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Cloning of Brain Aromatase Gene and Expression of Brain and Ovarian Aromatase Genes During Sexual Differentiation in Genetic Male and Female Nile Tilapia *Oreochromis niloticus*

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ABSTRACT A brain aromatase gene was identified from the Nile tilapia *Oreochromis niloticus*. The cDNA sequence of this gene differed from that of the ovarian aromatase gene previously reported from this species. Tissue specific expression for both brain and ovarian aromatase genes was examined in the tissues of adult tilapia. Brain aromatase mRNA was expressed in the brain, kidney, eye, ovary, and testis, but not in the liver and spleen. Ovarian aromatase mRNA was expressed in the brain, spleen, ovary, and testis but not in the eye, kidney, and liver. Differential aromatase gene expression between the sexes was investigated in all-male (XY) and all-female (XX) groups of tilapia fry from fertilisation throughout the sexual differentiation period. Semi-quantitative RT-PCR analysis revealed that the initiation of expression of both aromatase genes lay between 3 and 4 dpf (days post fertilisation) in both sexes. The level of brain aromatase mRNA gradually increased throughout the period studied with little difference between the sexes. This contrasted with marked sexual dimorphism of ovarian aromatase mRNA expression. In females, the expression level was maintained or increased gradually throughout ontogeny, while the level in males was dramatically down-regulated between 15 and 27 dpf. Subsequently, the level of ovarian aromatase mRNA expression fluctuated slightly in both sexes, with the expression in females always being higher than in males. These findings clearly suggest that ovarian aromatase plays a decisive role in sexual differentiation in this species and that this is achieved by down-regulation of the expression of this gene in males. *Mol. Reprod. Dev. 59: 359–370, 2001.*

Key Words: steroids; ontogeny; down-regulation; kidney

INTRODUCTION

A steroidogenic enzyme, cytochrome P450 aromatase, that catalyses androgens into oestrogens has been implicated in sexual differentiation of many vertebrates including fish (Piferrer et al., 1994; Guiguen et al., 1999; Kitano et al., 1999; Kwon et al., 2000a), amphibians (Yu et al., 1993), reptiles (Jeyasuria and Place, 1998) and birds (Elbrecht and Smith, 1992; Wartenberg et al., 1992). This enzyme is of particular interest in sexual differentiation of fish, since treatment of fish alevins or fry with exogenous sex steroid hormones can result in functional sex changes against genetic sex (Yamamoto, 1969). Inhibition of aromatase action by a chemical inhibitor mimics the sex-reversal effect of androgen treatments in some fish species (chinook salmon, *Oncorhynchus tshawytscha*, Piferrer et al., 1994; Nile tilapia *Oreochromis niloticus,* Kwon et al., 2000a) suggesting the involvement of this enzyme in fish sexual differentiation. Up-regulation of ovarian aromatase gene expression in female juvenile fish was also reported in other species (Japanese flounder *Paralichthys olivaceus,* Kitano et al., 1999; rainbow trout, *Oncorhynchus mykiss,* Guiguen et al., 1999). However, when, where and how this aromatisation differentially occurs in genetic females and males is not known.

The Nile tilapia, *O. niloticus*, is a good model species to elucidate sex determination and differentiation mechanisms in fish as well as being an important species in aquaculture. This species has an XX/XY chromosomal sex determination mechanism (Jalabert et al., 1974; Mair et al., 1991; Carrasco et al., 1999), although it has been shown that both environmental factors (Baroiller et al., 1995; Abucay et al., 1999; Kwon et al., 2000b) and secondary genetic factors (Mair et al., 1991; Hussain et al., 1994; Sarder et al., 1999) can also influence sex determination. It is possible to produce batches of XX fish, which should be all female, through gynogenesis (Penman et al., 1987) or crosses between neomales (hormonally masculinised genetic females, XX) and normal females (Jalabert et al., 1974), and also batches of XY fish, which should be all male, by crossing normal females to YY males (Scott et al., 1989). The

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profiles of sex steroids during early development have been studied (Rothbard et al., 1987; Hines et al., 1999) and the period of sex differentiation, on the basis of histological observation, has been proposed (Nakamura and Nagahama, 1985; Nakamura and Nagahama, 1989). The cDNA sequence of the ovarian aromatase gene is also available.(Chang et al., 1997).

The first detectable signs of sexual differentiation in this species on the basis of histological observations are known to be around 26–30 days post fertilisation (dpf) (Nakamura and Nagahama, 1985; Nakamura and Nagahama, 1989) and the sexually labile period lasts from first feeding (11 dpf) to 35 dpf (Pandian and Sheela, 1995) on the basis of the results from steroid hormone treatments. Gale et al. (1999) successfully masculinised tilapia fry by immersing them in 500 μg/l 17α-methyldihydrotestosterone (MDHT) solution for 3 hr at 10 and 13 dpf. Furthermore, when XX tilapia fry were treated with an aromatase inhibitor (AI) at different times during the proposed labile period, the group treated during the first week from first feeding (11–17 dpf) was most sensitive in terms of masculinisation (Kwon et al., 2000a) The sex reversal results imply that the actual process of sex differentiation in this species could begin earlier than suggested by the histological data. Further understanding of the labile period is necessary to study the exact mechanism of sex determination/differentiation.

