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Original Article

The possible contribution of pituitary hormones to the heterochronic development of gonads and external morphology in overwintered larvae of *Hynobius retardatus*

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ABSTRACT In *Hynobius retardatus*, most larvae in regions of low elevation metamorphose by autumn of the same year. However, larvae of some populations found in cold, mountainous ponds cannot metamorphose within the year and become aged, overwintered larvae. Gonadal development in larvae under the age of 1 year (larvae developed from eggs spawned in the same year) and in aged, overwintered larvae (spawned and hatched in previous years) was examined at the same developmental stage (stage 63, full-grown larval stage). The number of germ cells and the cross-sectional areas of the gonads were much larger in 2-season-overwintered (third year) larvae than in larvae under the age of 1 year. To obtain reliable probes for investigating the possible contribution of TSH, FSH and LH to metamorphosis and gonadal development, cDNAs for *Hynobius TSH*β, *FSH*β and *LH*β genes were cloned. Their expressions were analyzed by means of semi-quantitative RT-PCR in larvae under the age of 1 year and in 2-season-overwintered larvae. No differences were observed in expression levels of either *TSH*β or *LH*β between larvae under the age of 1 year and the overwintered larvae. In contrast, expression of *FSH*β was much higher in the overwintered larvae than in larvae under the age of 1 year. These results suggest that gonadal development proceeds gradually with age even in the overwintered larvae, but that metamorphosis is retarded, probably due to the larvae's cold habitat. Heterochronic development of gonads and external morphology has been demonstrated in *H. retardatus*, suggesting a potency for neotenic reproduction in this species.

KEY WORDS: *salamander, overwintered larvae, pituitary genes, neoteny, heterochrony*

Introduction

Plasticity in phenotypic response often provides a reproductive advantage over a genetically fixed response in organisms that exist in temporally and spatially varying environments. In many urodele species, there are populations with an alternative life-history pathway: metamorphosis versus paedomorphosis or neoteny (Lynn, 1961; Dent, 1968; Brandon, 1989; Whiteman, 1994). Most larvae transform into immature individuals that remain more or less terrestrial before reaching sexual maturity; however, some larvae attain sexual maturity with larval morphology. These individuals are referred to as metamorphs and paedomorphs (or neotenes), respectively. Such changes in developmental timing are known as heterochrony (Wakahara, 1996). The maintenance of alternative life-history pathways is an interesting evolutionary and ecological problem that has been the subject of a number of recent studies (Dzukic *et al.*, 1990; Harris *et al.*, 1990; Kalezic and Dzukic, 1990; Whiteman, 1994; Kalezic *et al.*, 1996; Ryan and Semlitsch, 1998), but that has not been the object of developmental, endocrinological or molecular research until now.

The salamander *Hynobius retardatus* has been reported to show neotenic reproduction in the specific environment of Lake Kuttara, a small volcanic lake in Hokkaido, Japan (Sasaki, 1924; Sasaki and Nakamura, 1937). Unfortunately, however, the neotenic population in Lake Kuttara is believed to be extinct due to the introduction of hatchery fish to the lake. We have demonstrated

Abbreviations used in this paper: CRH, corticotropic hormone-releasing hormone; DIG, digoxigenin; DTT, dithiothreitol; FSH, follicle stimulating hormone; GnRH, gonadotropin-releasing hormone; LAG, line of arrested growth; LH, luteinizing hormone; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; T_4, thyroxine; TRH, thyroid stimulating hormone-releasing hormone; TSH, thyroid stimulating hormone.

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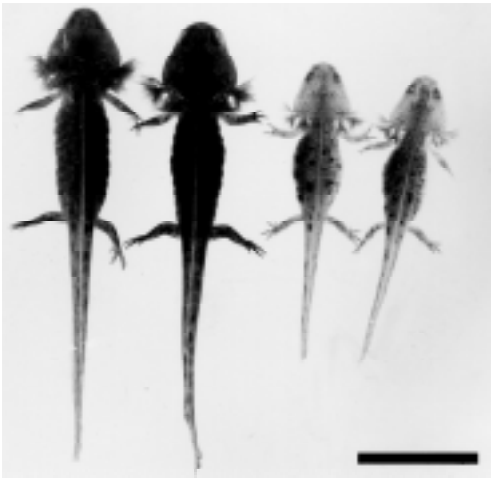

Fig. 1. External views of 2-season-overwintered, third-year larvae and larvae under the age of 1 year of *Hynobius retardatus*. *Overwintered larvae (left) were much larger in body length than larvae under the age of 1 year (right), although they were of the same developmental stage (stage 63; full-grown larvae). Bar, 20 mm.*

that the larvae of metamorphic populations can produce morphologically mature spermatozoa when metamorphosis has been arrested by goitrogens (Wakahara, 1994; Yamaguchi *et al.*, 1996) or thyroidectomy (Kanki and Wakahara, 1999). These observations suggest that gonadal development is independent of morphological metamorphosis in this species (Wakahara, 1996).

