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Effects of the Removal of Pineal Organ and Eyes on the Pigmentary Response in *Xenopus laevis*¹⁾

By

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(With 5 Text-figures)

It is well known in anurans that blinded tadpoles become dark in body color when they metamorphose (Rowlands, 1952; Takahashi and Imai, 1965). The phenomenon has been considered to be due to an excess release of MSH probably owing to some change in the hypothalamo-hypophysial axis resulting from degeneration of the optic nerve.

From the morphological study, the hypothesis has been presented that the amphibian pineal organ is photoreceptive (Eakin *et al.*, 1963; Oksche and von Harnack, 1963; Kelly and Smith, 1964; Charlton, 1968; Wakahara, 1968a), and this was confirmed electro-neurologically (Dodt and Heerd, 1962; Dodt and Jacobsen, 1963). It is also said that the pineal organ exerts a certain effect on the melanophore response or body color change (Bagnara, 1960, 1963, 1965; Charlton, 1966) and that it is the site of the production of "whitening hormone" or melatonin in anurans (Charlton, 1964).

Amphibian chromatic response has been explained by the bihumoral system consisted of the darkening hormone (MSH) and the whitening hormone (melatonin) (Hogben and Slome, 1931; Charlton, 1966). There is also another type of melanophores in the tail fin of *Xenopus laevis* larva which respond directly to light (Bagnara, 1957). In metamorphosed *Xenopus*, the body color is affected by serotonin, or 5-HT, secreted in the skin (Veerdonk *et al.*, 1961) or excitement.

In this study, the effect of the pineal organ on the pigmentary response was investigated using metamorphosing *Xenopus* which had previously been operated on the eyes and the pineal organ. Furthermore, circadian rhythm of the pigmentary response in the intact, blinded and pinealectomized animals was investigated in young adult *Xenopus*.

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Materials and Methods

All the animals, *Xenopus laevis*, used in this investigation were obtained from induced matings. They were fed on alfalfa powder throughout the larval stage and on chopped pork liver after they metamorphosed. The larvae were staged according to the table of Nieuwkoop and Faber (1956). Also the toads 2 or 3 months after metamorphosis were used as materials.

Prior to all surgery, anesthesia was made in MS222 (Sandoz) solution (1:3,000 in concentration). The eye balls were removed bilaterally of the larvae and toads by means of fine forceps and a pair of scissors. In order to obtain the pineal complex (the frontal organ *plus* pineal organ)-less animals, larval pineal complex was cauterized directly with a red hot needle. As the pineal complex consisted of the frontal organ buried in the skin of the head region and the pineal organ situated under the skull in the toads, the two structures were cauterized separately. All the animals survived through the surgery. Removal of the pineal organ was verified by observing serial sections through the cauterized region.

The meningeal melanophore preparation was made of the cranial bone of animals fixed in Helly's fixative. In order to evaluate the degree of melanophore response in such preparations, the melanophore index (MI) of Hogben and Slome (1931) was employed. The skin preparation was made of the dorsal skin of the animals fixed in Helly's fixative, dehydrated through an ascendant series of alcohol and sealed in balsam on a slide glass. The skin coloration index (SCI) was determined by measuring the per cent transmission of light with a photometer attached on the microscope.

Experiments

1. *Pigmentary response during the larval stage*

As a preliminary experiment, thirty animals at St. 40 were placed in the complete darkness, in order to know whether the larva can metamorphose without the aid of light. Feeding and water exchange were done under the red light (5-10 lux, for about 10 minutes a day). The result was clear: all the experimental larvae metamorphosed within the same period of time as the normal control completed metamorphosis (about 40 days). Their dermal melanophore was conspicuously contracted and the body color was extremely pale except in the tail fin, where the melanophores were fully expanded in the darkness. However, no significant difference was found in the number of dermal melanophores between the dark-adapted and the control animals, the result suggesting that the lighting condition gives little influence to the differentiation of the melanophore.

The morphological difference of the melanophores in the skin and the meninx was shown in Fig. 1 of the larval and metamorphosed animals reared in different lighting conditions. The dermal and meningeal melanophores of the larvae and the

meningeal melanophores of metamorphosed animals were expanded in the light (Fig. 1, a and e) and extremely contracted in the darkness (Fig. 1, b and f). The dermal melanophores of animals that had been reared and metamorphosed in the darkness were rather expanded (Fig. 1, d). The skin coloration of animals reared in the darkness got darker and darker until they became almost the same to or a little darker than that of the controls (Fig. 1, c) during the metamorphosis.

