Cloning and differential expression of estrogen receptor and aromatase genes in the self-fertilizing hermaphrodite and male mangrove rivulus, *Kryptolebias marmoratus*

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Abstract

The mechanisms underlying sex determination and differentiation in fishes are labile in response to environmental parameters. Sex-specific phenotypes are largely regulated by sex steroids, and the inhibition or the stimulation of aromatase can reverse sex as well as alter secondary sexual characteristics in fishes. Among vertebrates, the mangrove rivulus is the only known self-fertilizing hermaphrodite. Throughout most of its range, rivulus appear to exist as clonally reproducing hermaphrodites. However, outcrossing has been documented in Belize, where up to 25% of rivulus collected are males. The direct development of (primary) males occurs when embryos are incubated at 18 °C and hermaphrodites develop into secondary males when held at 28 °C. Given the importance of sex steroids, their receptors, and aromatase in sex determination and differentiation of fishes, we cloned, sequenced, and quantified the expression of estrogen receptors (*ER α* , *ER β*) and ovarian (*AroA*) and brain (*AroB*) aromatase genes. Hermaphrodites had increased *ER α* , *ER β* , *AroA*, and *AroB* gene expression in the liver, gonad, gonad, and brain respectively, compared to males. These data are consistent with the gene expression data reported for other species and are reflective of the presence of ovarian tissue in the hermaphrodites. Interestingly, we show the elevated expression of brain aromatase in the hermaphrodite brain. The role of the dimorphic expression of brain aromatase in the regulation of sex-specific characteristics is intriguing and requires further research. Because of the uniqueness of its reproductive biology, rivulus is an excellent model for elucidating the mechanisms regulating vertebrate sex determination and sexual differentiation.

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Introduction

Sex determination and sexual differentiation

Determination of female or male phenotype in mammals is under strict genetic control, which directs the development of ovaries or testes (Graves 1994). Differentiation of sexually dimorphic characteristics occurs mostly under the control of the sex steroid hormones, androgens and estrogens, which are synthesized primarily in the gonad (Wilson 1994).

Like mammals, most fish species have separate sexes (called gonochorism) and believed to have genetic sex determination. In contrast to mammals, the mechanisms underlying sex determination and differentiation in fishes vary widely and are labile in response to environmental parameters, including temperature and demographic structure of the local population (Devlin & Nagahama 2002). Furthermore, sex can be purposely manipulated via treatment with synthetic chemicals, a common practice in aquaculture (Donaldson 1996). Both sex determination and sexual differentiation are

altered in fishes exposed to endocrine disrupting chemicals (Damstra *et al.* 2002, Nagahama *et al.* 2004). A naturally occurring example of lability in sex determination and sexual differentiation is that of hermaphroditic fishes. Hermaphrodites either contain both ovarian and testicular tissues (called simultaneous hermaphroditism) or develop first as one sex and then change to the other (called sequential hermaphroditism) (Shapiro 1994).

Compared to sexual differentiation, little is known about the genetic and the molecular mechanisms that control sex determination in fishes and why genetic sex determination is relatively easier to override by environmental factors compared with mammals. Arguably, the best studied model of sex determination in fishes is the medaka, *Oryzias latipes*, in which the first sex determining gene, DNA motif on the Y chromosome (*DMY*), in a non-mammalian vertebrate has been identified (Matsuda *et al.* 2002). Analogous to the *SRY* gene in mammals, *DMY* expression has been documented in the Sertoli cells of the XY gonad during the time of sex determination. Two other genes, *DMRT-1b(Y)*

and the vasa homologs (primary germ cell markers) *vas-s* and *vas*, have also been associated with early events during sex determination of medaka and tilapia (*Oreochromis niloticus*). We know that the expression of *DMRT-1b(Y)* in medaka strictly correlates with the Y chromosome and treatment with exogenous estrogen has no effect on its expression (Scholz *et al.* 2003), and *vas-s* is predominantly expressed in oogenesis and *vas* in spermatogenesis in tilapia (Kobayashi *et al.* 2002), but the functional connection between these three genes remains unclear at this time.

Other researchers have focused on the primary germ cells as directors of sex determination. In gonochoristic species, ovarian tissue in females develops prior to spermatogenic tissue of males. This is hypothesized to be due to the induction of meiosis, resulting in ovarian tissue, or a block to meiosis in the primary germ cells, resulting in testicular tissue (Koubova *et al.* 2006). Injection studies of medaka embryos with *DMY* RNA caused a meiotic block compared to control fish (Kobayashi *et al.* 2004, Nagahama 2006). Recent research suggests a temporal relationship among *DMY*, *Sox9a*, and *DMRT-1b(Y)* (Kobayashi *et al.* 2004, Nakumoto *et al.* 2005, Nagahama 2006). While based on RNA and not on functional proteins, this pattern is strongly suggestive of a rudimentary signaling pathway, with *DMY* blocking meiosis, *Sox9a* regulating testicular tubule development, and *DMRT-1b(Y)* being important to spermatogenesis itself. To date, this information is only available in a single strain of the medaka.

Due to the interest in the aquaculture of ornamental and food fish species, much has been learnt about the differentiation of sex in fishes (Yamamoto 1969, Donaldson 1996, Devlin & Nagahama 2002). Sex-specific phenotypic differences are regulated by androgens, principally testosterone and 11-ketotestosterone, in male and 17 β -estradiol in female gonochorists and relevant, respective sex in sequential hermaphrodites. These sex steroids effect their mode of action through intracellular and membrane-bound receptors. Many studies using a suite of androgens or estrogens (in either aquatic exposures, or directly injected or implanted) have demonstrated the importance of these gonadally synthesized sex steroid hormones in sex determination and sexual differentiation. Fish can be readily sex-reversed by exogenous hormone treatment during phenocritical periods. These periods, where the gonad is bipotential, vary from embryonic to juvenile developmental stages. Adult fish can be masculinized or feminized by hormonal treatment, thus supporting the role of sex steroids in the maintenance of gametogenesis as well as the differentiation of secondary sex characteristics, including morphology, coloration, and behavior (Yamamoto 1969, Donaldson 1996, Devlin & Nagahama 2002). The importance of the relative concentration of these

sex steroids has been documented by the manipulation of aromatase, the enzyme that converts androgens to estrogens. Inhibition or stimulation of aromatase can reverse sex as well as alter secondary sex characteristics.

Reproductive biology of the mangrove rivulus

The mangrove rivulus (*Kryptolebias marmoratus*, synonym *Rivulus marmoratus*, referred in this paper as rivulus) is an excellent organism to study the mechanisms of sex determination and sexual differentiation because of the uniqueness of its reproductive biology. Among vertebrates, it is the only known self-fertilizing simultaneous hermaphrodite (Harrington 1961). For more information about the life history of the rivulus, see the review paper by Taylor (2000). The cytogenetics of rivulus has been studied, and Sola *et al.* concluded that the diploid chromosome number is 46 and the chromosomes are homomorphic (Sola *et al.* 1997). Most rivulus collected from the wild are selfing hermaphrodites, yet in some populations, males are found (only a few males have ever been collected in Florida, yet up to 25% of fish sampled in Carrie Bow Cay, Belize, are males); (Turner *et al.* 1992a). In addition to selfing, there is evidence of outcrossing, and the degree and prevalence in wild population is presently being investigated by others (Turner *et al.*, 1990 #1820, Lin & Dunson 1995 #1967, Murphy & Collier 1996 #2224, Turner, 1992 #2987, and Mackiewicz *et al.* 2006).

