Short communication

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Up-regulation of *P450arom* and down-regulation of *Dmrt-1* genes in the temperature-dependent sex reversal from genetic males to phenotypic females in a salamander

(12 pages of text, 2pages of tables, 1 page of figure legend and 2 pages of figures)

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Abstract When larvae of the salamander *Hynobius retardatus* were reared at a high temperature (28°C) during their thermosensitive period (TSP: from 15 to 30 days after hatching), all larvae developed to phenotypic females irrespective of their genetic sexes. *Hynobius* P450 aromatase (*P450arom*) and *Dmrt-1* cDNAs were isolated and their expression pattern was analyzed by competitive and conventional RT-PCR. While *P450arom* gene was expressed predominantly in the ovary, *Dmrt-1* was expressed exclusively in the testis. When larvae were reared at the female-producing temperature (28°C) during the TSP, strong expression of the *P450arom* and complete suppression of the *Dmrt-1* genes were induced in all experimental larvae. Up-regulation of the *P450arom* and down-regulation of *Dmrt-1* genes even in genetic males constitute a part of the molecular biological cascade for the temperature-dependent sex reversal from genetic males to phenotypic females in this salamander.

Keywords *Dmrt-1*, *P450arom*, salamander, sex differentiation, sex reversal,

Introduction

Basically, amphibian sex determination is controlled genetically. The genetic sex determination (GSD) in amphibians, however, can be overridden by environmental cues, such as temperature and exogeneously applied sex steroids (Dournon et al. 1990; Wallace et al. 1999). Recently we found that a salamander *Hynobius retardatus* showed temperature-sensitive sex reversal (genetic male to phenotypic female) when the larvae were reared at 28C°C (Sakata et al., 2005). The pattern of the sexual differentiation 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 urodeles are neither correlated with the phylogenetic relation nor with the combination of sex chromosomes, either male-heterogamy (XX/XY) or female-heterogamy (ZZ/ZW). It seems thus important to examine the expression pattern of a lot of genes involved in the sex determination and/or sexual differentiation, such as *P450arom*, *Dmrt-1*, *Sox-9*, *SF-1*, *Dax-1*, *Wt-1* etc. (see Morrish and Sinclair 2002) in each species, to understand the temperature sensitive sex reversal and sexual differentiation in urodeles.

P450 aromatase (*P450arom*) is a key enzyme to convert androgen to estrogen in many vertebrates. It was reported to be involved in not only normal development of ovary, but also temperature-dependent sex reversal (Chardard et al. 1995; Chardard and Dournon, 1999: Kuntz et al. 2003, 2004) in urodeles, TSD in many reptiles (Desvages et al. 1993; Murdock and Wibbels 2003a), and natural sex changes (Liu et al. 2004) and temperature-sensitive sex reversal (Kitano et al. 1999) in fishes. We have shown in *H. retardatus* that sexual dimorphism in the *P450arom* expression was observed as early as 15 days after hatching when the morphological differentiation of gonads had not been occurred (Sakata et al. 2005). These results showing a very early sexual dimorphism in *P450arom* even in undifferentiating gonads might support the pivotal role of the aromatase expression in sexual differentiation of gonads in *H. retardatus.*

Contrary to that, *Dmrt1*, a gene that contains the DM-domain (*Doublesex/Mab-3* DNA-binding motif), is considered to be one of the essential genes involved in the testicular differentiation cascade in mammals, birds, reptiles (Morrish and Sinclair 2002), amphibians

(Shibata et al. 2002) and fish (Marchand et al. 2000). Up-regulation of *Dmrt1* was observed in sex-reversed XX-neomales in a frog (Shibata et al. 2002) and in developing male gonad of a turtle incubated at the male-producing temperature (Murdock and Wibbels, 2003b).

In this study, the expression patterns of *H. retardatus P450arom* and *Dmrt1* were analyzed simultaneously using RNA samples extracted from individual larvae by competitive RT-PCR (*P450arom*) and conventional RT-PCR (D*mrt1*). *P450arom* mRNA was extensively expressed in female gonads, but not in the male ones. *Dmrt1* was expressed predominantly in male gonads, but not in the female ones. When the larvae were reared at the female-producing temperature during the TSP, *P450arom* was up-regulated but the *Dmrt1* was completely down-regulated in all larvae.

