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Nonylphenol modulates expression of androgen receptor and estrogen receptor genes differently in gender types of the hermaphroditic fish *Rivulus marmoratus*

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Abstract

To uncover the effect of estrogenic chemicals [4-nonylphenol (NP) and bisphenol A (BisA)] on the expression of androgen receptor (AR) and estrogen receptors (ERα and ERβ) in the hermaphroditic fish *Rivulus marmoratus*, we cloned the full length of the cDNAs encoding AR, ERα, and ERβ from gonadal tissue of *R. marmoratus* and analyzed the modulation of expression of these genes following exposure to estrogenic chemicals using real-time RT-PCR. *R. marmoratus* AR, ERα, and ERβ genes showed a high similarity to the relevant fish species on amino acid residues, respectively. Rm-ERα and Rm-ERβ cDNAs included a serine-rich region when compared to other teleost fish ER genes. Tissue-specific expression of Rm-AR and Rm-ERβ mRNAs in adult hermaphrodite *R. marmoratus* was high in the gonad, while Rm-ERα mRNA was high in the liver based on real-time RT-PCR. In addition, Rm-AR and Rm-ERα mRNAs increased along with developmental stage from stage 3 (5 dpf) to hatching, while Rm-ERβ mRNA increased from stage 2 (2 dpf). To uncover the effect of estrogenic chemicals on *R. marmoratus*, we exposed the fish to NP (300 μg/l) and BisA (600 μg/l) for 96 h. Significant down-regulation of Rm-AR, Rm-ERα, and Rm-ERβ mRNA was observed in gonadal tissue after exposure to NP but not BisA. In the liver, there were gender differences in gene expression after EDC exposure. These results demonstrate that expression patterns of the Rm-AR, Rm-ERα, and Rm-ERβ genes in the hermaphroditic fish, *R. marmoratus*, vary according to tissue and developmental stage as well as the specificity of environmental estrogenic chemicals. These genes can be useful as molecular biomarkers in assessing the potential impact of estrogenic compounds using this species as a model system.

Keywords: *Rivulus marmoratus*; Androgen receptor; Estrogen receptor α; Estrogen receptor β; Nonylphenol; Bisphenol A; Modulation; Expression

The molecular mechanism behind sex determination and differentiation in fish has yet to be well defined. Two research groups have independently isolated a sex-determination gene (the DMY gene) from Japanese medaka (*Oryzias latipes*) [1,2], but determinants may vary according to fish species. Unlike mammals, fish may convert their gender naturally or in response to chemical exposure (e.g., steroid hormones). Steroid hormones derived from the gonads play crucial roles in sexual differentiation, maturation, and behavior in vertebrates [3].

In fact, many gonochoristic species can undergo gonad sex inversion in either direction following sex steroid treatment early in development. Although the actual mechanisms involved are still unclear, steroid hormones have been implicated in natural sex change in fish. In addition, steroid hormone receptors are activated by steroid ligands,

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resulting in regulation of genes involved in reproduction and sexual differentiation. Estrogen receptor (ER) and androgen receptor (AR) genes are actively involved in this mechanism. Steroid hormone receptors may also be activated by steroid-mimicking endocrine-disrupting chemicals (EDCs) in the environment, altering sex differentiation [4]. However, little information is available on the effect of EDCs on expression of AR and ER genes in fish. Meucci and Arukwe [5] have suggested that ER and aromatase genes would be sensitive biomarkers for xenoestrogen effects, based on their research exposing Atlantic salmon to nonylphenol. Generally, in teleost fishes, AR plays a fundamental role at the time of sex differentiation, based on expression profiles between males and females. ERs influence early embryonic development and gonadal differentiation, suggesting a distinguished role for androgen and estrogens in sexual differentiation in fish [6]. Despite their importance, AR and ER genes have been cloned and examined in relatively few teleost fishes, including Japanese medaka (*O. latipes*) [7], fathead minnow (*Pimephales promelas*) [8], zebrafish (*Danio rerio*) [9], sea bass [6,10,11], and a few other species [12–18]. As a result, we still lack information on the structure of AR and ER genes, particularly from sexually diverse species.

The hermaphroditic fish *Rivulus marmoratus* has attractive features for genetic and environmental toxicology studies [19–22], including a relatively small body size (3–5 cm), short generation time (3–4 months), superior resistance to extreme environmental conditions (e.g., euryhaline, wide pH range, and wide temperature range), year-round spawning under controlled conditions, and genetic homogeneity [19,20]. With these advantages, *R. marmoratus* has been considered as a model bioassay organism for studying toxicogenomics [19–23]. In addition, several biomarker genes (e.g., vitellogenin, *CYP1A*, AhR, GSTα, c-*fos*, *ras*, choriogenin, aromatase, and others) have been cloned for *in vitro* study as well as *in vivo* testing at the molecular level to further develop *R. marmoratus* as an *in vitro* model test organism [19–22].

In this report, we examined the structure of AR, ERα, and ERβ cDNAs from *R. marmoratus* and demonstrated their expression patterns in different tissues, developmental stages, and/or sexual stages (i.e. hermaphrodite and secondary male) after exposure to EDCs using real-time reverse-transcriptase polymerase chain reaction (real-time RT-PCR). We discuss the role of these genes after exposure to NP and BisA in different genders of this fish species to better understand the modulation of steroid hormone genes and sex-related steroidogenic enzymes following EDC exposure.

Materials and methods

Chemicals. All chemicals used in this study were of molecular biology grade and were purchased from Sigma Chemicals, Inc. (St. Louis, MO), Qiagen (Valencia, CA), or Invitrogen, Inc (Carlsbad, CA) unless otherwise described. All oligonucleotide synthesis and DNA nucleotide sequencing were performed at Bionex Co. (Seoul, South Korea).

Fish rearing conditions and total RNA extraction. *Rivulus marmoratus* were reared and maintained at the aquarium of the Department of Molecular and Environmental Bioscience, Graduate School, Hanyang University, Seoul in Korea. The fish were maintained at 25 °C with 12 h/12 h light/darkness cycles and 10‰ salinity. Experimental fishes were anaesthetized on ice and sacrificed by decapitation. Tissues used for the study were quickly removed under sterile conditions and then homogenized in three volumes of TRIZOL® (Invitrogen, Paisley, Scotland, UK) with a tissue grinder. Total RNA from *R. marmoratus* was extracted according to manufacturer's instructions.

