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Authors: Matsui, Kumi, Takaichi, Shinichi, and Nakamura, Masahisa

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Morphological and Biochemical Changes in Carotenoid Granules in the Ventral Skin during Growth of the Japanese Newt *Cynops pyrrhogaster*

Kumi Matsui[1], Shinichi Takaichi[2] and Masahisa Nakamura[1]*

[1]*Department of Biology, School of Education, Waseda University, 1-6-1 Nishi-Waseda, Shinjuku-ku, Tokyo 169-8050, Japan*
[2]*Biological Laboratory, Nippon Medical School, Kosugi-cho 2, Nakahara-ku, Kawasaki 211-0063, Japan*

ABSTRACT—The color of the ventral skin of the Japanese adult newt *Cynops pyrrhogaster* is red, whereas that of the small juvenile newts at metamorphosis is creamy. Xanthophores in the red skin have many ring carotenoid vesicles (rcv) and a few homogenous carotenoid granules (hcg) , as reported earlier. To understand the reason for this change in coloration of the ventral skin of the newt, we carried out histological and biochemical studies to see whether the size and the number of carotenoid granules (hcg and rcv) in the xanthophores and also carotenoid content in the ventral skin change during the growth of this animal. By electron microscopic observation, only hcg were observed in the creamy skin of larvae at stage 59. The diameter of the hcg in the skin of the larvae was approximately 0.85 μm, but significantly decreased to 0.35 μm in the skin of the small juvenile newt. However, the number of the hcg/100 μm^2 of a xanthophore in the ventral skin was very low in the larva at stage 59, but increased in the small juvenile. The carotenoid content was very low in the creamy skin of small juveniles, but dramatically high in the red skin of the adult newts. In the red skin of the adult newt, many rcv (85%) and a few hcg (15%) were observed. However, the number of carotenoid granules (rcv and hcg)/100 μm^2 of a xanthophore in the red skin of adult newts was not different from that of hcg/100 μm^2 of a xanthophore in the creamy skin of small juveniles. The results, taken together, suggest that the increase in the size and the number of carotenoid granules and also carotenoid content in the ventral skin is very important for red body coloration during the growth of the Japanese newt *Cynops pyrrhogaster*.

Key words: carotenoid, carotenoid granule, ultrastructure, newt, *Cynops pyrrhogaster*

INTRODUCTION

Body coloration of animals is important for their behaviors (Edmunds, 1974; Endler, 1978). The body color of lower vertebrates is determined by the types of chromatophores in the skin (Bagnara *et al*., 1979). According to Bagnara (1966), melanophores appear first in the dorsal integument of a larva during development. Xanthophores, which have pteridines and/or carotenoids, appear second; and iridophores, containing purine crystals, or the so-called reflecting platelets, appear last. There are two ways for formation of the body coloration. One is that body color is produced by auto-synthesis of pigments. In fact, Ziegler *et al*. (2000) showed an active metabolic pathway of pteridine synthesis in the zebrafish. The other occurs by the uptake of pigments directly from foods. In amphibians, 3 kinds of pigment cells, i.e., melanophores, xanthophores and iridophores, are seen in the ventral skin of the juvenile after metamorphosis (Bagnara, 1998). In a previous study, we described the ultrastructure of carotenoid granules in xanthophores in the ventral skin of the wild Japanese adult newt *C. pyrrhogaster*, and found many rcv in them (Matsui *et al*., 2002). This suggests that the body coloration of the Japanese newt depends on the number and the carotenoid content of carotenoid granules (hcg and rcv) in these xanthophores in the ventral skin. However, it still remains unknown whether the size and the number of hcg and rcv and also the carotenoid content in the ventral skin change during the growth of this animal. Thus, histological and biochemical studies were carried out to understand the formation of the red coloration of the ventral skin of the adult Japanese newt *C. pyrrhogaster*.