The cytochrome (CYP19) P450 ovarian aromatase gene was the only known gene that codes for aromatase in fish until recently. However, brain type isoforms of aromatase gene have now been identified in goldfish *Carassius auratus* (Tchoudakova and Callard, 1998) and the Mozambique tilapia, *Oreochromis mossambicus* (Cruz and Canario, 2000). Tchoudakova and Callard (1998) proved that eukaryotic cells (COS 7 African green monkey kidney cells) transfected with goldfish brain or ovarian type aromatase gene constructs catalysed androgens to oestrogens, suggesting that there is little or no functional difference between the two isoforms in terms of aromatisation. It is well known that ovarian aromatase is involved in the reproductive cycle, especially in vitellogenesis (Nagahama et al., 1994). Brain aromatase in goldfish is also related to the reproductive cycle, showing seasonality and cyclicity in its expression (Gelinas et al., 1998), implying that both ovarian and brain aromatases are related to the steroidal events involved in reproduction. In the same context, it could be postulated that both brain and ovarian aromatase genes might be involved in the steroidal events in sexual differentiation. Prior to the present study no brain type aromatase gene had been identified in *O. niloticus*.

In this study, brain type aromatase gene in *O. niloticus* was identified and a partial cDNA sequence of this gene was determined. Tissue specific expression of both brain and ovarian aromatase gene in adult fish was studied. Despite much evidence that fish embryos at very early developmental stages respond to AI and steroid hormones resulting in sex-reversal (Piferrer et al., 1994; Gale et al., 1999; Kwon et al., 2000a), no attempts to investigate aromatase gene expression during these early stages had been made prior to this study. For the first time, expression of both brain and ovarian aromatase genes was investigated from fertilisation through the key gonadal differentiation period and beyond in genetic male (XY group) and female (XX group) Nile tilapia fry, using semi-quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

MATERIALS AND METHODS

Animals

Adult males and females (for each sex, n = 10) were used for cDNA library construction and Northern analysis. The brain and gonads were dissected from freshly killed animals, immediately frozen in liquid nitrogen and kept until use. For tissue specific expression, a further three adult males and three adult females were used. The brain, gonads, eyes, kidney, liver, and spleen from each fish were dissected out and processed in the same way as above.

For the study on ontogeny of differential aromatase gene expression between the sexes, all male (XY) and female (XX) groups of tilapia fry were produced by splitting a batch of eggs from a normal XX female and fertilising one-half with sperm from a YY male and the other half with sperm from an XX neomale, respectively. Eggs were manually stripped from ovulated females. Milt was collected into glass capillary tubes and used directly for fertilisation. After fertilisation, eggs were placed into a 1 L incubation jar. Samples were taken every day from fertilisation up to 10 dpf to examine the initiation of both brain and ovarian aromatase mRNA expression, and every 4 days from 11 to 43 dpf. The sampling processes were essentially the same as described above. Fish were weighed when they were sampled. After the sampling period, the remaining fish were grown on until they were sexed by the gonad squashing method (Guerrero and Shelton, 1974).

RNA Extraction

Total RNA was extracted using the method of Chomczynski and Sacchi (1987). Liver and egg samples were additionally treated with 4 M lithium chloride to remove glycogen from the pellets after the isopropanol precipitation (Puissant and Houdebine, 1990). The concentration of extracted RNA was estimated from the OD 260 value measured by a spectrophotometer. The quality was checked by the OD 260/280 ratio and electrophoresis. Total RNA was used for Northern analysis, reverse transcription and further extraction of mRNA.

PCR Cloning of Brain Aromatase cDNA

Poly(A)$^+$ RNA was purified from total RNA of brain tissue using an oligotex mRNA Mini Kit (QIAGEN). A brain cDNA library of *O. niloticus* was constructed from this Poly(A)$^+$ RNA using ZAP-cDNA Synthesis Kit (Stratagene).

TABLE 1. Primers Used for PCR Cloning of the Brain Aromatase Gene of *O. niloticus*. Size of PCR Products: 123bp (BF1→BR1), 218bp (BF2→BR2), 657bp (BF1→BR2), and 901bp (BF3→BR1)

Primers	Sequence (5′→3′)
Forward primer 1 (BF1)	GACTTTGCAACAGAGCTCAT
Forward primer 2 (BF2)	CCCAGTCGTTACTTCCAGCC
Forward primer 3 (BF3)	TAGAGCGTCAGAAGTCACTG
Reverse primer 1 (BR1)	AGCATGAAGAAGAGGCTGAT
Reverse primer 2 (BR2)	TCGTGGGATGAAGCGCATGG
Reverse primer 3 (BR3)	ACACTTCAGAGGACCTGCTC
Reverse primer 4 (BR4)	ATGAGCTCTGTTGCAAAGTC

DNA was purified from an aliquot of the cDNA library by proteinase K treatment and phenol–chloroform extraction, and used for PCR cloning. Three forward primers and four reverse primers (Table 1) were designed based on the brain aromatase cDNA sequence of the Mozambique tilapia, *O. mossambicus* (Cruz and Canario, 2000; GenBank accession number: AF135850). PCR was carried out using these primers and two universal primers, T7 and T3. The PCR conditions were 1 cycle at 94°C for 2 min, 60°C for 1 min, 72°C for 1 min; 40 cycles at 94°C for 1 min, 60°C for 30 seconds, 72°C for 1 min; and 1 cycle at 72°C for 6 min. The PCR products were digested by *Xho*I or *Eco*RI. Digested products were separated on low melting point agarose gels. Target fragments were purified from the gel using a glass fibre matrix column (Gel purification kit, Amersham Pharmacia Biotech, Inc.) and subcloned into T vector, modified from pBluescript II SK(+) by digestion with *Eco*RV to blunt the ends of the plasmid and adding dTTP to produce 3′ overhanging ends. Transformation of these ligates was performed using Epicurian Coli XL-Gold Ultracompetent cells (Stratagene) following the manufacturer's protocols. Transformed cells were plated on ampicillin agar medium with IPTG (isopropyl-1-thio-β-D-galactopyranoside) and X-gal (5-bromo-4-chloro-3-inodlyl-β-D-galactopyranoside) for blue/white color screening. White colonies were picked and screened by PCR with gene specific primers. Positive clones were sequenced using an ABI automated sequencer based on the cycle sequencing method (Applied Biosystems). A multiple sequence alignment program (ClustalW) was used to compare the deduced amino acid sequence from *O. niloticus* brain aromatase with *O. niloticus* ovarian aromatase and aromatases from other species.

