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Authors: Kouki, Tom, Kawamura, Kosuke, and Kikuyama, Sakae

Source: Zoological Science, 15(5) : 749-752

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.15.749

ZOOLOGICAL SCIENCE **15**: 749–752 (1998)

Lack of Inhibitory Control of Melanophore-Stimulating Hormone Secretion in the Larval Toad, *Bufo japonicus*

Tom Kouki, Kosuke Kawamura and Sakae Kikuyama*

Department of Biology, School of Education, Waseda University, Nishi-Waseda 1-6-1, Shinjuku-ku, Tokyo 169-8050, Japan

ABSTRACT—In general, both larval and adult amphibians are known to change their skin color depending on the background color. However, in the Japanese toad *Bufo japonicus*, the larvae were found to be devoid of the ability of responding to the background color. Melanin granules in the dermal melanophores were in a state of constant dispersion independently of the color of the background to which they were subjected. Pharmacological and histochemical studies revealed that in the toad tadpoles, melanophore-stimulating hormone is being released without inhibition, presumably because innervation of catecholaminergic fibers in the intermediate lobe is incomplete until they finish metamorphosis. Biological significance of the dark color of *Bufo* tadpoles is discussed in conjunction with another characteristic that they have a tendency to aggregate to form a dense black mass.

INTRODUCTION

Amphibian larvae as well as adults change their skin color in response to a light- or dark-colored background. In this response, aggregation and dispersion of melanin granules in the dermal melanophores play the most important role. The movement of melanin granules is dependent on circulating levels of α-melanophore-stimulating hormone (α-MSH) (Hadley and Bagnara, 1975). Secretion of α-MSH by the intermediate lobe is generally believed to be under inhibitory control by the hypothalamus (Jenks *et al*., 1993). Tadpoles of Bufonidae, however, exhibit invariably a dark color (Wassersug, 1973). During a series of experiments conducted with embryos and larvae of *Bufo japonicus* (Kawamura and Kikuyama, 1992, 1995; Kikuyama *et al*., 1993), we have noticed that their somatic cells are pigmented with melanin derived from the ovum in which a large amount of the pigment had been deposited during oogenesis, and that melanin granules in the dermal melanophores are in a state of constant dispersion irrespective of the color of the background to which they were exposed. This lead us to study the mechanisms underlying this phenomenon.

MATERIALS AND METHODS

Bufo japonicus tadpoles were hatched from eggs collected in the suburbs of Tokyo and fed boiled spinach in our laboratory at 22°C. The metamorphic stages were classified according to Iwasawa (1987).

In order to confirm that the toad larvae are lacking the ability to respond to the background, intact tadpoles at premetamorphic (stage 38), prometamorphic (stage 40), and climax (stage 43) stages as well as postmetamorphic juveniles were kept in white or black containers for 24 hr under constant illumination with a fluorescent light. At the same time, tail-bud embryos were hypophysectomized, reared until their hatch-mates reached metamorphic climax and subjected to a similar test. The melanophores in the dorsal and tail skin were observed under a microscope immediately after the animals were killed by decapitation and staged according to the melanophore index (MI) of Hogben and Slome (1931).

To examine the effects of dopamine agonist and MSH on dermal melanophores, 5 μg of bromocriptine (Sandoz), or 1 μg of αMSH (Bachem) was injected to intact prometamorphic (stage 40) tadpoles. Bromocriptine was dissolved in a small volume of ethanol, which was then diluted with 2000 volumes of saline. MSH was dissolved in saline. Control animals received vehicles only. Each injection volume was 0.01 ml. The melanophores in the tail or dorsal skin were observed 6 hr after the injection.

To investigate the melanophore response *in vitro*, tail segments (4 mm in length) cut from prometamorphic (stage 40) tadpoles were cultured at 22°C in petri dishes (3 cm diameter) containing 2 ml of Gey's culture medium (pH 7.2) diluted for amphibians. The medium was supplemented with αMSH (10^{-6} M) when required. The melanophores were observed after culturing for 12 hr.

