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**On the Daily Variation of Mitotic Rate in Thymic  
Lymphocytes of the South African Clawed  
Toad, *Xenopus laevis* (Daudin)**

By

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*(With 8 Text-figures)*

Many biological phenomena, in a variety of animals and plants, even in unicellular organisms (Saunders, 1977; Schweiger, 1977), are known to show an overt rhythmicity. The range of frequencies that had been found in these organisms extends from cycles of less than a second to cycles of a year or more, but the circadian rhythm with the frequency of about 24-hours has been the one most extensively investigated. For example, the daily variations of mitotic division in rodents have been noticed: in the cornea (Scheving and Pauly, 1967; Pauly *et al.*, 1976), alimentary tract (Sigdestad and Leshner, 1978; Scheving *et al.*, 1978), and epithelium of the ear (Bullough and Laurence, 1966; Scheving and Pauly, 1960). In amphibians, the daily rhythm of mitotic rate has been reported of the larval body epidermis of two urodeles, *Ambystoma tigrinum* (Chiakulas and Scheving, 1966) and *A. punctatum* (Scheving *et al.*, 1972). At present, however, only a relatively little information is available on the mechanism by which the rhythm is established in these animals.

The present study was undertaken to obtain information on the mitotic rate of lymphocytes in the thymic cortex of *Xenopus laevis*, in relation to the developmental stage of the animals under different light conditions. The thymic cortex was chosen as material for counting mitotic figures, because the area is predominantly occupied by the population of a single type of cell, lymphocytes, and because it was thought that their relatively high mitotic rate would provide more acceptable evidence for the daily rhythm, if any. An attempt was also made to examine the possible role of the photosensory system (the lateral eyes and the frontal organ or Stirnorgan) in synchronizing the somatic cell mitotic rate to external light condition.

### Materials and Methods

The animals used were the larvae and toadlets of the South African clawed toad, *Xenopus laevis*, which were reared in our laboratory. Fertilized eggs were obtained after both males and females were injected with human chorionic gonadotropin (Gnatropin, Teikoku Zoki Co., Tokyo) into their dorsal lymph sacs. Sometimes, artificially inseminated eggs were also used. The larvae were fed on soybean powder (Bonlact, Wakodo Co., Tokyo) during the first week and boiled alfalfa leaf powder thereafter. After metamorphosis, the animals were fed with pieces of beef liver. The animals were reared at  $23\pm 1^\circ\text{C}$ . Water exchange and feeding were done every other day during larval periods, or once a day after metamorphosis. Developmental stages were determined according to the Normal Table of Nieuwkoop and Faber (1956).

During the experimental periods, the light intensity was  $800\pm 50$  lux and the photoperiod was LD 12:12. For most animals in both control and experimental groups, the light was switched on automatically at 06:00 and off at 18:00. The animals in reversed light condition were exposed to the light from 18:00 to 06:00. Throughout the course of the experiments, 6 to 8 animals were sacrificed at 6-hr intervals during a 24-hr period. The animals were fixed *in toto* with Bouin's solution. After fixation, one of the paired thymuses was dissected in 70% ethanol, dehydrated in an ascending series of ethanol and embedded in paraffin (Tissue Prep, Fisher Scientific Co., U.S.A.). The serial sections of  $5\ \mu\text{m}$  in thickness were stained with Delafield's hematoxylin and eosin.

For each specimen, observation was made on three sections, the largest section and the other two  $50\ \mu\text{m}$  apart from it, respectively. The counts of mitotic figures (pro-, meta-, ana-, and telophases) were performed over the whole region of thymic cortex under the microscope with magnification of  $\times 1,000$ .

The area occupied by the thymic cortex was measured on these three sections by the paper cutting method. Under the microscope, the outline of the cortex was drawn with the aid of camera lucida on a paper with uniform thickness; the paper was then cut and weighed. Based on the number of mitotic figures and the width of the cortical area, the final figures of mitotic divisions were expressed as the number per  $6.25\times 10^4\ \mu\text{m}^2$  of the cortex.

