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Heterochronic Expression of Several Adult Phenotypes in Normally Metamorphosing and Metamorphosis-Arrested Larvae of a Salamander *Hynobius retardatus*

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ABSTRACT—Heterochronic expressions of several larval and adult phenotypes in *Hynobius retardatus*, which had been reported to show neotenic reproduction, were described in normally metamorphosing animals (controls) and goitrogen-induced, metamorphosis-arrested larvae. External gills, dorsal tailfins and Leydig's cells in the epidermis were completely diminished in the controls during and after metamorphosis, but fully remained in the metamorphosis-arrested larvae. Two types of dermal glands, mucous and serous glands, however, behaved differently from Leydig's cells, even though they constituted the same skin. The dermal gland cells in the controls appeared at a premetamorphic stage, gradually increased in number during the metamorphosis and fully developed after the metamorphosis. Those in metamorphosis-arrested larvae appeared much later than in the controls. Thus, aged, metamorphosis-arrested larvae had skin which consisted of larval type epidermis (Leydig's cells) and adult type dermis (mucous and serous glands). Contrary to these, a transition of globin subunits from larval to adult types occurred practically on the same time schedule in both the controls and metamorphosis-arrested larvae. These observations suggest that there are at least three types of organs or cells which behave differently during and after metamorphosis in *Hynobius retardatus*.

INTRODUCTION

Hynobius retardatus has been reported to show neotenic reproduction in a specific environment of Lake Kuttara (Sasaki, 1924; Sasaki and Nakamura, 1937). Unfortunately, however, the neotenic population in Lake Kuttara is believed to be extinct at present. It is therefore unclear whether the main population of *H. retardatus*, which is widely distributed throughout Hokkaido, has faculties of neotenous reproduction under specific environmental or certain experimental conditions. In this respect, we have recently demonstrated that the larvae can produce morphologically mature spermatozoa even in larval forms, when the metamorphosis has been arrested by goitrogens (Wakahara, 1994; Yamaguchi *et al.*, 1996), and that a transition of globin subunits from larval to adult types occurs on the same time schedule in both normally metamorphosing animals and metamorphosis-arrested larvae (Arai and Wakahara, 1993) or precociously metamorphosed animals (Wakahara *et al.*, 1994). These observations strongly suggest that the gonadal development and certain biochemical alterations from larval to adult types will be independent on the morphological metamorphosis in this species. Because these chronological separations or heterochronic expressions between germ cell and somatic cell developments will be fundamental causes of the neoteny (Gould, 1977), it seems important to investigate the heterochronic expressions among the gonadal development, several biochemical alterations from larval to adult phenotypes and the morphological metamorphosis for understanding the mechanism of metamorphosis of neotenic urodela.

Among various alterations, changes in molecular constituents of the body such as blood proteins (Frieden, 1961), keratins (Nishikawa *et al.*, 1992) and hemoglobin subunits (Cardellini and Sala, 1979; Kobel and Wolff, 1983; Jurd, 1985), and a pattern of nitrogen excretion (Weber, 1967; Wakahara *et al.*, 1994), were extensively studied in many amphibian species. Changes in skin (epidermal and dermal) cells (Heady and Kollros, 1964; Fox, 1985; Robinson and Heintzelman, 1987; Izutsu *et al.*,1993; Amano *et al.*, 1995), and in red blood cells (Ducibella, 1974a) were also described. Almost all studies have shown that those metamorphic changes from larval to adult phenotypes are regulated by complicated hormonal controls including thyroid hormones, prolactin, ACTH and corticoids (Dodd and Dodd, 1976; Rosenkilde, 1985). At present, however, little studies have done with respect to heterochronic expressions of adult phenotypes during the metamorphosis in amphibians.

In order to analyze possible mechanisms causing the chronological separation between the morphological metamorphosis and the gonadal development or certain biochemical alterations from larval to adult types in *Hynobius retardatus*, observations were extended to several organs and cell types, such as the external gills, dorsal tailfins, epidermal and dermal cells and red blood cells (RBCs).