Northern Analysis

Both brain and ovarian total RNA (10 μg each) were resuspended in 10 μl GFM buffer containing glyoxal, formamide, and MOPS, and incubated at 55°C for 15 min. After mixing with 1 μl of 10X loading buffer, these RNA samples were run on a 1% neutral gel in 1X MOPS buffer with a size marker alongside. The gel was then soaked for 30 min in 20X SSC. The RNA was transferred from the gel onto a positively charged nylon membrane (Hybond, Boehringer Mannheim) using the capillary transfer method in 20X SSC overnight. After transfer, the RNA on the membrane was immobilised by baking the membrane for 1 hr at 80°C.

The membrane was hybridised overnight at 68°C in 20 ml of Church–Gilbert solution (Church and Gilbert, 1984) with a brain aromatase cDNA probe. The probe (657bp) was generated by PCR using primer BF1 and BR2, and labeled with α-^{32}P dCTP by nick translation (Nick Translation System, Promega). The membrane was washed three times over 2 hr at 68°C in 0.1X SSC/0.1% SDS and subjected to autoradiography to visualise hybridisation signals.

The size of the brain aromatase RNA transcript was estimated by comparison of the distance between the gel well and the hybridisation signal on the X-ray film with the RNA size markers on the original gel (Sigma, 0.2–10 kb).

RT-PCR Analysis for Tissue Specific Expression of Aromatase mRNA

Total RNA isolated from the tissues of adult brain, kidney, eye, liver, spleen, and gonads was treated with RNAse free DNAse I (Boehringer Mannheim). Each RNA aliquot (5 μg) was reverse transcribed using MMLV-RT (Promega) following the standard procedure. The resultant cDNA (1 μl) was used as a template for subsequent PCR.

Several sets of ovarian aromatase gene primers were designed based on the *O. niloticus* ovarian aromatase cDNA sequence (Chang et al., 1997). After testing all of these primers, one pair (forward primer, OF: 5′-CTGAGAATGTGACGCAGTGC-3′; reverse primer, OR: 5′-CAGCAGTGTCACCAAAATGG-3′) was chosen for the gene expression experiment. The PCR product from these primers was 490bp in length, and subsequent sequencing revealed that the product corresponded to the reported *O. niloticus* ovarian aromatase cDNA sequence. As a control, actin primers (forward primer, AF: 5′-AATCGTGCGTGACATCAAGG-3′; reverse primer, AR: 5′-AGTATTTACGCTCAGGTGGG-3′; product size 392bp) were designed based on the actin cDNA sequence of a closely related species, *O. mossambicus* (GeneBank database accession number: Y18689). For the brain aromatase gene, primers BF1 and BR2 were used (Table 1). PCR was carried out according to the conditions described above. PCR products were run in a 1% agarose gel in 1X TBE buffer. PCR products (3 μl each) for the brain aromatase gene and the actin gene were loaded together into one lane for each tissue. In the same way, PCR products (3 μl each) for the ovarian aromatase gene and the actin gene were also loaded together into one lane for each tissue. PCR products were visualised on a UV transilluminator (Ultra-Violet Products).

Differential Expression of Brain and Ovarian Aromatase Genes During Ontogeny

To assay the aromatase expression levels between the sexes and developmental stages, a semi-quantita-

tive RT-PCR method was established. Total RNA, isolated from the whole body of male and female groups collected throughout ontogeny, was treated with RNAse free DNAse I (Boehringer Mannheim). Each RNA aliquot (5 μg) was reverse transcribed using oligo $d(T)_{15}$ following the standard procedure. The resultant cDNA was purified using a DNA purification kit (Boehringer Mannheim) to remove enzymes, unincorporated dNTP and oligo d(T) primer.

The purified cDNA (0.5 μl of each sample) was mixed with 3 μl of 0.05 μg/ml EtBr and spotted on the surface of a transparent disposable Petri dish to estimate the concentration of each sample. The Petri dish was inverted and exposed to a UV transilluminator. One cDNA sample was chosen as a standard. Using this cDNA, PCR was optimised to achieve a semi-quantitative response. The standard cDNA was subjected to serial dilution (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, and 1:2048). Using these dilutions as templates, a series of PCRs was conducted with different numbers of PCR cycles (25, 30, 35, or 40 cycles) and with different annealing temperatures (55, 58, 60, or 63°C) for different gene-specific primers. Through these PCRs, the range of cDNA concentrations that responded quantitatively was determined. The concentrations of all cDNA samples were adjusted by diluting or concentrating (using a mechanical concentrator) until the sample concentration fell into the detection range. The optimal number of PCR cycles was determined as follows: 25 cycles for the actin gene, 35 cycles for the brain and ovarian aromatase genes. The optimal annealing temperature was determined as 60°C for all three genes.

PCR was carried out for all samples (XX and XY groups at 4, 6, 8, 11, 15, 19, 23, 27, 31, 33, 37, 39, and 43 dpf) with the different gene primers (in triplicate). PCR products were run on 1% agarose gels in 1X TBE buffer with EtBr. The gel was briefly exposed to UV light to check the presence of PCR products and was then subjected to Southern hybridisation. The DNA on the gel was first denatured by soaking the gel for 45 min in 1.5 M NaCl/0.5 N NaOH with gentle agitation on a mechanical rotator and then neutralised by soaking for 45 min in 1M Tris (pH 7.4)/1.5 M NaCl at room temperature. The DNA was transferred onto a nylon membrane in 10X SSC overnight using the capillary transfer method. The membrane was dried and baked for 1 hr at 80°C.

