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Authors: Kobayashi, Makito, Tajima, Chihiro, and Sugimoto, Masazumi

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Effects of Androgens on the Development of Nuptial Coloration and Chromatophores in the Bitterling *Rhodeus ocellatus ocellatus*

Makito Kobayashi[1]*, Chihiro Tajima[2] and Masazumi Sugimoto[2]

[1]*Department of Biology, Division of Natural Sciences, International Christian University, Mitaka, Tokyo 181-8585, Japan*
[2]*Department of Biomolecular Science, Faculty of Science, Toho University, Miyama, Funabashi, Chiba 274-8510, Japan*

Sexually mature male bitterlings, *Rhodeus ocellatus ocellatus,* exhibit distinct nuptial color, whereas females maintain a body color similar to that of juveniles. In the present study, body color and chromatophores were compared between male and female bitterlings, and the effects of androgens on body color and chromatophore densities were examined in females to clarify the role of androgen in the development of nuptial coloration and chromatophores. Males showed green, blue, and red color in specific regions of their skin and red color on the dorsal and caudal fins; females showed a subdued silver body color. For chromatophores, small greenish-type iridophores were observed in the green color region in the skin of males, whereas females had large spindle-shaped silvery-type iridophores in corresponding regions. Many erythrophores were observed in males in blue and red color regions in the skin and red color regions in the fins, but females possessed xanthophores in corresponding regions. The melanophore density of the skin was not different between males and females, but the distribution of melanophores in the fins was different between them. Treatment with 11-ketotestosterone or methyltestosterone induced male-type nuptial coloration in the female skin and fins. The distribution of chromatophores in androgen-treated females was similar to that in sexually mature males: an increase in the number of greenish-type iridophores and erythrophores was also observed in the skin. These results indicate that androgen induces male-type nuptial coloration in the bitterling and that the responses of chromatophores to androgen differ with the type and distribution site of the chromatophores.

Key words: androgen, 11-ketotestosterone, methyltestosterone, nuptial color, melanophore, erythrophore, iridophore, bitterling

INTRODUCTION

Most teleost fish change their body color in response to background color or by a dependence on physiological conditions (Fujii, 1993, 2000; Sugimoto, 2002). Adaptation of body color to environmental conditions functions as protective coloration, while body color changes that reflect physiological conditions are used as communication signals among conspecific individuals. Nuptial color in fish is known to develop with sexual maturation, and some species of territorial males show this coloration as a signal of sexual maturity (Borg, 1994). Such body color changes are basically expressed by two categories of chromatophore responses: a rapid response caused by the immediate movement of pigments or platelets within the chromatophores, and a long-term response caused by changes in the density of chromatophores.

* Corresponding author. Phone: +81-422-33-3132;
Fax : +81-422-33-1449;
E-mail: makito@icu.ac.jp
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The neural and endocrine control of melanophore responses for adapting body color to a background color have been extensively studied in various species of fish (van Eys and Peters, 1981; Baker et al., 1984; Sugimoto et al., 2000; Sugimoto, 2002; Sugimoto et al., 2005). Although there are some studies on sexual dimorphism of body color or nuptial color of fish, few studies examine the difference in body color between male and female at the cellular level (Borg, 1994). In order to understand the development and regulation of body color, studies of body color at the chromatophore level are essential. It is also known that nuptial color in male fish is one of the male secondary sexual characters and induced by testicular androgens. Previous studies examined the effects of androgens on body color and chromatophores, but most of these studies used a synthetic androgen, methyltestosterone (MT) (Borg, 1994). In the present study, we used MT and 11-ketotestosterone (KT), a fish androgen, and compared their effects.

The rose bitterling, *Rhodeus ocellatus ocellatus*, is a small cyprinid species (Acheilognathinae) weighing 2–6 g, and sexually mature males of this species develop bright nuptial coloration, resulting in a clear difference in body

color between males and females; the latter keep the body color of juvenile fish. Besides distinct sexual difference in the body color, this species is also easy to maintain in the laboratory. Therefore, it is considered that this species could be a good model for the study of endocrine regulation of nuptial coloration and chromatophore development.