In order to demonstrate the distribution of monoaminergic nerve fibers in the intermediate lobe, the Falck-Hillarp method (Falck *et al*., 1962) was employed. The pituitaries attached to the brain tissue of prometamorphic (stage 40) tadpoles and of postmetamorphic juvenile toads were frozen in 2-methyl butane cooled by liquid nitrogen and dried for 6 days in a freeze-drying apparatus (Oka Science, Japan). The Falck-Hillarp reaction was performed with paraformaldehyde vapor (70% relative humidity) for 1 hr at 80°C. Tissues were vacuum-embedded in paraffin and 8-10 μm serial sagittal sections were heated above the melting point of paraffin. After cooling, they were mounted with coverslips using Entellan (Merck) and heated again up to 60°C for 1 hr. Sections were examined with a fluorescent microscope (Olympus FLM, Japan) equipped with an HBO 200/4 super pressure mercury lamp (Osram, Germany) with the use of an excitation filter BV and barrier filter Y-50.

* Corresponding author: Tel. +81-3-5286-1517;
FAX. +81-3-3207-9694.

RESULTS

Mean MI of intact tadpoles at various metamorphic stages and juveniles kept in black containers were invariably 5.0 ± 0 (n = 8). There was no difference in the degree of expansion of the pigment granules between the dorsal and tail melanophores. Likewise, all the premetamorphic, prometamorphic and climax tadpoles kept in a white containers remained black (MI = 5.0 ± 0, n = 8). However, in the juvenile toads kept in white containers, mean MI value was 1.2 ± 0.4 (n = 8). The mean MI of hypophysectomized tadpoles kept in either black or white containers was 1.0 ± 0 (n = 8).

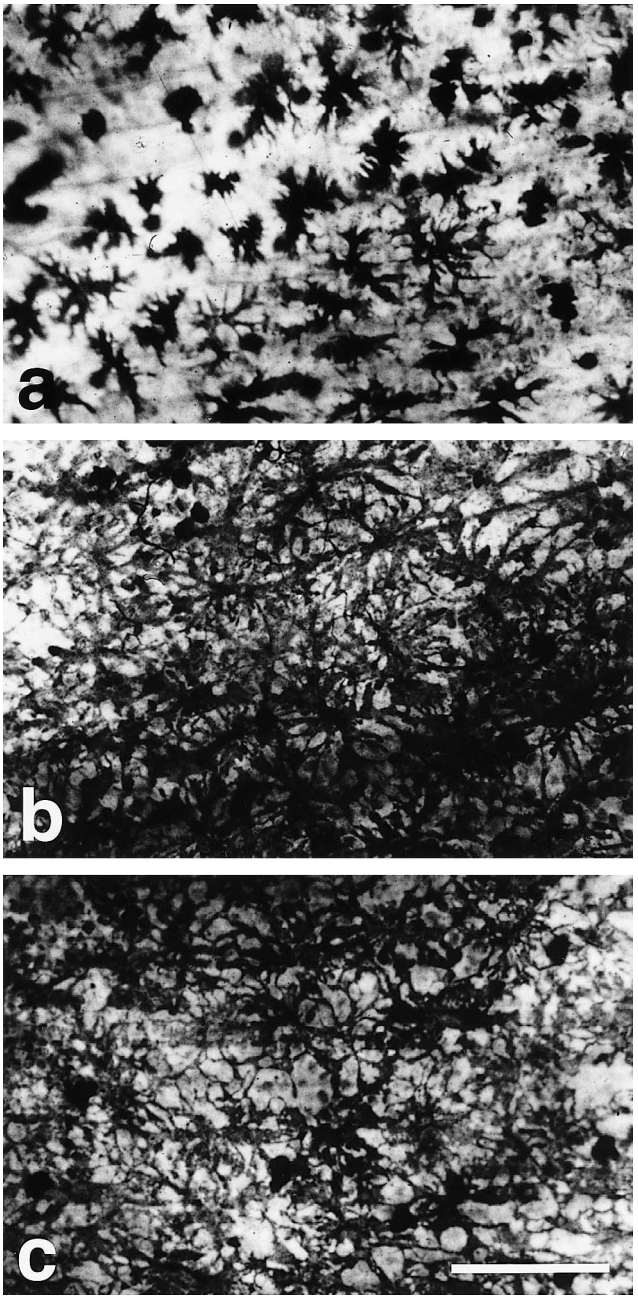

Fig. 1. Melanophores of the tail of the tadpoles injected with bromocriptine (**a**), bromocriptine plus MSH (**b**) or vehicle (**c**). Bromocriptine causes aggregation of the melanin granules *in vivo*. Bar, 50 μm.