As a hermaphroditic species, sex determination is plastic and has a strong environmental influence with temperature being known to affect sex determination (Harrington 1967 #3612, Harrington 1968 #3611). In the laboratory, hermaphrodites develop at normal culture temperatures of 25 °C. Primary males (direct development) can be produced when embryos are incubated at 18 °C, although the response is *not* all or none, i.e. <70% develop as males (Harrington 1967). Some hermaphrodites can be induced to develop into secondary males by holding them at 28 °C (Harrington 1968, 1971), presumably through degeneration of their ovarian tissue, although this transformation has not been studied systematically (Harrington 1975). Since there is evidence of outcrossing in the Belizean population, mating is presumed to occur between hermaphrodites and males (Turner *et al.* 1992a,b). Further, Belizean males are about the size of hermaphrodites, hence these are hypothesized to be secondary males; high water temperatures in the tropics may induce the herm→secondary male transformation. While hermaphrodites are reported to go through a juvenile female stage prior to ontogeny of testicular tissue (Soto *et al.* 1992, Cole & Noakes 1997), no one has ever observed or collected functional, adult females in the field or laboratory until recently. In our lab, 100%

juvenile rivulus ≤ 7 days post-hatching (dph) exposed to ethinyl-estradiol (1 ppb until 28 dph) and raised in untreated water until adulthood (110 dph), developed as females (unpublished data from EFO lab).

Given the importance of sex steroid hormones and their receptors, and the enzyme aromatase in sexual differentiation of fishes, we cloned and sequenced the estrogen receptor ($ER\alpha$ and $ER\beta$), cloned and partially sequenced ovarian and brain aromatase (*AroA* and *AroB* respectively) genes. Next, we compared the expression of *ER\alpha*, *ER\beta*, *AroA*, and *AroB* genes between the adult hermaphrodite and male rivulus. We hypothesized that there would be differential expression of these genes between these reproductive morphs and that those differences would reflect the presence of ovarian tissue in the hermaphrodite.

Materials and methods

Fish

Fourteen adult hermaphrodites and 14 primary males (age 11–12 months) were obtained from a breeding colony of rivulus maintained by EFO. Fish were maintained at $24 \pm 1^\circ\text{C}$, 16 h light:8 h darkness photoperiod, 15 ppt seawater (reverse osmosis water) plus Instant Ocean sea salts (Aquarium Systems, Inc., Menton, OH, USA) and fed brine shrimp (*Artemia salina*) nauplii daily *ad libitum*. Fish were chosen at random, anesthetized with 150 ppm MS-222 buffered with NaH_2CO_3 (Sigma), and euthanized by severing the spinal cord immediately posterior to the skull. The brain (including pituitary) and liver were excised and immediately snap-frozen in liquid nitrogen and stored at -80°C for quantitative PCR (QPCR, see below). After a section of the gonad was removed for histology (see below), the remainder was snap-frozen and stored as explained for the brain and liver.

Histological examination of the gonad

A sample of each gonad was fixed in 10% neutral buffered formaldehyde, dehydrated in an ethanol series, cleared in CitriSolv (Fisher Scientific, Hampton, NH, USA), and embedded in paraffin. The tissues were sectioned ventrally at $5\ \mu\text{m}$ on a rotary microtome and stained with hematoxylin and eosin-Y (Humason 1997). For each fish, three slides were chosen (from approximate third points in the sample) and stained. The gonadal tissue samples were analyzed for the presence of both ovarian and testicular tissues and only for testicular tissue in the hermaphrodites and male fish respectively (Fig. 1). Only reproductively active fish were acceptable for use in this study. Gonadal tissue was acceptable if oocytes, perinuclear follicles, and

Figure 1 Ventral sections of the rivulus hermaphrodite (A) and male (B) ovarian tissue (O), testicular tissue (T), and swim bladder (SB).

vitellogenic follicles were observed in the ovarian and spermatocytes, spermatatids, spermatozoa were observed in the testicular tissue (modified from (Grier 1981, Wallace & Selman 1981)).

Cloning and sequencing of *ER\alpha* and *ER\beta* genes

For the $ER\alpha$ and $ER\beta$, two conserved amino acid regions, YHYGVW and NKGME/DQ of fish $ER\alpha$ and $ER\beta$, were selected and their degenerate oligonucleotides were used as primers for PCR. As a template for PCR, first-strand cDNA was synthesized from $1\ \mu\text{g}$ of poly (A) RNA isolated from the gonad and the brain. After amplification, the amplified DNA fragment was subcloned with TA-cloning plasmid pGEM-T Easy (Promega), sequenced using a BigDye terminator Cycle Sequencing-kit (PE Biosystems, Foster City, CA, USA) with T7 and SP6 primers, and analyzed on the ABI PRISM 377 automatic sequencer (PE Biosystems). The 5'- and 3'-ends of the ER cDNA were amplified by rapid amplification of the cDNA end (RACE) using a

SMART RACE cDNA Amplification kit (BD Biosciences Clontech). For 5'-RACE of ER α , two primers were used: 5'-GAGCAGGAGCAGCACCTGTTTCAGGAGG-3' for the first round PCR and 5'-TGCTGCTGCGTCTCTCCCATCATGAC-3' for the second round PCR. For 3'-RACE of ER α , two primers were used: 5'-GCCACCGCCTCTCGCTTCCGCATGCTC-3' for the first round PCR and 5'-ACGGCGCGGCGGTGCAGAACATGCTGG-3' for the second round PCR. For 5'-RACE of ER β , two primers were used: 5'-GGTCTGCTTATTCGGCCCTGCGAGGAC-3' for the first round PCR and 5'-ACTCGCTCACCAGCGGGCTCCGATAG-3' for the second round PCR. For 3'-RACE of ER β , two primers were used: 5'-AATATGTGCCTCAGCTCCTCCGAGGGC-3' for the first round PCR and 5'-GACGGACGCTCTGGTGTGGGCCATCG-3' for the second round PCR.

Cloning and sequencing of *AroA* and *AroB* genes

For the *AroA* and *AroB* aromatase genes, two conserved amino acid regions, TACNYN and RYFQPFQ of the fish *AroA* and *AroB* aromatase genes, were selected and their degenerate oligonucleotides used as primers for PCR. As a template for PCR, first-strand cDNA was synthesized from 1 μ g poly (A) RNA isolated from the gonad and the brain. After amplification, the amplified DNA fragment was subcloned with TA-cloning plasmid pGEM-T Easy (Promega), sequenced using a BigDye terminator Cycle Sequencing-kit (PE Biosystems) with T7 and SP6 primers, and analyzed on the ABI PRISM 377 automatic sequencer (PE Biosystems). The 5'- and 3'-ends of the aromatase cDNA were amplified by rapid amplification of the cDNA end (RACE) using a SMART RACE cDNA Amplification kit (BD Biosciences Clontech). For 3'-RACE of *AroA*, two primers were used: 5'-AGGAGCAGCTGCAGAACCGGGACCTTC-3' for the first round PCR and 5'-AATGCCTCCGCTTCCACCCGGTGG-3' for the second round PCR. For 5'-RACE of *AroB*, two primers were used: 5'-CAGGAGACTGAGGGCGTCCACGTGTCC-3' for the first round PCR and 5'-GGCTGTCCAGGTGTTGGCTGTGGAGC-3' for the second round PCR. For 3'-RACE of *AroB*, two primers were used: 5'-GCCCCGACACGCTGTCCATCAGCCTC-3' for the first round PCR and 5'-ATGGTGGAGGAGATGAACGCCGCTTG-3' for the second round PCR.