In the present study, as a first step toward understanding development of nuptial color at the cellular level, we compared body color and type of chromatophores between sexually mature male and female bitterlings. We also examined the effects of androgens, KT and MT, on body color and chromatophore density in female bitterlings. Such studies on the development of fish chromatophores will not only elucidate mechanisms regulating body color change in fish but will provide basic information for fish aquaculture in which body color is highly related to commercial value (Bolker and Hill, 2000; Van der Salm et al., 2004; Kalinowski et al., 2005).

MATERIALS AND METHODS

Fish

Wild rose bitterlings (*Rhodeus ocellatus ocellatus*) were obtained from a local fish market in Saitama Prefecture in May. Most of the fish obtained were sexually mature. Males had nuptial color, and females had vitellogenic oocytes at autopsy. Male and female bitterlings weighing 2.0–5.3 g were kept in 60-liter glass stock tanks at 25°C under 16L-8D to maintain their sexual maturity (Asahina et al., 1983). Fish were fed 1.0% body weight of commercial fish feed for juvenile carp, Koi Crumble No. 3 (Nihon Nosanko, Yokohama, Japan).

Comparison of body color and chromatophores between sexually mature male and female bitterlings

Body color was observed and compared between sexually mature males and females. Close-up photographs of body parts were taken by using a dissecting microscope (SZX12, Olympus, Tokyo, Japan) with a digital camera (Coolpix 4500, Nikon, Tokyo, Japan) after fish were anesthetized with a 0.03% 2-phenoxyethanol solution (Wako, Osaka, Japan).

Chromatophores on the scales were observed and compared between sexually mature males and females, and included melanophores, erythrophores, xanthophores, and two types of iridophores (the small greenish-type and the large spindle-shaped silvery-type). Erythrophores and xanthophores in this species were distinguished by their red color and yellow color, respectively

For observation of chromatophores on the scales, fish were anesthetized in a 0.03% 2-phenoxyethanol solution, and scales were collected from five regions, A–E, of the trunk (Fig. 1A): A, the top of the nape; B, the lateral part of the nape; C, the anterior part of side of the body; D, the lateral part of abdomen; E, the caudal peduncle. Scales were immersed in a physiologically balanced solution (PBS; 125.3 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.8 mM $MgCl_2$, 5.6 mM D-glucose, 5.0 mM Tris, pH 7.4). Photomicrographs of each scale were taken with a microscope (Optiphot-2, Nikon) equipped with a digital camera (Coolpix 990, Nikon). Quantitative analysis of choromatophores was conducted in the androgen administration experiment.

Effects of androgens on body color and chromatophores in female bitterlings

A synthetic androgen, methyltestosterone (MT) (Sigma, St. Louis, MO, USA), or fish androgen, 11-ketotestosterone (KT) (Cosmo Bio, Tokyo, Japan), was administered to females, and the effects on body color and chromatophore density were examined. Experimental females were divided into four groups and fed with one of the following feeds for 30 or 50 days: feed containing MT at a concentration of 20 μg/g diet, feed containing KT at a concentration of 2.0 μg or 20 μg/g diet, or feed containing no androgen (control females). To make feed containing androgen, androgens were dissolved in ethanol, and the androgen solution and fish feed (Koi Crumble No. 3, Nihon Nosanko) were mixed to make feed of desired concentrations of androgen. The ethanol was evaporated at room temperature. Ethanol without androgen was used to prepare the control feed. The daily ration was 1.0% of body weight throughout the experiment. Males were fed with the control feed. Fish of each group were kept in 10-liter glass tanks (25°C under 16L-8D) for 30 days (males, control females, MT 20 μg/g diet, KT 2.0 μg/g diet, and KT 20 μg/g diet) or 50 days (MT 20 μg/g and KT 20 μg/g diet).

