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Original Article

P450 aromatase expression in the temperature-sensitive sexual differentiation of salamander *(Hynobius retardatus)* gonads

NATSUKO SAKATA, YOICHIRO TAMORI and MASAMI WAKAHARA*

Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo, Japan

ABSTRACT Sex differentiation of gonads in amphibians is believed to be controlled genetically, but altered epigenetically or environmentally. When larvae of the salamander *Hynobius retardatus* were reared at defined temperatures from hatching to metamorphic stages, a high temperature (28ºC) induced exclusively female gonads (ovaries), whereas intermediate (20 and 23ºC) or lower (16ºC) temperatures produced a 1:1 sex ratio of the morphological gonads. The thermosensitive period was determined to be restricted from 15 to 30 days after hatching, just before or when sexual differentiation occurred. *Hynobius P450 aromatase (P450arom)* cDNA was isolated from adult gonads and the partial nucleotide or deduced amino acid sequences were determined, showing a high level of identity with various vertebrate species. The *P450arom* gene was expressed predominantly in the adult ovary and brain, weakly in testis, but not in other somatic organs. A typical sexual dimorphism in *P450arom* expression was detected in normally developing larvae by a quantitative competitive RT-PCR; strong expression in the female gonads but very weak in male gonads. The dimorphism was detected much earlier than the morphological sexual differentiation of the gonads. When larvae were reared at the female-producing temperature (28ºC), strong expression was detected in all the temperature-treated larvae, suggesting that *P450arom* was up-regulated, even in genetic males. Our results confirm the importance of the *P450arom* regulation in the sexual differentiation of gonads and demonstrate that an up-regulation of *P450arom* is involved in the process of temperature-sensitive sex reversal in this species.

KEY WORDS: *P450 aromatase, GSD, TSD, sex differentiation, competitive RT-PCR, salamander*

Introduction

Most amphibians lack morphologically distinguishable sex chromosomes, but a number of experimental techniques have shown that amphibian sex determination is controlled genetically. Although approximately 50 species of amphibians show morphologically distinguishable sex chromosomes, sex-determining genes such as *SRY* in mammals (Gubbay *et al.*, 1990) and *DMY* in fish (Matsuda *et al.*, 2002) have not yet been described in amphibians (Swain, 2002; Wallace *et al.*, 1999). The genetic mechanism of sex determination (GSD) in amphibians, thus, can be overridden by environmental cues, such as temperature and exogeneously applied sex steroids (Dournon *et al.*, 1990; Wallace *et al.*, 1999). If the experimentally induced sex-reversed animals are fertile, breeding tests (e.g., cross between sex-reversed male (so called neomale) and original female and vice versa) can determine whether the species has male (XY) or female (ZW) heterogamy, even though the species has unrecognizable sex chromosomes (Hayes, 1998; Wallace *et al.*, 1999). The best documented example is *Pleurodeles waltl*, which is a urodele with ZZ male-ZW female genotypic sex determination. The newt, however, has been feminized by rearing larvae in estradiol (Gallien, 1954) or masculinized by rearing larvae at high temperature (Dournon and Houillon, 1984, 1985). Contrary to this, some urodelan species show somewhat different behavior: *P. poireti* which also has a ZZ/ZW type of GSD is feminized by high temperature treatment (Dournon *et al.*, 1984). *Triturus cristatus* which has a XX/XY type of GSD responds to larval treatments in much the same manner as *P. waltl*. It is feminized by estradiol (Wallace *et al.*, 1997) and can be masculinized at high temperature (Wallace and Wallace,

Abbreviations used in this paper: DMY, Y-specific DM-domain gene; GMC, gonad-mesonephros complex; GSD, genetic sex determination; P450 arom, P450 aromatase; SF-1, steroidogenic factor 1; RT-PCR, reverse transcription-polymerase chain reaction; SRY, sex-determining region Y; TSD, temperature-sensitive sex determination; TSP, temperature sensitive period.

***Address correspondence to:** Dr. Masami Wakahara. Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan. Fax: +81-011-706-4455. e-mail: chami@sci.hokudai.ac.jp

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2000). Similarly, it was reported in anurans that high temperatures caused masculinization in *Rana sylvatica* (XX/XY) (Witschi, 1956), *R. temporaria* (XX/XY) and *Bufo vulgaris* (ZZ/ZW). All of these studies demonstrated that the temperature-sensitive pattern of gonadal sex differentiation in amphibians did not correspond strictly to the pattern of heterochromosome (male or female heterogamy), or to the taxonomical groups. *Hynobius retardatus* is a salamander living in Hokkaido, northern Japan, adapted to the cold environment (Iwasaki and Wakahara, 1999). This salamander is characterized by a polyphenism of typical and broad-headed carnivorous morphs (Michimae and Wakahara, 2001; 2002) and by neotenic reproduction in a specific population (Wakahara, 1996). Both characteristics are ecological manifestation of the adaptation of this species to extremely cold environment. In this species, it was reported that the gonads were transformed from the female to the male features when larvae had been reared at a high temperature (Uchida, 1937). Contrary to the report, we have sometimes been bothered with a fact that gonads of the salamander tend to differentiate into ovaries and that the sex ratio of larvae inclines to female when laboratory experiments are conducted in summer season (Kanki and Wakahara, 2001), or when the larvae are reared at a high temperature. In this study, we examined the pattern of sexual differentiation of gonads in larvae of *H. retardatus* which had been exposed to distinctive temperature regimens. The results reported here demonstrate that the gonadal differentiation is altered to female phenotype irrespective of their original sexes by rearing larvae at a high temperature only during 15 to 30 days after hatching. P450 aromatase (*P450arom*) is a key enzyme to convert androgen to estrogen in many vertebrates (Simpson *et al.*, 1994). It is involved in female versus male differentiation in anurans (Kato *et al.*, 2004), as well as in urodeles (Chardard *et al.*, 1995; Chardard and Dournon, 1999). It was also reported to be involved in temperature-dependent sex reversal (Chardard *et al.*, 1995; Chardard and Dournon, 1999: Kuntz *et al.*, 2003a, b) in urodeles, in temperature-dependent sex determination (TSD) in many reptiles (Desvages *et al.*, 1993; Gabriel *et al.*, 2001; Murdock and Wibbels, 2003; Place *et al.*, 2001) and natural sex changes (Lee *et al.*, 2002; Liu *et al.*, 2004) and temperature-sensitive sex reversal (Kitano *et al.*, 1999) in fishes. In this study, *H. retardatus P450arom* cDNA was cloned and the expression pattern was examined in normally developing larvae and in larvae reared at the female-producing temperature. *P450arom* mRNA was extensively expressed in female gonads, but not in the male ones. It was up-regulated in all the larvae reared at the female-producing temperature, just opposite to the case in *Pleurodeles waltl* in which the aromatase was suppressed in larvae reared at the male-producing, high temperature (Kuntz *et al.*, 2003a, b). Our results suggest that the up-regulation of the *P450arom* is involved in the process of normal sexual differentiation of gonads and temperature-sensitive sex reversal in *H. retardatus*. This provides a new experimental system for investigation of sexual differentiation of gonads in urodeles.