QPCR

We quantified gene expression using QPCR performed on the ABI Prism 5700 Sequence Detection System with SYBR Green PCR Master Mix according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Unless noted, all supplies were purchased through Fisher Scientific. The brain, liver,

and gonad tissue stored at -80°C were transferred directly to 1 ml cold TRIzol (Invitrogen) and homogenized twice for 30 s each using a Psychotron NITON, NS 310E (Microtec Co., Ltd, Chiba, Japan). Following homogenization, 200 μ l of hydrated chloroform was added and the samples were vortexed and incubated at 4°C for 30 min. Samples were centrifuged and the aqueous phase was transferred to another tube containing 500 μ l of isopropanol, vortexed, and incubated at 4°C for 30 min. The supernatant was removed and 500 μ l of 80% ethanol was added to precipitate total RNA. Total RNA was purified with RNeasy mini kit following the manufacturer's instructions (Qiagen), quantified using a DU640 spectrophotometer (Beckman Coulter, Fullerton, CA, USA), and adjusted to a final concentration of $0.1\text{ }\mu\text{g}/\mu\text{l}$. An aliquot of purified RNA was run out on 1% agarose gel to assure quality.

Total RNA ($1.0\text{ }\mu\text{g}/\mu\text{l}$) was processed by attaching poly dT-linked primers (Invitrogen) to the poly (A) end of the mRNA following the manufacturer's instructions. cDNA was synthesized with SuperScript II RNase H reverse transcriptase (Invitrogen) and dNTP mix (Invitrogen) for 10 min at room temperature, 90 min at 42°C , and 10 min at 70°C . To run QPCR, SYBR Green PCR Master Mix, forward and reverse primers, and water were combined and gently vortexed. cDNA standards of 2 μ l (for each gene at 125, 25, 5, and $1\text{ }\mu\text{g}/\mu\text{l}$) or samples were combined with 48 μ l SYBR Green PCR Master Mix, forward and reverse primers, and water. Into each well of a MicroAmp 96-well reaction plate (Applied Biosystems), 15 μ l of this mixture was pipetted in triplicate for both standards and samples. QPCR cycles were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The primers were chosen to amplify short PCR products ($<100\text{ bp}$) with the assistance of Primer Express software (Applied Biosystems) and primer sequences were synthesized by Qiagen (Table 1). Melting curve analysis using ABI Prism 5700 Sequence Detection System software was performed to confirm the primer efficiency.

Data analysis

All sequences generated were searched for similarity using BLASTN and BLASTP of the National Center of Biotechnology Information. Multiple sequence alignments and construction of unrooted phylogenetic trees were performed using the CLUSTALW (Thompson *et al.* 1994) and TREEVIEW programs (Page 2000).

Estrogen receptor and aromatase gene expression were normalized by the expression of ribosomal protein L8. Data were checked for homogeneity of variance, transformed where necessary, and retested. Normalized gene expression in the hermaphrodite and the male

Table 1 Quantitative PCR primer pairs for estrogen receptors, gonadal and brain aromatase, and the ribosomal protein L8 (normalizing) genes

	Abbreviation	Forward	Reverse
Gene name			
Estrogen receptor- α	<i>ERα</i>	5'-CCCAGCGACCAA TCAGTGACT-3'	3'-TTGCGCACACCT CCTTTCA-5'
Estrogen receptor- β	<i>ERβ</i>	5'-TAAACGAACCTT CACCGCCA-3'	3'-CGGCCTCTTCAT GTCCTTCAT-5'
Aromatase-ovarian	<i>AroA</i>	5'-TGCAGGTTCTGG AGAGCTTCA-3'	3'-TGAAGCCGTCGA TGATGTCA-5'
Aromatase-brain	<i>AroB</i>	5'-GTGGTTGACTTC ACGATGCGT-3'	3'-TGCATGAGGCCA AGGTTGA-5'
Ribosomal protein (normalizing gene)	<i>L8</i>	5'-TGATAAGCCCAT CCTGAAGGC-3'	3'-TGCTCAACAGGA TTCATAGCCA-5'

tissues were compared using the *t*-test or where data remained heteroscedastic, using the Mann–Whitney *U*-test (StatView, v 5.0, Cary, NC, USA).

Results

Cloning and sequencing of *ER α* and *ER β* genes

To understand the effects of estrogens on the differentiation of rivulus, we tried to isolate the cDNA clone of ER. Using PCR techniques, partial DNA fragments were amplified from the rivulus gonad RNA. DNA fragments were obtained and sequence analysis showed that the fragments were classified into two kinds of clones, one similar to *ER α* and the other to *ER β* (data not shown). Next, using the RACE technique, we were able to clone the full-length rivulus *ER α* and *ER β* cDNA in the 5' and 3' directions, including the ATG start site and the TGA (*ER α*), or TAG (*ER β*) termination signals (Fig. 2; GenBank accession nos AB251458 and AB251457 for *ER α* and *ER β* respectively). Rivulus *ER α* has a 1743 bp open reading frame with 5' and 3' untranslated regions of 358 and 853 bp respectively (Fig. 2, data not shown). The deduced protein is 580 amino acids (aa) with a calculated molecular mass of 62 868.3 Da. *ER β* has a 1653 bp open reading frame with 5' and 3' untranslated regions of 783 and 779 bp respectively (Fig. 1, data not shown). The deduced protein is 550 aa with a calculated molecular mass of 61 268.2 Da.

Amino acid sequences of rivulus *ER α* and *ER β* show an overall homology of 35%. Using the nomenclature of Krust *et al.* (1986), rivulus *ER α* and *ER β* sequences can be divided into four domains based on their sequence homology to other steroid hormone receptors. Full-length amino acid sequences were used to derive phylogenetic tree data. The two ERs share 5% homology in the A/B domain, 95.4% in the C domain (the DNA-binding domain), 10.9% in the D domain, 51.8% in the E domain (the ligand-binding domain), and 11.1% in the F domain (Fig. 2A). Thus, domains C and E are highly conserved between rivulus *ER α* and *ER β* , whereas A/B, D and F domains show greater

variability. The overall comparison of both rivulus ER amino acid sequences with those of other fishes revealed that they were most similar to the ER sequences of the mummichog (*Fundulus heteroclitus*) at 78.7% for *ER α* and 83.0% for *ER β* and medaka (*O. latipes*) at 80.3% for *ER α* and 76.1% for *ER β* (data not shown).