The body color of experimental fish was compared at 20, 30, and 50 days of treatment, and photographs were taken at 20 and 50 days. Close-up photographs of body parts and scales from the five regions were taken as described above at 30 days of treatment. The numbers of melanophores and erythorphores were counted at 30 and 50 days of treatment, and the densities of these cells in the scale skin were calculated by using NIH imaging software. Two or three scales were isolated from each region of each fish, and five fish comprised each experimental group. The results are shown as means±SEM. Differences in means among groups were analyzed by ANOVA followed by the Tukey-Kramer HSD test. The level of significance was set at P=0.05.

RESULTS

Body color and chromatophores in sexually mature males and females

Photographs of sexually mature male and female bitterlings are shown in Fig. 1A and 1B. Males exhibited green, blue, orange, pink, silver, and black color on their body and fins as nuptial coloration, while females were of subdued silver and black coloration. Juveniles and females typically have a black spot on the dorsal fin, whereas mature males lack this spot (Fig. 1B).

The typical male nuptial color was observed as follows: orange color in the iris (Fig. 1C); black color on the dorsal fin, with orange spots on anterior part of the fin (Fig. 1D); green color on the nape (region A, Fig. 1E); and orange spots from the caudal peduncle to the caudal fin (region E, Fig. 1F). Compared to males, females did not develop these colors on the body and fins (iris, Fig. 1G; dorsal fin, Fig. 1H; region A, Fig. 1I; region E, Fig. 1J). Most females had a clear, black spot on the dorsal fin (Fig. 1H).

Chromatophores on the scales showed a different distribution between males and females. In males, greenish-type iridophores were observed in scales collected from the green colored area of the nape (region A, Fig. 1K, L), but silverly-type iridophores were observed in the scales of females (region A, Fig. 1Q, R). The number of melanophores in this green color area of the nape (region A) tended to higher in males than in females, but the difference was not significant when the density of this region was compared between males and females in the androgen administration experiment (Fig. 3).

Males had a bluish color in the lateral part of the nape (region B, Fig. 1A), but females had subdued silver in this region. When chromatophores in the scales collected from this region were observed, males had greenish-type iridophores, silvery-type iridopores, and erhythrophores (region B, Fig. 1M, N), whereas females had silvery-type iridophores and xanthophores (region B, Fig. 1S, T). The bluish part in