Several variations in larval life histories have been reported in *H. retardatus* (Iwasaki and Wakahara, 1999); in some specific populations in cold, mountainous ponds, larvae cannot metamorphose during the first, second or third years, so they metamorphose in the second, third or fourth year depending on the altitude of the pond. In almost all populations living in low-altitude regions of Hokkaido, however, spawning is observed in early spring, and hatched larvae metamorphose by autumn of the same year. If gonadal development proceeds independently of morphological metamorphosis (Wakahara, 1996), even in natural conditions, overwintered larvae could have more developed gonads than larvae under the age of 1 year at the same developmental stage.

In this study, we compared gonadal development in larvae under the age of 1 year and in overwintered larvae to confirm whether gonadal development proceeds gradually according to chronological age (even in the overwintered larvae), or whether it is instead substantially determined by the larvae's developmental stage. To analyze molecular mechanisms regulating metamorphosis and gonadal development in *Hynobius retardatus*, we cloned full-length cDNAs for the *TSHβ, FSHβ* and *LHβ* genes, and analyzed their expression in the pituitaries of larvae under the age of 1 year and of overwintered larvae. The results reported here demonstrate that gonadal development proceeds with chronological age even in the overwintered larvae whose metamorphosis is retarded. Also, we discuss the possible contribution of TSH, FSH and LH to facultative neoteny in this species.

Results

Age assessment of overwintered larvae

Figure 1 shows an external view of larvae under the age of 1 year and of aged, overwintered larvae at the same developmental stage (stage 63, full-grown larval stage). Both larvae displayed a typical larval form characterized by well-developed external gills and tail fin (Shi, 2000). The aged, 2-season-overwintered larvae (left) were much larger in body length than those under the age of 1 year (right) and resulted in larger juveniles (Iwasaki and Wakahara, 1999). The ages of the larvae were assessed by skeletochronology (Esteban *et al.*, 1996; Misawa and Matsui, 1999). Figure 2 shows histological sections of the humerus of a larva under the age of 1 year (Fig. 2A) and that of an overwintered larva (Fig. 2B). Although no lines of arrested growth (LAGs) were observed in the humerus of the larva under the age of 1 year, 2 LAGs were seen in that of the overwintered larva (Fig. 2B, arrows). Since LAGs are formed by the annual cessation of local osteogenesis during the winter season (Esteban *et al.*, 1996), the number of LAGs reflect the chronological age of the animal. This finding suggests that the larva shown in Fig. 2B was 2-season-overwintered, and was thus a third-year larva. The ages of overwintered larvae were precisely determined using this skeletochronological procedure. Larvae used in the following analyses were under the age of 1 year, 2-season-overwintered (third-year) or 3-season-overwintered (fourth-year) larvae, whereas their developmental stage was identical (stage 63).

Gonadal development in overwintered larvae

Gonadal development in the larvae was analyzed histologically. The numbers of each age group of larvae and their sex were as follows: under the age of 1 year, 18 (5 males and 13 females); 2-season-overwintered larvae collected in spring of the third year, 14 (7 males and 7 females); 2-season-overwintered larvae collected in autumn of the third year, 9 (5 males and 4 females); 3-season-overwintered larvae, 3 (1 male and 2 females). Figure 3 shows testicular growth in larvae under the age of 1 year (Fig. 3A) and in 2-season-overwintered larvae collected in autumn of the third year

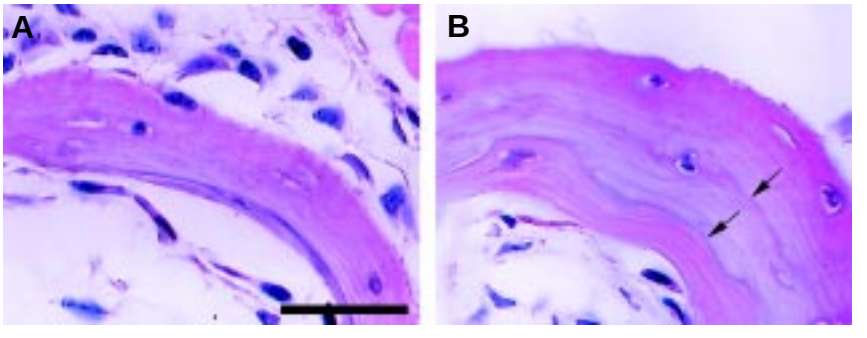

Fig. 2. Histological sections of the humerus of a larva under the age of 1 year and of an overwintered larva. **(A)** *No lines of arrested growth (LAGs) were observed in the larva under the age of 1 year.* **(B)** *In contrast, 2 LAGs (arrows) were observed in the overwintered larva , suggesting that it was 2-season-overwintered, and thus a third-year larva. Bar, 30 μm.*