Materials and Methods

Animals used the experiments

Experiment 1:

One hundred and fifty, newly hatched larvae of *Hynobius retardatus* were divided into 5 groups of 30 larvae each. They were allotted to 5 different temperature regimens, 20, 22, 24, 26 and 28°C, during the TSP: from 15 to 30 days, Sakata et al., 2005) respectively. Sixty days later, they were fixed with Bouin's fluid, and their gonads were processed for histological examination for determination of morphological gonadal sexes. 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 $p<0.001$.

Experiment 2:

Fifteen larvae each of 25, 30 and 35 days after hatching were used for examination of *P450arom* and *Dmrt-1* expression in normally developing larvae during the sexual differentiation. Because

the genetic sex of larvae used in the experiment of gene expression analysis was not known in advance in *H. retardatus*, their histological gonadal sexes were determined individually. The gonad-mesonephros-complex (GMC) was excised from individual larva, and the left half of the GMC was fixed in Bouin's fluid and then examined histologically. The corresponding right half was processed for RNA extraction and used for the conventional and competitive RT-PCR (Sakata et al. 2005). Thus it was possible to know the sex-specific and developmental stage-specific expression pattern of *P450arom* and *Dmrt-1* genes, respectively, in individual larvae.

Experiment 3:

Ten larvae each of 30, 35, and 40 days after hatching which had been reared at 28°C during the TSP were used to examine possible, thermosensitive changes in *P450arom* and *Dmrt-1* expression. The GMCs were excised from individual larvae, and then processed similarly to those described above.

cDNA cloning

cDNA for *Hynobius P450arom* was previously cloned (GenBank accession No. AB204518, Sakata et al. 2005). To obtain *Hynobius Dmrt1* cDNA, total RNAs were collected from adult testes 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). Sequences for the first degenerate primers set of dDMRT1 was (F) 5'-GCAGCGGGTGATGGCNGCNCAGGT-3' and (R) 5'-TCRCCRCCCAGCAGTCAAGATTCTGGC-3' [R (A or T); N (any)]. PCR condition was 30 seconds at 94°C for melting, 30 seconds at 45°C for annealing, 30 seconds at 72°C for extension. cDNA fragment of expectable length was amplified after 30 cycles of reaction. The

product was electrophoresed on 2% agarose gel. 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 specific primers set was designed.

RT-PCR

Single-stranded cDNA was generated from the total RNAs extracted from the GMCs of normally developing larvae and larvae reared at 28 during the TSP, with a SuperScript first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instruction. Competitive RT-PCR for *P450arom* was performed according to the procedure described previously (Sakata et al. 2005). Conventional RT-PCR was done for *Dmrt1*, using the specific primers set, (F)5'-TGCACTACCAAGGCAGCAG-3' and (R) 5'-CAGCTCTCAATAGAGGATGCACTCA-3'. cDNA fragment of *β*-actin was amplified as a control using a specific primers set of (F) 5'-AGAAGCTTGCGGTGGACAATGG-3' and (R) 5'-GACATCAGGAGGACCTGTATGCC-3'. The PCR products were separated on 2% agarose gels containing ethidium bromide.

Results and discussion

Experiment 1:

Gonads of all 146 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: 26 females out of 28 larvae examined (93%). 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 20, 22, 24 and 26°C at all (data not shown). The difference of only 2°C causes the sex reversal from genetic males to phenotypic females in this species. Unfortunately, however, larvae reared at higher temperatures than 28C could not survive in this species, and thus it was impossible to determine the effect of the higher temperatures.

Experiment 2:

A partial sequence for *Hynobius Dmrt-1* cDNA was amplified with RT-PCR, yielding 684-bp fragment. Predicted amino acid sequence deduced from the fragment is shown in Fig. 1. It contained a well conserved DM domain, male-specific motif and P/S rich region. Similarity of the partially cloned *Hynobius Dmrt-1* to those of other vertebrates at amino acid level was relatively low, 60, 61, 64 and 57% to human, chick, turtle, and frog, respectively.