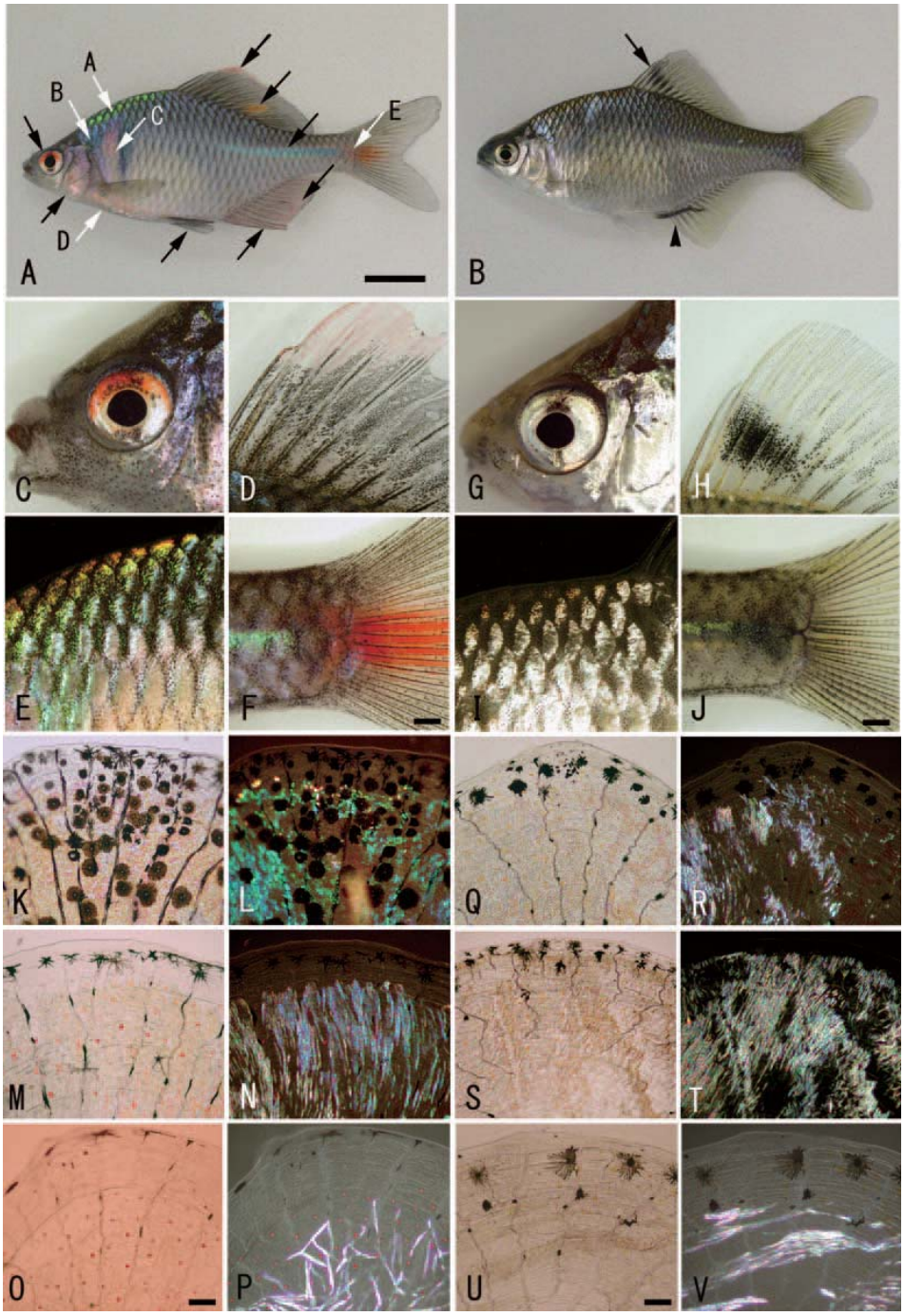

Fig. 1. Sexually mature male **(A)** and female **(B)** bitterlings (*Rhodeus ocellatus ocellatus*). Males show nuptial coloration. Arrows indicate the areas in which body color is different between males and females. Some mature males show orange spots in the anterior and middle parts of the dorsal fin, but most males have an orange spot only in the anterior part of the dorsal fin. White arrows indicate the regions where scales were collected for chromatophore observation. Females have a black spot on the dorsal fin. Arrowhead indicates the ovipositor. Scale bar, 1 cm. **(C–F)** Body parts of male bitterlings that show typical nuptial coloration. **(C)** Iris with orange color. **(D)** Anterior part of the dorsal fin with red spot. **(E)** Nape with green color. **(F)** Caudal peduncle with red spot. Scale bar, 1 mm. **(G–J)** Body parts of female bitterlings. **(G)** Iris with silver color. **(H)** Anterior part of the dorsal fin with a black spot. **(I)** Nape with silver color. **(J)** Caudal peduncle with dark color. Scale bar, 1 mm. **(K—V)** Chromatophores on the scales. **(K, M, O, Q, S, U)** Photographs taken with ordinary transmission optics. **(L, N, P, R, T, V)** Photographs taken under dark-field epi-illumination. **(K, L)** Male, region A; many melanophores (round or dendritic black cells) and greenish-type iridophores (round-shaped, blue or green colored cells in L) are evident. **(M, N)** Male, region B; melanophores, erythrophores (small red cells), silvery-type iridophores, and greenish-type iridophores are evident. **(N)** Scale showing blue color where silvery-type and greenish-type iridophores are present; cell margin with greenish-type iridophores is not clear in the photograph. **(O, P)** Male, region E; melanophores, erythrophores, and silvery-type iridophores are evident. Scale bar, 100 μm. **(Q, R)** Female, region A; melanophores and silvery-type iridophores **(R)** are evident; no greenish-type iridophores were observed. **(S, T)** Female, region B; melanophores, xanthophores (small orange cells), and silvery-type iridophores are evident. **(U, V)** Female, region E; melanophores, xanthophores, and silvery-type iridophores are evident. Scale bar, 100 μm.