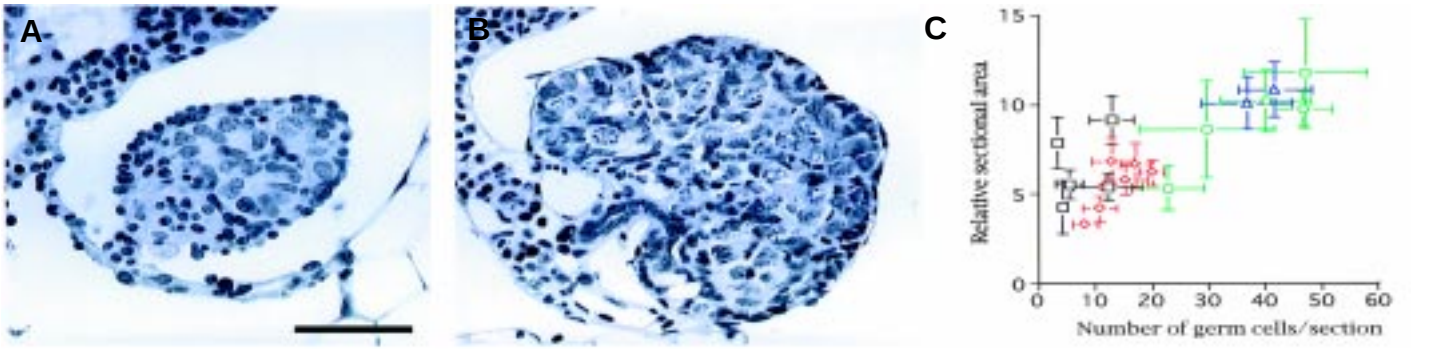

Fig. 3. Development of testes in larvae under the age of 1 year and in overwintered larvae at stage 63, full-grown larva. (A) *Histological section through the testis of a larva under the age of 1 year.* **(B)** *Histological section through the testis of a 2-season-overwintered larva collected in autumn of the third year. Several germ cells show the characteristic morphology of spermatocytes at leptotene to pachytene stages.* **(C)** *Comparison of the number of germ cells and the cross-sectional areas of testes in larvae under the age of 1 year (open, black squares), 2-season-overwintered larvae collected in spring of the third year (red plots), 2-season-overwintered larvae collected in autumn of the third year (green plots) and 3-season-overwintered larvae collected in spring of the fourth year (blue plots). Aged, overwintered larvae had larger testes with more germ cells than those under the age of 1 year. Bar, 50 μm.*

(Fig. 3B). The cross-sectional areas of the testes and the number of germ cells showed a tendency to increase in overwintered larvae more than in larvae under the age of 1 year. Especially, 2-season-overwintered (third-year) larvae collected in autumn and 3-season-overwintered (fourth-year) larvae had obviously larger testes with a greater number of the germ cells (Fig. 3C). Several germ cells in the third-year larvae had entered meiotic prophase (Fig. 3B). However, germ cells in larvae under the age of 1 year showed morphological characteristics of primordial germ cells or spermatogonia (Fig. 3A).

Figure 4 shows ovarian growth in overwintered larvae. The volume of the ovaries and the number of germ cells were also larger in 2-season-overwintered (third-year) larvae collected in autumn and in 3-season-overwintered (fourth-year) larvae than in larvae under the age of 1 year (Fig. 4C). Several germ cells in the third-year larvae were at meiotic prophase (pachytene to diplotene) and showed well-developed cytoplasm characteristic of primary oocytes (Fig. 4B). No cytoplasmic development was observed in the oocytes of larvae under the age of 1 year (Fig. 4A).

TSHβ, FSHβ and LHβ sequences of H. retardatus

Full-length cDNA sequences of *Hynobius TSHβ* were obtained from a cDNA library constructed from the pituitary gland of metamorphosis-arrested larvae reared in goitrogen (Kanki and Wakahara, 2000). FSHβ and LHβ cDNA were obtained by 5'- and 3'-RACE using partial cDNA sequences for FSHβ, which had been cloned previously (Kanki and Wakahara, 2000), and for LHβ, cloned in this study. The sequences encoded 141, 129 and 132 amino acid residues, respectively. Predicted amino acid sequences of these genes are shown in Fig. 5. Similarities of *Hynobius TSHβ*, *FSHβ* and *LHβ* to those of human and bullfrog were 62% and 53% for *TSHβ*, 62% and 56% for *FSHβ* and 44% and 72% for *LHβ*, respectively (Koide *et al.*, 1996; Okada *et al.*, 2000). Partially cloned cDNA sequences for *Hynobius TSHβ* $_{(35\text{-}127)}$ and