Cloning of R. marmoratus AR, ERα, and ERβ cDNA. To partially clone *R. marmoratus* AR, ERα, and ERβ fragments, we designed degenerative oligonucleotides from highly conserved regions after Clustal W analysis of multiple alignments including full-length AR, ERα, or ERβ cDNAs cloned to date, and used those primers to amplify the corresponding cDNAs from gonad cDNA. RT-PCR was carried out using 2 μM of each primer (degenerative primer sets for AR1/AR2, ERα1/ERα2, and ERβ1/ERβ2 are shown in Table 1) with the following conditions: 94 °C/4 min; 45 cycles of 98 °C/25 s, 55 °C/40 s, 72 °C/90 s; and 72 °C/10 min. The amplified PCR products were isolated from 1% agarose gels, cloned into pCR2.1 TA vectors, and sequenced with an ABI PRISM 3700 DNA analyzer.

Rapid amplification of 5′-end and 3′-end of R. marmoratus AR, ERα, and ERβ cDNA. To get full-length *R. marmoratus* AR, ERα, and ERβ cDNA, we used the GeneRacer kit (Invitrogen) according to the manufacturer's instructions. Briefly, to verify a 5′-unknown sequence, three primers (5′-GSP1, 5′-GSP2, and 5′-GSP3; Table 1) were designed. The first round of PCR amplification was carried out using specific primer 5′-GSP1 and the SNAP-purified product was sequentially re-amplified using the nested specific primer 5′-GSP2 together with 5′-RACE adaptor primer (AAP). The subsequent nested amplification was conducted using specific primers 5′-GSP3 primer together with 5′-RACE Adaptor primer (AUAP) in the following 50-μl reaction: 0.2 μg cDNA template (*R. marmoratus* gonad), 10 μM 5′-GSP1 primer and 5′-RACE Adaptor primer (AUAP), 10 μM of each dNTP, and LA *Taq* DNA polymerase (5 U/μl, TaKaRa).

To get 3′-unkown sequences of *R. marmoratus* AR, ERα, and ERβ cDNA, first-strand cDNA was synthesized using poly(A) mRNA with oligo(dT) anchor primer (Invitrogen) and *R. marmoratus* gonad mRNA. The 3′-RACE products of *R. marmoratus* AR, ERα, and ERβ cDNA were amplified by PCR using 3′-GSP1 primer and 3′-RACE Adaptor primer (AUAP) in the following 50-μl reaction: 0.2 μg cDNA template (*R. marmoratus* gonad), 10 μM 3′-GSP1 primer and 3′-RACE Adaptor primer (AUAP), 10 μM of each dNTP, and LA *Taq* DNA polymerase (5 U/μl). A second nested amplification was conducted using 3′-GSP2 and 3′-RACE Adaptor primer (Table 1). All PCR amplification conditions were the same as for the initial PCR (earlier section) except that annealing temperatures were 58 °C, 60 °C, and 62 °C for AR, ERα, and ERβ, respectively. The final PCR products were isolated from 1% agarose/TBE gels, cloned into pCR2.1 TA vectors and sequenced.

Phylogenetic analysis of R. marmoratus AR, ERα, and ERβ genes. Phylogenetic analysis was conducted using Bayesian analysis with Mr. Bayesian's program (Ver. 3.1.1) on the amino acid sequences of *R. marmoratus* AR, ERα, and ERβ gene products. All sequences from fishes except *R. marmoratus* AR, ERα, and ERβ genes were retrieved from GenBank. The retrieved sequences were aligned with Clustal X by the multiple alignment method [24]. Gaps and missing data were completely excluded from the data analysis. The generated data matrix was converted to nexus format and analyzed with Mr. Bayesian's program using the GTR model. The Markov chain Monte Carlo process was set to four chains and 1,000,000 generations were conducted. The sampling frequency was set to every 100 generations. After analysis, the first 1000 generations were deleted as burn-in processes and the consensus tree constructed. Phylogenetic trees were visualized with TreeView of PHYLIP [25].

Tissue-specific expression of R. marmoratus AR, ERα, and ERβ. To analyze the tissue expression of *R. marmoratus* AR, ERα, and ERβ mRNA, semi-quantitative RT-PCR was carried out using brain, eye, gonad, intestine, liver, muscle, and skin of adult *R. marmoratus*. Two micrograms of total RNA from the above tissues of *R. marmoratus* was

Table 1
Primers used in RT-PCR and RACE-PCR to clone cDNAs in *Rivulus marmoratus* androgen receptor (AR), estrogen receptor α (ERα), and estrogen receptor β (ERβ) genes

Gene target	Oligo	Primer sequence	Nucleotide position
AR	AF	5′-TYTCYGCSTGCGCCACAATCTCAGA-3′	374–398
	AR	5′-TCAAAGTACTTCTGACTYTTCARRCCCTC-3′	2029–2057
AR 3′-end	A3GSP1	5′-GCATCTTTCCCAGGAGTTTGTGCTG-3′	1938–1962
	A3GSP2	5′-GGAGTTCCTCTGCATGAAGGCCCTG-3′	1980–2004
AR 5′-end	A5GSP1	5′-AGTCTCCGAACTTTTGAA-3′	658–675
	A5GSP2	5′-TCCACAGGTTGCTTGTCTCGCTCG-3′	630–653
	A5GSP3	5′-ACAGCTCTGGCTGGCAGACGCGGG-3′	565–588
ERα	EF	5′-AGGGTCACAATGACTAYATGTGCCC-3′	723–747
	ER	5′-GCTCCATKCCTTTGTTGCTCATG-3′	1645–1667
ERα 3′-end	E3GSP1	5′-CGAAGTCACCATGATGACCCTG-3′	1111–1132
	E3GSP2	5′-GGCCACCGCCTCTCGCTTCCGC-3′	1384–1405
ERα 5′-end	E5GSP1	5′-GTTTGAGTTTGAGCAT-3′	1406–1421
	E5GSP2	5′-GCGGTGGCCAGCAGCATGTCG-3′	1372–1392
	E5GSP3	5′-GGAACACAGGATCGGTGGCTCGG-3′	1062–1084
ERβ	BF	5′-TTCAARAGGAGYATCCAARGRCACA-3′	855–878
	BR	5′-AGRCAGACGTACTCCTCCCKCTG-3′	1554–1576
ERβ 3′-end	B3GSP1	5′-GCAGGGAGGAGGGACAGTGTGTGGAGGGCA-3′	1465–1494
	B3GSP2	5′-CGACATGCTGCTGGCAGCCAC-3′	1508–1528
ERβ 5′-end	B5GSP1	5′-AGACTCAACTCTACAAAA-3′	1331–1348
	B5GSP2	5′-CATCATCATGCTGGCCTCTGTG-3′	1244–1265
	B5GSP3	5′-GCTGCAGCGTTCACGCCTCACAC-3′	991–1013