* Corresponding author: Tel. +81-3-5286-1518;
FAX. +81-3-3207-9694.
E-mail: nakamra@waseda. jp

MATERIALS AND METHODS

Animals

In all the experiments, the Japanese newt *C. pyrrhogaster* was used. Juvenile and adult newts were captured in Fukue Island in Nagasaki Prefecture from 1998 through 2002. The juvenile newts were divided into 3 groups in terms of their snout-vent length or SVL (the small, SVL<24.9mm; the medium, 25mm<SVL<34.9mm; and the large, 35mm<SVL<40.9mm). Eggs of laboratory-reared newts were spawned by injection of human chorionic gonadotropin (25 units/body) into the body cavity of mature females, each dose given at a 2-day interval until they spawn. Developmental stages were determined according to Okada (1947). Larvae were fed with only baby brine shrimp (*Artemia sp*), having canthaxanthin as their main carotenoid (85% of the total carotenoids; Krinsky, 1965).

Histological analysis

Ventral skins of newts were prepared, and the ultrastructure of the specimens was examined by transmission electron microscopy (Hitachi, model H-300, Tokyo, Japan), as reported elsewhere (Matsui *et al*., 2002).

Numbers of carotenoid granules (hcg and rcv) were counted in the sections of specimens from different stages of newts and presented as values/100 μm^2 of a xanthophore in the ventral skin. Sizes (diameters) of carotenoid granules were determined from TEM photos and analyzed statistically by using paired t-test. To compare sizes of the granules, we used mean values for statistical analysis.

Carotenoid analysis

The contents of carotenoids in the ventral skin of larvae and newts were determined by the methods of TLC and HPLC. Carotenoids for TLC analysis were prepared as described elsewhere (Matsui *et al*., 2002). HPLC analysis was carried out by the method of Takaichi and Ishidsu (1992). Pigments were extracted from the ventral skin of a newt with acetone, the solvent was evaporated, and the extract was washed twice with ethanol for dehydration. The pigments were dissolved in a small amount of the mixture of chloroform and methanol (v/v=3/1) and analyzed by the HPLC. The sample was loaded on a column (μBondapak C_{18}, 8 mm×100 mm, RCM type; Waters, Milford, MA, USA) and eluted with 100% methanol at a flow-rate of 2.0 ml/min at room temperature (Takaichi and Ishidsu, 1992). Absorption spectra and retention times were recorded by an HPLC system, equipped with a photodiode-array detector (Otsuka Electronics, model MCPD-3600, Osaka, Japan). Each peak was identified by compairing its retention time and absorption spectra with those of β-carotene, astaxanthin, canthaxanthin and zeaxanthin as standards (Takaichi and Shimada, 1992). Total quantity was calculated according to Buchwald and Jencks (1968).

RESULTS

Fig. 1 shows the color of the skin of a larva at stage 59 (a), a juvenile newt at metamorphosis (b), and small (left), medium (middle) and large (right) wild juvenile newts (c). As can be seen in Fig. 1, the color of the ventral skin of a larva at stage 59 and a small juvenile was creamy, whereas that of medium and large juveniles was red. Then, we examined hcg in the xanthophores in the skin of newts at different stages. As shown in Fig. 2a, a few hcg were observed in the creamy skin of larvae at stage 59, as indicated by the black arrowhead. Their average size and the number were 0.78±0.07 μm (mean±SEM) and 0.99±0.62/100 μm^2 of a xanthophore (mean±SEM), respectively. Only hcg were also observed in the creamy skin of newts at metamorphosis (Fig. 2b); and their average size and the number were 0.85±0.05 μm and 1.35±0.58/100 μm^2, respectively. In the skin of small, medium and large juveniles, only hcg were seen (Fig. 2d, 2e). Sizes of the hcg decreased dramatically