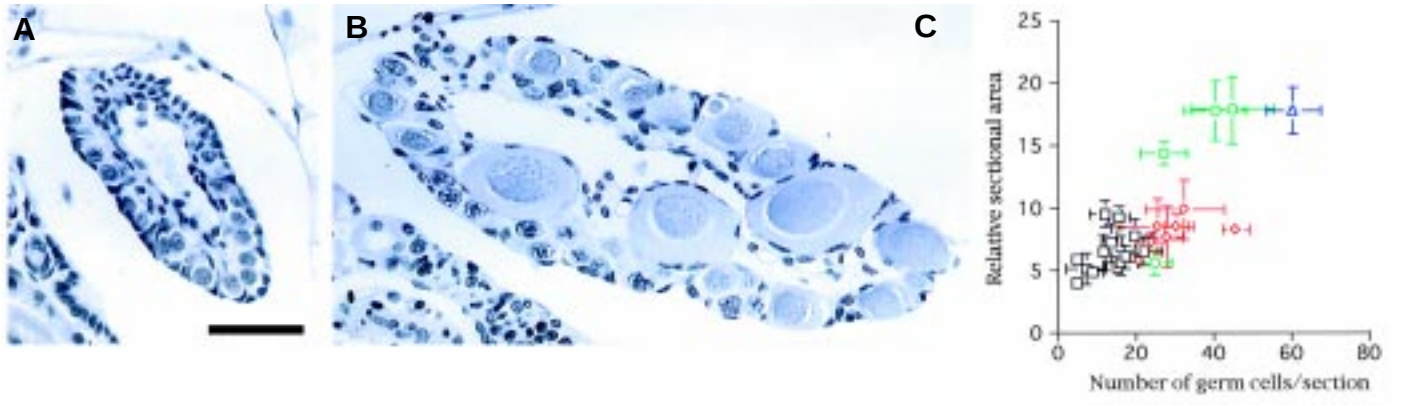

Fig. 4. Development of ovaries in larvae under the age of 1 year and in overwintered larvae at stage 63, full-grown larva. (A) *Histological section through the ovary of a larva under the age of 1 year.* **(B)** *Histological section through the ovary of a 2-season-overwintered larva collected in autumn of the third year. Several germ cells show the characteristic morphology of oocytes with well-developed cytoplasm at pachytene to diplotene stages.* **(C)** *Comparison of the number of germ cells and ovary cross-sectional areas in larvae under the age of 1 year (open, black squares), 2-season-overwintered larvae collected in spring of the third year (red plots), 2-season-overwintered larvae collected in autumn of the third year (green plots) and 3-season-overwintered larvae collected in spring of the fourth year (blue plots). Aged, overwintered larvae had larger ovaries with more developed germ cells than those under the age of 1 year. Bar, 50 μm.*