The membranes were hybridised at 68°C in 20 ml of Church-Gilbert solution with brain aromatase, ovarian aromatase, or actin probes in separate hybridisation chambers overnight. Each probe was generated by PCR using primer BF1 and BR2 for brain aromatase, OF and OR for ovarian aromatase and AF and AR for actin, and labelled with α-^{32}P dCTP by nick translation (Promega). The membrane was washed three times over 2 hr at 68°C in 0.1X SSC/0.1% SDS and subjected to autoradiography to visualise hybridisation signals.

Hybridisation signals were digitised using an image analysis system (Image-Pro PLUS, Media Cybernetics). The relative expression level of aromatase genes to that of actin was calculated and used to compare the level between sexes and different developmental stages. The data were shown as mean±SEM. Statistical differences of relative aromatase mRNA expression to actin mRNA between the sexes were determined by *t*-test ($P < 0.05$).

RESULTS

Identification of the Brain Aromatase Gene

A partial brain aromatase cDNA (1707bp) was isolated from the brain cDNA library of tilapia *O. niloticus* using primers based on the *O. mossambicus* brain aromatase cDNA sequence (Fig. 1). This cDNA sequence showed 97.0% identity to the *O. mossambicus* brain aromatase cDNA sequence and 67.9% identity to the *O. niloticus* ovarian aromatase cDNA sequence. The 5′ end of the *O. niloticus* brain aromatase cDNA was not successfully sequenced.

The identified sequence included a 1444bp open reading frame (ORF) (part of exon 1 and exon 2–9) and a 263bp 3′end untranslated region (3′UTR). The amino acid sequence for the ORF derived from this partial *O. niloticus* brain aromatase cDNA sequence showed 96.7% identity with the amino acid sequence of the corresponding region of *O. mossambicus* and 69.9% with that of goldfish brain aromatase (Fig. 2). This partial sequence contains an I-helix region, an Ozol's peptide region, an aromatase specific conserved region, and a heme-binding region. These regions showed high homology between different aromatase genes, whereas the termini of these aromatase genes showed very low homology.

Identities between brain type aromatases of different animals are higher than those between brain type and ovarian type aromatases within a species. To illustrate the phylogenetic relationships of *O. niloticus* brain aromatase and other reported aromatases, an evolutionary tree was constructed using the Neighbor-Joining method of Clustal X (Fig. 3). Fish brain aromatases clustered separately from fish ovarian aromatases and also from human and chicken aromatases. By Northern hybridisation with the brain aromatase probe, one single clear transcript was detected in brain but only very faintly in ovary (data not shown). The full-length size of the *O. niloticus* brain aromatase transcript was estimated to be 2.66 kb.

Tissue Specific Expression of Aromatase Genes

Tissue specific expression of both brain and ovarian aromatase mRNA was investigated in various adult tissues using RT-PCR (Fig. 4). Brain aromatase mRNA was expressed at high levels in both sexes in the brain and less so in the kidney, eye, ovary, and testis, but could not be detected in the liver or spleen. In contrast, ovarian aromatase mRNA was expressed strongly in the ovary and less so in the brain, spleen, and testis, but could not be detected in the eye, kidney, or liver. Actin mRNA, used as a control for gene expression, was

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1  TAGAGCGTCAGAAGTCACTGCTGTCCTGCTTCTTCTGCTGCTGTTGTTGCTGCTTTTCAC
2  CACCTGGAGACAAAGAAAACAGTCACACATACCAGGTCCTTTCTTCTTAGCAGGACTCGG
3  TCCAATTCTCTCCTACAGCAGATTCATCTGGTCTGGGATTGGAACAGCGTGTAACTACTA
4  CAACAACAAATATGGGAGCATTGTGCGGGTGTGGATAAACGGAGAGGAGACCCTGATTTT
5  GAGCAGGTCCTCTGAAGTGTACCACGTTTTGAGGAGTGCCCACTACACCTCCAGATTTGG
6  CAGCAAAAAAGGACTCGAGTGCATCGGCATGTACGGAAATGGTATCATTTTCAACAGTGA
7  TGTCCTGCTTTGGAAAAAAGTGAGAACATACTTTTCTAAAGCTCTGACTGGACCCGGCCT
8  GCAGAGGACCGTAGGAATCTGTGTGAGCTCCACAGCCAAACACCTGGACAACTTACAGGA
9  CATGACTGACCCCTCTGGACATGTAGATGCTCTCAATCTCCTGAGAGCCATCGTGTTGGA
10 CATCTCCAACCGGCTGTTCCTCAGAGTGCCGTTAAATGAGAAAGACTTCTTGACGAAAAT
11 TCACAACTACTTTGATACCTGGCAAACAGTTCTAATAAAACCAGATATATTCTTCAAGGT
12 TGGATGGCTGTACAACAAGCATAAGAGAGCAGCACAGGAGCTGCAAGATGCAATGGAGAG
13 CCTGCTTGAAGTTAAGAGAAAGATGATTCATGAAGCCGAGAAGCTGGACGACGAGCTCGA
14 CTTTGCAACAGAGCTCATCTTCGCCCAGAACCACGGAGAGCTATCAGCAGATAACGTCAG
15 GCAGTGTGTGCTAGAGATGGTGATCGCAGCCCCTGACACACTTTCCATCAGCCTCTTCTT
16 CATGCCGATGCTGCTGAAACAGAACCCGGACATAGAGCTGCAGCTAGTGGAGGAGATGAA
17 CACCATCTTGAATGAAAAAGACGTGGAAAATATCGATTACCAAAGCCTGAAGGTGATGGA
18 GAGCTTCATCAACGAGTCTTTGAGGTTTCATCCTGTGGTCGGTTTCACAATGAGGAAAGC
19 TCTGGAGGACAACGACATCGCAGGCACAAAAATCAAGAAGGGCACCAACATCATTCTCAA
20 CACTGGCCTCATGCACAAAACCGAATTCTTCCCCAAACCTGAAGAGTTCAACCACACGAA
21 CTTTGAAAAAACGGTACCCAATCGTTACTTCCAGCCCTTTGGCTGCGGGCCTCGTTCCTG
22 TGTGGGCAAACACATCGCCATGGTGATGATGAAGGCCATCCTGGTCACTCTCCTGTCTCG
23 GTACACTGTGTGTCCTCATCAAGGCTGCACACTCAGCAGCATCAAGCAGACCAACAACCT
24 GTCACAGCAGCCGGTGGAAGACGAGCACAGCCTGGCCATGCGCTTCATCCCACGAACGAA
25 ATAACCCCACGACAACAAGCTGCGAAACATAACAGCGCTTTTTACACTTCAGATGAAAGC
26 ATCTTGAAATGGGTGTAAAGGCCTTAATGAAGTCCTGTACTAGGAAATACTAACAGTAAA
27 TTTGTATTAACAGCACATGTACGCCAAATTCAACTTTACTAATCTTTAAAAAAATACACT
28 TGTTAAGTAGAGTTTTCTGATTCATGTTTTGACAATGTAAACAGCTCATACTTTTAATAA
29 AGATCCATTTGCAAAAAAAAAAAAAAA
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Fig. 1. Partial nucleotide sequence of tilapia *O. niloticus* brain P450 aromatase cDNA. A stop codon and a polyadenylation signal are underlined. The nucleotide sequence data and predicted amino acid sequence (Fig. 2) can be found in the GenBank database with Accession No. AF306786.