Based on the alignment results, phylogenetic analyses were carried out and a phylogenetic tree of *ER α* and *ER β* proteins was constructed using the Neighbor-Joining method (Fig. 2B) (Saitou & Nei 1987). The unrooted ER distance analysis grouped the *ER α* and *ER β* homologs on separate clades and both rivulus ERs with the corresponding orthologous ER protein of other teleost fish, with high bootstrap supports for both the ERs (Felsenstein 1985). The phylogenetic tree revealed that both *ER α* and *ER β* were most closely related to the mummichog and medaka ERs.

Cloning and sequencing of *AroA* and *AroB* genes

To isolate *AroA* and *AroB* cDNAs from rivulus, PCR-based amplification techniques were used. DNA fragments were obtained and sequence analysis showed that the fragments were classified into two kinds of clones: one is similar to *AroB*, and the other to *AroA* (data not shown). Next, using the RACE technique, we tried to clone the full-length rivulus *AroA* and *AroB*. We could obtain the *AroB* cDNA in the 5' and 3' directions including the ATG start site and the TAA termination signal (Fig. 3; GenBank accession no. AB251459). Rivulus *AroB* has a 1509 bp open reading frame with 5' and 3' untranslated regions of 815 and 218 bp respectively (Fig. 3A, data not shown). The deduced protein is 502 aa with a calculated molecular mass of 57 097.33 Da. We could also determine the terminal signal (TGA) and 3' untranslated region of *AroA*. Unfortunately, however, we could not determine the ATG start site by 5' RACE. Partial sequence of *AroA* has a 1293 bp open reading frame with 3' untranslated region of 419 bp (GenBank accession no. AB251460, Fig. 3B, data not shown).

A		B	
ATGTACCTTGAAGAGAGCGGGGCTCTGGAGGGTAGCTGTCTGGACTTCTAGAGGGG	60	ATGGCCCGCTCACCTCTCGGAGAAGCATCAGCGCTCTCGAGCTCCAAGAGGTGGAC	60
M Y P E E S R G S G G V A A V D F L E G	20	M A A V T S P E K H Q P L L Q L Q E V D	20
ACGTATGACTATGCTGCCCCACCTCGCCCTACTCTCTCTAGCAACCTCCACACT	120	TCCAGTCGGGTGCGCAGTCGCATCTGTCCCGGTCTCTCAGCTCTCCCTGCTGTGTC	120
T Y D Y A A P T P A P T P L Y S N S T T	40	S S R V A S R I L S P V L S S S P G L S	40
GGGTACTTCTCTCTCTGAGCGCCACGGACCCCGTCTAATGGCAGCTTCAGTCT	180	CAGAACCCAGCAGCCATCTGCATCCCGCTCTGTACACGACCTCGGCACGACTTC	180
G Y F S A P L D A H G P P S N G S L Q S	60	H E P S Q P I C I P T Y T D L G H D F	60
CTAGGAAGTGGGCAACTAGTCCACTGTGTGTTGTGCCACCAAGCTCAGCCCC	240	CCGCCATTCTTTCTACAGCCCGACCATCTTCAGCTACGCGGTTCGAGATTTCGGAG	240
L G S G P T S P L V F V P T S P R L S P	80	P P I S F Y S P T I F S Y A G P S I S E	80
TTTATGCACTCTCTGGTCACTATCTGGAACCGCTCAACACCCGCTACAGATCCAGC	300	CACCCCTCGGTGATCAGTGTGCTGAGCGCTCTTTGTTTGGCCGGTCCAGGACAGTG	300
F M H P A G H Y L E T A S T P V Y R S S	100	H P S V H Q S L S A S L F W P F G H G H V	100
CACGAGCAGCTCTCAGAGAGGACCACTGTGACCCCGTGACGAGGATGAGTGTGGG	360	GGGGTTCTGTACCCCTGCACCGCTCCGAGCCGAGCCAGCAGCAGGTGAGCAGTGC	360
H Q P A S R E D Q C D P R D E A C S V G	120	G G S V P L H R S Q T R A Q Q H G Q P V	120
GAGTTAGAGAGTCAAGCGCGCGGGGGTTTGAGATGGCAAGAGACAGTTCTGTGT	420	CAGAGTCCGTGGGACAGCGCTTAAACACAGTAAGAGTGGAGGAGGAGCTCCAGGAG	420
E L G G A A G A G A E G A M A K E T R F C	140	Q S P W D S V L T T S A A A R S S Q E	140
GCGGTGTGCGCAGCTACTGCTTCCGGGTACCACTACGGGTGTGCTCTCGAGGGCTGC	480	GTGAGGAGGCGGTGTGCTGTGCGCGGGAAGCCGACCTCCATTACTGCGCGGTGTGT	480
A V C S D Y A S G Y H Y G V W S C E G C	160	V E E A V V S S G G K A D L H Y C A V C	160
AAGGCTCTCTCAAGAGGAGCATTCAGGGTCACAATGACTATATGTGCCAGCGACCAAT	540	CACGACTACGCTCGGGGTACCACTACGGCGTCTGGTCTCGGAGGCTCGAAGGCTTC	540
K A F P K R S I Q G H N D Y M C P A T N	180	H D Y A S G Y H Y G V W S C E G C K A F	180
CAGTGTACTATTGACAGGAATCGGAGGAAGAGCTGCGAGCTTGTGCTCTTAGGAAGTGT	600	TTCAAGAGGAGATCCCAAGGACACAACGACTCATCTCTCCGCACTAATCAATGCACC	600
Q C T I D R N R N K G S C Q A C R L R K C	200	F K R S I Q G H N D Y I C P A T N Q C T	200
TACGAAGTGGTATGATGAAAGAGGTGTGCGCAAGACCGTGTGCGTCTTTGCGCGC	660	ATCGACAAAAACCGCGCAAGAGCTGCCAGGCTTCCGCTCTCGCAATGTATGAAGTT	660
Y E V G M M K G G V R K D R G R V L R R	220	I D K N R R K S C Q A C R L R K C Y E V	220
GACAAAAGACGGGAGGATTGGAGACAGAGCAAGGTTGCCAAAGCTTGGAGCACAAA	720	GGCATGACTAATGTGTTATTCGCAAGAGCGTGAAACTATCGGAGCCCGCTGGTGAGG	720
D K R R G G I G D R D K V A K G L E H K	240	G M T K C G I R K E R G M P P E L S V L R	240
GCAGCAGCAGTCATGATGGGAGGAGCGCAGCAGCAGCAGCAGCATCGGAGGAGGA	780	CGAGTGACGCTGTCTCTCGCAGGCGCAATAGCAGACAAAGCTGTGATGAGCA	780
A A A S H D G R R R S S S S S I G G G	260	R V T R L S S Q G R I S R P N V L T G P	260
GGAGGAGGAATCTCTGTTACTGAATCTTCTCTGAACAGGTGCTGCTCTGCTCCAG	840	GCGGAGAGTTCGTAAACGAACCTTACCGCCATCATGCCCGGAGCAGCTGATTGAG	840
G G G R S L N E P S P P S P P S P P S P P	280	A E S S L N E P S P P S P P S P P S P P	280
GGCGCGAGCCACCGATCTCTGTTCCCGTCAGAAGCTGAGCGACCTACACCGAAGTC	900	CGAATAATGAGGCGGAGCCACAGAGATCTACGTGATGAAGGACATGAAGAGCGCGTG	900
G A E P P I L C S R Q K L S R P Y T E V	300	R I M E A E P P E I Y V M K D M K R P L	300
ACCATGATGACCCCTCTCAGCAGATGCGCGACAGGAGTGTGTCACATGATGCGCTGG	960	ACGAGGACAGCGTATGATGTGCTGCTCACCAATCTGGCTGATGAAGAGCTGTTACATG	960
T M M T L L T S M A D K E L V H M I A W	320	T E A D V M M S L T N L A D K E L V H M	320
GCCAAGAAGCTTCAGGTTCTCTGAGTGTCTCTCCAGCATCAGGTTCTGCTGCTGGAG	1020	ATCAGCTGGGCAAAAGATTCCAGGCTTTGTGGAGCTCAGTCTCTGAGCAGGATCCAT	1020
A K K L P R G L K L L H D Q V L L L L E	340	I S W A K K I P G F V E L S L D Q V H	340
AGCTCGTGGTGGAGATCTCATGATCGGGCTCATCTGGAGGTCAATCACTGCCAGGA	1080	CTGTTGGAGTGTGCTGCTGGAGGTGTGATGATGGAGTGTGATGAGAGTCCGTGGAC	1080
S S W L E I L M I G L I W R S I H C P G	360	L L E C C W L E V L M M G L M W R S V D	360
AAGCTCATTTTTCACAGGACCTCATATTGGAAGAGGAGCGAGGCGAGTGTGGAGGT	1140	CACCCGGGAAACTCATCTCTCTCCGACCTCAGCTCAGCAGAGAGGAGGAGCTGC	1140
K L I F A G D L I L D R S E G D C V E G	380	H P G K L I F S P D L S L S R E E G S C	380
ATGGCGAGATCTTGACATGTCTGCTGCCACCGCTCTCGCTTCCGATGTCTCAAATC	1200	GTCAGGGCTTCGTGGAGATCTTCGACATGCTGATAGTGGAGCTCCGAGTGAGAGAG	1200
M A E I F D M L L A T A S R F R M L K L	400	V Q G F V E I F D M L I A A T S R V R E	400
AAACCCGAGGAATTTGCTGCCTTAAAGCCATCATCTGCTCAACTCTGGTGCCCTTTCT	1260	CTCAAACTGAAGAGGAGGAATACGTCTGCTCAAGGCCATGATCTCTCAACTCCAAT	1260
K P E E F R F L K A I I L L N S G A F S	420	L K L K R E E Y V C L K A M I L L N S N	420
TTCTGCACCGCAGCATGAGAGCCCTTCACAGCGCGCGCGGTGAGAACATGCTGGAC	1320	ATGTGCTCAGCTCTCCGAGGCGAGGAGCCTTCAGATGCTGCTCAAGCTGCTGCGC	1320
F C T G T M E P L L H D G A A V Q N M L D	440	M C L S S S E G S E D L Q S R S K L L R	440
ACCATGACGACCGCTCATCACACACATCAGCCAGTCTCGGTTCAGGAGCAG	1380	CTGCTGAGCGCGTGAACGAGCTCTGGTGTGGGCATGCCCAAGCGGCTTACCTTC	1380
T I T D A L I H H I S Q S G Y S V Q E Q	460	L L D A V T D A L V W A I A K S G L T F	460
GCGAGACGACGAGCGCTGCTCTGCTGCTCCACATCAGACACATGAGCAACAA	1440	CGGACAGTACACCGCTGCTCACTGCTCATGCTGCTCTCAACATCGCATGTC	1440
A R R Q A Q L L L L S H I R H M S N K	480	A R Q Q Y T R L A H L L M L L S H I R H V	480
GGCATGGAACATCTGTACAGCATGAAATGCAAGAACAAAGTGCCTCTGTACGACCTGTG	1500	AGCAACAAAGCATGGACACCTGCACTGATGAAGATGAAGAACATGTTGCTTTGTAC	1500
G M E H L Y S M K C K N K V P L Y D L L	500	S N K G M D H L H C M K M K N M V P L Y	500
CTGGAGATGCTGCTGCTCCAGCGCTCCACCGTCCAGTCAATCATCTCAGTCCCTGTCC	1560	GACCTGTGCTGGAGATGCTGGAGCCCATCATGACAGCTCCCGCTGCTCAGCGC	1560
L E M L D A H R L L H R P V K S S Q S L S	520	D L L L E M L D A H I M S R S L P H R	520
CAAGCTGAAAGAGTGCCCTCTCTCTCTCTCTCTGCTGCGGTGATGTGGCGGC	1620	CCGCTCAGACGGAGAGTGCCTCGCGCGGACCTTCAACACCTGGACTCCGACGAGCTT	1620
Q A E R V A P S S S S S C G D G G G	540	P A Q T E S A A A G P S N T W T P S S V	540
TCCTCTCTCGAGGTGCACTTCAGGACCTCGAGGATCAGTGAACCCGAGCAGAAC	1680	GGAGTGGAGGTGAAGCCGACTACTCAGAATAG	1653
S S A G C S S G P R G I S E N P S R T	560	G V G G E A Q Y S E * 550	
CGTCAAGCCGAGCGTCTGTCAGTACGAGGCTCCGCTCTGCTGACCAACGCGCTG	1740		
P S G P S V L Q Y Y G G S R P D C T N A L	580		