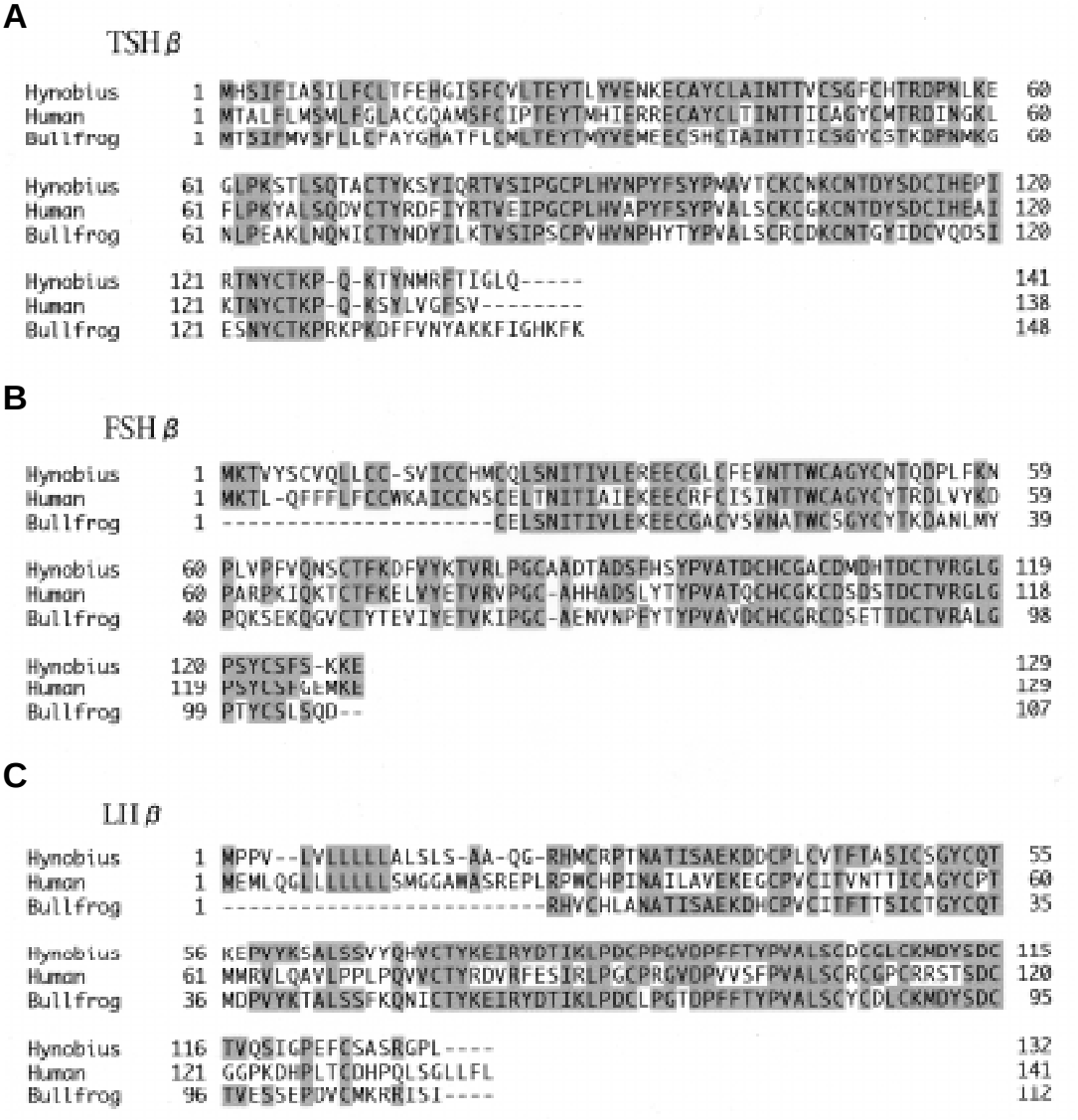

Fig. 5. Predicted amino acid sequences of *Hynobius* pituitary genes. *TSHβ* **(A)**, *FSHβ* **(B)** *and LHβ* **(C)** *subunits deduced from cloned cDNAs, and comparison with the alignment of TSHβ, FSHβ and LHβ subunits from human and bullfrog. Gray background indicates the same amino acid residues as in the* Hynobius *sequences. Similarities of* Hynobius *TSHβ, FSHβ and LHβ to those of human and bullfrog were 62% and 53% for TSHβ, 62% and 56% for FSHβ and 44% and 72% for LHβ.*

FSHβ $_{(42\text{-}115)}$ reported previously (Kanki and Wakahara, 2000) and *LHβ* $_{(48\text{-}100)}$ cloned in this study are completely included in the full-length sequences reported here. Specificity of the cDNAs obtained was confirmed by RT-PCR analyses using various tissues and organs of metamorphosed juveniles, such as the pituitary gland, brain, muscle, intestine, liver and spleen (Fig. 6). Positive signals of the expected length were detected only when cDNAs that were reverse-transcribed from the pituitary total-RNAs were amplified using gene-specific primers for *TSHβ*, *FSHβ* and *LHβ*, respectively. No positive signals were detected at all in the brain, muscle, intestine, liver or spleen. In contrast, positive signals for the *actin* gene as an internal control were detected in all organs and tissues examined.

Expression of TSHβ, FSHβ *and* LHβ *genes*

It was difficult to detect positive signals of *FSHβ* by the usual Northern blotting using a partial cDNA probe for *H. retardatus* *FSHβ* $_{(42\text{-}115)}$ because of the small amount of expression of this gene during larval stages (Kanki and Wakahara, 2000). Thus, semi-quantitative analyses were conducted by estimating the amount of cDNA amplified by RT-PCR with DIG-labeled RNA probes (Fig. 7). *Hynobius* actin mRNA was employed as an internal control for an amplification standard. Positive signals were easily detected on the blots of the RT-PCR products of each gene by this procedure (Fig. 7A). Independent amplifications from 3 different cDNA pools of pituitary glands for each group of larvae, those under the age of 1 year and overwintered larvae, were performed to confirm quantitative differences in the expression levels of those genes between the 2 groups. Expression levels of each gene were thus estimated semi-quantitatively by measuring amounts of cDNAs amplified with PCR using specific RNA probes conjugated with DIG. Intensities of signals of *TSHβ*, *FSHβ*, *LHβ*, and *actin* were measured by computer image-analyzer software (NIH-image), and the relative expression levels of *TSHβ*, *FSHβ* and *LHβ* to *actin* were compared between larvae under the age of 1 year and 2-season-overwintered larvae (Fig. 7B). The level of expression of

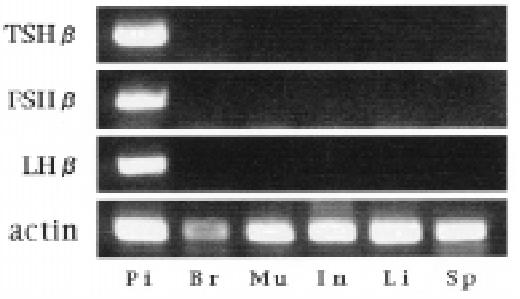

Fig. 6. (Left) Tissue-specific expression of *TSHβ*, *FSHβ* and *LHβ* genes. *The specificity of cDNAs was confirmed by RT-PCR analyses using the pituitary gland (Pi), brain (Br), muscle (Mu), intestine (In), liver (Li) and spleen (Sp). cDNAs amplified with PCR using gene-specific primers were electrophoresed in 1% agarose gels containing EtBr. After 30 PCR cycles, positive signals for TSHβ, FSHβ and LHβ were detected in the lanes of the pituitary gland, but no positive signals were detected in the lanes of other organs. Positive signals for the actin gene, used as an internal control, were detected in all lanes examined.*