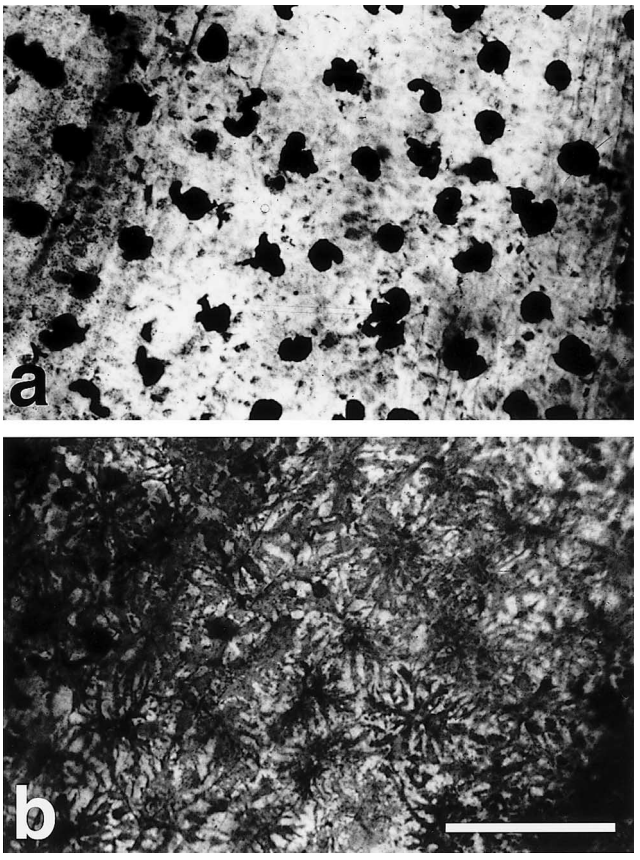

Fig. 2. Melanophores of the explants of tail segment incubated with (**b**) or without (**a**) MSH. When cultured without MSH, the melanin granules become aggregated. Bar, 50 μm.

In the prometamorphic tadpoles, the injection of bromocriptine caused lightening of the skin color (Fig. 1a), the mean MI being 2.6 ± 0.2 (n = 8) whereas, the larvae receiving MSH simultaneously with bromocriptine remained dark (Fig. 1b), the mean MI being 5.0 ± 0 (n = 8). In the specimens injected with MSH only or injected with vehicles (Fig. 1c) were invariably dark, the mean MI being 5.0 ± 0 (n = 8).

The tail segments cultured in the medium without MSH supplementation showed lightening of the color (Fig. 2a), the mean MI being 1.1 ± 0.1 (n = 8). In contrast, the tail segments cultured in the medium containing MSH remained dark (Fig. 2b), the mean MI being 5.0 ± 0 (n = 8).

Intermediate lobes from tadpoles (n = 5) at the prometamorphic stage emitted no formaldehyde-induced fluorescence, indicating the absence of catecholaminergic fibers (Fig. 3a). Whereas, the fluorescence was conspicuous in the intermediate lobes from metamorphosed juveniles (n = 5), showing a fine network of catecholaminergic fibers (Fig. 3b).

DISCUSSION

We have confirmed that the melanin granules in the dermal melanophores of toad larvae at various stages examined