Results

Sex ratio in natural population

Morphological characteristics of sexual differentiation of larval gonads in *Hynobius retardatus* are shown in Figure 1. From 0 to 10 days after hatching, developing genital ridges were formed by protruding from dorsal ceolomic epithelium with a few primordial germ cells surrounded by some somatic cells. Ten to 20 days after hatching, larvae had indifferent gonads nested with several germ cells. Sexual differentiation of gonads either to the ovary or testis was not observed yet. Morphological manifestation of gonadal sex differentiation occurred from 20 to 30 days of hatching. Developing ovaries were characterized by an ovarian cavity and by presence of growing oocytes at the cortex of the ovary.

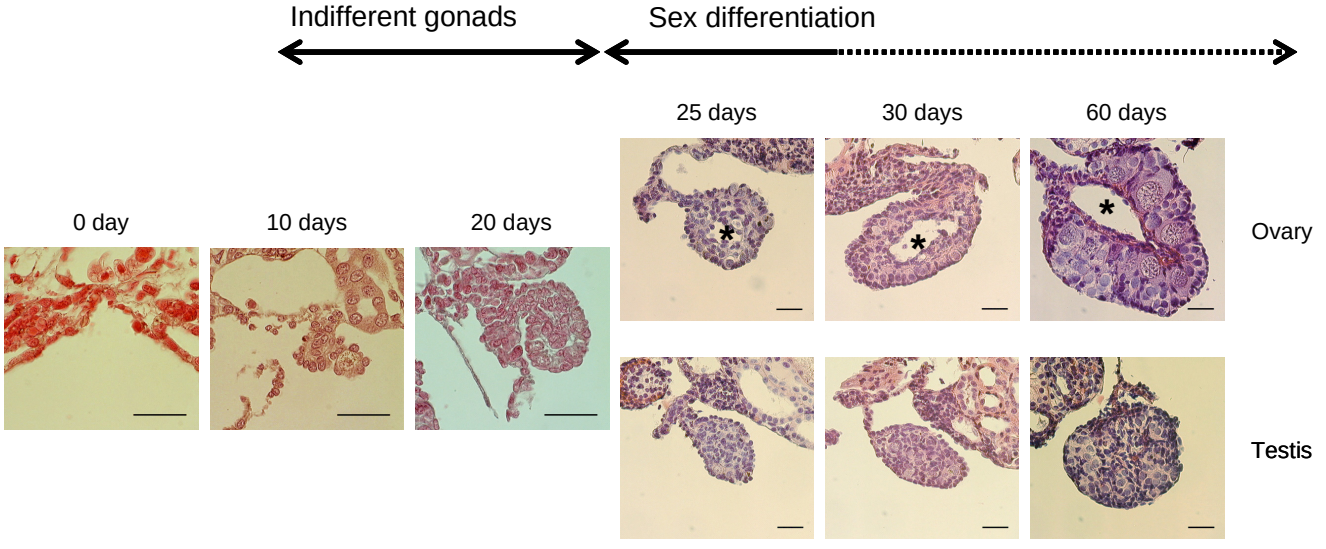


Fig. 1. Morphological sexual differentiation of gonads of *Hynobius retardatus* during early larval stages. *Morphological manifestation of gonadal sex differentiation occurred from 20 to 30 days of hatching. Developing ovaries were characterized by an ovarian cavity (asterisks) and by presence of growing oocytes at the cortex of the ovary. Contrary to this, testes were characterized by an accumulation of germ cells at the medulla of the developing gonads. The sexual differentiation of gonads in this species was established by 30 days after hatching at 20ºC, much earlier than the completion of the metamorphosis. Metamorphosis occurred around 60 days after hatching. Scale bars indicate 50 μm.*