MATERIALS AND METHODS

Animals

Fertilized eggs of *Hynobius retardatus* were collected from ponds or in small streams in the vicinity of Sapporo in the breeding season. Newly hatched larvae were reared at a room temperature either in aqueous solution of 0.02% thiourea and 0.04% sodium perchlorate (Wakahara, 1994) to arrest the metamorphosis (experimentals), or goitrogen-free medium (controls). They were fed with commercially available frog feed pellets (Oriental Kobo Co., No.2 for frog) or live *Tubifex*. After the controls metamorphosed (approximately 70 days after hatching), they were tranferred to a terrarium. Developmental stages were determined according to the normal table for *Hynobius nigrescens* (Iwasawa and Yamashita, 1991), a closely related species to *H. retardatus*.

Histology

At proper intervals, animals were fixed in Bouin's fixative, and skin of the head region was processed for histological observations. Serial sections of 8 μm in thickness were stained wtih Delafield's hematoxilin and eosin.

Measurements of developmental degrees of organs and cells

Sizes of the external gills and dorsal tialfin were directly measured in the fixed materials using sliding callipers at the level of 0.05 mm. In order to assess the developmental degrees of Leydig's cells and two types of the dermal glands, tentative indices were employed: these indices were caliculated from the number of Leydig's cells or dermal glands per unit area on histological sections. Number of Leydig's cells or dermal glands on every 5 section from the serial histological sections was counted and then the average number of them per unit area was caliculated.

Electrophoresis

Blood samples were collected directly from heart when the larvae were very small, or from tail when the animals were large enough for bleeding. Procedures for preparaton of hemolysates were described previously (Arai and Wakahara, 1993). After the amount of protein was determined using BCA Protein Assay Reagent (Pierce Chem. Co.), the samples were electrophoresed. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970), using 15% separating gels. All electrophoresed gels were stained with Coomassie Brilliant Blue. Electrophoretic profiles were photographed respectively and a transition of globin subunits from larval to adult types was analyzed by measuring a proportion of larval to adult globins using a computerized Image Analyzer (NIH-Image). For the convenience sake, the proportion of larval specific globins (designated as L1 on a SDS-PAGE, or as HL1, HL2 and HL3 on a two dimentional electrophoresis (Arai and Wakahara, 1993)) to total globins was caliculated (cf. Fig. 5 inset).

RESULTS

Changes in external morphology

Normal larvae metamorphosed approximately 70 days after hatching and transferred from aquatic to terrestrial habitats (see Fig. 2 in Wakahara *et al.*, 1994). The most conspicuous changes in the external morphology during the metamorphosis was disappearance of external gills and dorsal tailfin. Figure 1 shows developmental fates of external gills (Fig. 1A) and dorsal tailfin (Fig. 1B) in normally metamorphic animals and metamorphosis-arrested larvae in *Hynobius retardatus*. In normally metamorphic animals (i.e., controls), both organs gradually decreased in size during the metamorphosis and completely diminished after the metamorphosis. Contrary to these, in the metamorphosis-arrested larvae the external gills and dorsal tailfin still developed well during and after the period when the controls were metamorphosing and metamorphosed. At the end of the experiment (300 days after hatching), both organs remained in the metamorphosis-arrested larvae.

Changes in epidermal structures

Typical larval skin was entirely composed of an epidermis which was mainly occupied with Leydig's cells (Fig. 2A), epidermal specific cells in urodeles (Kelly, 1966). The Leydig's cells were extraordinarily large (approximately 70 μm in diameter) and contained a lot of large cytoplasmic granules which stained with hematoxylin. Dermal structures except for the basal lamina and melanophores were hardly seen in histological sections (Fig. 2A). On the other hand, adult skin was composed of an epidermis which was predominantly occupied by stratified squamous cells, and of dermal tissues which consisted of two types of dermal glands, mucous and serous glands (Fig. 2B). The mucous gland was composed of several gland cells, a central cavity and a duct opening to body surface. The secretory granules which stained faintly with eosin were very small and thus looked amorphous at the light microscopical level (Fig. 2B). The serous gland was also