expressed from all tissues investigated. No clear sexual differences were found in the pattern of either aromatase gene expression in adult tissues except for the gonads. Only the ovarian aromatase showed a consistent difference between the sexes in expression levels in the gonads. Brain aromatase expression in kidney and ovarian aromatase gene expression in spleen appeared to be sexually dimorphic in some samples, but the pattern was not consistent in other males and females studied.

Differential Expression of Brain and Ovarian Aromatase Genes During Ontogeny

Brain and ovarian aromatase gene expression was studied by semi-quantitative RT-PCR analysis in genetic male and female groups of *O. niloticus* from fertilisation to 43 dpf by which time sex is histologically discernible. The final sex ratio of the sampled groups showed 84.4% females in the genetic female group (XX) and 100% males in the genetic male group (XY).

The initiation of expression of both brain and ovarian aromatase mRNA lay between 3 and 4 dpf both in males and females (Fig. 5 and 6). Aromatase mRNA expression (both brain and ovarian type) was weak but detectable at 3 dpf and 40 cycles, but not at 35 cycles. However, from 4 dpf, it was expressed consistently within the detection range of this study. There were no significant sexual differences of brain or ovarian type aromatase gene expression levels up to 11 dpf.

The level of brain aromatase mRNA gradually increased through the period studied here with little difference between the sexes. No significant differences were found although the level in females was almost always higher than that in males (Fig. 5A and B). On the contrary, the change of ovarian aromatase mRNA expression through ontogeny was rather marked (Fig. 6A and B). While the expression level in females continued to increase from 11 to 31 dpf, the expression in males showed down-regulation from 11 dpf, resulting in significant differences between the sexes. The expression level in the males continued to drop through 15 and 19 dpf, and then showed some recovery at around 27 dpf. After that, the level of ovarian aromatase mRNA expression was fairly constant with some fluctuation in both sexes but the expression in females was always significantly higher than in males ($P < 0.05$).

Sequencing of the cDNA produced by the "ovarian" primers in the present study showed that this overlapped a homologous region of the brain aromatase cDNA but was clearly different from this and identical to the previously published ovarian sequence (Chang et al., 1997).

DISCUSSION

Brain Type Aromatase Gene

The partial cDNA sequence of brain type aromatase identified in this study was different from that of the