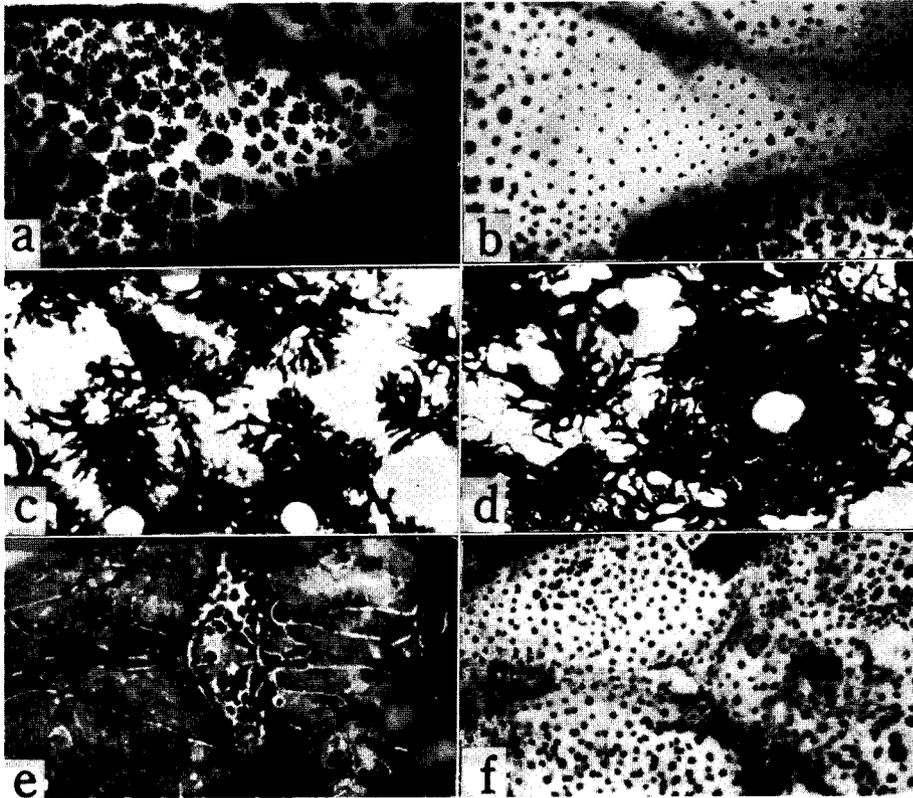


Fig. 1. Photomicrographs showing melanophore response in larvae and toads, *Xenopus laevis*, placed in various lighting conditions. Light-(a) and dark-(b) adapted dermal melanophores in larva. $\times 24$. Dermal melanophores of the toads that metamorphosed in the continuous light (c) and in the dark (d). $\times 130$. Light-(e) and dark-(f) adapted meningeal melanophores of the toads. $\times 18$.

The next series of experiments was performed to examine whether or how the eyes and the pineal complex (the frontal organ *plus* pineal organ) participate in the pigmentary response of the larva kept in light or dark. Thus, eighty larvae at St. 58-59 were divided into four groups of twenty specimens, each as follows: 1)

eye ball-less group (EBL), 2) pineal complex-less group (PCL), 3) eye ball-less and pineal complex-less group (EBL-PCL), and 4) intact group (INT). These four groups were further subdivided into two subgroups of ten animals and they were placed either in natural day-night condition or in complete darkness. After three weeks, all the animals metamorphosed, and they were examined in respect to the skin coloration and the MMI. The results are summarized in Fig. 2.

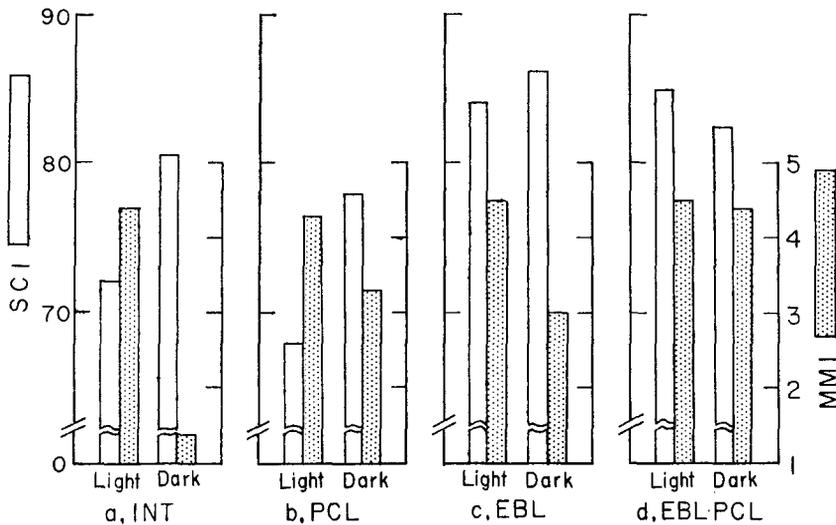


Fig. 2. Differences in the skin coloration index (SCI) and the meningeal melanophore index (MMI) of previously operated animals which were reared under the usual day-night condition (Light) or complete darkness (Dark). For further explanation, see text.