In order to know the expression pattern of *P450arom* and *Dmrt1* during gonadal differentiation, competitive RT-PCR (for *P450arom*) and conventional RT-PCR (for *Dmrt-1*) were done using developing GMCs of 45 larvae reared at the 20°C (normal controls). Normally developing larvae showed a balanced sex ratio (1:1). Fourteen (25 and 30 days after hatching) and 13 (35 days) larvae out of 15 larvae each were analyzed for gene expression. Table 1 shows sex-specific expression pattern of *P450arom* and *Dmrt-1* genes, respectively: *P450arom* was expressed extensively in female GMCs during the sexual differentiation period. The expression levels increased from $3.3x10^4$ to $20x10^4$ copies of *P450arom* mRNA/500 ng total RNAs in average during 25 to 35 days after hatching. Every female GMC expressed a considerable level of *P450arom* mRNA. Contrary to these, no mRNA of the *P450arom* was detectable in male GMC at all. These suggest that *P450arom* was not expressed in males during the gonadal differentiation period in this species.

The expression pattern of *Dmrt-1* was completely opposite to that of *P450arom*: almost all

males expressed *Dmrt-1* mRNA during the sexual differentiation period (16 of 20 larvae examined)(Table 1). In females, however, no signals were detected at all throughout the sexual differentiation of gonads (none of 21 larvae examined). Although the amount of the *Dmrt-1* mRNA could not be determined by the conventional RT-PCR, the sexual dimorphism in the *Dmrt-1* expression, specific expression in male but not in female GMCs in this species were confirmed. The sex-specific expressions of the *P450arom* (female) and *Dmrt-1* (male) are also shown in Fig. 2.

Experiment 3:

All gonads out of 30 larvae reared at the female-producing temperature developed to ovaries (100%), showing that sex reversal from males to phenotypic females occurred in all potential or genetic males (Table 2). All larvae reared at the female-producing temperature expressed *P450arom* mRNA at the high level comparable to that of the genetic females, $5.3x10^4$ ~ $1.4.7x10^4$ copies/500 ng total RNAs in average (Table 2). Because all GMCs of the experimental larvae expressed the *P450arom* (see range of the expression in Table 2), it was concluded that the *P450arom* was up-regulated even in genetic males when larvae were reared at the female-producing temperature. Contrary to this, *Dmrt1* was completely suppressed in almost all larvae (except one of 29 larvae examined) that had been reared at 28°C, suggesting that the *Dmrt1* was down-regulated by rearing larvae at the female-producing temperature, irrespective of genetic sexes.

A positive correlation between the up-regulation of *P450arom* but down-regulation of *Dmrt1* and morphological ovarian differentiation in *H. retardatus* was clearly demonstrated (Table 2). Thus, the temperature-dependent sex reversal or deviation to female phenotype in this species is explainable in terms of the *P450arom* and *Dmrt1* regulation. 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. 2003). 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 (28°C). Furthermore, the *Hynobius Dmrt1* was completely down-regulated in larvae reared at the female-producing temperature (Table 2). It is thus assumed that genetic males are sex-reversed to phenotypic females at least by two factors such as 1) an acceleration of ovarian differentiation under the influence of estrogens, and 2) a failure in testicular differentiation caused by down-regulation of *Dmrt1*.

Considering the phenomenon of temperature sensitivity of gonadal differentiation in *H. retardatus*, the main problem to be solved is to identify the thermosensitive factor(s) which occur in the regulatory cascade leading to activation or repression of transcription of the *P450arom* and *Dmrt1* genes. In this respect, Kuntz et al. (2004) recently hypothesized that aromatase could be a target of a temperature-sensitive sex reversing effect in the gonads but not in the brain in *Preurodeles waltl*. 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). Sexual dimorphism in the SF-1 protein, high level in female but low in male gonads was reported in an anura *Rana catesbeiana* (Mayer et al. 2002). Thus, it is absolutely necessary to examine the

possible involvement of the transcriptional factors, such as *SF-1*, *Sox-9* and *Dax-1* to the differentiation in *H. retardatus* gonads, to clarify the molecular mechanism of the temperature-sensitive sex reversal in this species.

Acknowledgements We thank Dr. Asato Kuroiwa, Center for Advanced Science and Technology, Hokkaido University, for a kind gift of *Dmrt1*-degenerate primers set.