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Figure 2 Nucleotide sequences of the rivulus ER α (A) and ER β (B), and the deduced amino acid sequences.

The deduced amino acid sequences of the rivulus aromatase genes were compared with other fishes (Fig. 4). The overall homology between rivulus AroA and AroB is 57.2%. The homologies between rivulus and those of other fishes, medaka, mummichog,

roach (*Rutilus rutilus*), zebrafish (*Danio rerio*), goldfish (*Carassius auratus*), and catfish (*Ictalurus punctatus*) are 83.2, 83.2, 63.1, 62.6, 59.7, and 59.0% respectively. The homologies between the AroB in rivulus and those of other fishes, medaka, mummichog, roach, zebrafish,

Figure 3 Structure (A) and phylogenetic tree (B) of the rivulus ERα and ERβ.

goldfish, and catfish, are 74.7, 74.0, 57.1, 55.9, 57.1, and 54.4% respectively. Based on the alignment results, phylogenetic analyses were carried out and a phylogenetic tree of AroA and AroB proteins was constructed using the Neighbor-Joining method (Fig. 4B; Saitou & Nei 1987). The unrooted aromatase distance analysis grouped the AroA and AroB homologs on separate clades and both rivulus aromatase genes with the corresponding orthologous aromatase protein of other teleost fish, with high bootstrap support for both aromatase genes (Felsenstein 1985). The phylogenetic tree revealed that both AroA and AroB were most closely related to the corresponding medaka and mummichog aromatase genes as with the ER genes (Fig. 5).

QPCR

For a summary of the expression of *ERα*, *ERβ*, *AroA*, and *AroB* genes in the brain, gonad, and liver of hermaphrodites and males, see Table 2. In the liver, *ERα* expression was 6.16× greater in hermaphrodites compared to males ($P=0.0004$, Fig. 6). Similarly, the

expression of *ERβ* gene was 2.21× greater in hermaphrodites compared to males in the gonad ($P=0.0002$, Fig. 6). No difference was observed in either *ERα* or *ERβ* expression in the brains between reproductive morphs.