Fig. 1N is an area where both greenish-type and silvery-type iridophores were observed at higher magnification (data not shown). In other regions, greenish-type iridophores were rarely observed in males, and iridophores were mostly silverly type (region E, Fig. 1O, P; regions C and D, photographs not shown). Greenish-type iridophores were rarely observed in the scales of females collected from the regions in which males showed green or blue color, and iridophores were mostly silvery type (region A, Fig. 1Q, R; region B, Fig. 1S, T; region E, Fig. 1U, V; regions C and D, photographs not shown).

Males had pink or red color in some regions in the body (regions C, D, and E, Fig. 1A), whereas females had a subdued silver color in these regions (Fig. 1B). A comparison between males and females of the chromatophores in scales collected from these regions showed that males had erythrophores, whereas females had xanthophores (region E, Fig. 1O, P, U, V; regions C and D, photographs not shown).

Effects of androgens on body color and chromatophores in female bitterlings

The body color of androgen-treated females developed

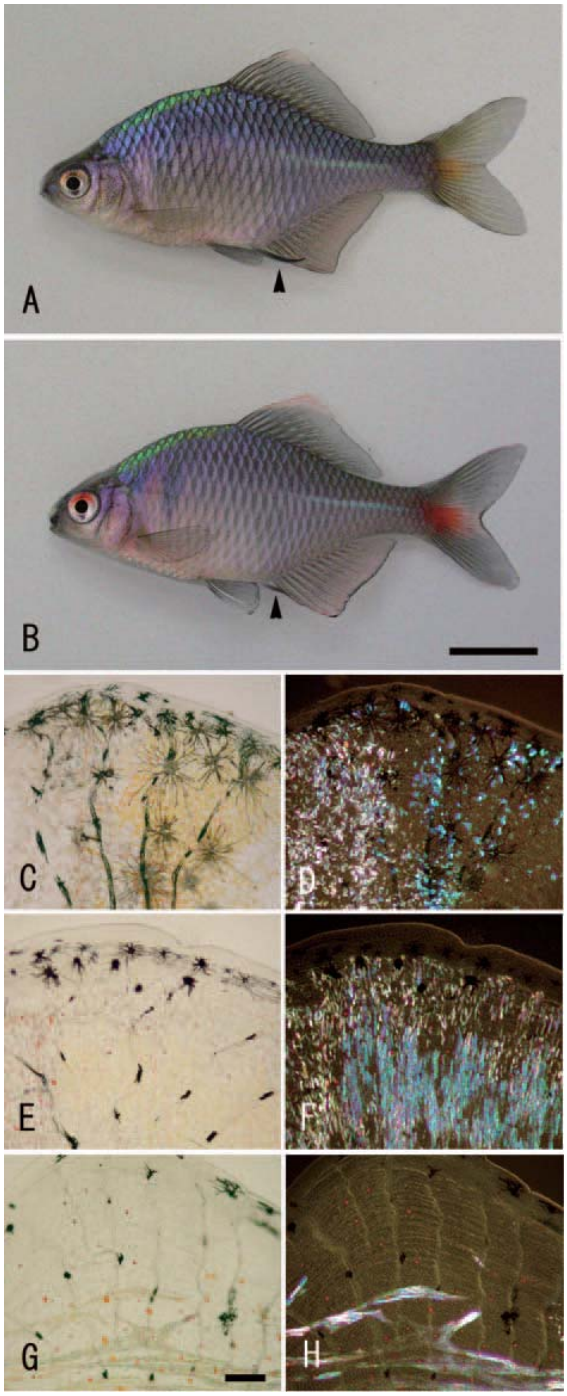

Fig. 2. Androgen-treated female bitterlings. Fish were administered methyltestosterone by diet (20 μg/g diet) for 20 days **(A)** or 50 days **(B)**. Male-type nuptial coloration developed in the females. Arrowheads indicate the ovipositor. Scale bar, 1 cm. Chromatophores on the scales of female bitterlings treated with methyltestosterone in the diet (20 μg/g diet) for 30 days. **(C, E, G)** Photographs taken with ordinary transmission optics. **(D, F, H)** Photographs taken under dark field epi-illumination. **(C, D)** Region A; melanophores and greenish- and silvery-type iridophores **(D)** are evident. **(E, F)** Region B; melanophores, erythrophores, greenish-type iridophores, and silvery-type iridophores **(F)** are evident; the scale shows blue color where silvery-type and greenish-type irodophores are present. **(G, H)** Region E; melanophores, erythrophores, and silvery-type iridophores are evident **(H)**. Scale bar, 100 μm.