reverse transcribed with an oligo(dT) primer and superscript™ III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. PCR amplification of *R. marmoratus* AR, ERα, and ERβ cDNA was carried out with the following conditions: 94 °C/4 min; 30 cycles of 94 °C/30 s, 55 °C/30 s, 72 °C/30 s; and 72 °C/7 min. Relative gene expression was calculated using *R. marmoratus* β-actin as an internal control following the procedures described in Lee et al. [19,20]. The resulting PCR products were separated on 1% agarose/TBE gels containing ethidium bromide and visualized on a Fluor-STM Multimager system (Bio-Rad), and the densitometric signals were measured using Bio1D® image analysis system software (Bio-Rad).

Developmental stage-specific expression of R. marmoratus AR, ERα, and ERβ mRNA. To check the level of the expression of *R. marmoratus* AR, ERα, and ERβ mRNA during developmental stages, semi-quantitative RT-PCR was carried out. Briefly, tissue samples were collected at 2, 4, 9, and 12 days after fertilization (Stages 1–4) as well as 5 h after hatching (Stage 5). At each sampling time, the eggs or hatched fish (10 individuals) were collected and homogenized in three volumes of TRIZOL® reagent (Invitrogen) with a tissue grinder. Total RNA was extracted following the manufacturer's instructions, and the isolated mRNA was stored at −80 °C until use. A semi-quantitative RT-PCR was carried out with conventional method as described in Lee et al. [19].

Modulation of expression of R. marmoratus AR, ERα, and ERβ mRNA after exposure to EDCs. To examine the effect of EDC exposure on *R. marmoratus* AR, ERα, and ERβ gene expression, we exposed fish to 300 μg/L NP and 600 μg/L BisA for 96 h in their water tank. NP and BisA were first dissolved in 100% dimethyl sulfoxide (DMSO; Sigma) and then diluted with ultrapure distilled water. The exposure concentrations of NP and BisA were chosen based on the results of previous studies [21,26]. The treatment water was replaced with freshly prepared treatment water 24 h after exposure. The exposed fish were maintained under a 12-h light/12-h dark photoperiod at 25 °C and were not fed during the exposure periods. At the end of the 96 h exposure, each tissue (brain, eye, gonad, intestine, liver, muscle, and skin) was sampled, rapidly homogenized in three volumes of TRIZOL® with a tissue grinder, and stored at −80 °C until use.

To establish EDC-screening methods using molecular biomarkers of *R. marmoratus*, expression levels of AR, ERα, and ERβ mRNA were measured after exposure to NP and BisA using real-time RT-PCR. For real-time RT-PCR, 2 μg of each RNA sample was reverse transcribed to cDNA using SuperScript™ III reverse transcriptase (Invitrogen) in a reaction volume of 20 μl. For real-time PCR amplification, each reaction included 1 μl of cDNA and 0.2 μM primers (real time RT-F/R and β-actin RT-F/R; Table 2). Reaction conditions were 94 °C/4 min; 35 cycles of 94 °C/30 s, 55 °C/30 s, 72 °C/30 s; and 72 °C/10 min. To confirm the amplification of specific product, cycles were continued to check the melting curve under the following conditions: 95 °C/1 min, 55 °C/1 min, and 80 cycles of 55 °C/ 10 s with 0.5 °C increase per cycle. SYBR Green from Molecular probe Inc. (Invitrogen) was used to detect specific PCR products. Amplification and detection of SYBR Green were performed with a MyiQ cycler (Bio-Rad). The Rm β-actin gene was used as a housekeeping reference to normalize expression levels between samples [19–21]. All data were expressed relative to β-actin to normalize for any difference in reverse transcriptase efficiency. All experiments were analyzed in triplicate. Threshold cycle (C_t) value (the PCR cycle number at which fluorescence was detected above threshold and decreased linearly with increasing input target quantity) was obtained from MyiQ cycler optical system software version 1.0 (Bio-Rad) and used to calculate ΔC_T values ($\Delta C_T = C_t$ of the housekeeping gene $- C_t$ of the target gene) of each sample. Fold change for the relative gene expression to DMSO control was determined by the $2^{-\Delta\Delta C_t}$ method [27].

To check the level of the gene expression of *R. marmoratus* AR, ERα, and ERβ mRNA in secondary males with degenerated ovaries, real-time RT-PCR was carried out as described above. Briefly, tissue samples were collected from secondary males. At each sampling time, gonads from two individuals were collected and homogenized in 3 volumes of TRIZOL® reagent (Invitrogen) with a tissue grinder. Total RNA was extracted following manufacturer's instructions, and the isolated mRNA was stored at −80 °C until use. Real-time RT-PCR was carried out with conventional method as described above.

Statistical analysis. The levels of the *R. marmoratus* AR, ERα, and ERβ mRNAs were recorded relative to β-actin gene expression as shown in Lee et al. [21]. All data were expressed as means ± SD and were examined by unpaired Student's *t*-test after normalization. Differences were considered significant at $P < 0.05$.

Table 2
Sequence of primer pairs used in semi-quantitative RT-PCR and real-time RT-PCR

Gene name (accession number)	RT-PCR primer (upper) Real-time PCR primer (lower) (upper, sense primer; lower, antisense primer)	Product size (bp)
Androgen receptor (DQ339105)	5′-GCTGCAGAAGGAAAACAGAAAT-3′ 5′-GGTGTTCGTTAAAGACGAGGTC-3′	556
	5′-GGCTGGACGTGGTCTACAACGACTC-3′ 5′-CACACGTGAGCGCACCGTAATG-3′	144
Estrogen receptor α (DQ339108)	5′-GGAAGAGCTGCCAGGCTTGTCGTCT-3′ 5′-TATGAGGTCCTGTGCAAAAATG-3′	542
	5′-TTGACAGGAATCGGAGGAAGAG-3′ 5′-CCTTGTCTCTGTCTCCAATCCC-3′	147
Estrogen receptor β (DQ339109)	5′-AAATCAATGCACAATCGACAAG-3′ 5′-GTTGAGGAGTATCATGGCCTTC-3′	697
	5′-GCAGCCACTTCTCGCTTTCG-3′ 5′-GCTCTCCAGCTCCTCTGTGGTTTG-3′	126
β-Actin (AF168615)	5′-CAAGGCCGGATTTGCTGGAG-3′ 5′-TCACCGGAGTCCATGACGAT-3′	420
	5′-CTTGCGGAATCCACGAGACC-3′ 5′-CCAGGGCTGTGATCTCCTTCTG-3′	149