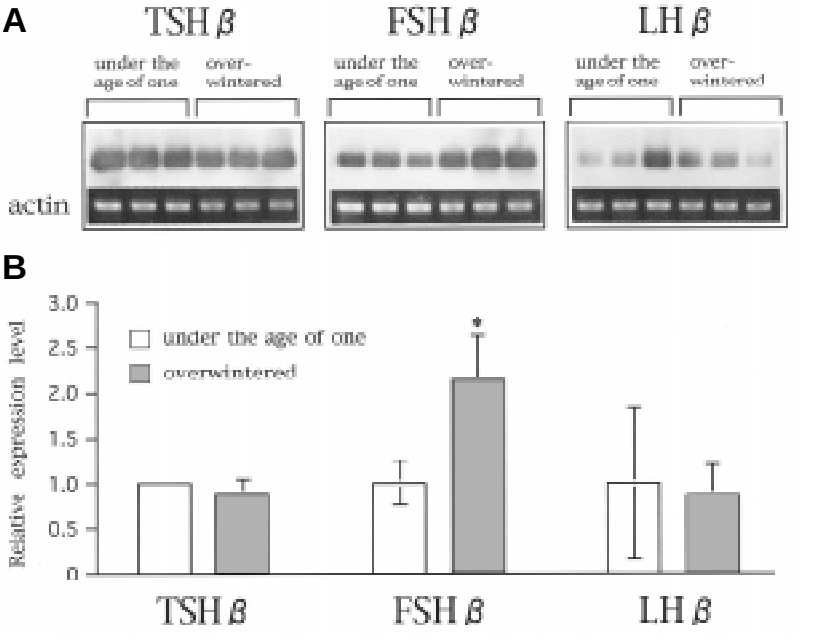

Fig. 7. (Right) Semi-quantitative analyses of expression levels of *TSHβ*, *FSHβ* and *LHβ*, in larvae under the age of 1 year and in 2-season-overwintered larvae. (A) *Three independent experiments using in total 18 larvae (9 larvae under the age of 1 year and 9 overwintered larvae) were performed. Total RNAs from 3 pituitary glands were pooled for each gene and amplified with RT-PCR using gene-specific primers. Hynobius actin mRNA was used as an internal marker for the amplification standard. cDNAs amplified with 25 reaction cycles were loaded, electrophoresed and blotted, and detected by specific RNA probes for TSHβ, FSHβ and LHβ.* **(B)** *Intensities of signals of TSHβ, FSHβ and LHβ, and actin cDNAs were measured by computer image-analyzer software (NIH-image), and the relative amounts of the hormone cDNAs to actin were calculated. The expression levels in larvae under the age of 1 year were standardized to 1.0. Expression of FSHβ in the overwintered larvae (grey column) was significantly higher than in the larvae under the age of 1 year (white column), whereas differences in TSHβ and LHβ expression were not significant. Asterisk represents $P < 0.05$ compared with the white column by the Student t-test.*

FSHβ was considerably higher in the 2-season-overwintered larvae than in the larvae under the age of 1 year. Expression levels of *TSHβ,* assessed by the relative amount of cDNAs amplified from the same cDNA pools as were used for *FSHβ,* were almost identical between larvae under the age of 1 year and the aged, overwintered larvae. Expression levels of *LHβ* on average were almost identical between the 2 groups.

Discussion

Gonadal development of overwintered larvae

Previously, we assessed the age of the overwintered larvae of *H. retardatus* by their differential body sizes within each pond (Iwasaki and Wakahara, 1999). In this study, however, the ages of overwintered larvae were determined by a skeletochronological procedure (Esteban *et al.*, 1996; Misawa and Matsui, 1999), permitting a more precise determination of age. Larvae born in cold ponds grow during the summer, but they cannot attain metamorphosis by autumn and hibernate from late autumn to the next spring in the muddy ground under the snow. The larvae repeat the cycle of growth in the summer and cessation or retardation of growth in the winter for several years according to the altitude and temperature (Iwasaki and Wakahara, 1999). Thus, lines of arrested growth (LAGs) formed in the humerus reflect their correct ages. Larvae that have 2 LAGs have already passed 2 winter seasons, and therefore are third-year larvae at the time of collection.

Two-season-overwintered larvae have larger gonads than those under the age of 1 year (Figs. 3,4) at the same developmental stage (stage 63, Fig. 1). This observation suggests that the gonads develop gradually with age even in overwintered larvae, but that metamorphosis is retarded in the overwintered larvae, probably due to their cold habitat (Moriya, 1979, 1983a,b; Iwasaki and Wakahara, 1999). Metamorphosis and gonadal development are generally regulated by different physiological mechanisms. Metamorphosis is regulated by the hypophyseal-thyroidal axes and gonadal development by the hypophyseal-gonadal axes, (Rosenkilde, 1985), but a certain overlapping of endocrinological regulation between the thyroidal and gonadal axes has been reported (Jacobs and Kuhn, 1988; Jacobs *et al.*, 1988). Growth and development of gonads independent of metamorphosis in overwintered larvae in *H. retardatus* suggests that the thyroidal and gonadal axes are regulated differentially in this species.

Contribution of TSH, FSH and LH to metamorphosis and gonadal development

In this study, we cloned cDNAs for the full-length genes *TSHβ*, *FSHβ* and *LHβ* in *H. retardatus* larvae and investigated the precise contribution of these hormones to metamorphosis and gonadal development in the natural habitat as well as under experimental conditions. The specificity of cDNAs used in this study was confirmed by RT-PCR analyses using the pituitary glands, brain, muscle, intestine, liver and spleen (Fig. 6). *TSHβ*, *FSHβ* and *LHβ* genes were expressed only in the pituitary gland. Although

extrapituitary expression of the *prolactin* gene has recently been reported in goldfish, African clawed frog and mouse (Imaoka *et al*., 2000), expression of *TSHβ*, *FSHβ* and *LHβ* in *H. retardatus* might be limited to the pituitary gland.