male-type nuptial coloration (Fig. 2A, B). The male-type coloration appeared to be more intense with a longer period of androgen treatment (Fig. 2A, B). When the effects of KT and MT were compared (20 μg, 30 days), MT seemed to be more potent (photograph not shown). Androgen-treated females developed green color on the nape; blue color on the anterior part of the side of the body, orange color in the iris; pink color on the opercula, abdomen, anterior part of the side of the body, and the anal fin; orange spots on the dorsal fin and caudal fin; and black color on the dorsal fin, ventral fin, and margin of the anal fin. The black spot on the dorsal fin disappeared after androgen treatment.

Chromatophores on the scales in the androgen-treated females showed a distribution similar to that of males with nuptial color: greenish-type iridophores appeared in the green color area on the top of nape (region A, Fig. 2C, D) and blue color area on the lateral part of the nape (region B, Fig. 2E, F). In the bluish part in Fig. 2F, both greenish-type and silvery-type iridophores were observed. In other areas, silvery-type iridophores were mostly observed (region E, Fig. 2G, H; regions C and D, data not shown). Many erythrophores were observed in regions B, C, D, and E (region B, Fig. 2E, F; region E, Fig. 2G, H; regions C and D, photographs not shown). Few xanthophores were observed.

Melanophore densities of the scale in the five regions were not different between males and females, and androgen treatment did not affect the density except in region D of the 20 μgKT-50 days group (Fig. 3). The erythrophore density was different between males and females, and androgens significantly increased the erythrophore density in regions D and E ($P<0.05$) (Fig. 3).

Some androgen-treated females ovulated during the

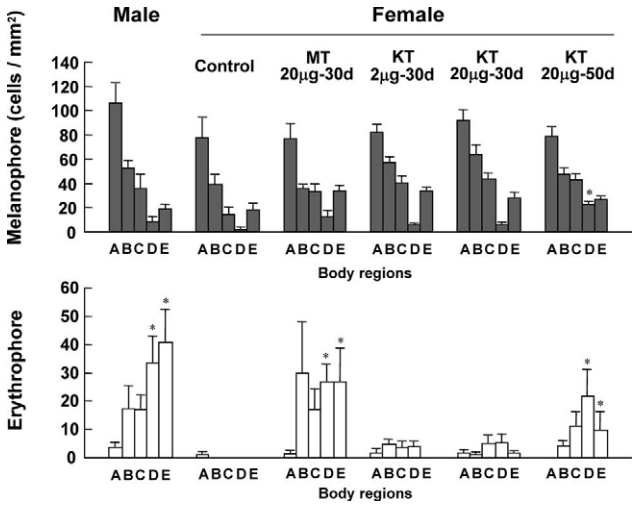

Fig. 3. Effects of androgens on the densities of melanophores and erythrophores in the scales of female bitterlings. Scales were collected from the five regions of the trunk indicated in Fig. 1A: A, top of nape; B, lateral part of nape; C, anterior part of side of body; D, lateral part of abdomen; E, caudal peduncle. MT, methyltestosoterone; KT, 11-ketotestosterone. Fish were fed either MT or KT at a dose of 2 or 20 μg/g diet for 30 or 50 days (d). Each column and bar indicate a mean and SEM (N=5). *, significance compared to control females ($P<0.05$).

experimental period, but did not engage in spawning behavior because of the absence of freshwater mussels as a spawning substrate.

