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Research Articles

Spermatogenesis is extraordinarily accelerated in metamorphosis-arrested larvae of a salamander, *Hynobius retardatus*

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Abstract. Laboratory experiments were conducted to induce neoteny in *Hynobius retardatus*, which had been reported to propagate in larval forms like axolotl. A large number of newly hatched larvae were reared in an aqueous solution of thiourea (TU) and sodium perchlorate (SPC) in order to arrest the metamorphosis. Gonadal development in the metamophosis-arrested larvae was compared with that in normally metamorphosing and metamorphosed controls. Metamorphosis-arrested male larvae produced morphologically mature spermatozoa approximately 4 months after hatching, when the gonads in the controls began to differentiate into testes, or to show the premeiotic proliferation of germ cells. Possible endocrine controls of these phenomena are discussed.
Key words. Neoteny; metamorphosis; goitrogens; *Hynobius*; spermatogenesis.

Neoteny is implicated in a wide variety of biologically important problems such as evolution and natural selection[1], heterochronic gene expression during ontogeny[2], and of course the physiology and endocrinology of metamorphosis[3]. A particular population of *Hynobius retardatus* (Hynobidae, Urodela) has been reported to show neoteny only in the specific environment of Lake Kuttara, a small volcanous lake not far from Sapporo[4,5]. Unfortunately, however, the neotenic population in Lake Kuttara is believed to be extinct. It is therefore unclear whether the main population of *H. retardatus*, which is widely distributed throughout Hokkaido, can become sexually mature without undergoing metamorphosis or can show neoteny under certain experimental conditions. If neotenic reproduction in *H. retardatus* can be induced in laboratory conditions, it would be useful as a novel experimental animal in addition to axolotl[6,7]. In the present study, a large number of newly hatched larvae of *H. retardatus* were reared in an aqueous solution of thiourea (TU) and sodium perchlorate (SPC), potent goitrogens, to determine whether the larvae can become sexually mature without undergoing or completing metamorphosis. Gonadal development in male larvae whose metamorphosis was completely arrested was much faster than in normally metamorphosed animals.

Materials and methods

Fertilized eggs of *Hynobius retardatus* were collected from several ponds or small streams in the vicinity of Sapporo during the breeding season. The embryos were reared in aerated tap water at room temperature until they hatched (approximately 10 d). Hatched larvae were subgrouped and reared either in an aqueous solution of 0.02% thiourea (TU) and 0.04% sodium perchlorate (SPC), or in goitrogen-free medium as controls. All media were supplemented with antibiotics (50 IU penicillin G and 25 µg streptomycin sulfate/ml) to avoid infectious diseases. They were fed with commercially available frog feed pellets (Oriental Kobo Co., No. 2 for frogs) for younger larvae or live *Tubifex* for larger ones. Rearing water was changed every day, just before feeding. After the controls metamorphosed, the juveniles were transferred to a terrarium and fed daily with live *Tubifex*. Developmental stages of the metamorphosis-arrested and normally metamorphosing larvae were determined according to the normal table for *Hynobius nigrescens*[8], a closely related species to *H. retardatus*. Each month, ten animals of metamorphosis-arrested and controls were anesthetized in MS222 (1:2000) (Sandoz) and then fixed with Bouin's fixative. Their gonads and thyroid glands were embedded in paraffin, serially sectioned, and stained with Delafield's hematoxylin and eosin. To assess degrees of the development of gonads, sectional areas were measured on every ten sections after taking photographs.

Results

Figure 1 shows external views of metamorphosis-arrested larvae which were reared in 0.02% TU and 0.04% SPC solution and controls reared in goitrogen-free water and in a terrarium after metamorphosis at room temperature. All controls metamorphosed 70 d after the hatching, and they turned to a terrestrial habitat. In contrast, anatomical metamorphosis was effectively arrested in the goitrogen-treated animals. The