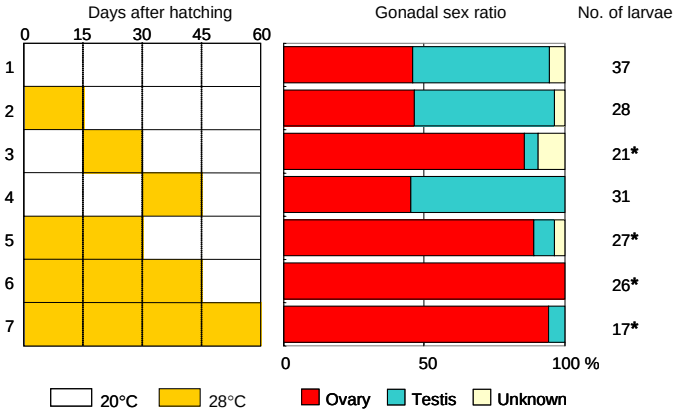


Fig. 2. Thermosensitive period during gonadal sex differentiation in *H. retardatus*. *Temperature regimens of experiment (left) and the sex ratios of gonads at the end of experiments (right). Newly hatched larvae were reared at either 28ºC (female-producing temperature) or 20ºC (balanced sex ratio-inducing temperature) in specific time schedule from hatching to metamorphic stages. Seven types of the temperature regimens are illustrated in the left. At the end of experiments, gonad-mesonephros complexes (GMCs) were fixed and examined for gonadal sexual differentiation. «Unknown» in the figure indicates gonads whose sexes could not be determined by histological observation as either testis or ovary showing indifferent, hermaphrodite and/or intersex. The sex ratios were significantly deviated to female in Exp. no.3, Exp. no. 5, Exp. no. 6 and Exp. no. 7. The sex ratios in the controls (Exp. no.1) and larvae in Exp. no. 2, Exp. no. 4 were nearly 1:1. Thermosensitive period was restricted from 15 to 30 days after hatching in this species. Asterisks on numerals show significant difference (p<0.001) from expected, balanced sex ratio, 1:1.*

Contrary to this, testes were characterized by an accumulation of germ cells at the medulla of the developing gonads. The sexual differentiation of gonads in this species was established by 30 days after hatching at 20ºC (stage 53), much earlier than the completion of the metamorphosis (60~70 days after hatching at 20ºC, Arai and Wakahara, 1993). The sex ratio of the larvae that had been captured at the natural ponds was analyzed according to the morphological characteristics described above. The sex ratio of larval *Hynobius retardatus* in natural population was not deviated (n.s. in χ^2 test), from the expected, balanced ratio of 1:1 (35 males and 44 females out of 79 larvae so far examined histologically). The sex ratio suggesting that the sex ratio in this species was controlled to be 1:1 in the wild population.

Temperature effects on developing gonads

Table 1 shows the temperature effects on developing gonads of *H. retardatus.* Two hundred larvae were allocated to 4 treatments of the specific temperatures (16, 20, 23 and 28ºC) 50 larvae each. Some of them were accidentally lost during the course of experiment (from 0 day of the hatching to the completion of metamorphosis or to 70 days after hatching). Main reduction of the number of larvae was due to frequent cannibalism among larvae in rearing tanks (Wakahara, 1995, 1997; Michimae and Wakahara, 2001). Because the cannibalism occurred randomly (either to cannibalize or to be cannibalized) irrespective of sexes (Wakahara, 1995), cannibalism did not affect the resultant sex ratio of the survived larvae. Gonads of all 133 larvae that survived for experimental period were examined histologically and their gonadal sexes were determined. High temperature (28ºC) induced much more females than males: 41 females out of 43 larvae examined (95.3%). This deviation of the sex ratio from expected natural sex ratio was statistically significant (p<0.001, χ^2-test). Contrary to this, no deviation of the sex ratio was observed in larvae that had been reared at 16, 20 and 23ºC.

To determine the possible thermosensitive period, larvae were reared at either 28ºC (female-inducing temperature) or 20ºC (balanced sex ratio-inducing temperature) in specific time schedules from hatching to metamorphic stages (Fig. 2). All of the gonads of the experimental larvae (totally 194) were examined histologically at the end of experiments and their gonadal sexes were determined. The sex ratios of the controls (continuously reared at 20ºC, Exp. no. 1), larvae that had been reared at 28ºC from 0 to 15 days (Exp. no. 2) and from 30 to 45 days (Exp. no. 4) after hatching, were nearly 1:1. Contrary to these, the sex ratios were significantly deviated to female in larvae that had been reared at 28ºC from 15 to 30 days (Exp. no. 3), from 0 to 30 days (Exp. no. 5), from 0 to 45 days (Exp. no. 6) and from 0 to 60 days (Exp. no. 7) after hatching. These results convincingly showed that the thermosensitive period was restricted from 15 to 30 days after hatching in *H. retardatus*. This was in agreement with the fact that the morphological manifestation of gonadal sex differentiation occurred from 20 to 30 days after hatching (Fig. 1).

P450 aromatase sequence of *Hynobius retardatus*

A partial sequence for *P450arom* cDNA of *Hynobius retardatus* was amplified with RT-PCR using a set of primers F1 and R3, yielding 650-bp cDNA fragment. Predicted amino acid sequence deduced from the fragment is shown in Figure 3. Because degenerate primers used at first in this experiment were designed in reference to common sequences conserved in many vertebrates, the cDNA fragment obtained represented approximately 50% of the full length of cDNA. Similarities of the partially cloned *P450arom* gene of *H. retardatus* to those of other vertebrates at the amino acid level were thus relatively high, 85% to human, 86% to chick, 88% to turtle, 88% to *Xenopus* and 88% to newt aromatase, respectively (Fig. 3). Tissue-specific expression of the *P450arom* was examined by a conventional RT-PCR (Fig. 4) using RNAs extracted from several adult organs. The *Hynobius P450arom* gene was expressed predominantly in adult ovary and brain, weakly in testis, but not in the intestine, liver, kidney and lung.

TABLE 1

SEX RATIO OF GONADS IN *HYNOBIUS RETARDATUS* REARED AT DIFFERENT TEMPERATURES FROM HATCHING TO METAMORPHOSIS

Temperature	No. of embryos used	No. of larvae examined	Morphological gonadal sex				
			female	male	unknown	% female	χ^2-test *
16ºC	50	38	15	17	6	39.5	ns
20ºC	50	20	8	12	0	40.0	ns
23ºC	50	30	13	17	0	43.3	ns
28ºC	50	43	41	1	1	95.3	p<0.001

*significance of the deviations of sex ratio from the balanced sex ratio (1:1) was analyzed.