The expression of *AroA* gene in the hermaphrodite gonad was 28.8× greater compared to that measured in the male gonad ($P<0.0001$, Fig. 7). We measured a 2.73× increase in *AroB* gene expression in the hermaphrodite compared to male brain ($P=0.007$, Fig. 7). No differences in *AroA* or *AroB* expression were observed between the reproductive morphs in the liver.

Discussion

We tested the hypothesis that gene expression would differ between rivulus hermaphrodites and males and that expression would reflect the presence of ovarian tissue in the hermaphrodites. In this paper, we show reproductive morph- and tissue-specific differences since hermaphrodites had increased *ERα*, *ERβ*, *AroA*, and *AroB* gene expression in the liver, gonad, gonad, and brain respectively, compared to males.

In contrast to mammals, which have two ERs, there are three known ERs in teleost fishes (Hawkins *et al.* 2000). These include *ERα* and two isoforms of *ERβ*: *ERβ1* (*ERβa* or *Esr2b*) and *ERβ2* (also *ERβb* or *Esr2a*, and formerly *ERγ*; Filby & Tyler 2005, Hawkins *et al.* 2005, Pinto *et al.* 2006). The two *ERβ* isoforms arose from a gene duplication following the split between tetrapods and teleosts (Hawkins *et al.* 2000).

ERα expression has been measured in a number of species, including Atlantic croaker, zebrafish, sea bream (*Sparus auratus*), and the fathead minnow (*Pimephales promelas*) (Menuet *et al.* 2002, Filby & Tyler 2005, Hawkins *et al.* 2005, Pinto *et al.* 2006). In this study, expression of *ERα* in rivulus was strongest in the liver and this finding is in agreement with the previous studies. *ERα* has a strong affinity for estradiol and is upregulated in the liver by estradiol in females and estrogen-exposed male fish, where it induces vitellogenin synthesis (Filby & Tyler 2005). Given the presence of ovarian tissue, the increased expression of hepatic *ERα* in the hermaphrodites compared to the males makes sense.

ERβ1 and *ERβ2* expression has also been characterized in the Atlantic croaker, zebrafish, sea bream, and the fathead minnow (Menuet *et al.* 2002, Filby & Tyler 2005, Hawkins *et al.* 2005, Pinto *et al.* 2006). In this study, gonadal *ERβ* expression was upregulated in the hermaphrodite compared to the male rivulus. Again, these results are consistent with other studies, where 17β-estradiol synthesized mainly by the ovarian follicular granulosa cells has a stimulatory effect through the

A		B	
ATGATGCAACACGAGGTGCTCAGCGTCGSCCAACCGTGGTTCTTGTCTGTCTGAAGTC	60	ACGGCTTGTAATTACTACAAACAAGTACGAGACATGGTGAGAGTGTGGATCAATGGA	60
M M Q P E V L S V G Q T V V S C L S E V	20	T A C N Y Y N K K Y G D M V R V W I N G	20
ACCTCCCTGCTGCTGCTGTTCTTCTGCTGATCACCGCATGGAGCGCAACACCACTCA	120	GAGGAGACCTGATCCTCAGCAGGGCATCAGCAGTTTCATCACGTCTCAGGAGTGGCAAA	120
T S L L L L L L F L L I T A W S R T H H S	40	E E T L I L S R A S A V H H V L R S G K	40
CACACCCAGGTCTCTCTCTGCGCAGGATCTGGTCCGATCTGTCTACAGCAGGTTT	180	TATACGTACGCTTCGGGAGCCAGCAGGGATCTCCGTGCTCGGCGATGAGAGAGGC	180
H T P G P S F L A G L G P I L S Y S R F	60	Y T S R F G S Q Q G L R L G M N E R	60
ATTGAGTGGGATGGAAACGCGTCACTACAAACCGCAATATGGCAGCATTGTG	240	ATCATATTCACACAAACGCTGCTCTGTGGAACACATACGCACTATTTCGCAAGCC	240
I W T G I G T A C N Y Y N R K Y G S I V	80	I I F N N N V T L W K N I R T Y F A K A	80
AGGTTGTGGATTAATGGGAGAGACCTTCATTTGAGCAAGTCTGCGGCGTGTACCAT	300	CTGACGGTCCGAACATGAGCAGACCGTGGAGGTTTGTGTCTCGTCCACGAGCTCTAC	300
R V W I N G E E T F I L S K S A V Y H	100	L T G P N M Q Q T V E V C V S T Q S H	100
GTGTTGAGAAAGGATACATCTGAGGTTCTGGGAGCAGAAAGGCTCAAGTGATT	360	CTGGACGACCTGGACAGTCTGACTCATGTAGAGCTCTCAGCTTGTGCTGCGCTGACCGTG	360
V L R S L D N Y I C R F G S R R G L K C I	120	L D D L D S L T H V D V L S L L R C T V	120
GGGATGGAGGGCAGGGGATCATTTTCAACAGTGATGTCCACTCTGGAAAAAGTGAGG	420	GTGGACATCTCCACAGGCTCTTCTGGAGCGTCTCTCGATGAGAAAGAGCTGTCTGTG	420
G M E G Q G I I F N S D V P L W K K V R	140	V D I S N R L F L D V P L D E K E L L L	140
ACGTACTTTTAAAGCTGAGCGCCCGCCTTAGGAGGACAGTGAGGATCTGTGTG	480	AAGATTCAAGAGTCTTTGACACGTGGACGCGTGTGATCAACCTGACGTCTACTTC	480
T Y F S K A L S G P S L R R T V R I C V	160	K I Q K Y F D T W Q S V L I K P D I C V	160
AGCTCCACAGCAACACCTGGACGCTGAGGACGTGACCGACCTCTGGACACGTG	540	AGGTTACGCTGGATTACGAGAAACAAGCCGACGCCAGGAGCTGAGCAAGCCATC	540
S S T A K H L D S L Q D V T D P S G H V	180	R F S W I Q Q K H K A A A Q E L Q D A I	180
GACGCCCTCAGTCTCTGAGAGCGATGATGGTGGACATCTCAACAGGCTGTCTCTCAGG	600	GAGGCCCTGTTGAGCAGAAAGCGGGAATGGAGCAGGACAGAGAAATGGACAAACAT	600
D A L S L L R A I V V D I S N R L F L R	200	E G L V E Q K R R E M E Q A E K L D N I	200
GTGCTCTGAATGAGAAGGACCTGTGATGAAGATCCAGAACTACTTTGAGACCTGGCAA	660	AACTTCGTGAGATCTCATCTTTGACAAAAACCGCGGAGCTGTCCGCCGACAACTG	660
V P L N E K D L L M K I Q N Y F E T W Q	220	N F A A D L I F A Q N H G E L S A D N V	220
ACGGTCTGATAAAGCTGAGCTTTCTTCAAGCTCGGATGGCTCTACAACAAACAAG	720	AGSCAGTGCATGCTGGAGATGGTGTATCGCCGCCCGGACACCTTGCATCAGCTGTTC	720
T V L I K P D V F F K L G W L Y N K H K	240	R Q C M L E M V I A A P D T L S I S L F	240
AGAGCAGCCGAGGCTGAGAGCAGATGGAGGCTGCTGGAAGTGAAACGAAAGATG	780	TTTACTGCTGCTCTCTCAACAGAAATCCAGATGTGGAGCTGCTGAGAGATC	780
R A A Q L E Q D E M E S L L E V K R K M	260	F M L L L L K Q N P D V E L Q L L G E I	260
ATCGATGACGACAGAGCTGACGATGATCGGACCTTCCGACAGAGCTCATCTTCCTG	840	GACGCTGTCTGATGAGGAGCAGCTGAGAAACCGGACCTTCAAGAACTGAGAGTCTG	840
I D D A E K L D D D R D F A T E L I F A	280	D A V V G E E Q L Q N R D L Q K L Q V L	280
CAGAAATCACGGGGAGCTCTCAGCAGCAACGTCAGGCACTGCTGCTGGAGATGGTGATC	900	GAGAGCTTCATCAACGAATGCTCCGCTTCCACCGGTTGGTGGATCACCATTGCGCCGG	900
Q N H G E L S A D N V R Q C V L E M V I	300	E S F I N E C L R F H P V V D F T M R R	300
GCAGCCCCGACACGCTGTCTCAGCCTCTTCTTATGCTGATGCTGCTCAACAGAAC	960	GCCCTTCTGATGACATCATCAGCGCTTCAGGATCAAGAGGACCAACATCATCTG	960
A A P D T L S I S L F F M L M L L K Q N	320	A L S D D I I D G F R V R K G T N I I L	320
CCTGACGTGGAGCTGAAGATGGTGGGAGGATGAACGCGCTTGAATGGGAAAGCCATA	1020	AACACGGCCGGATGCACCGGACCGAGTTCTTCCACAGAGCCAGAGTTACGCTGGAG	1020
P D V E L K M V E E M N A A L N G K A I	340	N T G R M H R T E F F H R A Q E F S L E	340
GAGGACCTGGATTACGAAGATTAAGATGAGGAGCGTTTCATCAGCGAGTGTCTGAGG	1080	AACCTCAGAAAGATGCCCTCGCCGCTACTTCCAGCGTTTGGTTCTGGGCCCCCGCC	1080
E D V D Y E G L K V M E R F I S E C L R A	360	N F Q K N A P R R Y F Q P F G S G P R A	360
TTTCAACCTGTGTTGATCTCAGATGGCTTAAGCTCTGGAGGAGGACAACTGACGCGC	1140	TGCGTTGGCAACACATCGCATGGTATGATGAATCCATCTCTGTTGACATCTCTGGGT	1140
F H P V V D F T M R K A L E E D N I D G	380	T G V G K H I A M V M M K S I L V T L L G	380
ATGAAATCAGGAAGGCAACAACATCTTCAACCTTGGCCTCATGCACAAGACGAG	1200	CAGTTCCTGCTGTGCCCCCCCGGACCTGACCTTGACTGCTCTCCGACAGCAACAAC	1200
M K I R K G T N I I L N L G L M H K T E	400	Q F S V C P P R D L T L D C L P Q T N N	400
TTCTTCCCCAACCCAGAGGTTCAACCTGACGAACCTCGACAACAGCGTGGCCAGTCGC	1260	CTGTCCACGACCGGTGGAGCACCAGGAGGAGGAGGAGGCTGACGATGAGGTTCTTA	1260
F F P K P K E F N L T N F D N T V P S R	420	L S Q Q P V E H Q E E A G G L S M R F L	420
TTCTTCCAGCCCTTTGGCTGCGGGCCGCTCTGCTGCTTGGCAACATATGCCATGGTG	1320	CCCCGACAGAGAGGAGGTGGCCCTGCGCTCGA	1293
F F Q P P F G C G P R S C V G K H I A M V	440	P R Q R G R W P S P * 430	
ATGATGAAGGCCATCTTGATCAGCTGCTGCTGCTGTACACGGTGTGTCCCGCAAGGC	1380		
M M K A I L I T L L S R Y T V C P R Q G	460		
TGCACGCTGGACGATCGCGGACGACCAACAACTGTGCGACGAGCTGTGGAGGACGAG	1440		
C T L D S I R Q T N N L S Q Q P V E D E	480		
CTCGGCTCGCATGCGCTTATCCCTGTAACCGAAACAAAGCGCAGTCACTCCGAA	1500		
L G L A M R F I P R T Q K Q S A V N S E	500		
ACAGAATAA 1509			
T E * 502			