Results and discussion

Cloning and sequence analysis of R. marmoratus AR cDNA

The partial fragment of *R. marmoratus* AR cDNA amplified by RT-PCR was approximately 1683 bp long. The 5′- and 3′-ends of *R. marmoratus* AR obtained by 5′- and 3′-RACE were products of 588 and 494 bp, respectively. The resulting full-length *R. marmoratus* cDNA was 2473 bp cDNA containing 2229 bp of open reading frame (ORF) that encoded 742 aa. *R. marmoratus* AR contained a poly(A) tail, but no clear polyadenylation signal (AATAAA) was found in the 3′-UTR (GenBank DQ339105). The deduced amino acid sequence of *R. marmoratus* AR was aligned with AR sequences of other fish using Clustal W, and a BLAST search (www.ncbi.nlm.nih.gov) revealed the highest homology with cyprindontiformes and belloniformes species such as the killifish *Gambusia zaffinis* (70% identity) and Japanese medaka *O. latipes* (69% identity), while goldfish *Carassius auratus* (46% identity) showed the lowest similarity. The deduced amino acid sequence of *R. marmoratus* AR had a theoretical p*I* of 6.83 and a calculated molecular weight of 81.7 kDa, and also contained several functional motifs such as DNA-binding regions, a c4 zinc finger region, and a hormone ligand-binding domain, typical of all nuclear hormone receptors (Fig. 1). Like sea bass AR, *R. marmoratus* AR included two zinc finger motifs: the proximal box (GSCKV) for specifically binding the response element and the distal box (ASKND) for recognition of the spacing between half-sites of the response element and dimerization [6]. The DNA binding region and hormone ligand-binding domain of *R. marmoratus* AR also shared the highest amino acid identities (48.7%) as compared to AR in fish species (Fig. 1).

Cloning and sequence analysis of R. marmoratus ERα and ERβ cDNA

To identify partial fragments of *R. marmoratus* ERα cDNA, we amplified a product of approximately 944 bp using RT-PCR with degenerate primers (EF/ER) from *R. marmoratus* gonad, and cloned the amplimer to pCR2.1 vector for sequence analysis. The 5′- and 3′-ends of *R. marmoratus* ERα were obtained by 5′- and 3′-RACE with *R. marmoratus* ERα-specific primers (E3GSP1-E5GSP3; Table 2) resulting in products of 1150 and 828 bp, respectively. The full-length of *R. marmoratus* ERα cDNA was 2212 bp with a 1875 bp ORF, encoding 624 aa. This *R. marmoratus* ERα cDNA encoded polyadenylation signals (AATAAA) with a poly(A)$^{+}$ tail in the 3′-UTR (GenBank DQ339108), and had biochemical characteristics of a theoretical p*I* of 8.34 and a calculated molecular weight of 67.8 kDa. Also, we obtained the alternative splicing form of *R. marmoratus* ERα by using 3′-RACE RT-PCR with *R. marmoratus* ERα specific primers (E3GSP2) with product of 556 bp (data not shown). This amino acid sequence was shorter (549 aa) than the original full-length (624 aa) *R. marmoratus* ERα sequence. This shorter sequence was registered with GenBank (DQ361028) as an alternative splicing type of *R. marmoratus* ERα. Interestingly, we obtained the *R. marmoratus* ERα subtype by using 3′-RACE RT-PCR in this study, but did not examine whether the *R. marmoratus* ERα subtype belonged to the ERα family as reported in teleost