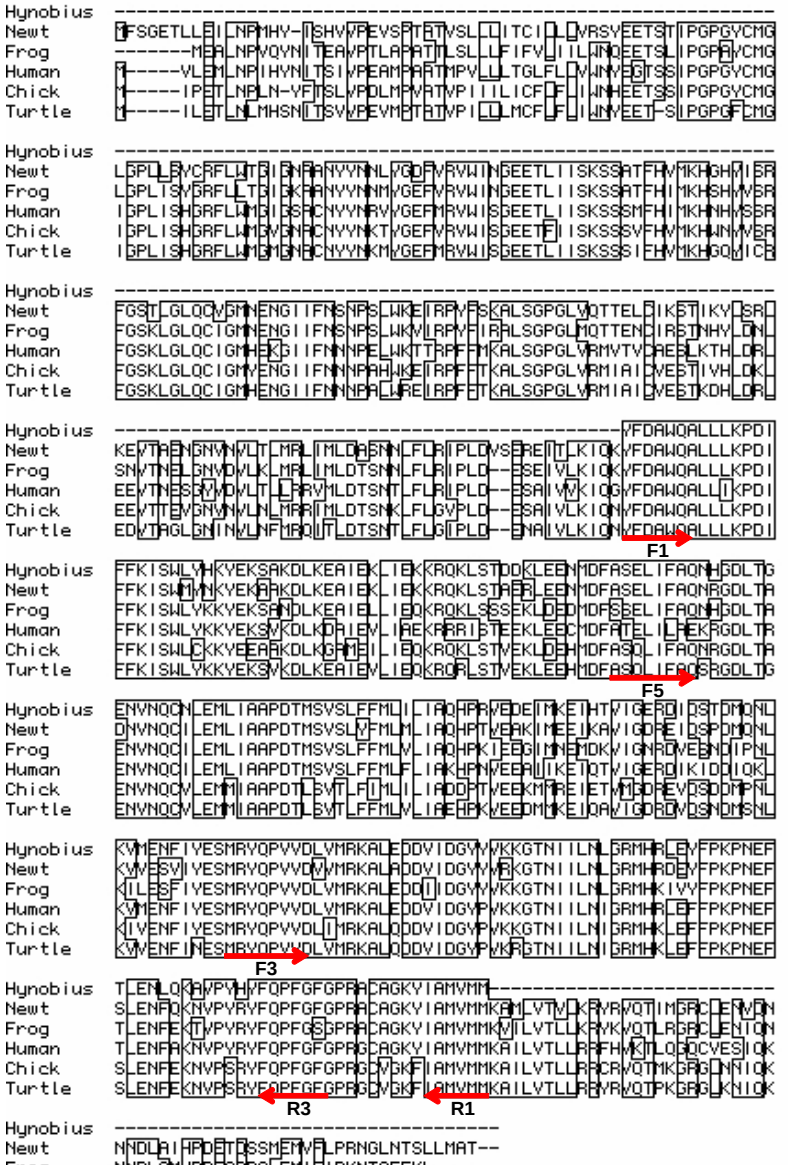


Fig. 3. Predicted amino acid sequence deduced from the partially cloned cDNA fragment of *H. retardatus* P450 aromatase. *Proportional comparison to the alignment of other vertebrate P450 aromatase is shown. Partial sequences of* Hynobius (*GenBank accession No. AB204518), newt (AB164064-1), Xenopus (BC079750-1), human (Y07508-1), chick (M73303-1) and turtle (AY029233-1) aromatase are aligned from top to bottom. Identical residues are enclosed by square boxes. Arrows indicate the sequences used as primers for conventional and competitive RT-PCRs.*

Sexual dimorphism in aromatase expression

Because it was not possible to quantify the *P450arom* expression level in larval gonads by the conventional RT-PCR, a quantitative competitive RT-PCR was conducted to determine approximate number of copies of *P450arom* mRNA. Figure 5 shows a general concept for the competitive RT-PCR. According to this procedure, it was possible to quantify the expression level of a specific mRNA at the level of 10^3 copies/500 ng total RNA. Normally developing larvae were collected every 5 days, 7 larvae each, from just after hatching to the metamorphosis. The GMCs from every larva that had been numbered individually were divided into left and right halves; the left halves were fixed in Bouin's fixative and processed for histological examination for morphological gonadal determination and the right ones were used for the extraction of RNAs and the competitive RT-PCR. Figure 6 shows a sexual dimorphism in expression level of *P450arom* between female and male gonads. In female, 10^4~10^5 copies of *P450arom* mRNA / 500 ng total RNA was detected as early as 25 days after hatching. A higher expression (over 10^5 copies/500 ng total RNA) was consistently observed in the ovaries 35 days after hatching onward. Contrary to this, the expression level could not be determined in male gonads until 45 days after hatching, because of a very weak expression or under the limit of detection level. The expression level of the *P450arom* in the testes was under $5x10^3$ copies/500 ng total RNA even in later development. Interestingly, the expression dimorphism was detected much earlier than the morphological sexual differentiation of gonads: a half of indifferent gonads in larvae of 15 and 20 days after hatching expressed the *P450arom* mRNA at the level of 10^3~10^4 copies (Fig. 6A), but other half did not.