In the intact animals, both the skin coloration and the MMI showed a marked difference between the animals metamorphosed in the usual day-night condition (INT-Light animals) and those metamorphosed in darkness (INT-Dark animals) (Fig. 2, a). INT-Light animals had a medium skin color (SCI, about 72) and dispersed meningeal melanophore (MI, about 4.5), whereas INT-Dark animals displayed much darker skin color (SCI, about 80) and a remarkable contraction of meningeal melanophore (MI, about 1.4).

Among the pineal complex-less animals (PCL), those metamorphosed under the usual day-night condition (PCL-Light animals) showed a paler body color (SCI, about 68) and more dispersed meningeal melanophore (MI, about 4.3) than those metamorphosed in darkness (PCL-Dark) which showed a darker skin color (SCI, about 78) and intermediately dispersed meningeal melanophore (MI, about 3.3) (Fig. 2, b). Although the skin coloration and the meningeal melanophore index in the PCL and INT were almost identical in usual light, they were signi-

ificantly different in dark. The result indicates that removal of the pineal complex from the larva maintained in the darkness brought about a certain effect on the animals, making their meningeal melanophores unable to contract under the continued darkness.

Among the blinded animals which had been reared and metamorphosed under the continual darkness, both the EBL-PCL and EBL animals were remarkably dark (SCI, about 82 and 86, respectively) (Fig. 2, c and d). The meningeal melanophores of the EBL-PCL-Dark animals were almost fully expanded (MI, about 4, 5), whereas that of the EBL-Dark animals was only intermediately expanded (MI, about 3.0). In the usual day-night condition, however, no significant difference was found to exist of the pattern of the SCI and MMI between the EBL-PCL and the EBL animals (Fig. 2, c and d), both having dark skin (SCI, about 85) and expanded meningeal melanophores (MI, about 4.5).

Furthermore, between the EBL-PCL-Light and the EBL-PCL-Dark animals, no significant difference was found of the SCI and the MMI (Fig. 2, d).

2. Pigmentary response in metamorphosed *Xenopus*

In order to establish the pigmentary response in toads after metamorphosis, darkening of the body color induced by removal of eye and the circadian rhythms of color change in the skin and meninx were investigated. Two groups of twenty eye ball-less (EBL) toads and two groups of twenty intact (INT) toads were subjected to either continuous illumination (about 200 lux) or complete darkness, respectively. They were killed on the 0, 2nd, 8th, 16th and 32nd day, each time four intact and four EBL animals from both groups. Only the skin preparations were made. The results of this experiment are illustrated in Fig. 3.

The eye-less animals operated after metamorphosis became dark when they were placed under continuous light (SCI, about 66 up to 86). The body darkening became evident about 1 week after the operation, and even the skin of the belly became dark about 1 year later. In the EBL toads reared in constant darkness, little change occurred in the skin coloration. For more than 1 month after the operation, it was almost similar to that of the light-adapted intact animals (Fig. 3). Furthermore, the light-adapted EBL toads became darker more rapidly than the EBL animal which had been reared under the usual day-night condition. These results seem to show that the body darkening induced by removal of the eye balls depended upon the lighting condition under which the animals were placed.

The dark-adapted intact toads became dark to some extent within the first two days and that condition lasted about one month.

It was examined in the next series of experiments whether there occurred the circadian rhythm of color change in the skin and meninx of the EBL animals that had been reared under the usual day-night condition for two months. Twenty intact and EBL toads were prepared, kept for two months in the usual laboratory condition, and were left in an outdoor pond to be subjected to natural day-night condition for a week before they were sacrificed for examination. They were killed

at 14:00, 20:00 of a day and at 2:00, 8:00 and 14:00 of the following day, each time 4 intact and 4 EBL toads, and the skin and meningeal melanophore preparations were made. The results are summarized in Fig. 4.