This is the first report to demonstrate that there are no differences in expression levels of *TSHβ* between larvae under the age of 1 year and 2-season-overwintered, third-year larvae (Fig. 7). Production and secretion of thyroxine (T_4), a major metamorphic hormone in amphibians, is regulated by TSH (Dodd and Dodd, 1976; Sakai *et al*., 1991). Thus the same expression level of *TSHβ* reflects a similar concentration of T_4 in larvae, and results in similar metamorphic stages. This is consistent with the identical developmental stages of both groups of larvae used in this study (stage 63). In contrast, we detected a significant difference in expression levels of *FSHβ* between larvae under the age of 1 year and 2-season-overwintered larvae (Fig. 7). This finding might explain the larger testes and ovaries observed in the overwintered larvae. If more and more FSH is being secreted according to their age and body size, rather than to their developmental stage, FSH should be responsible for the gradual development of the gonads in the overwintered larvae.

Production and secretion of amphibian TSH, FSH and LH are regulated by the hypothalamic hormones CRH (Denver, 1993; 1996) and GnRH (Stamper and Licht, 1990; Oguchi *et al*., 1997). It is, therefore, absolutely necessary to obtain several additional molecular probes such as the *TH receptor(s)*, *5'-deiodinase* (Galton, 1989), *TRH*, *CRH*, and *GnRH* genes in *H. retardatus* to elucidate further the controlling mechanisms of metamorphosis and gonadal development and, thus, of neoteny.

Possible mechanism of neoteny in H. retardatus

Neoteny in salamander species is roughly classified into 3 categories: permanent (*Siren*, *Necturus*), axolotl (*Ambystoma mexicanum*) and facultative neoteny (*A. tigrinum*, *A. gracile*) (Frieden, 1981). Among those 3 modes of the neoteny, mechanisms of facultative neoteny have been analyzed in many species. In *A. tigrinum*, Norris *et al*. (1973; 1977; 1985) have extensively analyzed the effects of thyroid, pituitary and steroid hormones on gonadal development and metamorphosis. The neoteny reported in *H. retardatus* (Sasaki, 1924; Sasaki and Nakamura, 1937) must be facultative, in which animals metamorphose or not, depending on the environment. Although the low temperature of Lake Kuttara is a candidate causal factor for neoteny in this species (Moriya, 1983a), nobody has succeeded in inducing experimental neoteny or observing any sign of gonadal maturation by raising the larvae at lower temperatures (4 °C for 3 years, Wakahara, unpublished observation; 6 °C for 6 months, Yamashita *et al*., 1990). In natural habitats, however, aged, overwintered larvae of *H. retardatus* living in cold ponds at high altitude show gradual gonadal development without completion of morphological metamorphosis (Figs. 3,4). This finding is consistent with our previous observations that gonadal development and possibly gonadal maturation are independent of metamorphosis in *H. retardatus* (Wakahara, 1994; Wakahara and Yamaguchi, 1996; Kanki and Wakahara, 1999), suggesting a genetic potency for neotenic reproduction in this species. At present, however, molecular and endocrinological mechanisms of the chronological separation of morphological and biochemical metamorphosis, or heterochrony, are entirely unknown. Proximate linkages between gene expression, physiological mechanisms and ecologically important phenotypes may yield comprehensive explanations for the ultimate factors for neoteny in urodeles.

Materials and Methods

Animals

Eggs and larvae under the age of 1 year and aged, overwintered larvae of *Hynobius retardatus* were collected from ponds at Jozankei (800 m elev.) and Asari (1000 m elev.) in the vicinity of Sapporo. Three age groups of larvae were concurrently observed in 1 pond (Iwasaki and Wakahara, 1999). Hatched larvae under the age of 1 year were reared in the laboratory until use when they developed to stage 63 (full-grown larval stage, just prior to the onset of metamorphosis). They were reared under the natural daylight condition at the room temperature. Developmental stages were determined according to the normal table for *Hynobius nigrescens,* a species closely related to *H. retardatus* (Iwasawa and Yamashita, 1991). Aged, overwintered larvae at stage 63 were chosen for analyses. Ages of larvae were determined by skeletochronology (Esteban *et al*., 1996; Misawa and Matsui, 1999), as described below.

cDNA cloning

To obtain a partial sequence for *Hynobius LHβ*, procedures similar to those employed previously for cloning *TSHβ* and *FSHβ* (Kanki and Wakahara, 2000) were applied. One microgram of the extracted total RNA was reverse-transcribed using the SuperScript II RT (GIBCO BRL, Gaithersburg, Md., USA). By referring to cDNA sequences of *LHβ* of other vertebrates (Koide *et al*., 1996), we designed appropriate degenerate primers for synthesizing cDNAs corresponding to LHβ mRNA in *H. retardatus*. Sequences of the primers used and annealing temperatures were:
forward, 5'-AT(ACT)TG(CT)GC(ACGT)GG(ACGT)TA(CT)TG-3';
reverse, 5'-A(AG)(ACGT)GC(ACGT)AC (ACGT)GG(AG)(AT)A-3';
annealing temperature, 50°C. cDNA fragments of the expected length were amplified after 2 sets of 30-cycle reactions. These products were electrophoresed on 2% agarose gel, yielding 161-bp fragments. These fragments were ligated with pBluescript (Stratagene, La Jolla, CA, USA) vector and transformed with *Escherichia coli* DH5-α competent cells (TOYOBO, Tokyo, Japan). After blue selection and cloning of the vectors, both strands of nucleotide sequences of the inserts from positive clones were determined using an ABI PRISM auto-sequencer (Perkin Elmer, Pomona, CA, USA).