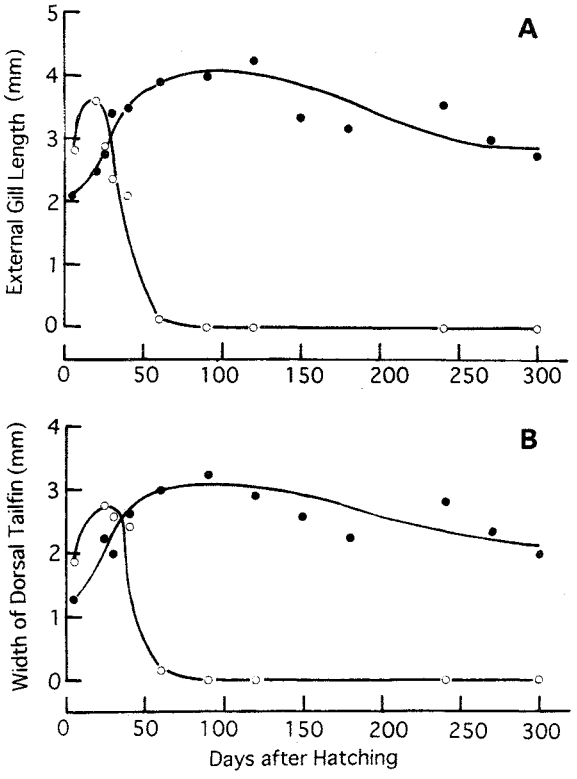

Fig. 1. Developmental fates of external gills (A) and dorsal tailfin (B) in normal controls (open circles) and in metamorphosis-arrested larvae (closed circles) of *Hynobius retardatus*. Both organs completely diminish during metamorphosis in the controls, but develop and remain in the metamorphosis-arrested larvae.

composed of several gland cells which contained secretory granules of various size (1-10 mm in diameter). The secretory granules were stained densely with eosin.

Figure 3 shows developmental changes in Leydig's cells in the controls and metamorphosis-arrested larvae. The Leydig's cells were main constituents of larval epidermis, and thus already observed at the hatching stage. They developed thereafter during the premetamorphic stage. Similarly to the external gills and dorsal tailfin, Leydig's cells in the controls gradually disappeared from the epidermis and were replaced

