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

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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 exogenously 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, 1997).
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 20°C, much earlier than the completion of the metamorphosis. Metamorphosis occurred around 60 days after hatching. Scale bars indicate 50 μm.
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 20°C (stage 53), much earlier than the differentiation of gonads in this species was established by 30 germ cells at the medulla of the developing gonads. The sexual differentiation of testis or ovary showing indifferent, hermaphrodite and/or intersex. The sex ratios of gonads of all 133 larvae that survived metamorphosis or to 70 days after hatching (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 20°C, Exp. no. 1), larvae that had been reared at 28°C 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 28°C 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.

### 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 28°C) 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; Michmae 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 (28°C) 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, χ²-test). Contrary to this, no deviation of the sex ratio was observed in larvae that had been reared at 16, 20 and 23°C.

To determine the possible thermosensitive period, larvae were reared at either 28°C (female-inducing temperature) or 20°C (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 20°C, Exp. no. 1), larvae that had been reared at 28°C 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 28°C 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).

### Table 1

<table>
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<tr>
<th>Temperature</th>
<th>No. of embryos used</th>
<th>No. of larvae examined</th>
<th>Morphological gonadal sex</th>
<th>χ²-test *</th>
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<tr>
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<td>50</td>
<td>38</td>
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<td>30</td>
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<td>43.3</td>
</tr>
<tr>
<td>28°C</td>
<td>50</td>
<td>43</td>
<td>female: 41, male: 1, unknown: 1</td>
<td>95.3</td>
</tr>
</tbody>
</table>

*significance of the deviations of sex ratio from the balanced sex ratio (1:1) was analyzed.
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 the 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 halves 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.
P450arom gene expression in a TSD salamander

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.

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 10⁴ to 10⁶ 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.

Fig. 6. Expression pattern of P450 aromatase during normal development (A) and in larvae reared at the female-producing temperature (28°C) 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 28°C 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.

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 (30°C) 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 30°C 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
other words, a sexual dimorphism in the expression of \(P450\text{arom}\) in normally developing larvae was clearly shown in our results of the quantitative competitive RT-PCR (Fig. 6A). \(P450\text{arom}\) 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 \(H. \text{retardatus}\). The temperature-dependent sex reversal or deviation to female phenotype in this species is also explainable in terms of the \(P450\text{arom}\) regulation. Figure 6B demonstrated convincingly that \(P450\text{arom}\) was up-regulated even in genetic males when reared at the female-producing temperature. The role of \(P450\text{arom}\) in ovarian development is similar to the sex differentiation and sex reversal in \(Pleurodeles \text{waltl}\) (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 \(P. \text{waltl}\) (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 \textit{in vitro} treatment of indifferent gonads by the aromatase inhibitor made male phenotype in \textit{Xenopus laevis} (Miyata and Kubo, 2000). In \(P. \text{waltl}\), 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 \(H. \text{retardatus}\): \(P450\text{arom}\) gene expression is up-regulated and the resulting aromatase activity may bring about a high level of estrogens when the larvae of \(H. \text{retardatus}\) are reared at the female-inducing temperature (28°C). 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 \(P450\text{arom}\) 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 \(P450\text{arom}\) even in undifferentiating gonads (Fig. 6A) might support the pivotal role of the aromatase expression in sexual differentiation of gonads in \(H. \text{retardatus}\). Considering the phenomenon of temperature sensitivity of gonadal differentiation in \(H. \text{retardatus}\), 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 \(P450\text{arom}\) 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 \(H. \text{retardatus}\) 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 \(H. \text{retardatus}\) is different from those in the newts \(Pleurodeles \text{waltl}\) that has ZZ male-ZW female sex determination mechanism and \(Triturus \text{crsitatus}\) 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 \(H. \text{retardatus}\) is similar to that in \(P. \text{poireti}\) 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 \(Rana \text{rugosa}\), 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 \(R. \text{rugosa}\). 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 determinent 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 \(H. \text{retardatus}\) 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 (Iwasa and Wakahara, 1999). They were stored at 4°C 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 28°C, 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 (Kanaki 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 χ² 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 20°C (a balanced sex ratio-inducing temperature) and 28°C (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 20°C (as a control), 2) from 0 to 15 days at 28°C and then shifted down at 20°C from 15 to 60 days, 3) from 0 to 15 days at 20°C, then shifted up at 28°C from 15 to 30 days and then shifted down at 20°C again until 60 days, 4) from 0 to 30 days at 20°C and then shifted up to 28°C from 30 to 45 days and again shifted down to 20°C until 60 days, 5) from 0 to 30 days at 28°C and then shifted down at 20°C from 30 to 60 days, 6) from 0 to 45 days at 28°C and then shifted down at 20°C from 45 to 60 days and 7) from 0 to 60 days at 28°C 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 (F1-R3) and degenerate forward-specific reverse (F1-R3) sets. By referring to cDNA sequences of *H. retardatus* mRNA in *H. retardatus*. Sequences of the first primers set and RT-PCR condition were: forward (F3); 5'-TAY AGN TAY CAR CCN GTI GTN GA-3'; reverse (R1), 5'-ATN GGC ANG GTN ATG ATG AA-3' [Y(C or T); R (A or T); H (not G)]; l (inosine); N (any), 30 seconds at 94°C for melting, 30 seconds at 45°C for annealing, 30 seconds at 72°C 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 GTT G3-3’ 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 GTG CTT G-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 TTT CGG TGG ACA ATG G-3’ and (R) 5’-GAC ATC AGG AGG ACC TGT ATG CC-3’ (Kanaki 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 (28°C), 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 -80°C individually as well and used for the quantitative RT-PCR. Thus, a sexual dimorphism in the *P450arom* 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 GTG CTT G-3’) and reverse; 5’-350R (GAC TTC GTT GCT TTC CAG TT)+R3 (TTC CAG CCA TTT GTT G-3’). This set of primers was expected to produce a fragment of 254 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 -80°C 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 (decimilal dilution from 10^-6 to 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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References