EBL toads exhibited a clear circadian rhythm both in the skin coloration and the meningeal melanophore index. They became paler in the nighttime (SCI, under 75) and darker in the daytime (SCI, about 85). Also, the meningeal melano-

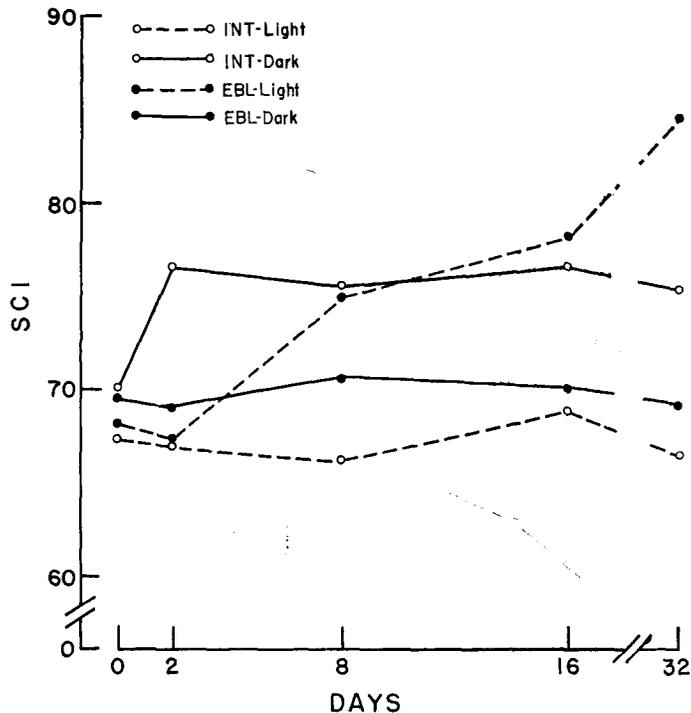


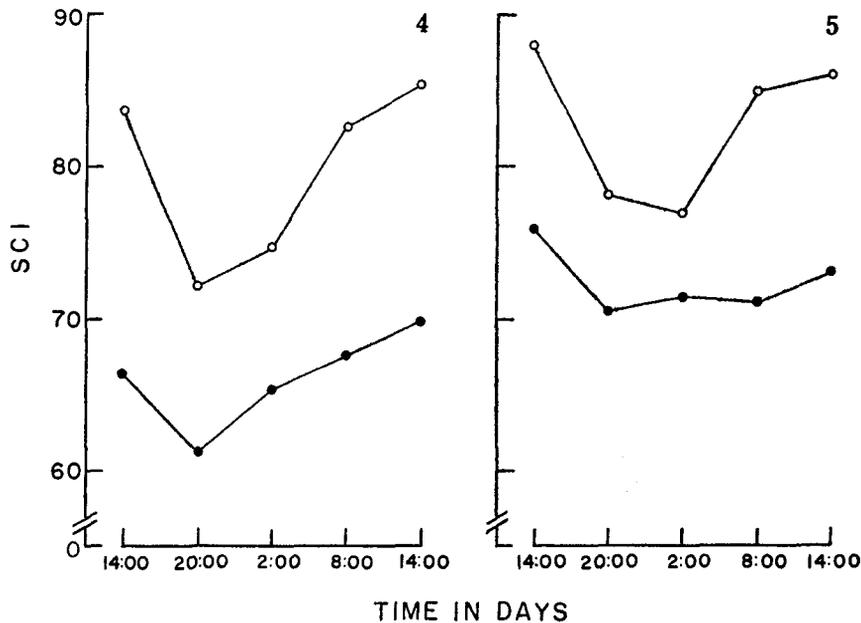
Fig. 3. Changes in the SCI of blinded (dots) and intact (circles) toads kept in continuous light (broken lines) or complete darkness (solid lines).

phore of these animals contracted in the night and expanded in the day. The intact animals, however, became paler to some extent at the beginning of night, and the circadian rhythm in color change was not evident in the skin. In the EBL animals as well as in intact ones, the meningeal melanophore showed the circadian rhythm of color change, expanding during the daytime and contracting during the nighttime.

In the next step of this experiment, the pineal complex was removed in order to determine whether the complex participates in the circadian rhythm of color change in the skin of the darkened EBL toads. Two experimental groups consisted

of twenty eye ball-less-and-pineal complex-less (EBL-PCL) toads were prepared from another batch, placed for about two months after the operation in the laboratory, and left in the outdoor pond for a week before they were sacrificed. They were killed at 14:00, 20:00 of day and at 2:00, 8:00 and 14:00 of the next day, each time 4 PCL and 4 EBL-PCL animals, and the skin preparations were made. The results of this experiment are illustrated in Fig. 5.