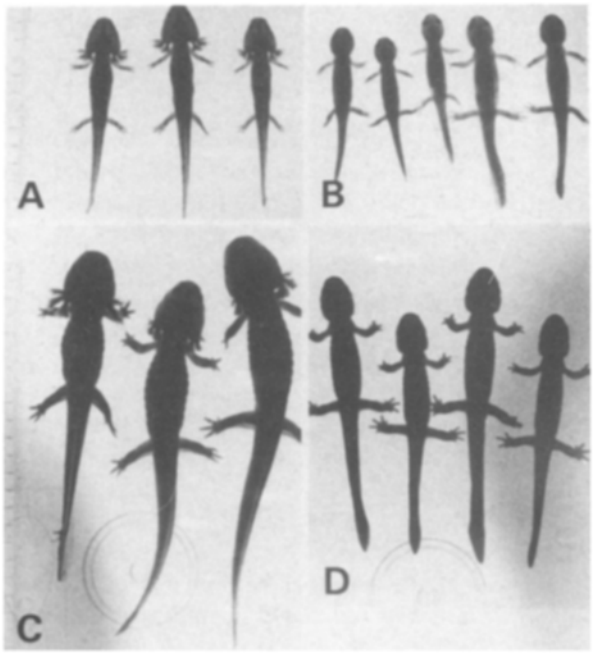

Figure 1. External morphology of metamorphosis-arrested *Hynobius retardatus* which were reared in an aqueous solution of thiourea and sodium perchlorate (*A* and *C*) and controls reared in goitrogen-free medium (*B* and *D*).
A Goitrogen-treated larvae at stage 63 (60 d after the hatching).
B Metamorphosed controls of the same age as in *A*
C Goitrogen-treated larvae at stage 64 (210 d after the hatching). Note external morphology characteristic of the larval form.
D Control animals at the same age as in *C*.

developmental progress of goitrogen-treated larvae was approximately identical to the control larvae until they reached stage 63, fully grown, premetamorphic larvae. After that, however, development of the goitrogen-treated larvae did not progress: an average developmental stage of the goitrogen-treated larvae was stage 63 when the controls metamorphosed (fig. 1A and B). At the end of this experiment (9 months after hatching), the average developmental stage of metamorphosis-arrested larvae was stage 64.8 (fig. 1C and D). This indicated convincingly that the goitrogens employed in this experiment inhibited metamorphosis at the morphological level through blockage of the activity of thyroid glands in the treated animals[9]. Thyroid glands in normally developing animals showed typical follicular structures with a large amount of colloidal substances (fig. 2A and B), while thyroid glands in goitrogen-treated animals showed extraordinarily developed follicular epithelium without colloidal substances at all (fig. 2C and D).

In contrast to the arrested development or morphological metamorphic events, the goitrogen-treated larvae grew successfully: average total length of metamorphosis-arrested larvae was 40 mm at hatching stage, 56 mm when all controls metamorphosed (70 d after hatching), and 112 mm at the end of this experiment (9 months after hatching).

Figure 3 shows testicular development in the metamorphosis-arrested and normally metamorphosing and

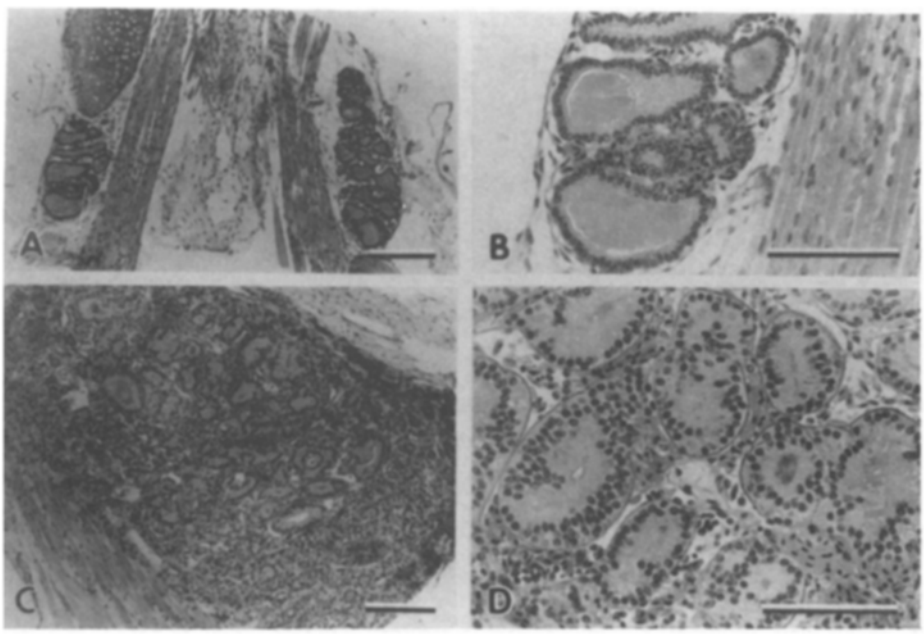

Figure 2. Histology of thyroid glands of a goitrogen-treated larva (*C* and *D*) and a control (*A* and *B*). Thyroid glands in controls show typical follicular structures with large amount of colloidal substances. Extraordinary enlargement of the entire thyroid gland is characteristic of the goitrogen-treated larvae (*C*). Follicular structures without any amount of colloidal substances are observed (*D*), suggesting functional atrophy in these animals.
Delafield's hematoxilin and eosin stain. Scale bars: 200 μm in *A* and *C*; 100 μm in *B* and *D*.