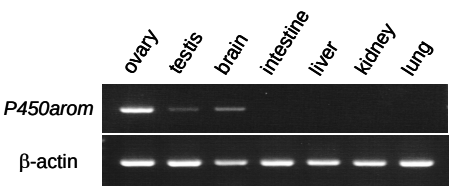
Aromatase expression in larvae reared at the female-producing temperature

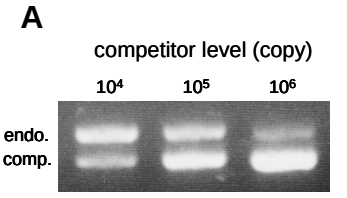
Quantitative, competitive RT-PCR was done using developing gonads of 70 larvae reared at the female-producing temperature (28ºC), during the temperature-sensitive period, i.e., from 15 to 30 days after hatching. Ten larvae each were collected every 5 days, from hatching to 60 days after hatching, numbered and divided into left and right halves. The left halves of their gonads were examined histologically to know their gonadal sexes. The right halves were used for the quantitative RT-PCR. All gonads out of 70 treated larvae (100%) developed to ovaries, showing that sex reversal from males to phenotypic females occurred in all potential or genotypic males. The results of the competitive RT-PCR are shown in Figure 6B. There was no dimorphism detected in the expression of *P450arom* at all. Considerable level of the *P450arom* expression was detected in every larva as early as 20 days after hatching. The expression levels increased gradually as larvae developed and strong expression was observed in all larvae of 35 days after hatching. The developmental pattern of the *P450arom* expression in larvae reared at 28ºC (Fig. 6B) was identical to that in female larvae reared at 20ºC (Fig. 6A), suggesting that the *P450arom* was up-regulated even in genetic males when larvae were reared at the female-producing temperature.

Discussion

High temperature made female phenotype of gonads in H. retardatus

Gonadal sexes of larval *Hynobius retardatus* deviated to female when they were reared at a high temperature from hatching stage





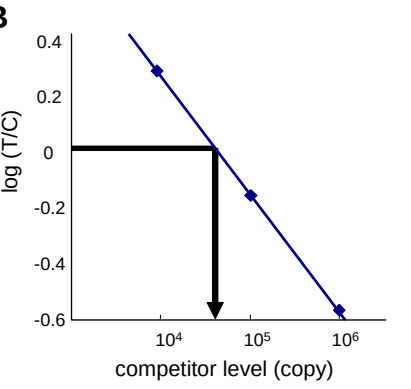


Fig. 4 (Left). Expression pattern of *P450 aromatase* in adult organs of *H. retardatus*. *RNAs extracted from adult ovary, testis, brain, intestine, liver, kidney and lung were amplified with a conventional RT-PCR using F5-R3 primers, respectively.* P450arom *was expressed predominantly in adult ovary and brain, weakly in testis, but not in other somatic organs. β-actin was used as an internal marker.*

C = PCR products for competitor
T = PCR products for *P450arom*

Fig. 5 (Right). General concept for quantitative competitive RT-PCR. (A) *Typical electrophoregram showing PCR products for endogenous P450arom and for competitor following a competitive RT-PCR. In this experiment, 3 sets of dilution of competitor (from 10^4 to 10^6 copies) and a standard concentration of total RNA (500 ng) from tissues were added to the RT tubes and then PCR was performed. The PCR products for the competitor (comp., 393 bp) run faster than those of the endogenous* P450arom *(endo., 460 bp).* **(B)** *Standard curve obtained from (A). Number of copies of endogenous* P450arom *was estimated from the point where the curve crossed the 0 level of vertical line that means expected concentration of* P450arom *identical to that of the competitor.*

to the completion of the metamorphosis, whereas the sex ratio of larvae reared at intermediate or lower temperatures was nearly 1:1 (Table 1). This finding is completely different from that reported previously in this species (Uchida, 1937). He reported that the gonads of larvae reared at a high temperature (30ºC) showed several degrees of transformation from the female to the male features. Since that observation has been still referred in recent review articles (e. g., Wallace *et al.*, 1999), it has been widely accepted that *H. retardatus* shows masculinization when reared at a high temperature. However, because 1) the number of larvae employed in his study was relatively small (totally 76 larvae), 2) the ages (developmental stages) of larvae used were not controlled (from 21 mm to 47 mm in body length at the beginning of the experiment), 3) time and duration of the exposure to 30ºC were rather random (from 7 to 71 days), 4) no proper controls were employed and 5) no statistical analyses were done, the conclusion of the previous observation was not reliable at the level of current developmental biology. Contrary to that, our results convincingly demonstrated that a high temperature made the gonads female type (Table 1) and that the thermosensitive period was restricted from 15 to 30 days after hatching (Fig. 2). The same results have been obtained in our laboratory using various populations of *H. retardatus* living throughout Hokkaido (data not shown). Exposure of larvae to a high temperature in Uchida's experiment was much later than the thermosensitive periods (from 15 to 30 days after hatching) we have found in this study.

Possible role of aromatase in gonadal sex differentiation

A positive correlation between the expression of *P450arom* gene and morphological ovarian differentiation in *H. retardatus*, in