For statistical analysis, the *t*-test was used.

### Results

The thymus of larval *Xenopus laevis* can be divided roughly into two parts, the outer cortex and the inner medulla. The cortex consists mainly of densely packed lymphocytes, whereas the medulla is composed of the epithelial cells and their derivatives such as the myoid cells, cysts and giant cells, and relatively few small-sized lymphocytes, all of which are loosely packed there (Fig. 1). Many mitotic lymphocytes at metaphases are seen predominantly in the cortex of colchicine-treated animals (Fig. 2); in the present study, however, all the counting of mitotic

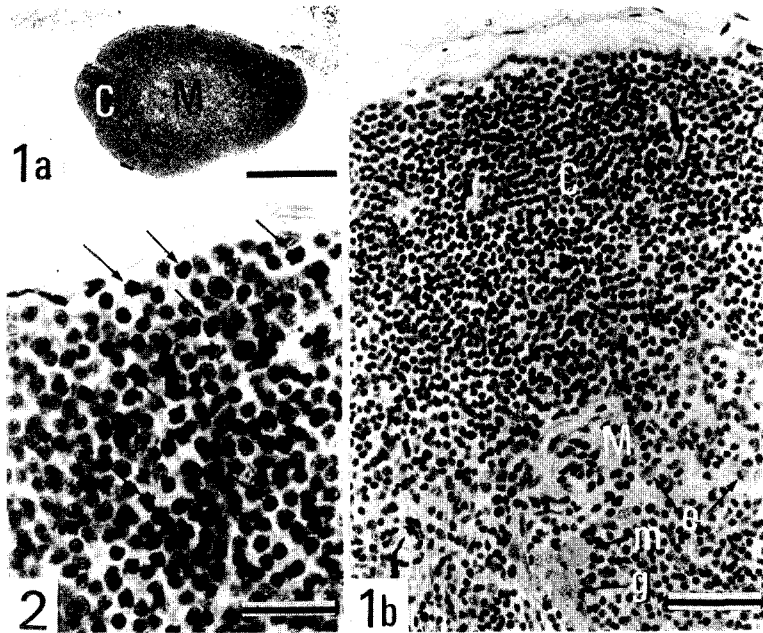


Fig. 1. a. A cross section of a thymus of a larva at stage 58/59. The deeply stained cortex (C) is clearly distinguishable from the paler medulla (M). Bar, 500  $\mu\text{m}$ .  $\times 30$ . b. A part of the thymus of the larva at stage 58/59. e, epithelial cells; g, giant cell; m, myoid cell. Bar, 50  $\mu\text{m}$ .  $\times 300$

Fig. 2. The cortical region of the thymus of the larva at stage 58/59 after immersion in colchicine solution (10 mg/100 ml) for 12 hours. Many lymphocytes (arrows) are seen to be arrested at metaphase showing somewhat picnotic appearance. Bar, 20  $\mu\text{m}$ .  $\times 750$

figures was made in the cortex of animals which had received no colchicine treatment.

#### *Animals in normal light condition*

Forty larvae at stage 50/51 (10-day old), selected from the stock aquaria were placed on the "normal" light condition (12 hours of light from 06:00 to 18:00 hours; LD 12:12) for 12 days. Starting at 13:00, on the 13th day, they (then at stage 53/54) were sacrificed at 6-hour intervals six to eight animals at each time respectively. Daily variation of mitotic rate of their thymic cortex is evident (Fig. 3), showing the highest peak at midnight and the lowest at the middle of light phase. The difference between the values at 01:00 and at 13:00 of the following day was statistically significant, whereas the difference between the values at 01:00 and the first 13:00 was not. Although the values obtained at a given time showed a considerable variation among individuals, the tendency of lower mitotic rate at