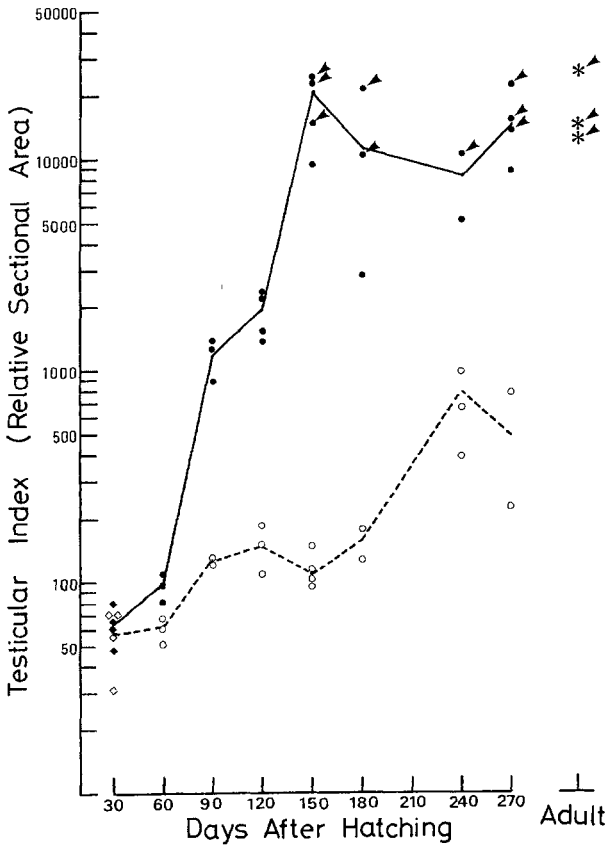

Figure 3. Testicular development in metamorphosis-arrested larvae (closed marks, solid line) and normally metamorphosing and metamorphosed animals (open, broken line). The ordinate (testicular index) shows relative average sectional area of each testis in a logarithmic scale. Each point shows a value in one individual. Squares on 30 d after hatching indicate indifferent gonads. Asterisks on the right show the degree of the testicular development in ordinary adult males at the breeding season. Arrowheads indicate the testes with morphologically mature spermatozoa.

metamorphosed *Hynobius*. In order to assess degrees of the gonadal development conveniently, average sectional areas of the testis in both animals were compared. No differences in the development of testes were detected until the controls metamorphosed, when germinal ridges were nested with germ cells which showed mitotic figures frequently (fig. 4A). During the summer season (90–120 d after hatching), the testes in metamorphosis-arrested larvae grew larger, whereas control testes showed no conspicuous growth. Sectional areas of the testis in metamorphosis-arrested larvae became ten times larger than the one in controls. Four months after hatching, morphologically mature spermatozoa were observed in the testis of metamorphosis-arrested larvae (fig. 4C and D), while the testis of controls was still at the initial stages of testicular differentiation (fig. 4A and B). The degree of gonadal development in metamorphosis-arrested larvae 4 months after hatching was identical to the sexually mature testis of an ordinary adult male captured in the breeding season (fig. 4E and F). By the end of this experiment (9 months after hatching), none of the controls had testes with morphologically mature spermatozoa.

In contrast to the testicular development, ovarian development in metamorphosis-arrested larvae was almost identical to that in normally metamorphosing and metamorphosed animals (fig. 5). Previtellogenic oocytes were detected in the developing ovaries approximately 90 d after hatching both in metamorphosis-arrested and metamorphosed larvae. The volume of ovaries grew slowly as a function of time. Maximal diameter of oocyte observed in 9-month-old females was 350 µm in both metamorphosis-arrested and controls. In the germinal vesicle of the previtellogenic oocytes, diplotene chromosomes were frequently observed. Thus, oogenesis in metamorphosis-arrested larvae and metamorphosed controls was at a very early phase of meiosis.

Discussion

Athough several attempts have been made to induce neoteny in *Hynobius retardatus*, or to investigate causal factor(s) of it[10–12], nobody has succeeded in obtaining sexually mature neotenic forms in laboratory conditions. It is demonstrated in this study that males at least of *H. retardatus* become sexually mature without completing metamorphosis. Thus, the independent development of gonads (including differentiation of germ cells) and somatic tissues, in other words, heterochrony of the developmental rate of the reproductive system and of soma, the essential mechanism of urodelan neoteny[1], was confirmed in this species.