DISCUSSION

The results of the present study indicate that male-type nuptial coloration in the bitterling is developed by the effects of androgen, although it is not clear whether the androgen directly acts on the chromatophores or via other cells. The disappearance of nuptial color after castration in another species of bitterling, *Acheilognathus intermedium* (presently *Tanakia lanceolata*), supports the involvement of androgens in the development of nuptial coloration in bitterlings (Tozawa, 1929). The present study also indicated that the responses to androgen were different among types and regions of chromatophores.

Melanophores, erythrophores, and xanthophores were observed in the skin and fins, and two types of iridophores were observed in the skin. Males had greenish-type iridophores in the area of the nape that shows green or blue color, whereas females had silvery-type iridophores in the corresponding area. These greenish-type iridophores seem to be essential for the expression of green or blue nuptial coloration. Androgen treatment of females induced green color in the nape, and iridophores of greenish type replaced those of silvery type in this region.

Many erythrophores were observed in red, orange, and pink areas of the skin and fins in males and androgen-treated females, but intact females had few erythrophores and more xanthophores. The present study clearly demonstrated that androgen induces the development of erythrophores. It is not yet known whether androgen-induced erythrophores develop independently from xanthophores or are derived from xanthophores. Oshima et al. (1996) found that prolactin (PRL) and melanophore-stimulating hormone (MSH) induced dispersion of pigments in erythrophores and xanthophores in vitro in male rose bitterlings, and suggested that these pituitary hormones are involved in the development of nuptial coloration. It should be examined whether PRL and MSH increase the density of erythrophores and other chromatophores.

Regardless of clear differences in body color, it is interesting that the distribution of melanophores in the scale skin was not different between males and females, and the melanophore density in the scale skin was not influenced by androgen treatment, indicating that these melanophores are insensitive to androgen, unlike greenish-type iridophores; however, melanophores in the fins responded to androgens. The black spot on the dorsal fin, a typical character in females, disappeared after androgen treatment due to a change in the distribution of melanophores. This black spot appeared again one month after androgen treatment ceased, even though red nuptial coloration was still present (data not shown). Development of melanophores in the fin, which causes fin darkening, was also observed following androgen treatment. These results suggest that bitterling has two types of melanophore, androgen sensitive and insensitive, localized in the fins and skin, respectively. Examination of the expression of androgen receptors would partially clarify the difference between these two types of melanophores. In addition to different responsiveness to androgens, it should be examined whether responses to other hormones, such as MSH, MCH, and norepinephrin, are different between the two types of melanopore. It has been reported in salmonid fishes that androgen stimulates body-color darkening, and that growth hormone induces blackening of the fin margin during the parr-smolt transformation (Ikuta et al., 1985; Komourdjian et al., 1976). Endocrine regulation on chromatophores may thus be different between salmonids and cyprinids.

The present study found that fish androgen and synthetic androgen had similar effects on the development of chromatophores. It is known that sexually mature male fish have high blood levels of testosterone and KT. KT is mostly a male-specific testicular sex steroid, but testosterone is produced in both the testis and the ovary. High circulating levels of testosterone are observed in sexually mature fishes of both sexes. Testosterone is an aromatizable androgen that plays a role in feedback effects on the brain-pituitary-gonad axis (Kobayashi et al., 1988; 1996), and KT is a non-aromatizable androgen which regulates male sex behavior and male secondary sexual characters (Borg, 1994). Although testosterone and KT sometimes show different physiological effects, MT, a potent analog of testosterone, showed an effect similar to KT at the peripheral level in the present study. Both KT and MT induced nuptial coloration in female bitterlings, but these androgens did not seem to affect the activity of the brain-pituitary-gonad axis of the females, since some MT- and KT-treated females ovulated during the experimental period (data not shown). Similar results have been reported in female goldfish, *Carassius auratus*, in which KT induced male-type sex behavior but did not inhibit the occurrence of ovulation (Stacey and Kobayashi, 1996; Kobayashi et al., 1997).

In summary, the present study demonstrated the effects of androgen on the development of male-type nuptial coloration and the development of greenish-type iridophores, erythrophores, and melanophores in a bitterling. It also showed that chromatophores differ in response according to their type and site. Further cellular and molecular studies are necessary to elucidate endocrine regulation of the development of nuptial color in fish.

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