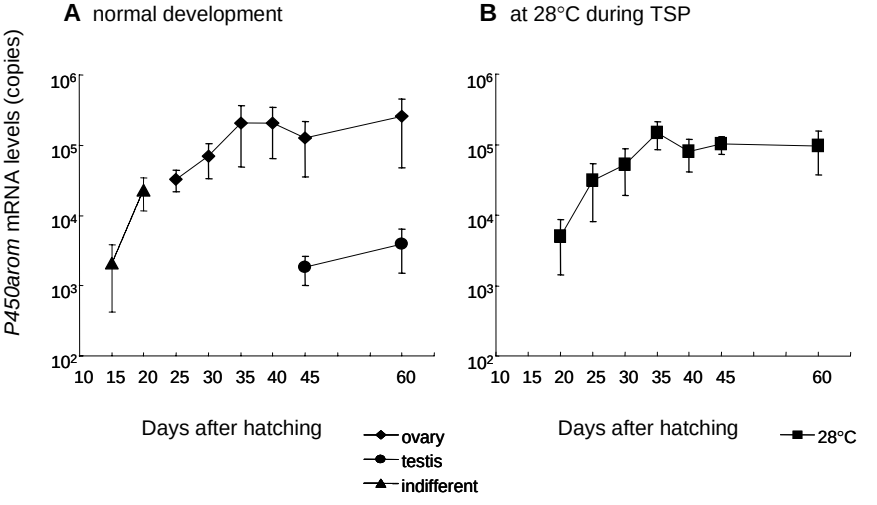


Fig. 6. Expression pattern of *P450 aromatase* during normal development (A) and in larvae reared at the female-producing temperature (28ºC) from hatching to the metamorphic stages (B). (A) *Gonad-mesonephros complexes (GMCs) in normally developing larvae were divided into left and right halves. The left halves were processed for histological observation for individual morphological gonadal differentiation. The right ones were used for the competitive RT-PCR. In ovaries,* P450arom *was detected as early as 25 days after hatching. Higher expression was consistently observed in the ovaries after 35 days after hatching. In testes, the expression level was much lower than in ovaries and could not be determined until 45 days after hatching, because of a very weak expression or under the limit of detection level. The expression dimorphism was detected much earlier than the morphological differentiation of gonads: one half of indifferent gonads (15 and 20 days after hatching) expressed the* P450arom*. Each point and bar represent an average of 7 independent determinations and standard deviation, respectively.* **(B)** *All larvae out of 70 that had been reared at 28ºC had ovaries, demonstrating that the sex reversal (male to female) was induced by the high temperature.* P450arom *was detected predominantly in all the larvae and the pattern of the expression was identical to that in normally developing female larvae (A), suggesting that the* P450arom *was up-regulated by high temperature even in the genetic males. Each point and bar represent an average of 10 independent determinations and standard deviation, respectively. TSP, temperature sensitive period.*

other words, a sexual dimorphism in the expression of *P450arom* in normally developing larvae was clearly shown in our results of the quantitative competitive RT-PCR (Fig. 6A). *P450arom* mRNA was expressed much more extensively in female gonads, at least 100-times more, than in male gonads, suggesting a positive involvement of aromatase activity in ovarian differentiation in *H. retardatus*. The temperature-dependent sex reversal or deviation to female phenotype in this species is also explainable in terms of the *P450arom* regulation. Figure 6B demonstrated convincingly that *P450arom* was up-regulated even in genetic males when reared at the female-producing temperature. The role of *P450arom* in ovarian development is similar to the sex differentiation and sex reversal in *Pleurodeles waltl* (Chardard *et al*., 1995; Chardard and Dournon, 1999), even though the direction of sex reversal by a high temperature is completely different between the two species. The larvae were sex reversed to males by rearing at a high temperature as well as by treating with aromatase inhibitors. Because aromatase has been proposed to play a key role in ovarian differentiation in *P. waltl* (Chardard *et al*., 1995), the inhibitors promoted or resulted in testicular differentiation by inhibition of the conversion from androgens to estrogens (Chardard and Dournon, 1999). Furthermore, it was reported that *in vitro* treatment of indifferent gonads by the aromatase inhibitor made male phenotype in *Xenopus laevis* (Miyata and Kubo, 2000). In *P. waltl*, the aromatase activity was suppressed in gonads of ZW larvae reared at male-inducing, high temperatures (Chardard *et al*., 1995; Kuntz *et al*., 2003a, b). Similar suppression of aromatase gene expression was observed in the temperature-dependent sex reversal of genetic females to phenotypic males in Japanese flounder reared at high temperature (Kitano *et al*., 1999). Contrary to these, the reverse scenario is expected in *H. retardatus* : *P450arom* gene expression is up-regulated and the resulting aromatase activity may bring about a high level of estrogens when the larvae of *H. retardatus* are reared at the female-inducing temperature (28ºC). It is thus assumed that genetic males are sex-reversed to phenotypic females under the influence of estrogens. This scenario is in accordance with the facts that sexual dimorphism in the *P450arom* expression was observed as early as 15 days after hatching (Fig. 6A) when the morphological differentiation of gonads had not been occurred (Fig. 1) and that differentiating gonads are easily modified to ovaries by treatment of larvae with estradiol-17β, but not to testes by treatment with testosterone in this species (unpublished observation). It has been hypothesized that estrogen production may play a pivotal role in the sex determination of reptiles with TSD. This hypothesis has been furthered by studies that have shown higher aromatase activity in the developing ovaries in some reptiles (Desvages *et al*., 1993). In other reptiles, however, it was reported that no differences were detected in aromatase gene expression levels between male- and female-producing temperatures during the thermosensitive period. After the thermosensitive period, aromatase mRNA levels increased in females, suggesting that the expression of aromatase may not be a pivotal regulatory step in the sex determination cascade (Murdock and Wibbels, 2003). These findings lead us to the study on regulation mechanisms of the aromatase gene. Our results showing a very early sexual dimorphism in *P450arom* even in undifferentiating gonads (Fig. 6A) might support the pivotal role of the aromatase expression in sexual differentiation of gonads in *H. retardatus*. Considering the phenomenon of temperature sensitivity of gonadal differentiation in *H. retardatus*, the main problem to be solved is to identify the thermosensitive factor which occurs in the regulatory cascade leading to activation or repression of transcription of the *P450arom* gene. In this respect, one of the transcriptional factors of the aromatase gene, steroidogenic factor-1 (*SF-1*), has been isolated recently in some amphibians (Kawano *et al*., 2001; Mayer *et al*., 2002). Examination of the possible involvement of the *SF-1* to the thermosensitive differentiation in *H. retardatus* gonads is absolutely necessary to clarify the molecular mechanism of this phenomenon.