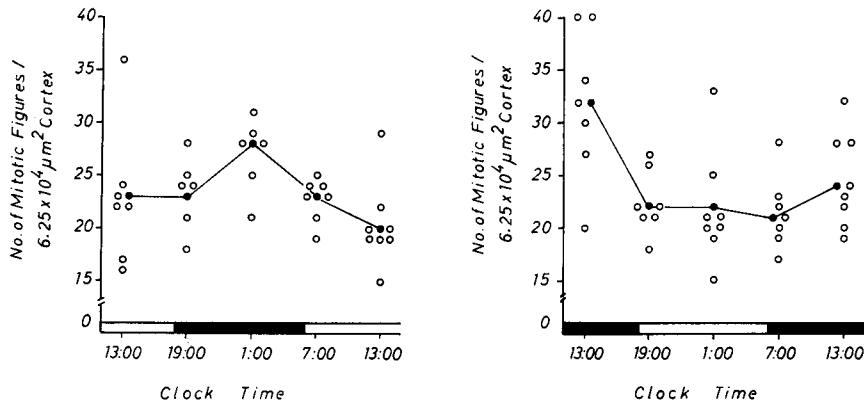


Fig. 3. (Left) Mitotic rate of lymphocytes in thymic cortex of stage 53/54 *Xenopus* larvae which had been placed under normal light condition for 12 days from stage 50/51. Open circles, values for each larva; dots, average values.

Fig. 4. (Right) Mitotic rate of lymphocytes in thymic cortex of stage 53/54 larvae that had been placed in the reversed light condition for 9 days since when they were at stage 50/51. Open circles, values for each larva; dots, average values.

light phase and higher rate at dark phase was evident, nevertheless.

#### *Animals in reversed light condition*

In order to know whether the mitotic rate in thymic cortex is dependent on the light condition, one hundred and twenty larvae at stage 50/51 from the stock aquaria were placed in the "reversed" light condition (12 hours of light from 18:00 to 06:00 hours; DL 12:12). They were sacrificed on the 10th (then stage 53/54), 15th (then stage 55/56) and the 21st (then stage 58/59) day at 6-hour intervals, respectively, six to eight animals at each time.

Prominent daily fluctuation in the mitotic rate rhythm was seen in the larvae subjected to reversed light condition for 9 days (Fig. 4). The difference between the values at 13:00 of the first day and 01:00 was statistically significant ( $p < 0.01$ ), whereas the difference between the values at 01:00 and the second 13:00 was not. Nevertheless, the animals showed an inverted pattern of daily mitotic variation, tending to have a high rate in dark phase and a low rate in the light phase. The daily fluctuation was more evident in older larvae subjected to this reversed light condition for 14 days (Fig. 5), while in the larvae at stage 55/56, the difference between the values at 01:00 and 13:00 was statistically significant ( $p < 0.001$ ). Again, the peak took place in scotophase and the trough in photophase, regardless of the clock time of day.

In the metamorphosing larvae (stage 58/59), the daily mitotic variation became obscure (Fig. 6). This is not due, however, to decreased mitotic rate in the dark phase, but to an equally high rate of mitosis during the light phase.