References

Chardard D, Desvages G, Pieau C, Dournon C. (1995) Aromatase activity in larval gonads of *Pleurodeles waltl* (Urodele, Amphibia) during normal sex differentiation and during sex reversal by thermal-treatment effect. Gen Comp Endocrinol 99: 100-107

Chardard D, Dournon C (1999) Sex reversal by aromatase inhibitor treatment in the newt *Pleurodeles waltl*. J Exp Zool 283: 43-50

Desvages G, Girondot, M, Pieau C (1993) Sensitive stages for the effects of temperature on gonadal aromatase activity in embryos of the marine turtle *Dermochelys coriacea*. Gen Comp Endocrinol 92: 54-61

Dournon C, Guillet F, Boucher D, Lacroix JC (1984) Cytogenetic and genetic evidence of male sexual inversion by heat treatment in the newt *Preurodeles poireti.* Chromosoma 90: 261-264

Dournon C, Houillon CH, Pieau C (1990) Temperature sex-reversal in amphibians and reptiles. Int .J Dev Biol 34: 81-92

Kawano K, Furusawa S, Matsuda H, Takase M, Nakamura M (2001) Expression of steroidogenic factor-1 in frog embryo and developing gonad. Gen Comp Endocrinol 123: 12-22

Kitano T, Takamune K, Kobayashi T, Nagahama Y, Abe S-I (1999) Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (*Paralichthys olivaceus*). J Mol Endocrinol 23: 167-176

Kuntz S, Chesnel A, Duterque-Coquillaud M, Grillier-Vuissoz I, Callier M, Dournon C, Flament S, Chardard D (2003) Differential expression of P450 aromatase during gonadal sex differentiation and sex reversal of the newt *Pleurodeles waltl*. J Steroid Biochem Mol Biol 84: 89-100

Kuntz S, Chesnel A, Flament S, Chardard D (2004) Cerebral and gonadal aromatase expressions are differently affected during sex differentiation of *Pleurodeles waltl*. J Mol Endocrinol 33: 717-727

Liu X, Liang B, Zhng M (2004) Sequence and expression of cytochrome P450 aromatase and FTZ-F1 genes in the protandrous black porgy (*Acanthopagrus schlegeli*). Gen Comp Endocrinol 138: 247-254

Marchand O, Govoroun M, D'Cotta H, McMeel O, Lareyre J, Bernot A, Laudet V, Guiguen Y (2000) *DMRT1* expression during gonadal differentiation and spermatogenesis in the rainbow trout, *Oncorhynchus mykiss*. Biochim Biophys Acta 1493: 180-187

Mayer LP, Overstreet SL, Dyer CA, Propper CR (2002) Sexually dimorphic expression of steroidogenic factor 1 (SF-1) in developing gondas of the American bullfrog, *Rana catesbeiana*. Gen Comp Endocrinol 127: 40-47

Miyata S, Kubo T (2000) *In vitro* effects of estradiol and aromatase inhibitor treatment on sex differentiation in *Xenopus laevis* gonads. Gen Comp Endocrinol 119: 105-110

Morrish BC, Sinclair AH (2002) Vertebrate sex determination: many means to an end. Reproduction 124: 447-457

Murdock C, Wibbels T. (2003a) Cloning and expression of aromatase in a turtle with temperature-dependent sex determination. Gen Comp Endocrinol 130: 109-119

Murdock C, Wibbels T (2003b) Expression of *Dmrt1* in a turtle with temperature-dependent sex determination. Cytogenet Genome Res 101: 302-308

Sakata N, Tamori Y, Wakahara M (2005) P450 aromatase expression in the temperature-sensitive sexual differentiation of salamander (*Hynobius retardatus*) gonads. Int J Dev Biol 49: 417-425

Shibata K, Takase M, Nakamura M (2002) The *Dmrt1* expression in sex-reversed gonads of amphibians. Gen Comp Endocrinol 127: 232-241

Wallace H, Badawy GMI, Wallace BMN (1999) Amphibian sex determination and sex reversal. Cell Mol Life Sci 55: 901-909

Wallace H, Wallace BMN (2000) Sex reversal of the newt *Triturus cristatus* reared at extreme temperatures. Int J Dev Biol 44: 807-810

Figure legends

Fig.1. Predicted amino acid sequence deduced from the partially cloned cDNA fragment of *Hynobius Dmrt1*. Proportional comparison to the alignment of other vertebrates *Dmrt1* is shown. Partial sequences of *Rana rugosa* (Genbank accession No. 2821303A), human (AL136365-1), chick (AF211349-1), turtle (AY316537-1) are aligned from top to bottom. Identical residues are enclosed by square boxes. DM domain, male-specific motif and P/S-rich region are underlined.