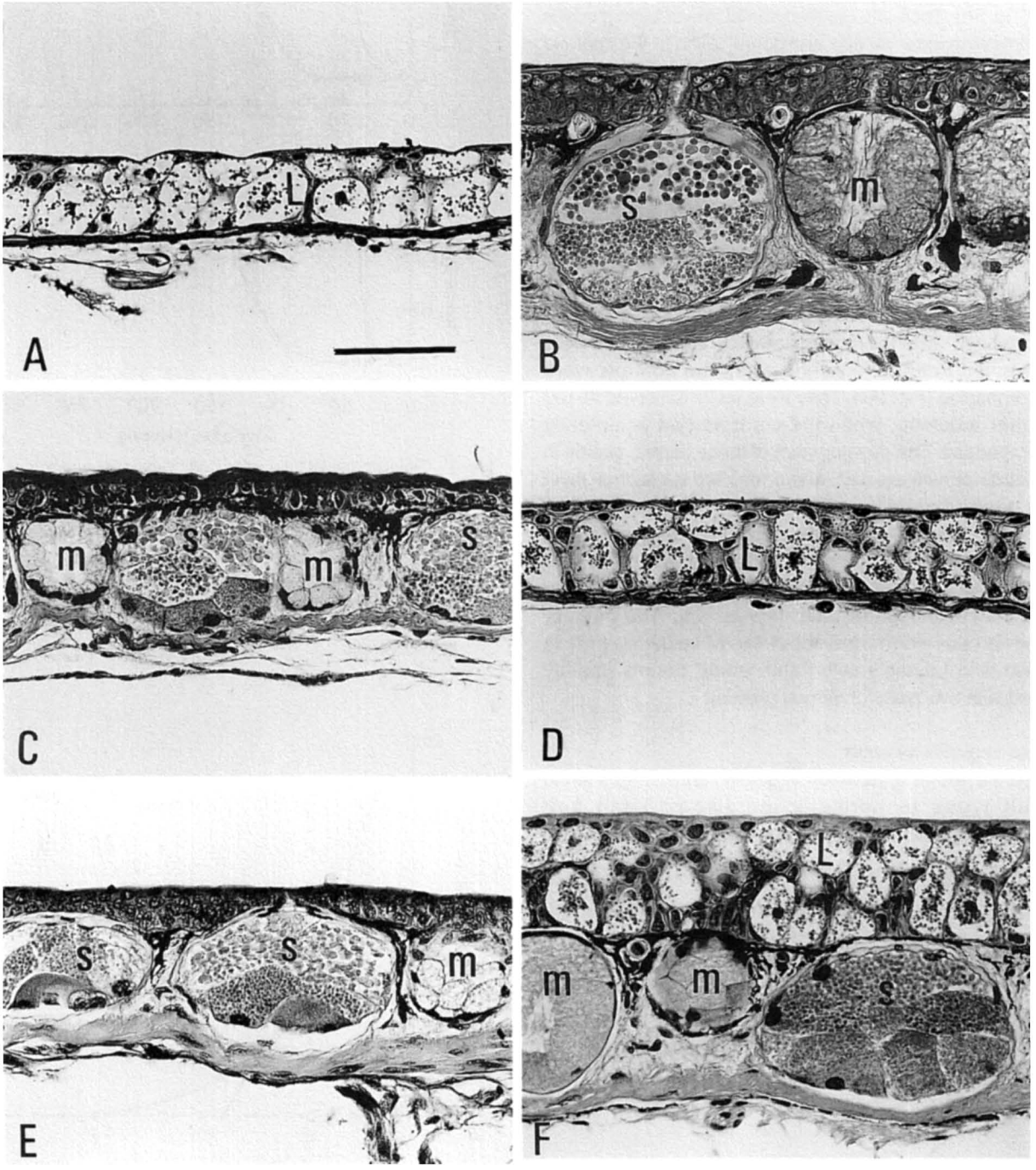

Fig. 2. Histology of head skins of normal controls (A, B, C and E) and of metamorphosis-arrested larvae (D, F). (A) Typical larva (25 days after hatching). The skin is almost composed of an epidermis which is mainly occupied with Leydig's cells. (B) Typical adult (sexually mature male). The skin is composed of an epidermis which consists of stratified squamous cells and a dermis which is occupied with well developed dermal glands; mucous and serous galnds. (C) Control juvenile just after metamorphosis (90 days after hatching). (D) Metamorphosis-arrested larva at 90 days after hatching. Epidermis is composed of larval specific Leydig's cells, and no dermal glands are observed. (E) Control juvenile at 270 days after hatching. (F) Metamorphosis-arrested larva at 270 days after hatching. Leydig's cells are still main constituents of the epidermis and well developed dermal glands occupy the dermis. L, Leydig's cell; m, mucous gland; s, serous gland. Hematoxylin-eosin stain. Bar indicates 100 μm.

by stratified squamous cells during the metamorphosis (Fig. 2C). On the one hand, they were well developed in the metamorphosis-arrested larvae during the period when the controls were metamorphosing and metamorphosed (Fig. 2D). At the end of the experiment, Leydig's cells were still main constituents of the epidermis in the metamorphosis-arrested larvae (Fig. 2F). Thus, the developmental fate of Leydig's cells was identical to external gills and dorsal tailfin in the controls and metamorphosis-arrested larvae.