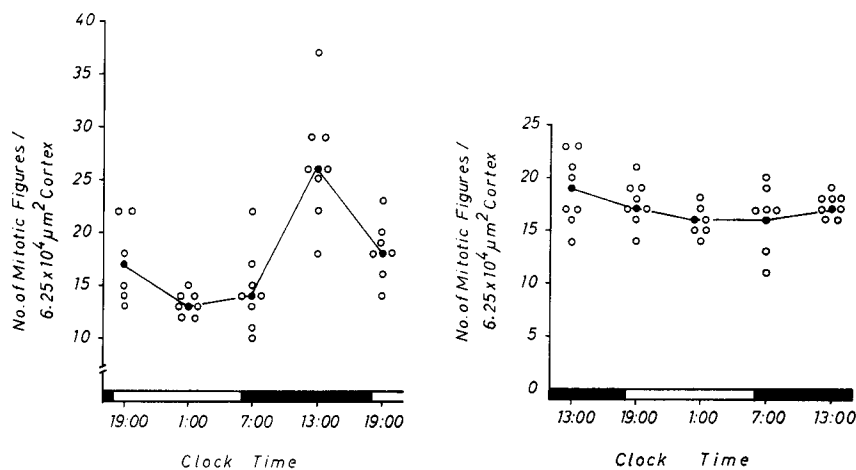


Fig. 5. (Left) Mitotic rate of lymphocytes of the larvae that had been placed in the reversed light condition for 14 days (from stage 50/51 to 55/56). Open circles, values for each larva; dots, average values.

Fig. 6. (Right) Mitotic rate of lymphocytes in metamorphosing larvae that had been placed in the reversed light condition for 20 days. Open circles, values for each larva; dots, average values.

It was also found that the over-all 24-hour mitotic rate is highest in the larvae at stage 53/54 and, thereafter, decreases gradually as the animals grow.

#### *Effects of removal of the lateral eyes and/or the frontal organ*

From the series of experiment just described, it became evident that the daily variation of mitosis is heavily, if not entirely, dependent on the environmental light condition. Therefore, in the next series of experiments, efforts were concentrated to decide if the lateral eyes or the frontal organ (Stirnorgan) are involved in synchronization of mitotic rate rhythm to the light condition. Two hundred larvae at stage 49/50 were placed in the reversed light condition of DL 12:12, and on the 8th day 120 larvae (then at stage 52/53) were blinded by surgically enucleating their eyeballs with Wecker's scissors without anesthesia. Seven days after the operation, both blinded and intact larvae, then at stage 55/56, were sacrificed at 6-hour intervals. Forty such blinded larvae had their frontal organ removed on the next day, with the aid of razor blades and watchmaker's forceps under anesthesia with 1:2,500 MS 222 (Sandoz, Basel). After the frontal organ was removed, the light schedule was again shifted to normal light condition (12 hours of light from 06:00 to 18:00 hours; LD 12:12). After 35 days of normal light condition, both the blinded and the blinded-and-frontal organ-less toadlets, which had simultaneously metamorphosed about 17 days before, were sacrificed at 6-hour

intervals. The data were expressed as relative values to the over-all 24-hr mean mitotic rate in each group.

The blinded larvae under the reversed light condition showed a daily variation of mitosis almost identical to that of intact control animals under similar condition (Fig. 7). Difference between the values at 13:00 and at 01:00 in these two groups of animals was statistically significant ( $p < 0.001$ ). However, after 35 days in the normal light condition, the magnitude of the rhythm became relatively narrower in two experimental groups as well as for the intact animals (Fig. 8). Nevertheless, the mitotic rate in all three groups of toadlets tended to show high values in the mid-dark phase and low values in the mid-light phase, respectively. Therefore, it is concluded that the fluctuating pattern of mitosis of thymic lymphocytes depends on the given light condition, whether the animals have their lateral eyes and frontal organ or not.