Figure 4 Nucleotide sequences of rivulus brain-type aromatase (A), ovarian-type aromatase (B) and the deduced amino acid sequences.

ER on the ovarian development, oogenesis, and seasonal ovarian recrudescence (Devlin & Nagahama 2002). Both isoforms of ER are known in the gonads of both sexes in fishes and mammals and there is no general agreement at this time about the relative importance of one isoform over another. All three isoforms of ER are known to be expressed in various brain regions and expression patterns differ in the Atlantic croaker compared to the zebrafish. Using both an analysis of nucleic and amino acid sequences, as well as the immunolocalization of ER α , ER β 1, and ER β 2

transcripts to distinct regions of the brain (preoptic area, hypothalamus, and cerebellum) in the Atlantic croaker (*Microgogonias undulatus*) and distinct yet overlapping brain regions in the zebrafish gives support to the hypothesis that these three forms of ER have specialized functions and enable different regulatory roles of reproduction and behavior (Menuet *et al.* 2002, Hawkins *et al.* 2005). In this study, there was no difference in the expression of ER β in the brain and we were not able to find evidence of a third ER in rivulus.

Figure 5 Alignment of the amino acid sequence (A) and phylogenetic tree of rivulus aromatase genes (B), *AroA* and *AroB*.

Table 2 Normalized expression of estrogen receptors and ovarian and brain aromatase genes for brain, gonad, and liver

	Abbreviation	Tissue	Normalized gene expression mean (S.E.M.)		Herm versus male
			Herm	Male	
Gene name Estrogen receptor- α	$ER\alpha$	Brain	0.021(0.005)	0.015(0.003)	NS
		Gonad	0.186(0.168)	0.027(0.043)	NS
		Liver	1.904(0.392)	0.309(0.088)	$P=0.0004$
Estrogen receptor- β	$ER\beta$	Brain	0.220(0.055)	0.145(0.023)	NS
		Gonad	1.702(0.135)	0.769(0.174)	$P=0.0002$
		Liver	0.326(0.133)	0.846(0.571)	NS
Aromatase-ovarian	$AroA$	Brain	0.043(0.008)	0.032(0.003)	NS
		Gonad	1.670(0.145)	0.058(0.023)	$P<0.0001$
		Liver	0.002(0.0004)	0.011(0.007)	NS
Aromatase-brain	$AroB$	Brain	4.536(1.01)	1.663(0.161)	$P=0.007$
		Gonad	0.776(0.268)	0.446(0.122)	NS
		Liver	0.005(0.001)	0.01(0.007)	NS

P values as shown and NS, non-significant, values at $P>0.05$.