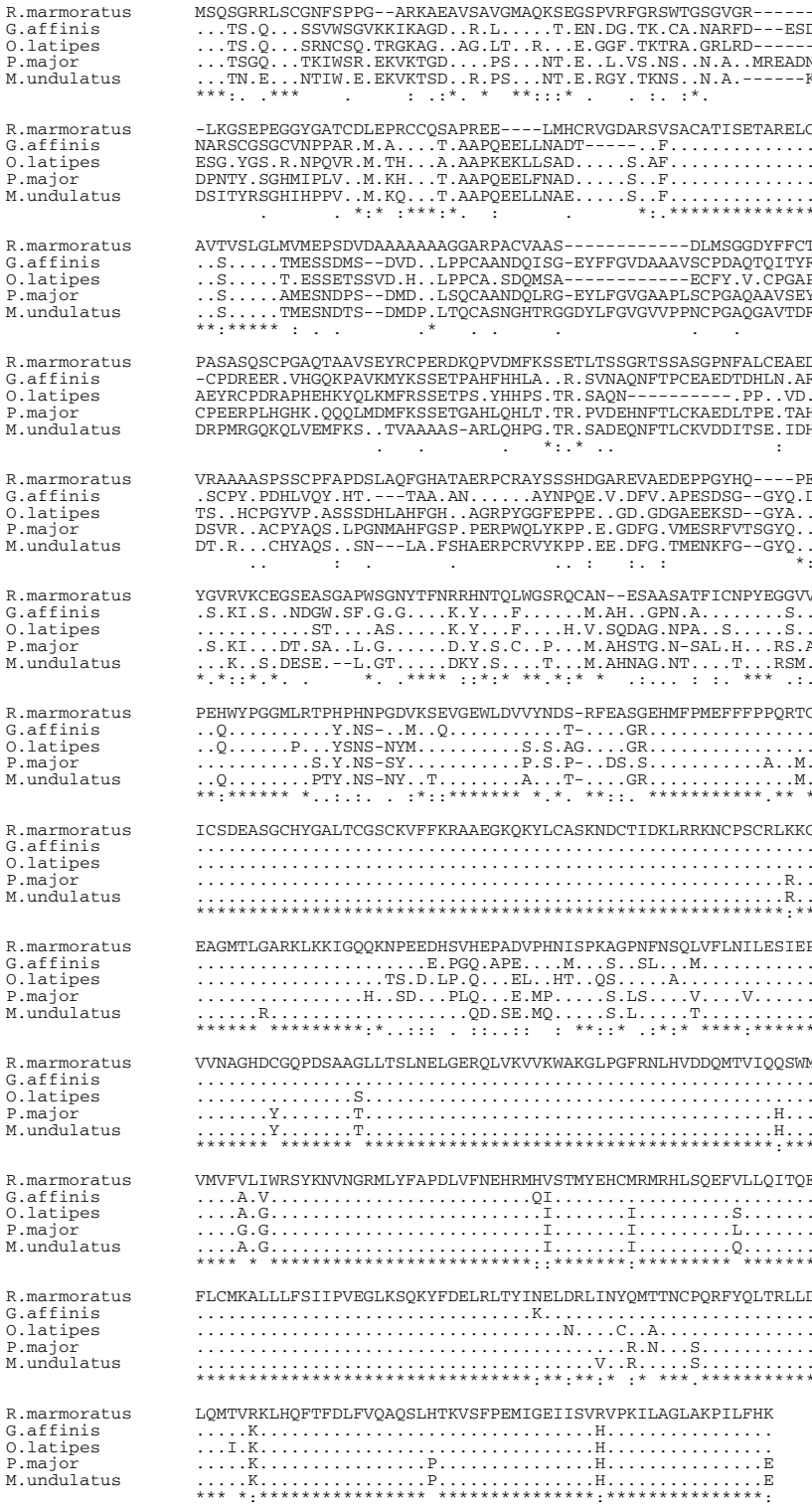

Fig. 1. Multiple alignment of *Rivulus marmoratus* AR with several teleost species using Clustal W. The AR sequences used for alignment were *Rivulus* AR (DQ339105), *G. affinis* (AB099303.1), *Oryzias latipes* (AB076399.1), *P. major* (AB017158.1), and *Micropogonias undulatus* (AY701761.1). Identical amino acid residues between species are indicated by asterisks, while conservative substitutions are indicated by dot-aligned sequences.

species. However, previous studies have reported the presence of long and short ERα transcripts at the 5′-end in rainbow trout and channel catfish [14,15]. Based upon a BLAST search using the deduced amino acid sequence of *R. marmoratus* ERα subtypes (two forms), it was not possible to classify ERα subtypes of fish species at the present time. This result suggests the possibility that *R. marmoratus* ERα splice variants exist. *R. marmoratus* ERα showed

81% (*Fundulus heteroclitus* ERα) to 82% identity (*O. latipes* ERα) with other fishes, and only 53% identity with the fathead minnow *P. promelas* ERα using Clustal W (Fig. 2). Sequence analysis demonstrated that *R. marmoratus* ERα consisted of major functional regions, including an estrogen receptor region, DNA-binding regions containing c4 zinc fingers, and a hormone ligand-binding domain typical of all nuclear hormone receptors. In addition, *R. marmoratus* ERα contained a serine-rich region in the C-terminal region. Like sea bass ER, *R. marmoratus* ERα possessed two activation boxes [10]: the P box (EGCKAFF) and the D box (PATNQ), which is a highly conserved zinc-finger motif. In addition, the *R. marmoratus* ERα protein sequence contains an activation function (AF)-2 domain, DLLLEML, indicating transcriptional activation [28].

To isolate partial fragments of *R. marmoratus* ERβ cDNA, we amplified approximately 721 bp by RT-PCR with degenerate primers (BF/BR) with *R. marmoratus* gonad cDNA. Using this sequence information, the 5′- and 3′-ends of *R. marmoratus* ERβ were obtained by RACE with *R. marmoratus* ERβ specific primers (B3GSP1-B5GSP3; Table 2) resulting in products of 1013 and 961 bp, respectively. The full-length *R. marmoratus* ERβ cDNA consisted of 2469 bp, of which the first 242 nucleotides made up the 5′-untranslated region, followed by 1995 bp of ORF for the ERβ protein, and a 232 nucleotide 3′-untranslated region. This cDNA encoded a poly(A)$^{+}$ tail, but no polyadenylation signals were clearly found in the 3′-UTR (GenBank DQ339109). The deduced amino acid sequence of *R. marmoratus*