The dermal melanophore of the EBL-PCL animals exhibited a clear circadian rhythm of behavior, contracting in night (SCI, under 80) and expanding in day (SCI, over 85). On the other hand, in the PCL animals as in the intact ones their dermal melanophore showed no circadian rhythm of behavior.



Figs. 4 and 5. Circadian changes in the SCI of the operated toads. Fig. 4, blinded (circles) and intact (dots) animals. Fig. 5, blinded-and-pinealectomized (circles) and pinealectomized (dots) animals.

Discussion

From the results obtained in the present experiment, it is evident that the *Xenopus* pineal complex possesses the potency to contract the larval meningeal melanophore in the darkness. The conclusion was drawn by comparing the difference in meningeal melanophore index between the INT-Dark and the PCL-Dark animals and the difference between the EBL-Dark and EBL-PCL-Dark animals. It

seems to support the hypothesis presented by Bagnara (1960, 1963), who observed inhibition of blanching reaction by pinealectomy in larval *Xenopus* and concluded that melatonin or a similar melanophore contracting substance is released from the pineal organ in the absence of light. The production or at least presence of melatonin in *Xenopus* pineal organ have been established by means of incorporations of radioactive precursor of melatonin in larva (Axelrod *et al.*, 1965) as well as in adult (Charlton, 1964). Charlton (1966) found that removal of the pineal organ in larval and adult *Xenopus* abolishes the primary and the secondary responses of color change.

During the larval stage the dermal melanophores except those in the tail fin are conspicuously contracted in the darkness. However, the SCI, the skin coloration index, is considerably high of the toads placed in the darkness. The body darkening reaction induced by placing adult *Xenopus* in darkness is a particular one comparable to the "excitement darkening". It is well known that the excitement induces body lightening in the major amphibian groups but darkening in *Xenopus* (Burgers and van Oordt, 1962). It is possible when a normal toad is placed more than two days in the darkness that a melanocyte-expanding-substance, most likely serotonin (Veerdonk *et al.*, 1961), is released from the skin and makes the body darker to some extent, as if the animal is exposed to ether-vapor. It has been pointed out that the melanin content of the dorsal skin of *Rana temporaria* increases as the frog is kept in darkness (Dawes, 1941). The "darkening effect" of the darkness in adult dermis permits the inference that some change in integument occurred during metamorphosis, whereas the pattern of the response of the meningeal melanophore remained unchanged through metamorphosis.

It has been noticed that the EBL (eye ball-less) toads fail to become darker when they are placed in the darkness, whereas the EBL larvae are found to become darker in the darkness after metamorphosis. The body darkening reaction induced by removal of eyes in adult *Xenopus* seems to depend upon the lighting condition because relatively small amount of MSH is produced and/or released. On the contrary, in the animals whose eyes have been removed at larval stage, excess MSH seems to be produced and released in consequence of a complete degeneration of the optic nerve during metamorphosis (Takahashi and Imai, 1965), when the integumental change occurred, so that animals become dark independently of the lighting condition.

Experiments on the circadian rhythm of color change of the skin indicate a possibility that the dermal melanophore responds directly to light, since not only the EBL but also the EBL-PCL animals become considerably pale in nighttime. This whitening reaction can be provoked neither by the "whitening hormone" produced in the pineal organ nor by a stimulus transmitted through the retinal pathway, because both eyes and pineal organ were removed over two months before the observation. The direct response of the melanophores to light or darkness, such as has been stated in the tail fin of *Xenopus* larva (Bagnara,

1957), indicates existence of a mechanism in the amphibian chromatic response which is different from the bihumoral system consisted of the whitening and the darkening hormone. The present author reported previously a distinct circadian rhythm in the morphology of the subcommissural organ (SCO), a structure closely related to the pineal organ in adult *Xenopus* (Wakahara, 1968b). At present, however, no evidence was produced to establish the relationship between the two rhythms.

Summary

Young adult *Xenopus* which had previously been operated during the larval stage on the eyes and the pineal organ were kept in various lighting conditions. In the dark the melanin granules in meningeal melanophores of intact animals were almost completely concentrated whereas those of the pinealectomized toads were intermediately dispersed. The blinded-and-pinealectomized animals had high values in both SCI (skin coloration index) and MMI (meningeal melanophore index) in either dark or light.

The circadian rhythm of pigmentary response in the dermal melanophores persisted in the blinded and the blinded-and-pinealectomized toads, suggesting the possibility that the melanophores are photosensitive in the absence of other major photoreceptors.

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