Fig. 2. Expression patterns of *P450arom* and *Dmrt-1* in larval *Hynobius retardatus*. GMCs of larvae 35 days after hatching were divided into left and right halves. Left halves were examined histologically for determination of their gonadal sexes, and the corresponding right halves were analyzed for gene expression. RNAs extracted from the GMCs were amplified with competitive (for *P450arom*) and conventional (for *Dmrt-1*) RT-PCR. *P450arom* was expressed predominantly in female GMCs, but not in male ones. Contrary to these, *Dmrt-1* was exclusively expressed in male GMCs, but not in female ones.

Figure 1.

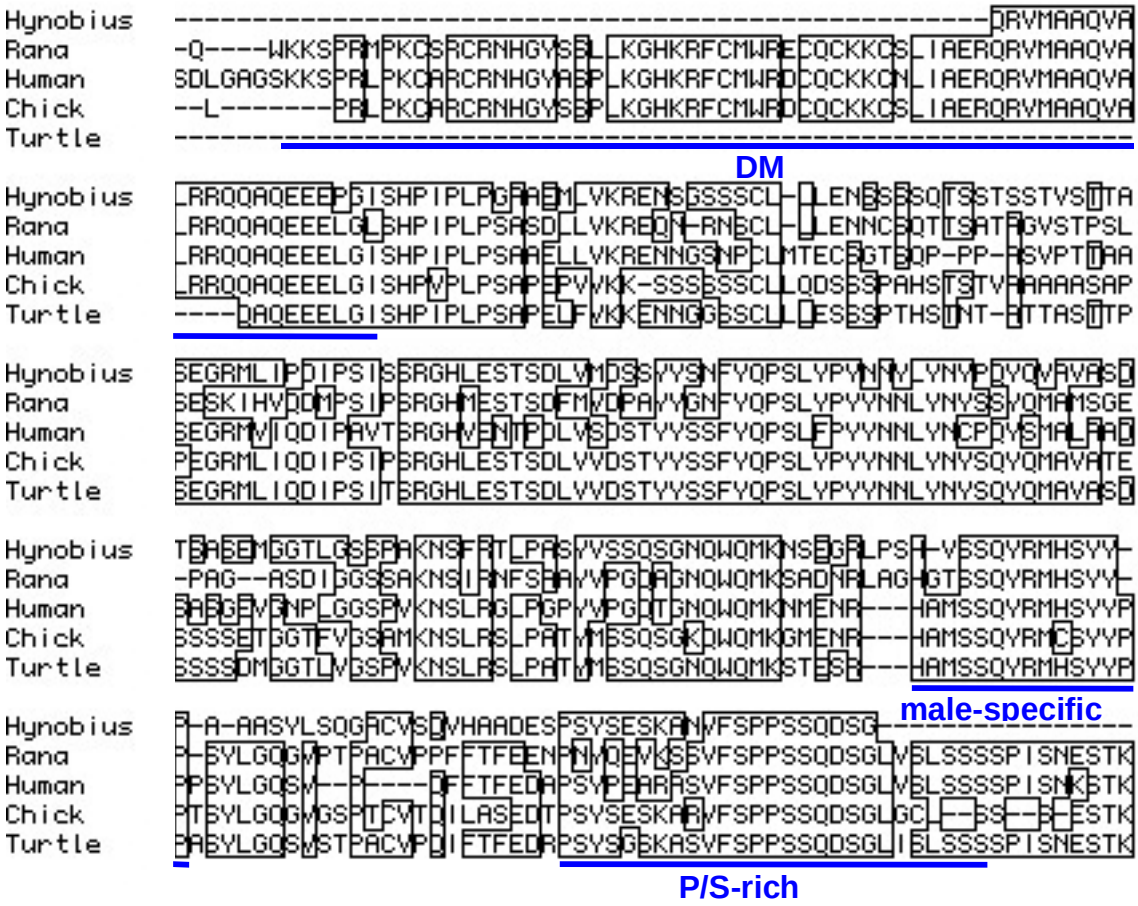

Figure 2.