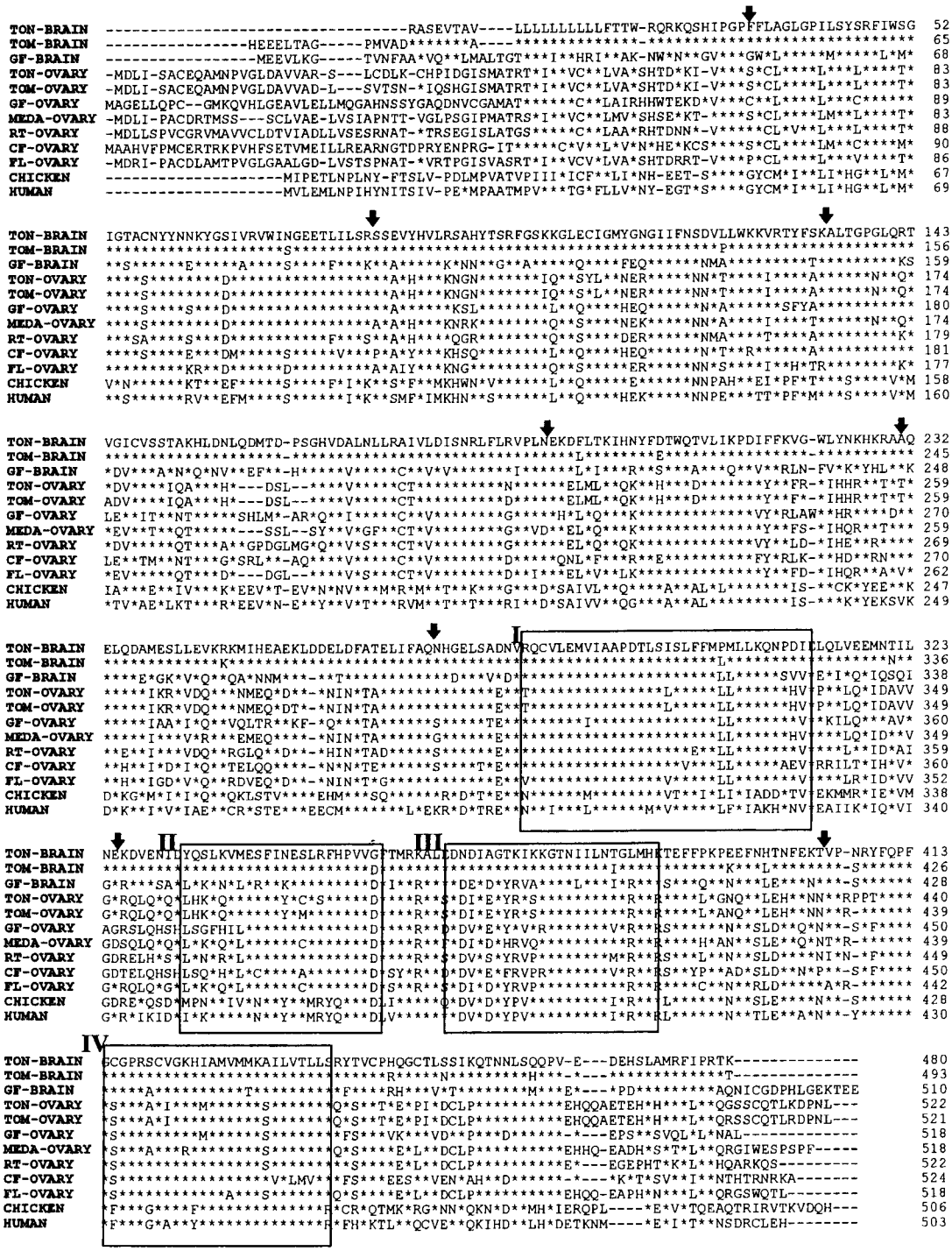

Fig. 2. Alignment of *O. niloticus* brain P450 aromatase amino acid sequences (deduced from the partial cDNA sequence) with sequences of the brain and ovarian P450 aromatase from other animals. CF: catfish *I. punctatus*, FL: flounder *P. olivaceus*; GF: goldfish *C. auratus*; MEDA: medaka *O. latipes*; RT: rainbow trout *O. mykiss*; TOM: Mozambique tilapia *O. mossabicus*, TON: Nile tilapia *O. niloticus* (references for each animal are in the text). The amino acid sequences that are identical to that of TON-Brain are marked by an *. The regions within boxes indicate an I-helix region (I), an Ozol's peptide region (II), an aromatase specific conserved region (III) and a heme-binding region (IV), respectively. Putative exon-intron boundaries are indicated by arrows.

ovarian type aromatase identified previously (Chang et al., 1997) in the Nile tilapia. In fish, aromatase genes have been cloned from rainbow trout ovary (Tanaka et al., 1992), catfish ovary (Trant, 1994), medaka ovary (Tanaka et al., 1995), *O. niloticus* ovary (Chang et al., 1997), goldfish ovary (Tchoudakova and Callard, 1998), goldfish brain (Gelinas et al., 1998), flounder ovary (Kitano et al., 1999) and *O. mossambicus* ovary and

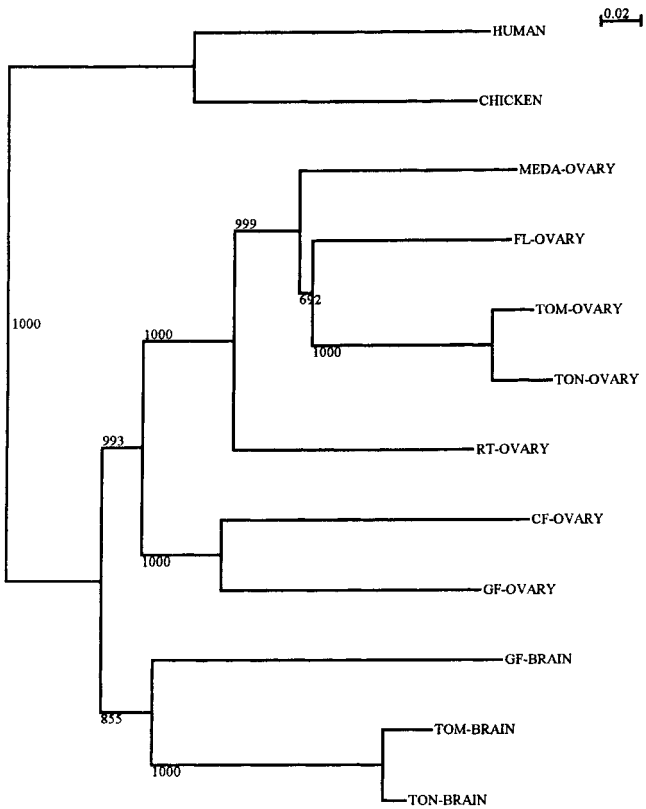

Fig. 3. Phylogenetic tree of P450 aromatase proteins. The tree was constructed using Clustal W multiple sequence alignment program and deduced amino acid sequences of P450 aromatase forms in chicken ovary, CF (catfish *I. punctatus*) ovary, FL (flounder *P. olivaceus*) ovary, GF (goldfish *C. auratus*) brain and ovary, human placenta, MEDA (medaka *O. latipes*) ovary, RT (rainbow trout *O. mykiss*) ovary, TOM (tilapia *O. mossabicus*) brain and ovary, and TON (tilapia *O. niloticus*) brain and ovary (for references see the text). The numbers at the branch points indicate the number of times that this branch was observed out of 1,000 times bootstrap clustering.