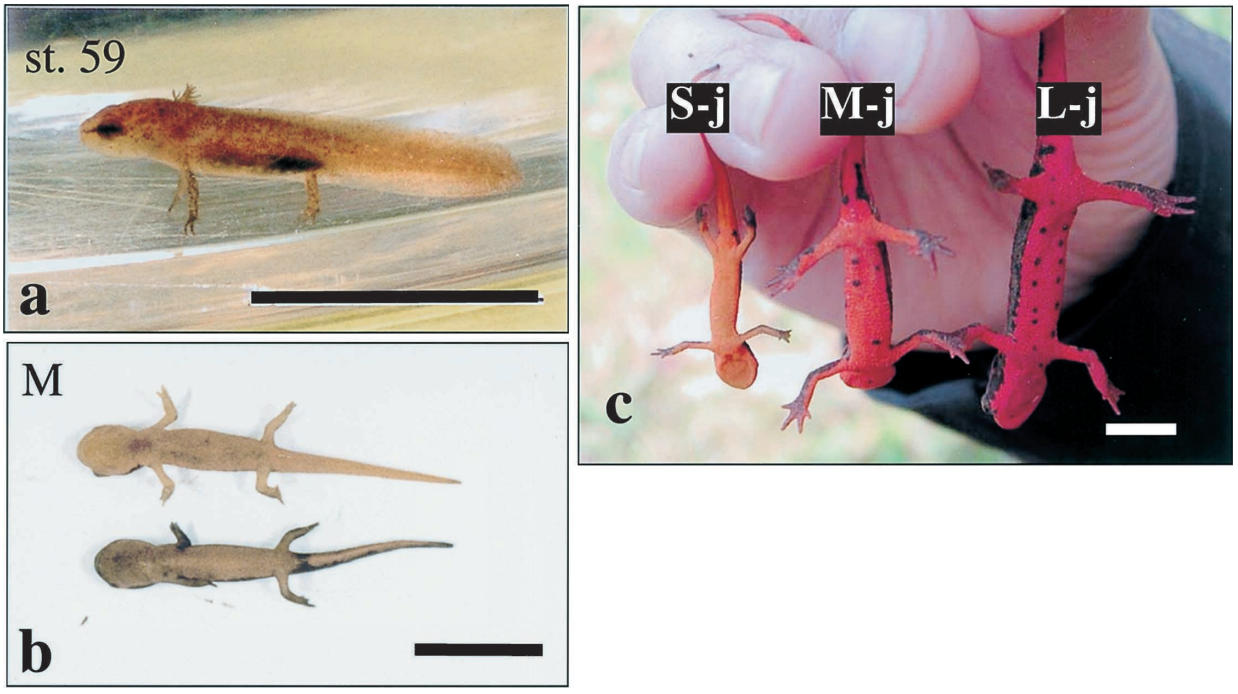

Fig. 1. Color of the ventral skin of a larva, a juvenile at metamorphosis, and juveviles of 3 sizes of the Japanese newt *Cynops pyrrhogaster.* (a) A larva at stage 59. (b) A newt at metamorphosis. (c) Small (left), medium (middle) and large (right) juveniles. Bar=1 cm.