In contrast to most mammals, which have a single aromatase gene, there are two isoforms of aromatase in most teleosts and these are thought to be the result of gene duplication (Simpson *et al.* 1994, Callard *et al.* 2001, Pellegrini *et al.* 2005). As with the ERs, aromatase expression occurs in many tissues including brain, pituitary, gonads, and adipose tissue. However, ovarian aromatase, AroA, is predominantly expressed in the ovary, and brain aromatase, AroB, in the brain. Also unique to teleosts is the magnitude (2–3 orders of magnitude greater than in mammals) of AroB expression and activity in the telencephalon, preoptic area, and hypothalamus of the teleost brain (Callard *et al.* 1978, Pellegrini *et al.* 2005). In the brain, furthermore, AroB expression typically exceeds the expression of AroA (Callard *et al.* 2001).

In this study, we isolated two aromatase cDNAs, encoding AroA and AroB. AroA cDNA of rivulus was cloned by PCR-based method; it encodes a protein of 430 amino acids with molecular mass of 57.1 kDa. In addition, the full-length AroB cDNA was isolated from brain RNA using the RACE technique, and the deduced protein was 502 amino acids with a calculated molecular mass of 58.6 kDa. All fish AroB proteins reported so far have in a range of 488–508 amino acid sequences (Gelinas *et al.* 1998, Halm *et al.* 2001, Valle *et al.* 2002). The amino acid sequence of rivulus AroB has no signal peptide; however, this amino acid sequence is of a membrane protein, which has a transmembrane helix in the N-terminal region (GQTVVSLSEVTSLLLLLFLLIT). This transmembrane domain is found in wrasse AroB

Figure 6 Expression of estrogen receptors, $ER\alpha$ and $ER\beta$, genes in the rivulus brain, gonad, and liver.

Figure 7 Expression of gonadal and brain aromatase, AroA and AroB, genes in the rivulus brain, gonad, and liver.

(VTVFLLLMVLLLLFT; Choi *et al.* 2005). Unfortunately, we could not determine the full sequence of AroA of rivulus. With the information of the full-length sequence, we will be able to better understand the functional structure and evolution of aromatase.

Alignment results of each aromatase subtype showed that the rivulus aromatase shared the highest homology with Cyprinodontiformes aromatases, such as mummichog and medaka. All compared aromatase sequences have higher conservation in functional regions common to both aromatase subtypes. Phylogenetic analysis shows that teleost group separates into two clear clusters, one of them containing the AroA and the other AroB. Rivulus aromatases also belong to each cluster form and this result is consistent with other teleost phylogenetic analysis, such as rainbow trout (Valle *et al.* 2002) and wrasse (Choi *et al.* 2005).

In rivulus, we measured greater AroA expression in the gonad of the hermaphrodite compared to the male gonad. These data are in agreement with research on other species and makes sense given the presence of ovarian tissue in the hermaphrodite (Callard *et al.* 2001). In goldfish and zebrafish, AroA expression was greater in the ovary compared to the testis. Japanese eels (*Anguilla japonica*) have only one aromatase gene (Jeng *et al.* 2005). Although making a direct comparison to rivulus is difficult due to this fact, it is interesting to note that the male eels have no measurable aromatase expression in the testis compared to the ovary, where expression is measurable.

The results discussed thus far are in accord with the findings of labs investigating other species. It is comforting to know that, in this initial study of gene expression in rivulus, data from the *ERs* and *AroA* genes are consistent with gene expression data reported for other species in the same tissues. Interestingly, the data from this study document the elevated expression of AroB in the hermaphrodite versus male brain.

The functional significance of the dimorphic expression of AroB is unclear at this time. The elevated expression of AroB in hermaphrodites compared with males may be due to different regulatory requirements of an organism with an ovotestis or simply a testis. Most of the hermaphrodites studied in detail are sequential, which are similar to gonochores in that temporally they have functional ovaries that transition into functional testes and vice versa. Prior to these data on rivulus, what we know about the endocrine control of reproductive physiology and/or ethology of simultaneous hermaphroditic fish is found in one published study on the belted sandfish (*Serranus subligarius*; Cheek *et al.* 2000). This study was limited to measuring the circulating concentrations of sex steroids during the reproductive season, daily spawning cycles, and

contains no information regarding the expression of ERs or aromatase enzyme genes. Nothing is known about the mechanisms regulating simultaneous development of ova and spermatozoa in the same gonad. Sex-specific brain differences in the regulation of gametogenesis are thought to be ultimately modulated by neurotransmitters upstream of GnRH-secreting neurons, i.e., to be controlled by the frequency and amplitude of GnRH release, but there are few published data for this hypothesis in gonochores or sequential hermaphrodites (Parhar *et al.* 2001, Miranda *et al.* 2003). Clearly, more research needs to be done to examine the differential expression of AroB and its potential role in the regulation of gametogenesis in the rivulus.

In mammals, aromatase is expressed in the neurons (Balthazart *et al.* 2003). In teleosts, AroB is known to be synthesized in the radial glial cells of the midshipman (*Porichthys notatus*), rainbow trout (*Oncorhynchus mykiss*), and zebrafish (Forlano *et al.* 2001, Menuet *et al.* 2002, 2003). Radial glial cells have been shown to play a role in neurogenesis and local synthesis of estradiol has been hypothesized to regulate neurogenesis in adult electric fish (*Apteronotus leptorhynchus*) and three-spine sticklebacks (*Gasterosteus aculeatus*; Pellegrini *et al.* 2005). While co-localization of ER and AroB would seem logical, thus far, these functionally related proteins are co-localized to brain/pituitary regions, but have not been found in the same cells (Menuet *et al.* 2002, 2003). The high levels of AroB in the teleost brain have been hypothesized to enable cell proliferation necessary for requisite sex-specific behaviors and gametogenesis during recrudescence in seasonal reproduction (Pellegrini *et al.* 2005). It seems unlikely that self-fertilizing rivulus hermaphrodites would have these requirements. In transitioning sequential hermaphrodites, elevated AroB levels may enable cellular proliferation in sex-specific neurons of the new sex (Pellegrini *et al.* 2005). Secondary male rivulus, which transition from functional selfing hermaphrodites to functional males at 28 °C might experience an increase in AroB to enable this new brain architecture. The thought that all the hermaphrodites in this experiment were undergoing transition to secondary males is also untenable, given the young age of these fishes and the temperature at which they were housed (24 ± 1 °C).

One of the weaknesses of the study was the lack of aromatase activity data, i.e., we have no knowledge of the amount of functional enzyme. Future research should examine the aromatase protein catalytic activity together with brain aromatase mRNA quantity to conclude a dimorphism in neuroestrogen synthesis and the potential regulatory role it may play in the reproductive ethology and physiology of the rivulus.

In conclusion, this is the first study to examine gene expression in the only self-fertilizing vertebrate hermaphrodite known, the mangrove rivulus, *K. marmoratus*. Rivulus is an exciting model for studies on sex determination and sexual differentiation because of the uniqueness of its reproductive biology. This research has laid the groundwork for future investigations of the role of sex steroid hormones in the differentiation of the brain, pituitary, liver, and gonad in the various and intriguing reproductive morphs of this model system.

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Animal care and use statement

All research contained in this manuscript was conducted in full compliance with the Florida Atlantic University Institutional Animal Care and Use Committee (permit #A0601).

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