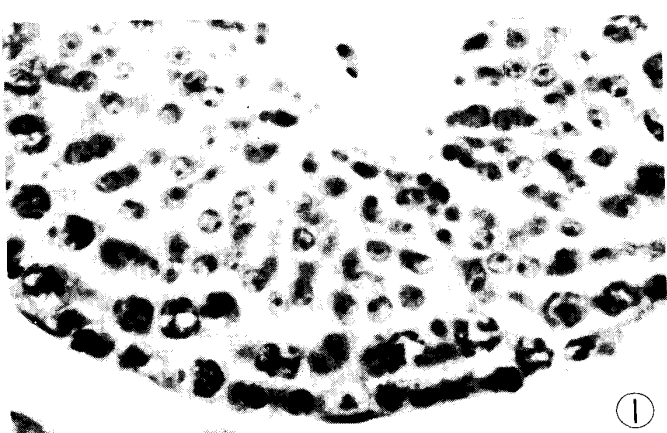


PLATE II

Figures 9–12 are photomicrographs of partial cross sections of mouse seminiferous tubules shown irregular cellular associations. hematoxylin-eosin stained. $\times 500$.

Fig. 9 Transversal section of tubule containing two typical cellular associations corresponding respectively to stage 3 (right area) and stage 5.

Fig. 10 Photograph shows an incomplete cellular association. The generation of elongated spermatids which are supposed to be present at stage 4 are completely absent.

Fig. 11 The secondary spermatocytes (\downarrow) which may require for stage 4 only are present in the cellular association belonging to stage 8 of the cycle.

Fig. 12 Photograph shows the occurrence of atretic tubule. Degenerate cells (\downarrow) are seen and elongated spermatids are missing from tubule appeared to be stage 7.