It has been reported that the axolotl, a famous neotenic form of *Ambystoma mexicanum*, is not biochemically neoteneous for at least two normally metamorphic events: the production of new hemoglobin polypeptide subunits and inhibition of the synthesis of larval subunits[13]. Since neotenic axolotls produce thyroid hormones at a very low level, and accomplish a number of cryptic metamorphic processes[14], different somatic tissues or cells will behave differently in response to the thyroid hormones. Similarly, a transition from larval to adult hemoglobin subunits has been shown to occur in the same time schedule in metamorphosis-arrested and normally metamorphosing *Hynobius retardatus*[9]. This suggests convincingly that even in *H. retardatus* biochemical metamorphosis proceeds independently of the morphological metamorphosis, like in the axolotl.

Although direct measurements of the plasma concentration of thyroid hormones, TSH, GTH and prolactin in goitrogen-treated larvae were not done in this study, the level of the thyroid hormones must be low, or practically zero, because of the almost complete inhibition of metamorphosis (fig. 1) and the histology which shows a hypertrophy in follicular epithelium without colloidal substances suggesting a functional atrophy of the thyroid glands (fig. 2). Since gonadal development is basically controlled by GTH from the pituitary gland, the

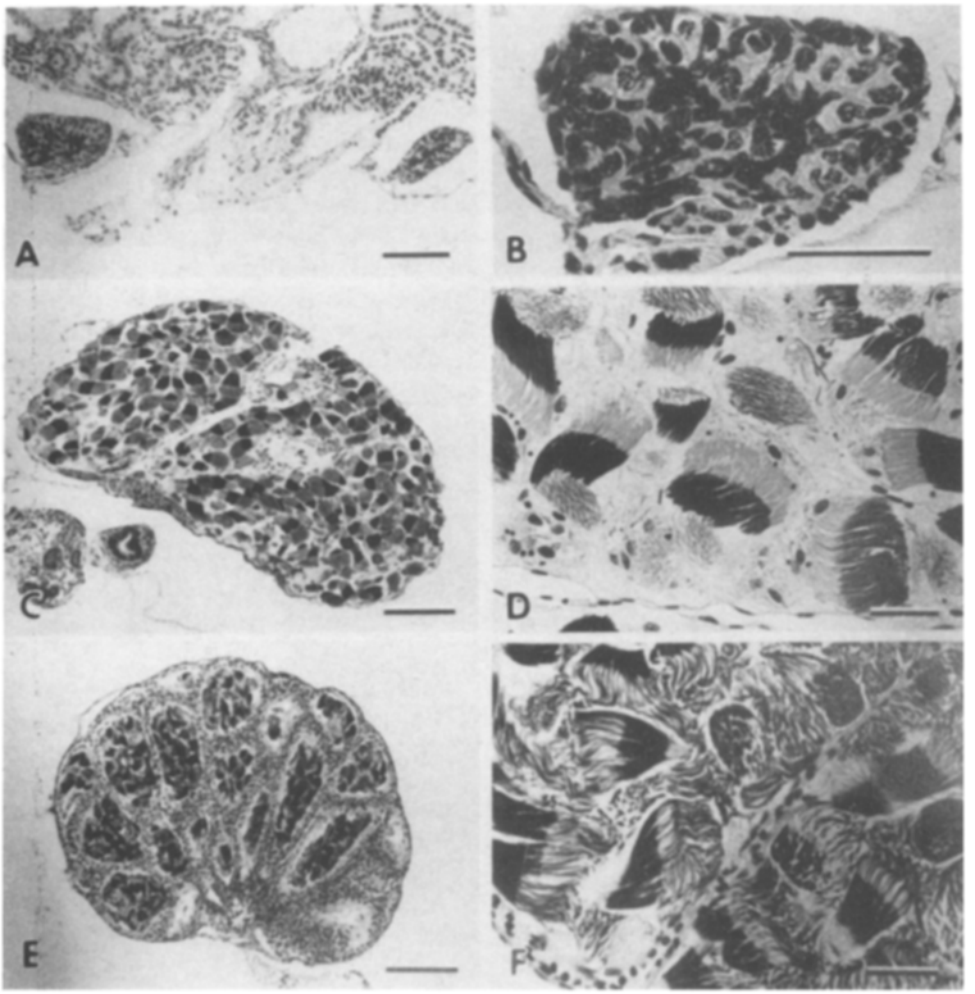

Figure 4. Histology of developing testes of a metamorphosis-arrested larva (*C* and *D*), of a control (*A* and *B*) at the same age as the metamorphosis-arrested larva, and of a sexually mature, ordinary adult male (*E* and *F*).
A A cross section of immature testis from a control animal at 120 d after hatching (2 months after metamorphosis). *B* An enlargement of *A*: a lot of mitotic figures of germ cells are to be seen.
C A cross section of testis from a metamorphosis-arrested larva at 120 d after the hatching (stage 64), showing extraordinarily precocious development of the testis. *D* An enlargement of *C*: many bundles of morphologically mature spermatozoa are clearly to be seen.
E Sexually mature testis from an ordinary adult male just prior to spawning, captured at the breeding season. Note that the size of this testis is identical to the one in the metamorphosis-arrested larva (*C*). *F* An enlargement of *E*: no differences are observed in the morphology of the spermatozoa between *D* and *F*.
Delafield's hematoxylin and eosin stain. Scale bars: 50 μm in *B*, *D* and *F*; 100 μm in *A*; 200 μm in *C* and *E*.