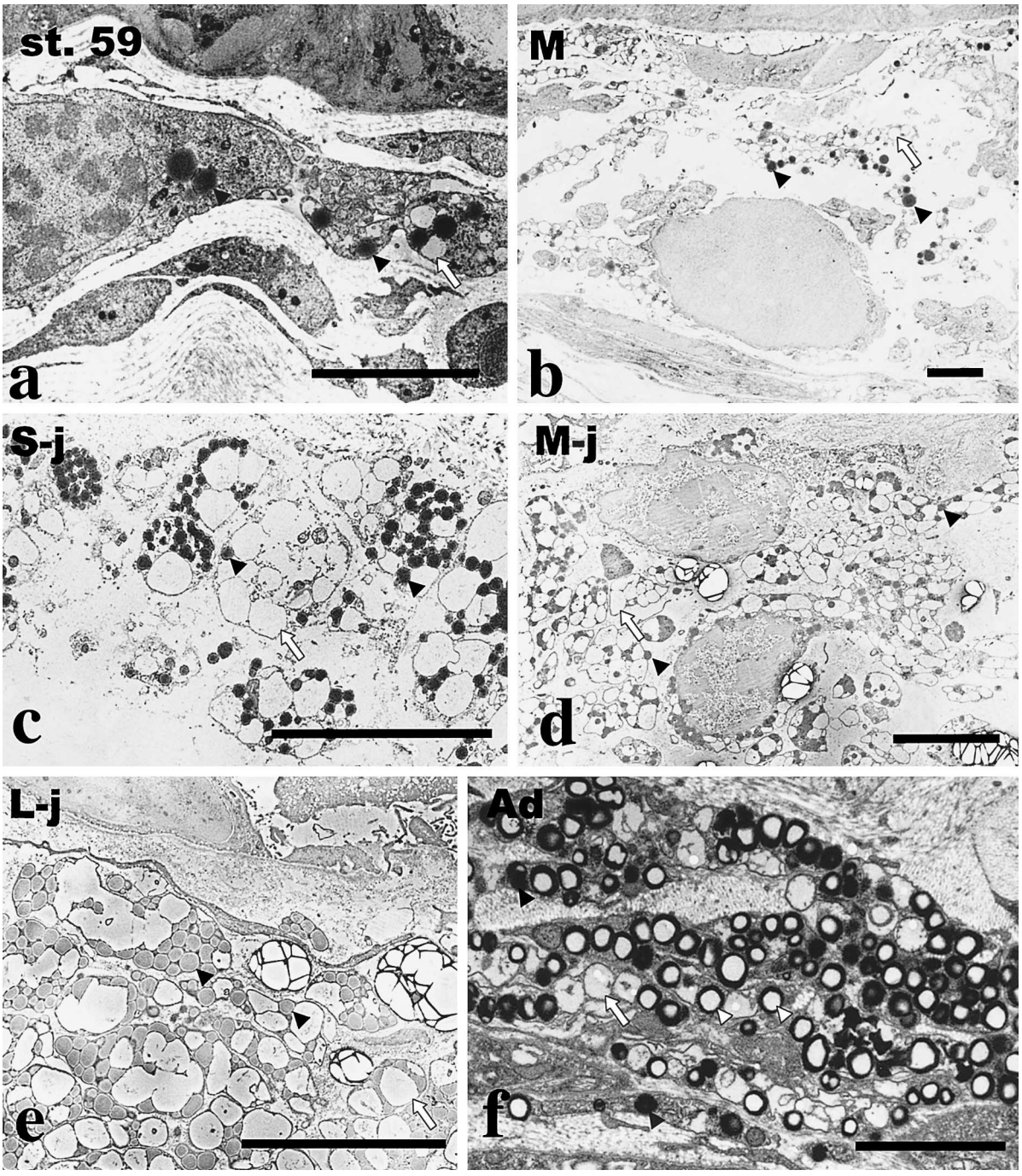

Fig. 2. Ultrastructures of hcg and rcv in a xanthophore in the newt skin. (a) A larva at stage 59. (b) A juvenile at metamorphosis. (c) A small juvenile. (d) A medium juvenile. (e) A large juvenile. (f) An adult newt. Black and white arrowheads indicate hcg and rcv, respectively. A pterinosome is indicated by the white arrow. Bar=5 μm.

to 0.35–0.45 μm, but the number dramatically increased to 99–101/100 μm^2. However, in the skin of adult newts, many rcv and a few hcg were found (Fig. 2f). As shown in Table 1, average size of carotenoid granules (rcv and hcg) in the red skin of adult newts was larger (0.46 μm) than that of hcg in the creamy skin of small juveniles (0.35 μm) ($p<0.05$). However, the number of rcv and hcg in the skin between adult newts and small juveniles was not different (117±17 *vs* 101±13/100 μm^2), as shown in Table 1 and Fig. 3. In addition, average sizes of hcg in the ventral skins of larvae at stages 44 to 59 and juveniles at metamorphosis were relatively large (0.70–0.85 μm), but dropped significantly in the creamy skin of small juveniles (0.35 μm) ($P<0.05$) (Table 1). Then, the size of carotenoid granules increased in the red skin of medium and large juveniles and adult newts (0.45–0.47 μm) ($p<0.05$) (Table 1). Moreover, total area of carotenoid granules was 0.77 μm^2/100 μm^2 of a xanthophore in the ventral skin of juveniles at metamorphosis when the out-

Table 1. Number (N) and Size (S) of carotenoid granules in the ventral skin of growing newts at different stages. Total area (μm^2) of carotenoid granules was calculated by $\pi(S/2)^2 \times N$, assuming a circular outline of carotenoid granules in Fig. 2. The letter n means the number of animals. Values are presented by the mean±SEM.