Changes in dermal structures

Figure 4 shows developmental changes in dermal glands (A, mucous gland; B, serous gland) in the controls and metamorphosis-arrested larvae. In the controls, these glands began to be observed 25 days after hatching (stage 60, premetamorphic stage), and then developed rapidly during the metamorphic stages. After the metamorphosis, they were major constituents of the dermal tissues (Fig. 2C, E). In the metamorphosis-arrested larvae, however, these glands were not observed during the period when the controls were metamorphosing (Fig. 2D). They were firstly observed at 100 days after hatching, when the controls had completely metamorphosed. The development of these dermal glands in the metamorphosis-arested larvae reached a maximal level at 160 days of hatching. At the end of the experiment, morphological properties and the degrees of development of these glands in the metamorphosis-arrested larvae (Fig. 2F) were identical to those in the controls (Fig. 2E). Thus, the aged metamorphosis-arrested larvae after 160 days of hatching had skin which consisted of "larval" epidermis (mainly occupied with Leydig's cells) and "adult" dermis (mainly occupied with two types of dermal glands).

Changes in globin subunits

Figure 5 shows a transition of globin subunits from larval to adult types in normally metamorphosing and metamorphosed animals, and metamorphosis-arrested larvae.

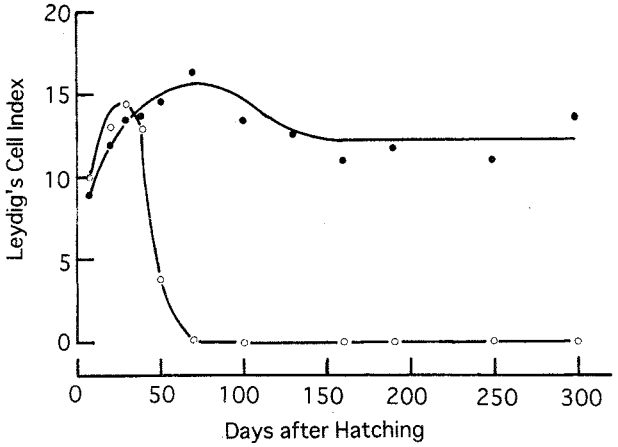

Fig. 3. Developmental fate of Leydig's cells in the epidermis in normal controls (open circles) and metamorphosis-arrested larvae (closed circles). The Leydig's cells diminish during metamorphosis in the controls, but not in the metamorphosis-arrested larvae.

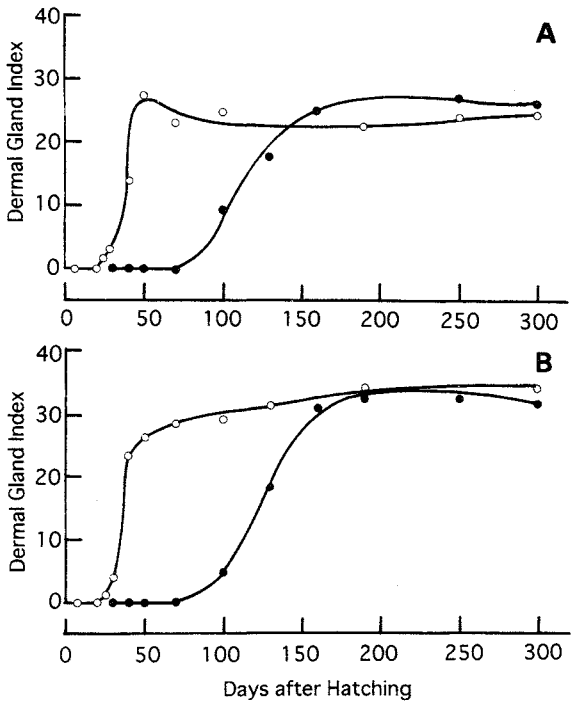

Fig. 4. Developmental fates of mucous glands (A) and serous glands (B) in the dermis of normal controls (open circles) and of metamorphosis-arrested larvae (closed circles). Both glands begin to develop at a premetamorphic stage (stage 60, 25 days after hatching) in the controls. In the metamorphosis-arrested larvae, however, they appear much later than in the controls. After 160 days of hatching, the developmental degrees of both glands in the metamrophosis-arrested larvae become identical to the controls.