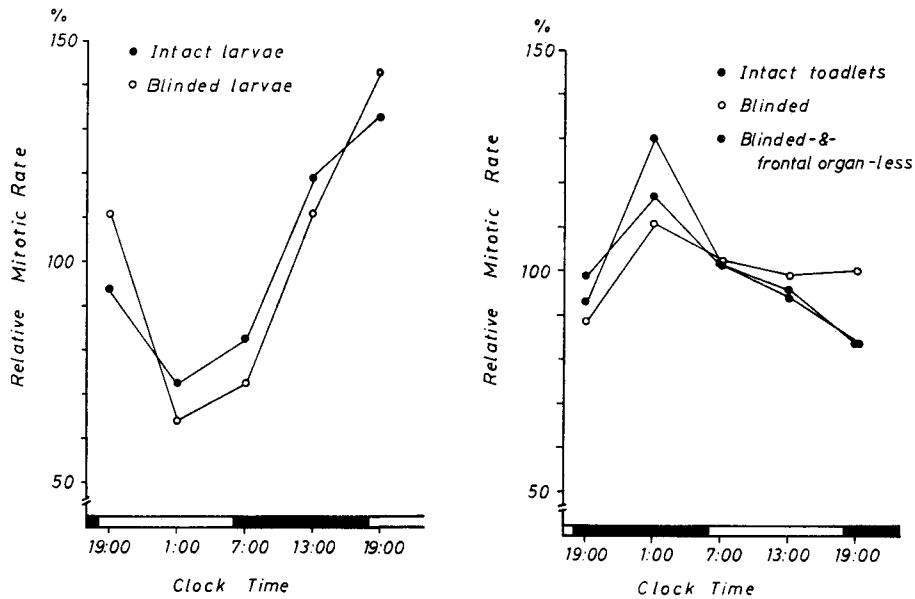


Fig. 7. (Left) Mitotic rate of thymic lymphocytes in the larvae (stage 55/56) that had been kept in the reversed light condition. The blinded larvae had their eyes removed 8 days previously. Each value represents the average of six to eight animals.

Fig. 8. (Right) Mitotic rate of thymic lymphocytes in the toadlets (about 17 days after metamorphosis) that had been kept under normal light condition for 35 days after they had spent 14 days of their larval life (from stage 49/50 to stage 55/56) in reversed light condition.

### Discussion

It was found that the lymphocytes in thymic cortex of *Xenopus* show an overt daily rhythmicity of the mitotic rate: the highest peak takes place during the period from mid-dark to early-light phase, and the trough during the period from mid-light to early-dark phase, regardless of the clock time. The results coincide well with the rhythm of mitotic rate in tail-fin epidermis of the same animals (Wakahara, 1972).

It was also found that the magnitude between the peak and the trough of a rhythmic cycle is variable depending on the developmental stage of animals. The highest magnitude occurred in the larvae at stage 55/56, becoming less prominent during metamorphosis (stage 58/59), and in the toadlets the magnitude of the rhythm was narrow. Pauly *et al.* (1976) reported in mouse thymus that the rhythm in DNA synthesis showed synchronization to a daily 4-hour restricted feeding schedule. In this connection, it is interesting to note that anuran does not take food during metamorphosis.

A considerable amount of work has been accumulated concerning the role of pineal organ in the circadian rhythm of physiological activities of animals. Wakahara (1972) found that the disappearance of the rhythmicity of mitotic rate in tail-fin epidermis was realized only when the blinded *Xenopus* larvae had their pineal organ cauterized, suggesting that photic stimuli were first perceived by the pineal organ and transmitted to the subcommissural organ, while the product(s) of the latter affected the mitosis in the epidermis. The present study indicated that neither the frontal organ nor the lateral eyes are the site of first perception of the photic stimuli, since removal of these structures failed to eliminate the daily rhythm in mitotic rate of lymphocytes. Thus, the attempt to decide whether the frontal organ rather than the pineal organ is the site of direct light perception was unsuccessful.

### Summary

The mitotic rate of lymphocytes in thymic cortex was measured on larvae and toadlets of *Xenopus laevis* that had been kept in the normal (LD 12:12) or reversed (DL 12:12) light condition.

1. The mitotic rate showed a prominent daily fluctuation, with the peak during the dark phase and the trough during the light phase, regardless of the clock time of day.

2. The rhythmicity was shown most prominently in the larvae at stage 55/56, becoming less prominent during metamorphosis (stage 58/59).

3. Surgical removal of the lateral eyes and frontal organ (Stirnorgan) of the larvae failed to restrain the animals from synchronizing the daily variation of mitotic rate to external light condition.

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