	Larvae (st. 44) n=2	Larvae (st. 55) n=2	Larvae (st. 59) n=3	Metamorphosis n=4	Small juveniles n=3	Medium juveniles n=3	Large juveniles n=3	Adult n=2
N (Mean±SEM)	0.22 ± 0.06	0.92 ± 0.18	0.99 ± 0.62	1.35 ± 0.58	101.50 ± 13.21	99.25 ± 16.11	101.67 ± 24.48	117.33 ± 17.13
S (μm) (Mean±SEM)	0.83 ± 0.03	0.70 ± 0.10	0.78 ± 0.07	0.85 ± 0.05	0.35 ± 0.02	0.47 ± 0.02	0.47 ± 0.05	0.46 ± 0.02
Total area (μm^2) $\pi(S/2)^2N$	0.12	0.35	0.47	0.77	9.76	15.78	17.63	19.49

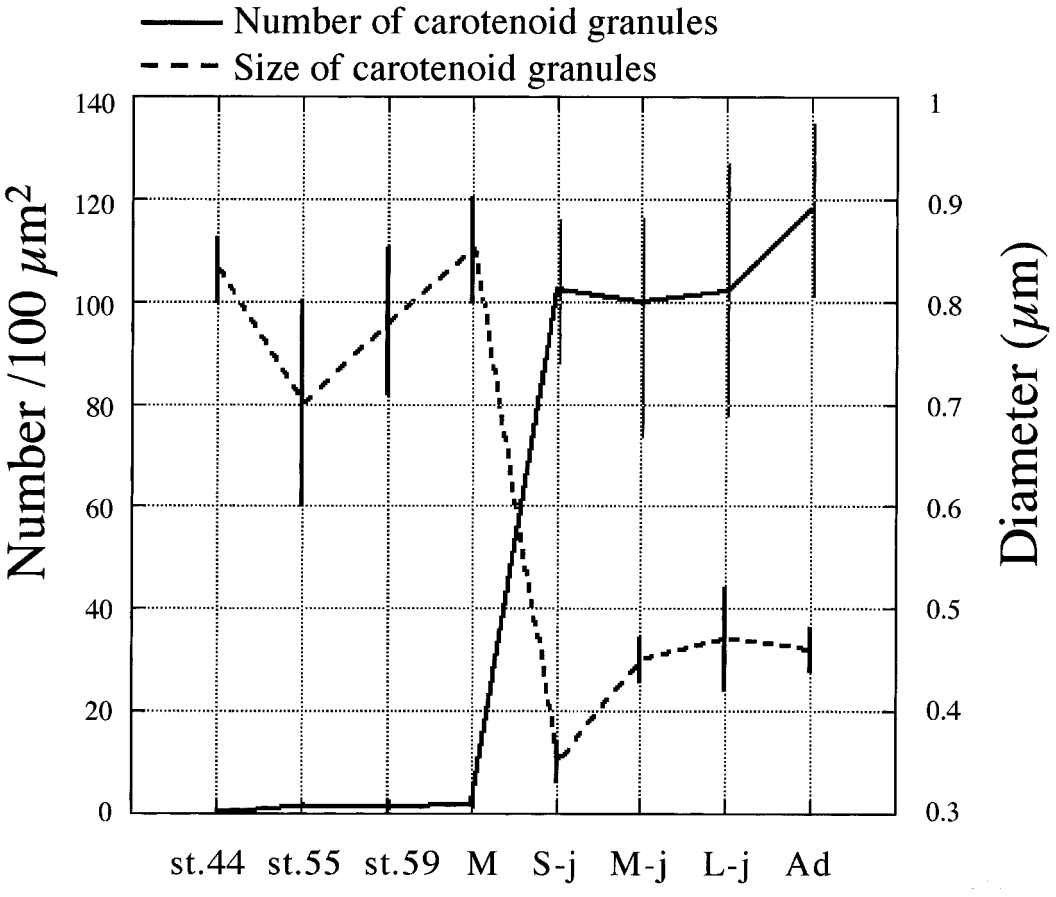

Fig. 3. Relationship between the number and size of carotenoid granules. Dotted and solid lines indicate the number and size of carotenoid granules, respectively. Numbers of granules in several areas of 100 μm^2 in the sections are counted and plotted in the figure. Vertical lines incicate the mean±SEM. M, a juvenile at metamorphosis. S-j, a small juvenile. M-j, a medium juvenile. L-j, a large juvenile. Ad, an adult newt.

line of carotenoid granules was assumed to be circular, but significantly increased to 9.76 μm^2 in small juveniles and then to 19.49 μm^2 in adult newts ($P<0.01$) (Table 1).