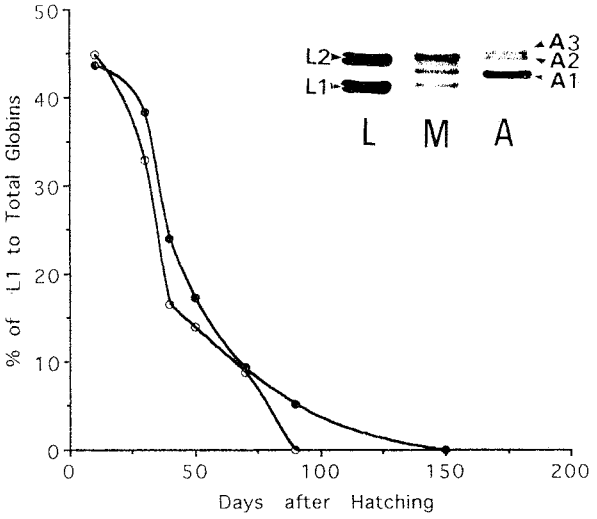

Fig. 5. Transitions of globin subunits from larval to adult types in normal controls (open circles) and metamorphosis-arrested larvae (closed circles). Chronological patterns of the globin transition are almost identical even in the controls and metamorphosis-arrested larvae. Inset: Electrophoregram of globin subunits on SDS-PAGE. L, typical larva; M, metamorphosing larva; A, typical adult. Proportions of L1 to total globins are caliculated using a computerized image analyzer (NIH-Image).

Because larval globins were detected on SDS-PAGE as two distinctive bands (L1 and L2, Fig. 5 inset) and a proportion of L1 to the total globins was caliculated, the proportion was approximately 0.5 in the typical larval hemoglobin, then gradually decreased during the metamorphosis, and finally became to 0 in the typical adult hemoglobin. Larval type globins were gradually decreased and replaced by adult types at almost the same rate both in the controls and metamorphosis-arrested animals. At 90 (controls) or 150 days (metamorphosis-arrested larvae) after hatching, the larval bands were completely diminished, in other words, the adult globins were exclusively expressed.

DISCUSSION

Larval organs vs adult organs

In anurans, three types of cells or organs, which behave differently during metamorphosis, are classified: larval-specific, larva-to-adult and adult-specific (Yoshizato, 1989, 1992). It seems, thus, possible to identify these three types of cells or organs even in urodeles. External gills and dorsal tailfin are considered to be "larval" organs, because they degenerated completely in the controls but fully remained in the metamorphosis-arrested larvae (Fig. 1). Disappearance of them in the controls is dependent on circulating thyroid hormones (Weber, 1967; Dodd and Dodd, 1976). Leydig's cells in the epidermis are also considered to be "larval" cells because the developmental fate of these in the controls and metamorphosis-arrested larvae was identical to the external gills and dorsal tailfin (cf. Figs. 1 and 3). This assumption is consistent with the previous observation that thyroxine treatment hastens the loss of the Leydig's cells in urodele larvae (Dodd and Dodd, 1976). On the other hand, the dermal gland cells are considered to be "adult" or "larva-to-adult" cells because they are initially observed at the premetamorphosis (statge 60) in this species or at the prometamorphic stages in other urodeles (Fox, 1985), then develop rapidly during the metamorphosis, and become major constituents of the dermal tissues after the metamorphosis (Fig. 2B, C, E). In the metamorphosis-arrested larvae, dermal glands appeared much later than in the controls (Fig. 4). Thus, the skin of aged, metamorphosis-arrested larvae consists of a "larval" epidermis which is mainly occupied with Leydig's cells and an "adult" dermis occupied with well developed dermal glands (Fig. 2F). These findings suggest that the development of the dermal glands is controlled by the other regulatory mechanism than that of the epidermis, even though the dermal glands are believed to originate from downgrowths of undifferentiated epidermal cells which later differentiate into the mucous and serous glands (Bovbjerg, 1963; Fox, 1985). Since we have no specific probes at present to identify larval or adult epidermis and dermis in *Hynobius* like in *Xenopus* (Nishikawa *et al.*, 1992), further analyses could not be done.