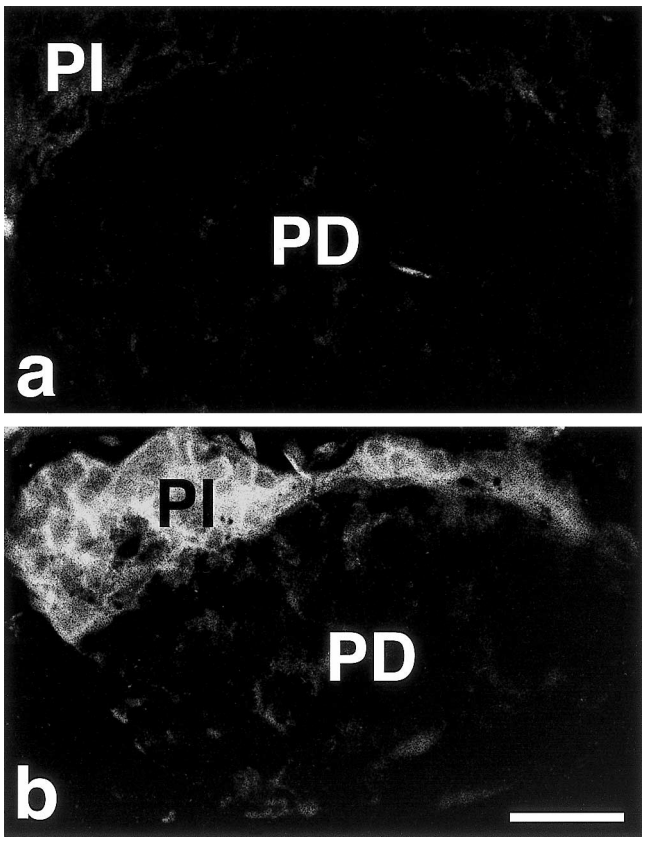

Fig. 3. Formaldehyde-induced fluorescence of catecholaminergic nerve fibers in the intermediate lobe of hypophysis. At prometamorphic stages, no catecholaminergic fibers are detected (**a**), whereas a fine network of Falck-Hillarp-positive nerve endings is observed in postmetamorphic juveniles (**b**). PD, distal lobe; PI, intermediate lobe. Bar, 20 μm.

are in a state of constant dispersion and that this is not affected by the color of the background to which they were exposed. Appearance of nearly complete background response in metamorphosed juveniles indicates that the background adaptation mechanism develops in association with metamorphosis.

The movement of the pigment granules in the melanophores of these animals seems to be dependent on MSH, since the dispersion of the melanin granules does not occur in the hypophysectomized specimens. The fact that the melanin granules in the melanophores of the explanted tail segments become concentrated unless MSH is not added to the culture medium also supports this view.

Injection of bromocriptine (dopamine agonist) to the prometamorphic larvae attenuated the expansion of melanin granules, and simultaneous injection of MSH kept the pigment granules in an expanded state. This suggests that the inhibitory control system of the release of MSH is not developed in the larvae. In fact, histochemical study revealed that innervation of catecholaminergic fibers, which is considered to be essential for the inhibition of the release of MSH, does not occur in the intermediate lobe of the prometamorphic larvae but that in the intermediate lobe of the postmetamorphic specimens, a well developed fiber network can be seen. The locus of the cell bodies sending fibers to the intermediate lobe of this species had not been clear until recently. In this respect, it is of interest to note that a group of catecholaminergic neurons in the preoptic recess organ develops during metamorphic climax, while catecholaminergic neurons in the paraventricular organ and nucleus infundibularis dorsalis already exist in premetamorphic tadpoles (Kikuyama *et al*., 1979). Recently, we have demonstrated that the cell bodies projecting fibers to the intermediate lobe are located in the rostral part of preoptic recess organ (PRO) since in the juveniles of which primordium of the rostral PRO had been removed at the open neurula stage the skin color remained constantly black and catecholaminergic fibers were scarcely found in the intermediate lobe (Kouki *et al*., 1998). In *Bufo japonicus* adults, dopamine seems to be a major factor for the inhibition of the release of MSH, since pimozide (dopamine antagonist) induces darkening of the skin color of the white-adapted toads (unpublished observation). It is also the case in the bullfrog, *Rana catesbeiana* which exhibit a proper background response as early as at premetamorphic stage. In the intermediate lobe of the bullfrog larvae, well developed catecholaminergic fibers are seen. In these animals, pimozide induces darkening and bromocriptine induces lightening of the skin color. Moreover, α-methyl-*p*-tyrosine, an inhibitor of catecholamine synthesis, blocks the white background adaptation (Miyakawa *et al*., 1982).

It has been pointed out that tadpoles of Bufonidae tend to aggregate to form a dense black mass in shallow water or near the bottom with hundreds to thousand individuals participating (Liu, 1950; Wager, 1965; Wassersug, 1973). Formation of aggregates in *Bufo* tadpoles with dark color is usually assumed to be an adaptation for absorbing the maximum available solar radiation (Wassersug, 1973). On the other hand, dark color of *Bufo* tadpoles and their tendency to aggregate have often been thought to make them highly conspicuous and easy prey for predators. Experimental comparative palatability tests on live tadpoles from various families, however, revealed that the least palatable is Bufonidae (Heusser, 1971; Wassersug, 1971). Possible explanation for this unpalatability may be that secretions from glandular cells, which are specifically found in the skin of *Bufo* larvae (Pfeifer, 1966) serve for the transmitter of unpalatability and/or that predators regard the dense black mass of tadpoles as a single large-sized animal that is hard to attack.

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(Received May 14, 1998 / Accepted May 25, 1998)