Finally, we analyzed the contents of carotenoids in the whole ventral skin of a juvenile and an adult. Contents of total carotenoids in the ventral skin were determined by the methods of TLC and HPLC. The results are shown in Fig. 4. The content of total carotenoids was low in the creamy skin of small juveniles (<2 μg/total ventral skin), but was very high in the red skin of the adult newts (approximately 35 μg/total ventral skin) (Fig. 4). The coefficient correlation was $y = 0.049 \cdot e^{(0.12x)}$ ($r=0.84$).

DISCUSSION

The ventral skin of the wild Japanese newt *C. pyrrhogaster* is creamy at metamorphosis, but turns red as the animal matures. Previously we described the ultrastructure of

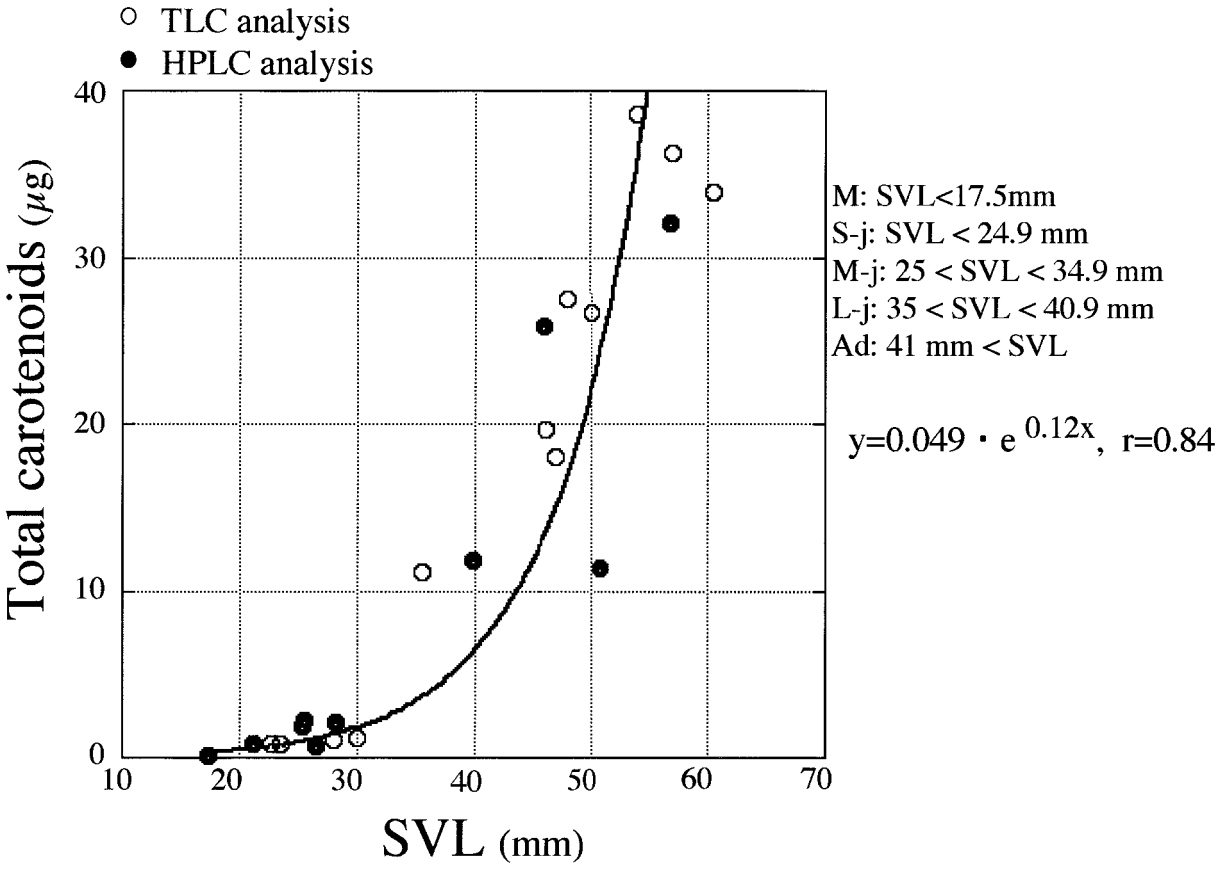

Fig. 4. Change in the content of total carotenoids in the ventral skin of newts. SVLs of newts at different stages are shown on the right side of the panel. Abbreviations at different stages are the same as described in the legend to Fig. 3. A regression curve was obtained according to Beyer (1976). (○) The carotenoid content measured by the TLC method. (●)The carotenoid content measured by the HPLC method.