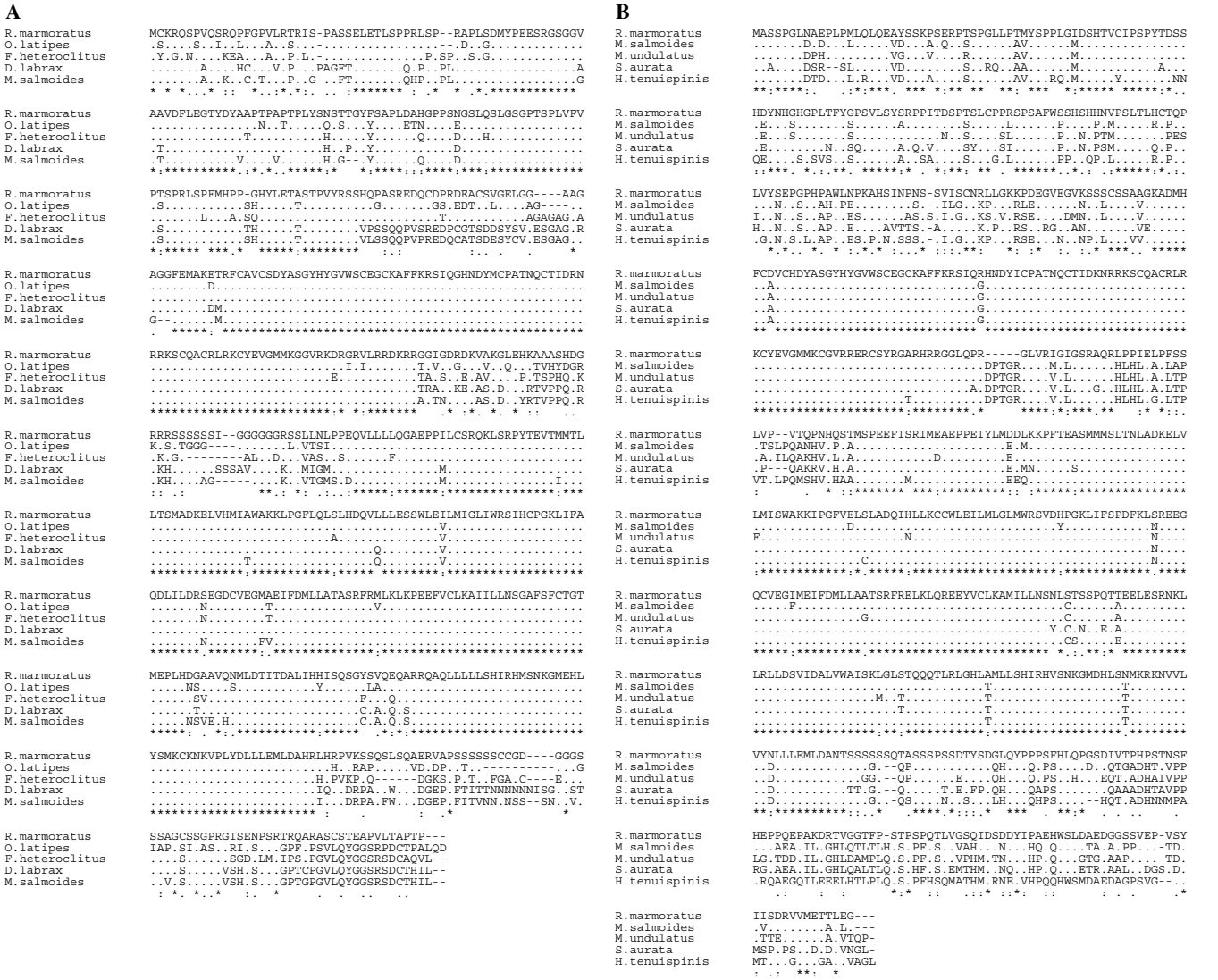

Fig. 2. (A) Multiple alignment of *Rivulus marmoratus* ERα from several teleost species using Clustal W. The ERα sequences used for alignment were *R. marmoratus* ERα (DQ339108), *Oryzias latipes* (AB033491.1), *Fundulus heteroclitus* (AB097197.1), *Dicentrarchus labrax* (AJ505009.1), and *Micropterus salmoides* (AF253062.2). (B) Multiple alignment of *R. marmoratus* ERβ from several teleost species using Clustal W. The ERβ sequences used for alignment were *R. marmoratus* ERβ (DQ339109), *Micropterus salmoides* (AY211021.1), *Micropogonias undulatus* (AF298181.1), *Sparus aurata* (AJ580048.1), and *Halichoeres tenuispinis* (AY305027.1). Identical amino acid residues between species are indicated by asterisks, while conservative substitutions are indicated by dot-aligned sequences.

ERβ encodes 664 amino acid residues with a theoretical p*I* of 6.52 and a calculated molecular weight of 73.6 kDa. We aligned fish ERβ proteins with Clustal W, and *R. marmoratus* ERβ showed the highest identities to large mouth bass *Micropterus salmoides* ERβ (80% identity) and Atlantic croaker *Micropogonias undulatus* ERβ (77% identity), while it showed the lowest similarity to the spiny dogfish *Squalus acanthias* (42% identity) (Fig. 2). *R. marmoratus* ERβ also had major functional regions in the ORF such as DNA-binding regions for c4 zinc fingers, a hormone ligand-binding domain, and a serine-rich region in the C-terminus. *R. marmoratus* ERβ included the same zinc-finger D and P-box sequence elements as ERα.

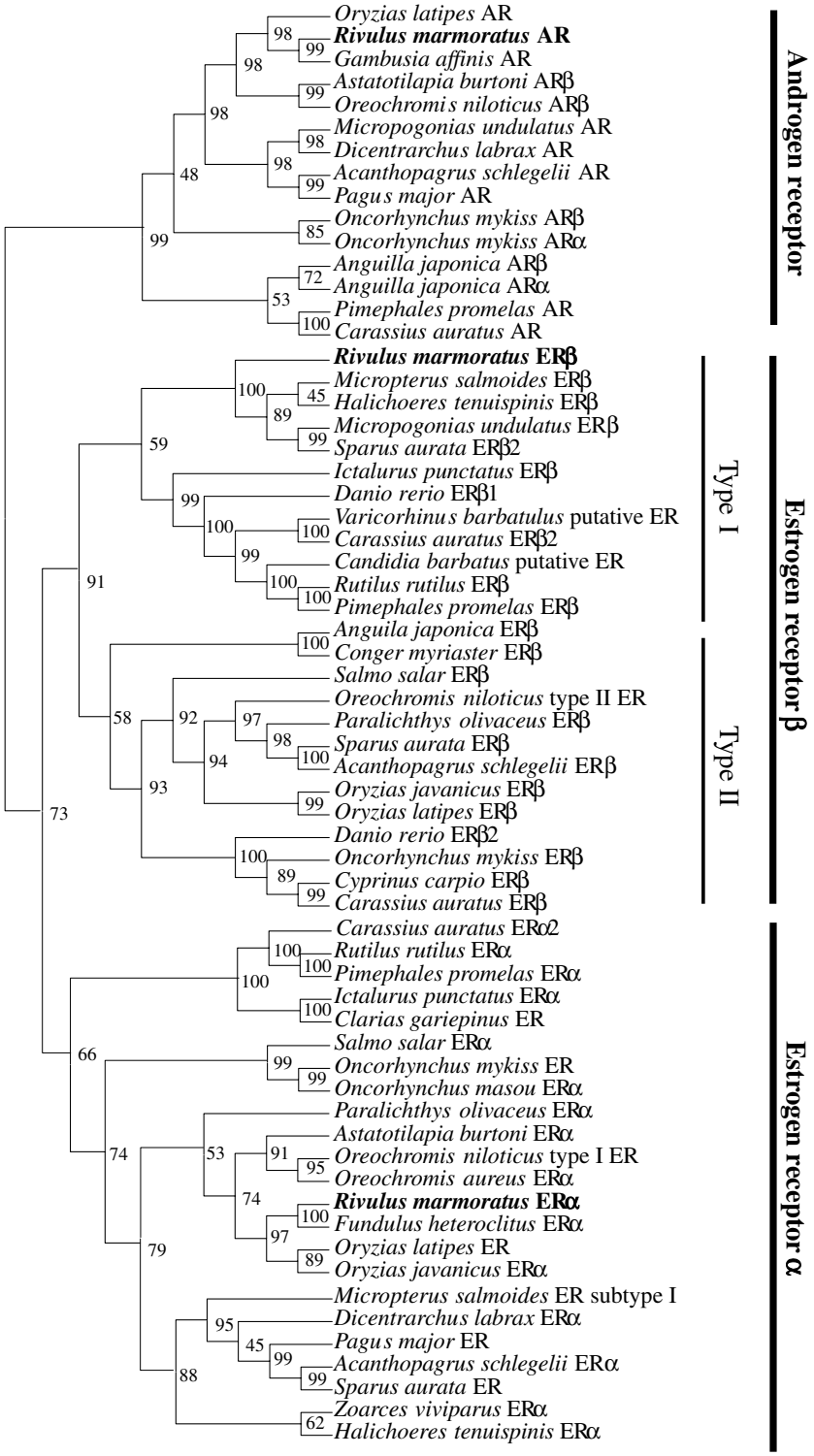

Fig. 3. Phylogenetic tree for *Rivulus marmoratus* AR, ERα, and ERβ genes. The tree was constructed by Bayesian method after Clustal X alignment.