Contribution of sex chromosomes to sexual differentiation in amphibians

Direction of the sex-reversal from male to female by rearing larvae at a high temperature in *H. retardatus* is different from those in the newts *Pleurodeles waltl* that has ZZ male-ZW female sex determination mechanism and *Triturus crsitatus* that has XX female-XY male sex determination (Wallace *et al*., 1999), both of which have been reported to show more males at higher temperatures (Chardard *et al*., 1995; Dournon *et al*., 1990; Wallace and Wallace, 2000). The pattern of the temperature-sensitive differentiation of gonadal sex in *H. retardatus* is similar to that in *P. poireti* which has ZZ/ZW type of GSD and is feminized by a high temperature treatment (Dournon *et al*., 1984). All these results indicate that the direction of thermosensitive sex deviation in amphibians are not correlated with the combination of sex chromosomes, either male-heterogamy (XX/XY) or female-heterogamy (ZZ/ZW). In this respect, it is worthy to note that regional races of *Rana rugosa*, a frog in Japan, show very interesting sex-determining system (Miura *et al*., 1996; Ohtani *et al*., 2000). It is divided into three or four geographical races on the basis of the karyotype of the sex chromosomes: one in which heteromorphic sex chromosomes occur in the female sex (ZZ/ZW-system), another in which they are present in males (XX/XY-system) and the remaining in which no heteromorphism is seen in either sex, but with XX/XY sex determining system. Extensive studies including genetics, cytogenetics and experimental morphology suggest that the genetic sex determination evolved from ZZ/ZW to XX/XY in *R. rugosa*. Ohtani *et al*. (2000) speculated that the putative female-determining gene(s) are located on the W chromosome as advocated by Hills and Green (1990). Wallace *et al*. (1999) envisaged that the Z chromosome carries a recessive male determinant which could be an inactive mutation of the corresponding W-linked gene needed for female differentiation. At present, however, it seems premature to conclude possible contribution of sex-determining gene(s) on the sex chromosomes to sexual differentiation in amphibians. Almost all studies suggesting that environment influences sex determination and/or differentiation in amphibians have been conducted at temperatures out of the range normally experienced by the species under study and these effects probably do not occur under natural conditions. Nevertheless, these types of experiments have contributed to experimental analyses on the involvement of genetic and environmental factors in gonadal differentiation (Hayes, 1998; Wallace *et al*., 1999), possible evolution of heteromorphic sex chromosomes such as XX/XY and ZZ/ZW (Ohtani *et al*., 2000; Sims *et al*., 1984) and search for putative sex-determining gene(s) and/or sex-specific sequences (Coriat *et al*., 1994; Kawano *et al*., 2001; Shibata *et al*., 2002) in amphibians. Because *H. retardatus* has been reported to have no morphologically distinctive sex

chromosomes (Makino, 1932) and because no sex-determining gene(s) or sex-specific sequences have been described in amphibians yet, it is not possible to determine which sex is heterogamy either at the morphological or at the molecular levels. Rather, cross test will be promising between sex-reversed male and normal female or vice versa in *H. retardatus*.

Materials and Methods

Animals

Fertilized eggs of *Hynobius retardatus* were collected from several ponds in the vicinity of Sapporo during breeding season (Iwasaki and Wakahara, 1999). They were stored at 4ºC until use. After hatching, the larvae were fed with live freshwater oligochaetes (*Tubifex*) or frozen red worms. Developmental stages were determined according to the normal table for *Hynobius nigrescens*, a species closely related to *H. retardatus* (Iwasawa and Yamashita, 1991).

Determination of temperatures affecting sex differentiation of gonads

Two hundred, newly hatched larvae were divided into 4 groups of 50 larvae each. They were allotted to 4 different temperature regimens, 16, 20, 23 and 28ºC, respectively. Sixty to 70 days later, they were fixed with Bouin's fluid and their gonads were processed for histological examination for determination of morphological gonadal sexes (Kanki and Wakahara, 2001). The significance of the deviations of sex ratio in each experimental group from the theoretical, or balanced sex ratio (1:1) was analyzed by the χ^2 test. Statistical significance was defined as $p<0.001$.

Determination of thermosensitive periods

The critical period of thermolabile sex differentiation in gonads of *H. retardatus* was examined by rearing larvae at 20ºC (a balanced sex ratio-inducing temperature) and 28ºC (a female-inducing temperature) with different time regimens for 60 days. Newly hatched larvae were allotted to 7 groups of 20 to 40 larvae each, as follows: 1) from 0 to 60 days at 20ºC (as a control), 2) from 0 to 15 days at 28ºC and then shifted down at 20ºC from 15 to 60 days, 3) from 0 to 15 days at 20ºC, then sifted up at 28ºC from15 to 30 days and then shifted down at 20ºC again until 60 days, 4) from 0 to 30 days at 20ºC and then shifted up to 28ºC from 30 to 45 days and again shifted down to 20ºC until 60days, 5) from 0 to 30 days at 28ºC and then shifted down at 20ºC from 30 to 60 days, 6) from 0 to 45 days at 28ºC and then shifted down at 20ºC from 45 to 60 days and 7) from 0 to 60 days at 28ºC continuously. The time-temperature regimens and the number of larvae examined are shown in result section (Fig. 2). At the end of the experiments, they were fixed with Bouin's fluid and their gonads were processed for histological examination.