brain (Cruz and Canario, 2000). Alignment of the newly identified *O. niloticus* brain aromatase amino acid sequence to these previously reported aromatases in fish shows brain type aromatase genes are more closely related to each other in different fish species than to ovarian aromatase genes from any fish species. This

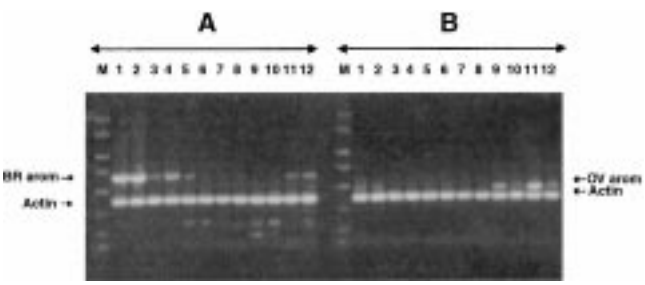

Fig. 4. Tissue specific aromatase gene expression in various tissues of adult *O. niloticus*. **(A)** brain aromatase product (BR arom) and actin product (Actin) (3μl each) were loaded together (odd numbers and even numbers indicate female and male, respectively). Tissues are brain, kidney, eye, liver, spleen, and gonads (from left to right); **(B)** ovarian aromatase (OV arom) and actin gene product, in the same order as for the brain aromatase and actin samples. M: size marker (50–2,000bp).

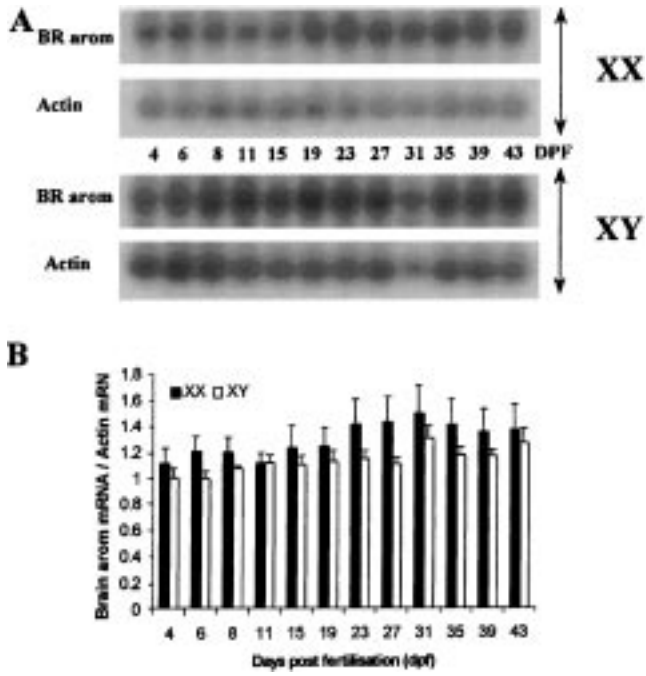

Fig. 5. Differential expression of brain aromatase mRNA (BR arom) during ontogeny in male and female groups. **(A)** RT-PCR products were hybridised with gene specific probes and detected by autoradiography (representative of three RT-PCRs); **(B)** Relative expression of brain aromatase mRNA to actin mRNA (Actin). Each bar represents the mean ± SEM

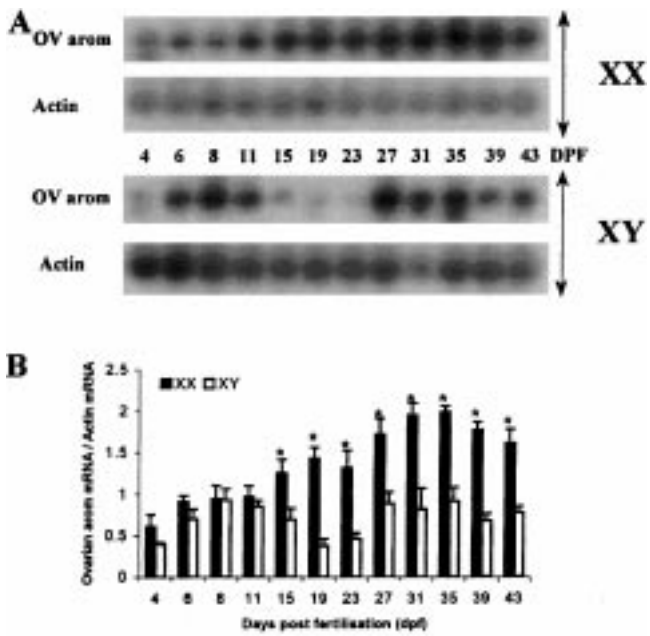

Fig. 6. Differential expression of ovarian aromatase mRNA (OV arom) during ontogeny in male and female groups. **(A)** RT-PCR products were hybridised with gene specific probes and detected by autoradiography (representative of three RT-PCRs); **(B)** Relative expression of ovarian aromatase mRNA to actin mRNA (Actin). Each bar represents the mean ± SEM *indicates significant difference between the sexes ($P < 0.05$).

strongly suggests that brain type aromatase genes differ from ovarian aromatase genes at least in fish. In addition, both types of aromatase genes in fish clustered separately from human and chicken aromatase genes in a phylogenetic tree. This suggests that, during evolution, fish brain aromatase branched out from ovarian aromatase (or vice versa) after the divergence of bony fishes from the line of mammals and birds.

The size of the *O. niloticus* brain aromatase transcript was estimated as 2.6–2.7 kb by Northern analysis. This is similar to those from *O. niloticus* ovary (2.6 kb, Chang et al., 1997), rainbow trout ovary (2.6 kb, Tanaka et al., 1992) and medaka ovary (2.6 kb, Tanaka et al., 1995), but much shorter than those from catfish ovary (3.4 kb, Trant, 1994) and goldfish brain (3.0 kb, Gelinas et al., 1998). There thus appears to be no great size difference between brain and ovarian aromatase mRNA in *O. niloticus*.