plasma concentration of GTH is assumed to be much higher in metamorphosis-arrested male larvae than in controls. The goitrogen-treated larvae may produce and release an exceptionally large amount of TRH and/or TSH, as a result of a lingering shortage in circulating thyroid hormones. Since the chemical structures of GTH and TSH are very similar throughout the vertebrates, and TSH has been reported to show an activity as GTH in some bony fishes[15], there is a possibility that TSH in *H. retaradatus* has a GTH activity. The second possibility is that the excess TRH stimulates the synthesis and release of GTH via an unknown mechanism.

Whereas precocious testicular development was observed in goitrogen-treated male larvae (fig. 3), no differences in ovarian development were found between metamorphosis-arrested female larvae and metamorphosed juveniles so far examined in this study (fig. 5). These facts may suggest that ovarian growth before vitellogenesis proceeds steadily without GTH activity, and are consistent with the observation that is takes longer for oocytes to develop to maturation than for spermatozoa[16] in axolotl. A lot of studies have been conducted to examine the effects of various antithyroid substances upon the development of the gonads in amphibians[17]. No convincing explanation, however, has been provided for the precocious gonadal development in goitrogen-treated animals. Thus, studies on GTH and TSH synthesis and release in goitrogen-treated larvae

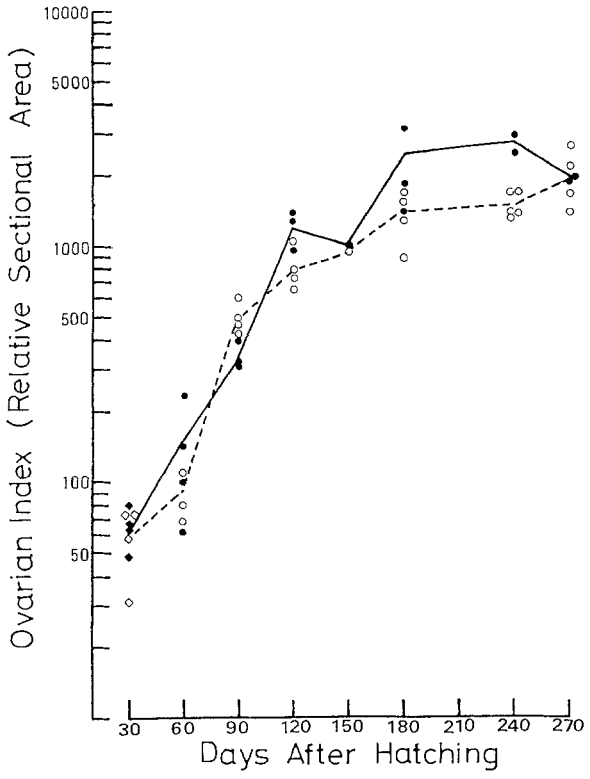

Figure 5. Ovarian development in the metamorphosis-arrested larvae (closed marks, solid line) and controls (open marks. broken line). The ordinate (ovarian index) shows relative average sectional area of each ovary in a logarithmic scale. Squares on 30 d after hatching indicate indifferent gonads. No differences are detected in the ovarian development between goitrogen-treated larvae and controls.

are necessary and are now progressing using modern technology.

There are no convincing data showing the age when this salamander attains sexual maturity in the wild. In *H. lichenatus*, a closely related species to *H. retardatus*, it was reported that the first indication of spermatogenesis was observed in the testes of 2-year-old males[18]. By rearing the larvae in goitrogens, it is possible to shorten the time required for sexual maturation, at least in males. This will help to make this salamander a new experimental animal, like axolotl. The experimentally-induced larval forms with morphologically mature spermatozoa produced in this study do not spawn mature gametes, nor propagate in the larval forms. The stage of the study will be to obtain neotenic individuals which can spawn mature gametes in laboratory conditions.

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