P450 aromatase cDNA cloning

Total RNAs were collected from adult ovaries with ISOGEN RNA extraction reagent (Nippon Gene). Poly(A)+mRNA was purified from total RNAs with OligotexTM-d30 (Super) mRNA purification kit (Takara) according to manufacturer's instruction. One microgram of the poly(A)+RNAs was reverse-transcribed using a SuperScript first-strand synthesis system for RT-PCR (Invitrogen). To obtain partial sequence for *Hynobius P450arom*, we designed 2 types of primers set for RT-PCR; degenerate forward-degenerate reverse (F3-R1) and degenerate forward-specific reverse (F1-R3) sets. By referring to cDNA sequences of *P450arom* of other vertebrates, we designed the first degenerate primers set for synthesizing cDNA corresponding to a part of *P450arom* mRNA in *H. retardatus*. Sequences of the first primers set and RT-PCR condition were: forward (F3), 5'-ATG AGN TAY CAR CCN GTI GTN GA-3'; reverse (R1), 5'-ATH GCN ATG GTN ATG ATG AA-3' [Y(C or T); R (A or T); H (not G); I (inosine); N (any)], 30 seconds at 94ºC for melting, 30 seconds at 45ºC for annealing, 30 seconds at 72ºC for extension. cDNA fragments of expectable length were amplified after 40 cycles of reaction. These products were electrophoresed on 2% agarose gel, yielding 254 bp fragment for *H. retardatus* aromatase. The fragment was ligated with PGEM-T vector (Promega) and transformed with *Escherichia coli* XL-1 - competent cells (TOYOBO). After the blue selection and cloning of the vectors, both strands of nucleotide sequences of the inserts from positive clones were determined using ABI PRISM auto sequencer. According to the sequence obtained, the second set of primers was designed; a degenerate primer (F1) 5'-TAY TTY GAY GCN TGG CAR GC-3' and a specific primer (R3) 5'-TTC CAG CCA TTT GGT TTT G-3' were designed for a longer sequence. The RT-PCR product using F1 and R3 was sequenced, yielding a 650 bp fragment of *Hynobius P450arom* cDNA (Fig. 3).

RT-PCR

Single-stranded cDNA was generated from the total RNAs extracted from several organs of adult *H. retardatus* with a SuperScript first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instruction. To amplify cDNA fragment of *Hynobius P450arom*, the specific primers set, (F5) 5'-A TCT GAA TTA ATT TTT GCT CAG-3' and (R3, described above), was used. cDNA fragment of β -actin was amplified as a control using a specific primers set of (F) 5'-AGA AGC TTG CGG TGG ACA ATG G-3' and (R) 5'-GAC ATC AGG AGG ACC TGT ATG CC-3' (Kanki and Wakahara, 2001). The PCR products were separated on 2% agarose gels containing ethidium bromide.

Quantitative, competitive RT-PCR

A quantitative competitive RT-PCR was done using gonads of 118 normally developing larvae and of 70 larvae reared at the female-producing temperature (28ºC), during the temperature-sensitive period, i.e., from 15 to 30 days after hatching. Seven larvae each from normally developing larvae were collected every 5 days from hatching to 60 days after hatching and numbered individually and their GMCs were excised on ice. The left halves of their gonads were individually fixed in Bouin's fluid and then examined histologically to know their gonadal sexes. The corresponding right halves were immediately frozen at -80ºC individually as well and used for the quantitative RT-PCR. Thus, a sexual dimorphism in the *P40arom* expression was determined precisely. In the high temperature-treated experiments, 10 larvae each were collected every 5 days, from hatching to 60 days after hatching, numbered and then the GMCs were divided into right and left halves. The GMCs were treated similarly to the procedures described above.

A competitive RT-PCR was conducted to quantify the *P450arom* mRNA in developing gonads of controls and experimental larvae by using Competitive DNA Construction Kit and Competitive RNA Transcription Kit (Takara). The competitor was designed to be identical to a portion of normally occurring *P450arom* mRNA, except that included some deletion. Due to the deletion, the competitor could be visualized on a agarose gel at a different location than the naturally occurring *P450arom* following RT-PCR (cf. Fig. 5). In that way, the competitor RNA could be included as an internal standard (at a known concentration) in the RT-PCRs. Thus, the competitor provided a means of quantifying the amount of mRNA in a particular sample (Murdock and Wibbels, 2003).

In order to develop a *P450arom* competitor molecule, a set of primers was specifically designed so that a small deletion (approximately 70 bp) could be made in the F5-R3 fragment for the endogenous mRNA. The primers for DNA competitor production were; forward: 5'-SP6-promoter sequence (ATT TAA GGT GAC ACT ATA GAA GAC) + F5 (A TCT GAA TTA ATT TTT GCT CAG) + F (GTA CGG TCA TCA TCT GAC AC)-3' and reverse; 5'-350R (GAC TTC GTT GCT TTC CAG TT)+R3 (TTC CAG CCA TTT GGT TTT G)-3'. This set of primers was expected to produce a fragment of 393 bp, 67 bp shorter than the product for original *P450arom* mRNA (PCR product with F5 and R3, 460 bp). The competitor DNA fragment was synthesized according to the manufacturer's instruction. RNA competitor was produced by a transcription of the DNA competitor

fragment that had SP6 promoter sequence, using Competitive RNA Transcription Kit (Takara). For the RNA synthesis, linearized DNA template, SP6 RNA polymerase and each NTP were mixed according to the manufacturer's protocol. Synthesized competitor RNA was isolated and pelleted by conventional procedures. The RNA pellet was then resuspended in nuclease-free water and the concentration was determined using a spectrophotometer. The RNA solution was diluted to a specific concentration and then stored at -80ºC for future use in *P450arom* competitive RT-PCRs. Competitive RT-PCR was conducted using SuperScript first-strand synthesis system for RT-PCR (Invitrogen), with the primers F5 and R3 described above. A standard dilution set of *P450arom* competitor (decimal dilution from 10^6~10^2 copies/tube) was used to examine each sample from normally developing larvae and experimental animals described above.

For each of experimental samples, a standard amount of total RNA (500 ng) was added to each RT reaction tube. Additionally, the standard dilution set of the competitor was added to the RT tubes. As a control tube, no competitor was added. PCR condition was nearly identical to that described above. Following amplification, the PCR products were separated by 3% agarose gel electrophoresis and the competitor and endogenous *P450arom* band intensities were quantified in each lane using NIH Image. These intensities were used to generate a standard curve for estimating the amount of endogenous *P450arom* mRNA in each sample (Fig. 5).

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