Morphological vs biochemical metamorphoses

Contrary to the morphological or anatomical metamorphosis discussed above, the transition of hemoglobins from larval to adult types occurred basically on the same time schedule both in the controls and metamorphosis-arrested larvae (Fig. 5), suggesting that the transition occurred independently on the thyroid hormones (Arai and Wakahara, 1993; Wakahara *et al.*, 1994). This is consistent with an earlier observation that the hemoglobin transition in *Xenopus laevis* was determined more by chronological ages, or size, or some other independent factors, rather than the hormonal control by thyroxine (MacLean and Turner, 1976).

Although the transition of globin subunits from larval to adult types occurs basically on the same time schedule in both controls and metamorphosis-arrested larvae, a transition of nitrogen excretion from ammonotelism (larval) to ureotelism (adult) in the metamorphosis-arrested larvae occurs a little later than in the controls (Wakahara *et al.*, 1994). The heterochronic expression in the transition of nitrogen excretion pattern in the controls and metamorphosis-arrestred larvae is similar to the development of the dermal glands, which appear in the skin of the metamorphosis-arrested larvae much later than in the controls (Fig. 4). These chronological differences in phenotypic expression have been explained by possible differences in the sensitivity to thyroid hormones among various tissues or cells in axolotl (Jurd, 1985). Provided that this is also true even in *Hynobius retardatus*, a higher concentration of thyroid hormones needs for morphological or anatomical metamorphosis such as disappearance of the external gills, dorsal tailfins and Leydig's cells in the epidermis, but a lower concentration for the transition of nitrogen excretion pattern and for the development of the dermal glands. Thyroid hormones will not need for, or have little effects on the transition of globin subunits from larval to adult types (Arai and Wakahara, 1993; Wakahara *et al.*, 1994). Number of thyroid hormone binding sites or recepters per cell may be different at different developmental stages (Yoshizato and Frieden, 1975; Moriya *et al.*, 1984) or among various tissues or cell types. Molecular biological approaches on the spatio-temporal expression of thyroid hormone receptors will answer the possible differences in the sensitivity to thyroid hormones among various tissues or cells in *H. retardatus*.

Relationship to neoteny

Neoteny in salamander species is roughly classified into three categories as permanent neoteny (*Siren*, *Necturus*), axolotl (*Ambystoma mexicanum*) and facultative neoteny (*A. tigrinum*, *A. gracile*) (Frieden, 1981). Since it was reported that the neotenic individuals of *Hynobius retardatus* which had been captured at Lake Kuttara metamorphosed under the laboratory condition (Sasaki, 1924), it is reasonable to assume that the neoteny in *Hynobius* must be a facultative neoteny, in which animals metamorphose depending on the environments. Low temperature in Lake Kuttara is a candidate for causal factor(s) of the neoteny in this species, but nobody has succeeded to restore the neotenic population (Moriya, 1983; Yamashita *et al.*, 1991), except for a precocious maturation of the testis in metamorphosis-arrested larvae (Wakahara, 1994;

Yamaguchi *et al*, 1996). Because the precocious maturation of testis and formation of spermatozoa in the metamorphosis-arrested larvae might be induced by an extraordinarily large amount of TSH resulting from a hypertrophy of TSH cells or from "thyroidectomy cells" in the pituitary gland following goitrogen-treament (Yamaguchi *et al*., 1996), that was not a true neoteny.

Separation of morphological metamorphosis from the biochemical 'metamorphosis' such as a transition of larval to adult hemoglobins (Ducibella, 1974b; Jurd, 1985; Arai and Wakahara, 1993), or heterochronic experssion between germ cell and somatic cell developments (Gould, 1977; Wakahara, 1994) will be fundamental causes of neoteny in urodeles. Thus, an experimental production of neoteny in this species depends on further elucidation on cellular and molecular mechanisms which bring about the separation between morphological and biochemical metamorphoses, or which govern a heterochronic expression of some adult phenotypes.

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