Full-length cDNAs for *Hynobius TSHβ* were obtained from a cDNA library constructed from the pituitary gland of metamorphosis-arrested larvae that had been reared in goitrogen (Kanki and Wakahara, 2000). *FSHβ* and *LHβ* genes were obtained by 5'- and 3'-RACE of the partial sequences of $FSH\beta_{(42\text{-}115)}$ cloned previously (Kanki and Wakahara, 2000) and of $LH\beta_{(48\text{-}100)}$ cloned in this study. A cDNA PCR library kit (TAKARA, Tokyo, Japan) was used to synthesize the adapter-ligated pituitary cDNA. 5'- and 3'-fragments were amplified with the adapter primers and primers located within the partial sequences obtained. To select the correct fragment of each gene, PCR products were subjected to Southern blot analysis using the RNA probes for each gene. Positive fragments were cloned and sequenced as described above.

The RT-PCR analyses were performed to confirm the specificity of the cDNAs obtained. Total RNAs extracted from the pituitary gland, brain, muscle, intestine, liver and spleen of the metamorphosed juveniles were reverse-transcribed to cDNAs. After 30 PCR cycles using the gene-specific primers, the PCR products were electrophoresed in 1% agarose gel containing ethidium bromide, and then photographed with a UV transilluminator.

DIG-labeling of RNA probes

To synthesize DIG-labeled sense- and anti-sense probes for *Hynobius TSHβ*, *FSHβ* and *LHβ*, cloned plasmids that included full-length sequences were treated with *Eco*RI or *Hind*III. After the linearized plasmids were

purified, RNAs were synthesized in the transcription buffer containing DIG RNA labeling mix (Boehringer Mannheim, Mannheim, Germany), 20 units RNase inhibitor, 40 units RNA polymerase (T3 or T7) and 0.1 M DTT, for 2 h at 37 °C. The synthesized DIG-labeled RNA probes were precipitated with ethanol twice and then dried. The amount of each RNA probe was determined by dot blotting with standard RNA samples. These probes were diluted to a concentration of 0.4 μg/μL and stored at –80°C with 20 units RNase inhibitor.

Semi-quantitative RT-PCR analysis

Nine pituitaries from larvae under the age of 1 year and 9 from 2-season-overwintered larvae were divided into 3 respective pools of 3 pituitaries each. Total RNA from each pool of 3 pituitaries was isolated by RNA extraction medium, ISOGEN (Nippon Gene, Tokyo, Japan). RT-PCR against the pituitary RNAs was performed using a cDNA synthesis kit (TAKARA, Tokyo, Japan) followed by 23 or 25 cycles of reaction with gene-specific primers, respectively. Sequences of the gene-specific primers used were *TSHβ*: forward, 5'-GTCAGAATGCATTCCATCTTCATAGCG-3', reverse, 5'-TCAAGAATGCACGTGTCTACTGAAGTC-3', annealing temperature, 59 °C; *FSHβ*: forward, 5'-TGCCTGCGAAACAACTTGAACAC-3'; reverse, 5'-CCTAACGTACAGATTTATTTTTATTACACAGG-3'; annealing temperature, 55 °C; and *LHβ*: forward, 5'-CTCTCCTCCGTAACCTCCG-3', reverse, 5'-CAGCTACTCTAAGCTACACCAC-3', annealing temperature, 57 °C. Amplified cDNAs were electrophoresed in 1% agarose gel, and then denatured in 0.5 M NaOH, 1.5 M NaCl, 15 min (2 times). After a wash with distilled water, the gels were neutralized in 0.5 M Tris, 3 M NaCl, pH 7.5, 15 min (2 times). cDNAs amplified with RT-PCR using gene-specific primers were blotted to Hybond-N^+ nylon membranes (Amersham Life Science, Buckinghamshire, UK). They were hybridized with gene-specific RNA probes labeled with DIG. Alkaline phosphatase-conjugated anti-DIG (Boehringer Mannheim), 1:3000, was used to detect the hybridized RNA probes. To obtain an internal control gene for an amplification standard, we designed appropriate primers for amplifying *Hynobius* actin cDNA. The sequences of the primers used were forward, 5'-GACATCAGGAAGGACCTGTATGCC-3' and reverse, 5'-AGAAGCACTTGCGGTGGACAATGG-3'.

Histology

Gonads and forelimbs were removed from larvae and fixed in Bouin's fixative. They were embedded in Tissue-Prep (Fisher Scientific, Fair Lawn, NJ, USA). Serial sections were stained with hematoxylin and eosin. To assess degrees of development of testes and ovaries, the sectional areas of the gonads were measured on 5 typical sections (8 μm thick) of each gonad with computer image-analyzer software (NIH image, see Kanki and Wakahara, 1999). The number of the germ cells was also counted. To assess the ages of the larvae used, sections through the humerus were observed, and the number of lines of arrested growth (LAGs, Esteban *et al.*, 1996; Misawa and Matsui, 1999) was counted.

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