carotenoid granules in the red ventrum of wild and laboratory-reared Japanese adult newts (Matsui *et al*., 2002). In the electron-microscopic study we found many rcv (85%) and a few hcg (15%) in the red skin of wild adult newts. The red skin of a wild adult newt contained a large amount of carotenoids, but the yellow skin of a laboratory-reared adult newt did not (Matsui *et al*., 2002). Therefore, the amount of carotenoids in the ventral skin seems to be very critical for producing the red ventral coloration of the Japanese newt *C. pyrrhogaster*. This hypothesis is supported by the present findings that 3 factors (size, number and carotenoid content of carotenoid granules in the ventral skin) change during the growth of the Japanese newt *C. pyrrhogaster*. It is well-known that the body coloration of amphibians is determined by chromatophores (melanophores, xanthophores, and iridophores) in the body skin. Xanthophores in the ventral skin of the newt cannot synthesize carotenoids (Britton, 1983; Bagnara, 1998). Therefore, the cells must uptake carotenoids through foods to produce the red ventral color. However, as the mechanism is still unclear, it will be important to investigate what kinds of foods juvenile newts eat routinely and what sorts of pigments are contained in these foods.

It should be noted that only hcg were seen in the creamy skin of small juveniles but that many rcv and a few hcg were observed in the red skin of the adult newts. The average size of rcv and hcg in the red skin of adult newts is larger than that of hcg in the creamy skin of small juveniles (Table 1 and Fig. 3). Furthermore, the number of rcv and hcg/100 μm^2 of a xanthophore in the red skin of adult animal was very similar to that of hcg/100 μm^2 of a xanthophore in the creamy skin of the small juvenile, indicating that the number of rcv is increased whereas that of hcg is decreased. However, this does not mean that hcg are replaced by rcv. Also, the amount of carotenoids in the red skin was much greater than that in the creamy skin. Thus, the size and the number of carotenoid granules, as well as the carotenoid content in the granules, are important factors for the red coloration of the ventral skin of the Japanese newt. As reported previously (Matsui *et al*., 2002), β-carotene and lutein were retained on the filter paper, but astaxanthin was not, when carotenoids on the paper were treated with OsO_4 and propylene oxide. It is evident that the amount of carotenoids in rcv and hcg in the red skin of the adult newt was much greater than that in hcg in the red skin of the large juvenile (see Fig. 2e, 2f, Fig. 4). It is, therefore, very likely that rcv were formed from hcg after some carotenoids in the hcg had been extracted during TEM process-

ing. It should also be noted that hcg in small juveniles appeared before carotenoids began to accumulate. As far as we know, this is the first report showing that the appearance of carotenoid granules in a pigment cell precedes the accumulation of carotenoids in them during the growth of the newt.

The Japanese newt *C. pyrrhogaster* has pterinosomes containing pteridines in xanthophores in the ventral skin. A few kinds of pteridines have been found in the skin of larvae, but not adults, of the Japanese newt *C. pyrrhogaster* (Obika, 1963). The skin of the adult newt instead contains a large amount of riboflavin (Obika, 1963). As it can be seen in Fig. 2, many pterinosomes were present in the skin of larval, juvenile and adult newts. However, we do not know at the present time how pterinosomes are involved in producing the red coloration of the ventral skin of the newt. The color of the ventral skin of the wild axolotl *Ambystoma mexicanum* is creamy. In the skin of this animal, no carotenoid was detected (Frost *et al*., 1984). The American newt *Notophthalmus viridescense* and the toad *Bombina orientalis* have xanthophores (erythrophores) in their skin, in which many carotenoid granules are present (Forbes *et al*., 1973; Frost and Robinson, 1984). Further studies are required to explain how carotenoids and pteridines are involved in the red coloration of the ventral skin of the Japanese newt *C. pyrrhogaster*. Nevertheless, the size, the number and the carotenoid content of hcg and rcv need to be increased for the red body coloration of the Japanese newt *C. pyrrhogaster*.

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