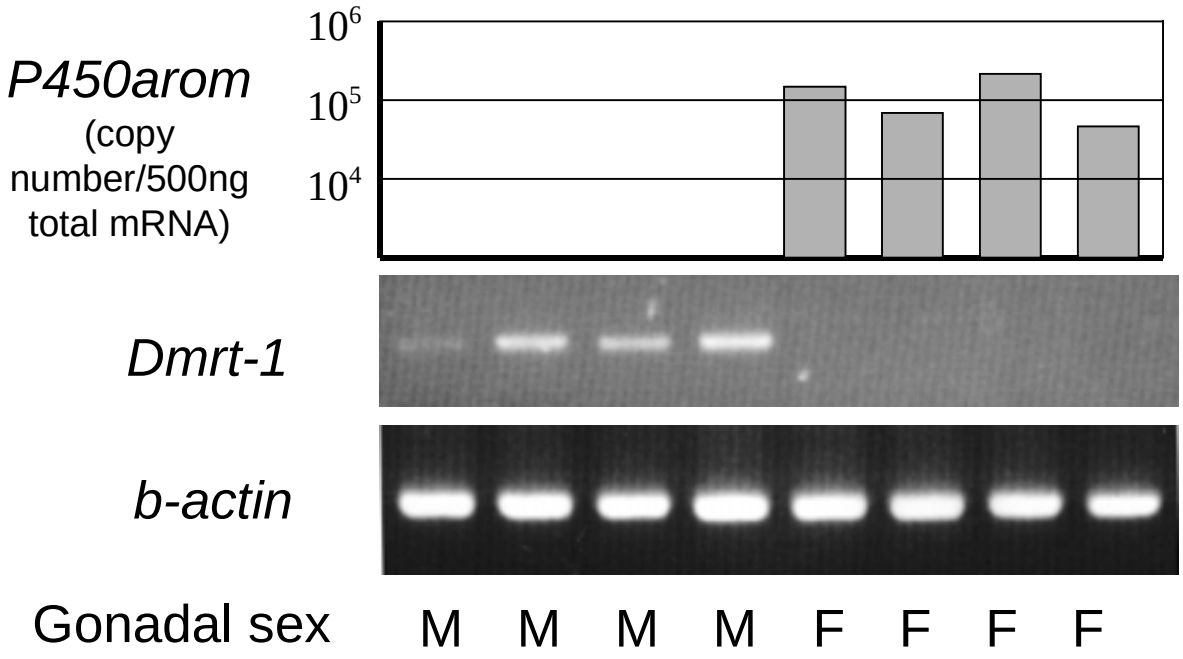
P450arom
(copy number/500ng total mRNA)
10^6
10^5
10^4
Dmrt-1
b-actin
Gonadal sex
M M M M F F F F

Table 1. Expression of *P450arom* and *Dmrt-1* during the sexual differentiation in normally developing *Hynobius* larvae which were reared at 20C

Days after hatching	No. of larvae used	Gonadal sex*	Expression of *P450arom*** (copies x 10^4) average	range	Expression of *Dmrt-1**** (no. of larvae)
25	15	ovary (7)	3.3	2.3~5.9	0
		testis (7)		ND	7
30	15	ovary (7)	6.9	2.5~12.1	0
		testis (7)		ND	4
35	15	ovary (7)	20.6	5.5~44.3	0
		testis (6)		ND	5

* gonadal sexes were determined by histological examination of the left halves of the GMCs from each larva. Sex ratio was nearly 1:1 in this experiment. Numerals in parenthesis showed no. of larvae examined in gene expression.

** expression level of *P450arom* was estimated by competitive RT-PCR. Average copy numbers of mRNA/500 ng total RNAs and their range, of larvae examined. ND, not detected.

***number of larvae expressing the *Dmrt-1* detected by RT-PCR, of larvae examined.

Table 2. Expression of *P450arom* and *Dmrt-1* in *Hynobius* larvae which were reared at the female-producing temperature (28) during the TSP (from 15 to 30days after hatching)

Days after hatching	No. of larvae used	Gonadal sex*	Expression of *P450arom*** (copies x 10^4) average	range	Expression of *Dmrt-1**** (no. of larvae)
30	10	ovary (10)	5.3	2.3~13.1	1
35	10	ovary (10)	14.7	7.3~28.6	0
40	10	ovary (10)	8.0	4.3~15.5	0

* gonadal sexes were determined by histological examination of the left halves of the GMCs from each larva. All larvae developed to female in this experiment. Numerals in parenthesis showed no. of larvae examined in gene expresion.

** expression level of *P450arom* was estimated by competitive RT-PCR. Average copy numbers of mRNA/500 ng total RNA and their range, of larvae examined.

***number of larvae expressing the *Dmrt-1* detected by conventional RT-PCR, of larvae examined.