Phylogenetic analysis of R. marmoratus AR, ERα, and ERβ

To uncover the relationship of *R. marmoratus* AR, ERα, and ERβ genes with homologues from other fishes, we conducted a phylogenetic analysis. As shown in Fig. 3, the resulting phylogenetic tree contained a clade for each of the AR, ERα, and ERβ gene families. The AR gene family showed a closer relationship to ERβ, indicating that ERβ may be derived from AR proteins by a gene duplication event. Regarding the AR gene, Sperry and Thomas [13] suggested that Atlantic croaker (*M. undulatus*) AR was divided into AR1 and AR2 subtypes, each with different biochemical properties and steroid binding specificities in brain and ovary. In addition, the presence of two AR subtypes has been further supported in rainbow trout [11] and Japanese eel [12]. However, only one AR gene subtype has been successfully cloned so far in *R. marmoratus*.

Tissue-specific expression of R. marmoratus AR, ERα, and ERβ

To check tissue-specific expression of AR, ERα, and ERβ in adult hermaphrodite *R. marmoratus*, we carried out semi-quantitative RT-PCR. Expression of AR and ERβ mRNA was high in gonadal tissue, while ERα mRNA was high in the liver, gonad, and brain, in order (Fig. 4). Most tissues showed very low expression of AR mRNA, except for the gonad, and there was no significant expression in the eye or intestine.

Socorro et al. [16] reported that sea bream ERα was the dominant estrogen receptor in sea bream liver, testis, and heart, while the ERβ gene was expressed in all tissues analyzed except gills. RT-PCR examination of tissue-specific expression of AR in adult sea bass males and females revealed that AR mRNA was expressed at high levels in the testis of males but at low levels in the female brain, ovary, and liver [6]. In the case of hermaphrodite fish species such as the protogynous wrasse, *Halichoeres trimaculatus*, AR and ER were expressed in relatively high levels in the gonad and brain of each sex type, and were also expressed in varying amounts in gill, liver, muscle, and brain [17].

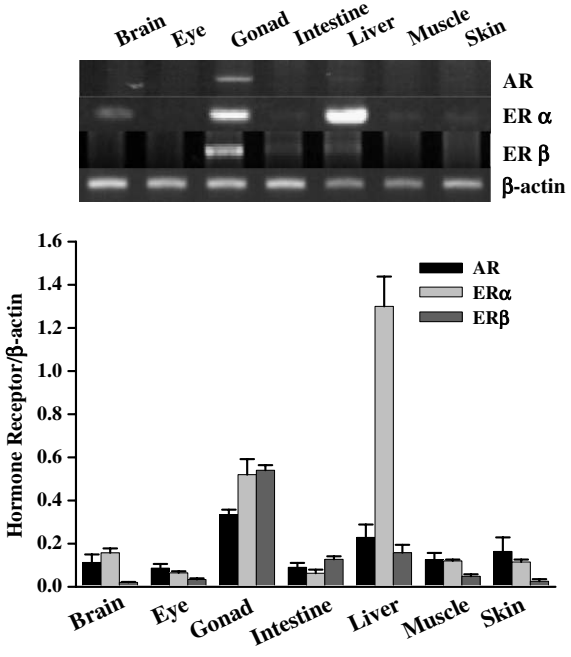

Fig. 4. Tissue-specific expression of *Rivulus marmoratus* AR, ERα, and ERβ genes.

Developmental stage-specific expression of R. marmoratus AR, ERα, and ERβ genes

To examine expression pattern of *R. marmoratus* AR, ERα, and ERβ mRNA during early embryogenesis, we carried out semi-quantitative RT-PCR. AR and ERα mRNA increased along with developmental stage from stage 3 (9 days after fertilization) to hatching, but ERβ mRNA increased at stage 2 (4 days after fertilization) (Fig. 5). Expression level of AR mRNA has been studied in the *H. trimaculatus* at different developmental stages [17] and in the protandrous black porgy, *Acanthopagus schlegeli* [18]. Changes in AR expression played an important role in controlling the sex differentiation process between males and females in both fishes.

Expression pattern of R. marmoratus AR, ERα, and ERβ genes after EDC exposure

To examine gene expression of *R. marmoratus* AR, ERα, and ERβ genes after exposure to EDCs such as NP and BisA, we carried out real-time RT-PCR using brain, gonad, and liver tissues after checking gene expression patterns of other tissues (e.g., eye, intestine, muscle, and skin) (Fig. 6).

After exposure to BisA (600 μg/l), ERα mRNA was significantly up-regulated in the brain, gonad, and liver, while AR was down-regulated in the liver (Fig. 6A). Androgen binds with the AR to regulate expression of an array of target genes that are important in male development and fertility [29]. Thus, BisA may interfere with the binding of androgen in the liver.

Gene expression of AR, ERα, and ERβ in the liver was different between hermaphrodites and secondary males following NP exposure (Fig. 6B and C), indicating that the role of NP varies according to gender type. Tanaka and Grizzle [26] showed that NP inhibited oogenesis and influenced gonadal differentiation and development in *R. marmoratus*. Although NP primarily acts by binding to estrogen receptors, it may have other mechanisms of action. Recently, NP has been shown to both enhance pregnane X receptor-mediated transcription in COS-7 cells [30] and to weakly bind androgen [31] and progesterone [32] receptors in mammalian *in vitro* assays. Other reports describe significant elevation of ER transcriptional activity in BisA and NP-exposed fish [33,34].

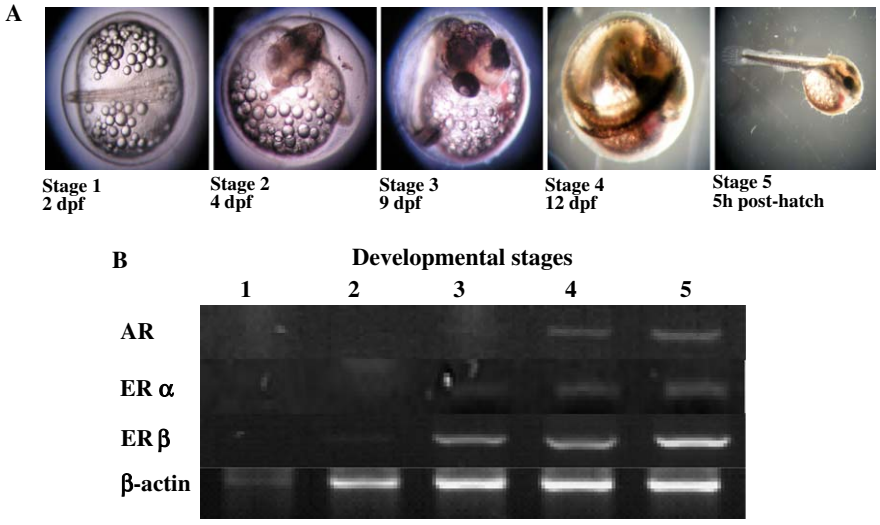

Fig. 5. Developmental stage-specific expression of *Rivulus marmoratus* AR, ERα, and ERβ genes. (A) Developmental stage of *Rivulus marmoratus* used in this study. Each stage was observed under stereomicroscope (40×) after fixation with glycerol. (B) Developmental stage-dependent expression of AR, ERα, and ERβ genes using semi-quantitative RT-PCR.

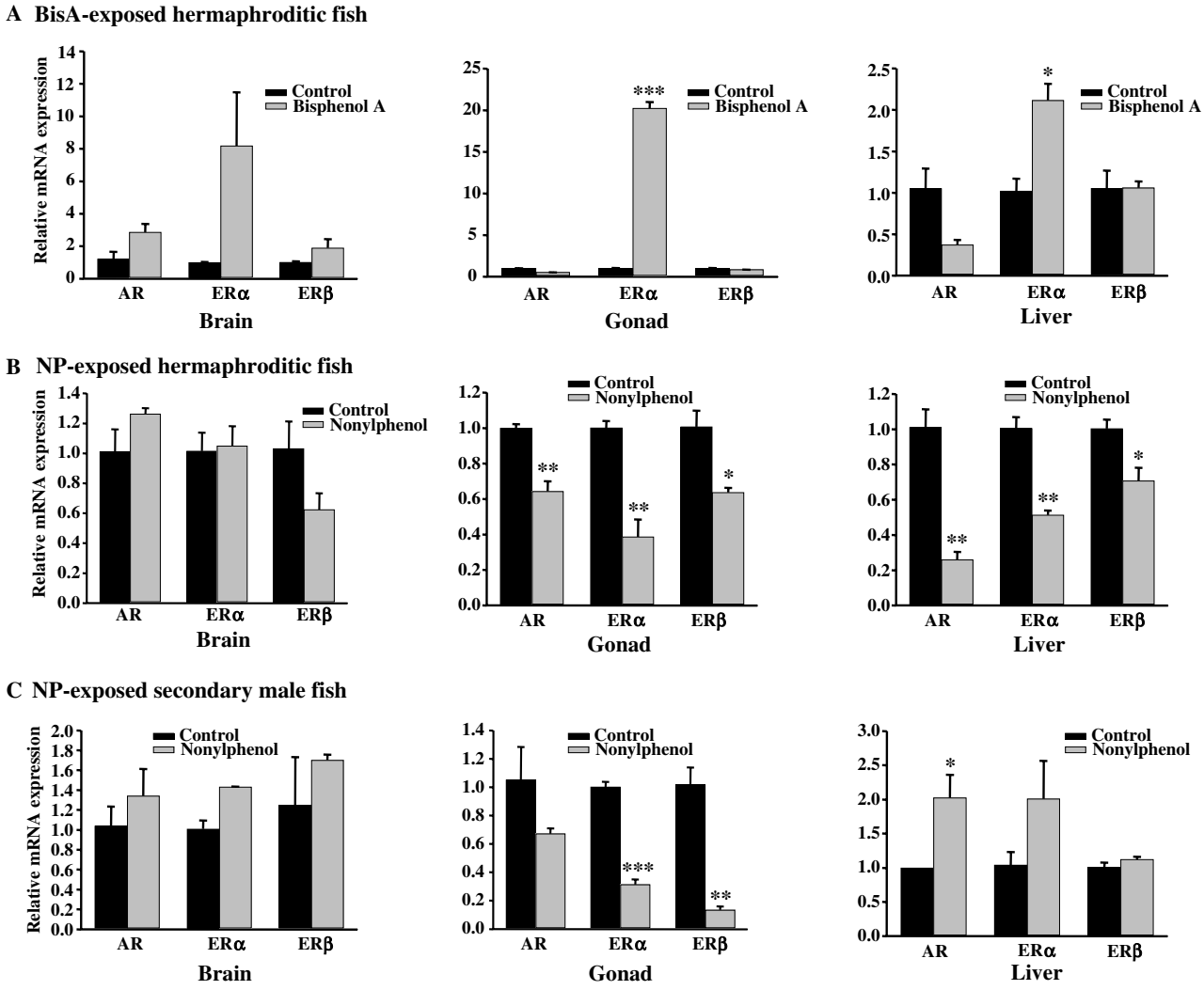

Fig. 6. Gender-specific expression of *Rivulus marmoratus* AR,ERα, and ERβ genes in the brain, gonad, and liver after exposure to bisphenol A and 4-nonylphenol. (A) BisA-exposed hermaphroditic fish, (B) NP-exposed hermaphroditic fish and (C) NP-exposed secondary male fish. The experiment was analysed in triplicate, and all data was shown as mean ± S.D. The symbols (*, **, and ***) indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Taken together, the hermaphroditic fish *R. marmoratus* may have unique system of AR, ERα, and ERβ gene expression following EDC exposure according to gender type if the estrogen and/or androgen may affect the molecular endocrinology system in fish model.

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