Tissue Specific Aromatase Gene Expression

The presence of aromatase activity is considered as an indicator of oestrogen synthesis (Norris, 1997). Aromatase activity is correlated with the expression level of aromatase mRNA (Chang et al., 1997; Gelinas et al., 1998). Thus, expression of aromatase mRNA should indicate oestrogenic activity in animal tissues. Expression of aromatase genes in vertebrates has been observed in a variety of cells and tissues including the ovary, testis (Silberzahn et al., 1988), placenta (Kellis and Vickery, 1987), brain and pituitary (Pasmanik and Callard, 1989), adipose tissue (Lueprasitsakul and Longcope, 1991), central nervous system (Gelinas and Callard, 1993), and skin fibroblasts (Stillman et al., 1991). The results from the present study suggest some other putative steroidogenic (oestrogenic) tissues based on aromatase gene expression.

Tetrapod vertebrates possess a discrete adrenal gland that is known to have steroidogenic activity, mainly producing adrenocorticosteroids, and the kidney of these animals is known to be non-steroidogenic. In teleosts, however, the adrenal gland resides within the kidney (Milano et al., 1997) and hence steroidogenic cells are found in the kidney of teleosts. Androgen and oestrogen synthesis was detected in *O. niloticus* kidneys (Watts et al., 1995). A steroidogenic enzyme, 3β-hydroxysteroid dehydrogenase (3β-HSD) was also detected in the head kidney of rainbow trout by immunohistochemical method (Kobayashi et al., 1996). In spite of this evidence for steroidogenic activity, aromatase mRNA had not been detected in fish kidneys before the present study (catfish: Trant et al., 1997; flounder: Kitano et al., 1999). Trant et al. (1997) speculated that multiple or different transcripts of aromatase are present in nongonadal tissues in fish. In the present study, ovarian aromatase mRNA was not detected in the kidney, but brain type aromatase mRNA was consistently expressed in the kidney in all males and females. Considering that both brain and ovarian forms of aromatase convert androgen to oestrogen (Tchoudakova and Callard, 1998), it can be suggested that the brain type aromatase gene is responsible for the previously reported oestrogenic activity in teleost kidneys. However, the physiological role of the oestrogen that is formed in fish kidney remains unknown.

The spleen has not generally been considered as a major steroidogenic tissue. Human fetal spleen was the only spleen known to express aromatase in vertebrates until recently (Price et al., 1992). However, the spleen of *O. niloticus* in this study showed strong expression of the ovarian aromatase gene. The expression of the ovarian aromatase gene in fish spleen has also been reported in the Japanese flounder *P. olivaceus* (Kitano et al., 1999). Possible steroidogenic activity was also suggested in mouse spleen (Morohashi et al., 1999). SF1 (also called Ad4BP) was originally identified as a steroidogenic, tissue-specific transcription factor. Morohashi et al. (1999) detected SF1 immunoreactive cells in the spleen of mouse. In their study, the presence of an mRNA encoding cholesterol side-chain cleavage P450 was also detected in mouse spleen. All these results above indicate that the spleen in vertebrates is capable of producing steroid hormones from precursors, although at the present time there is no explanation available for the function of locally produced steroids in the spleen.

The liver is generally considered as a non-steroidogenic tissue in vertebrates, although aromatase mRNA was detected in human fetal and adult liver (Harada et al., 1993; Toda et al., 1994; also see review by Simpson et al., 1994). In fish, no ovarian aromatase mRNA has been detected so far in the liver (catfish, Trant et al., 1997; goldfish, Gelinas et al., 1998; flounder, Kitano et al., 1999). In the present study, neither brain type nor ovarian type aromatase mRNA was detected in the liver of *O. niloticus*. Thus, it seems unlikely that teleost liver has oestrogenic activity, unless there is another tissue specific isoform of aromatase gene in fish liver.

In this study, low but consistent expression of brain aromatase mRNA and very weak and inconsistent expression of ovarian aromatase mRNA were detected in the eyes of tilapia. Similarly, Tchoudakova and Callard (1998) reported expression of both brain and ovarian type aromatase mRNA in goldfish retina. However, the pattern of expression of the two different genes was reversed. In goldfish, expression of ovarian aromatase in the retina was 25-fold higher than that of brain aromatase. Direct comparison would not be appropriate since the present study was not quantitative and also the sampling methods may have been different.

As expected, brain and ovarian aromatase mRNA were most abundant in the brain and in the ovary, respectively. Brain aromatase mRNA was expressed in both ovary and testis and ovarian aromatase mRNA was expressed in both male and female brains. Tchoudakova and Callard (1998) reported that ovarian aromatase was expressed in goldfish brain, but brain aromatase was not expressed in goldfish ovary. In this study, brain aromatase gene was hardly detectable by Northern analysis, but it was repeatedly detected by

RT-PCR from individual to individual in all ovaries and testes examined in *O. niloticus*. The expression of ovarian aromatase in the brain is in general agreement with all previous studies (Trant et al., 1997; Tchoudakova and Callard 1998; Kitano et al., 1999).

Differential Expression of Brain and Ovarian Aromatase Genes During Ontogeny

The significance of up-regulation of aromatase during sexual differentiation has previously been proposed in three fish species. In *O. niloticus*, Guiguen et al. (1999) showed that aromatase activity in the gonads of a control group (i.e., all female group produced by crossing an XX neomale to a normal female) was much higher at 64 dpf than that of a masculinised XX group induced by dietary administration of a steroidal AI (ATD, 1, 4, 6-androstatriene-3-17-dione). However, at this time, the higher aromatase activity in the female group is likely to be the result of gonadal differentiation rather than the cause, as it has been shown that phenotypic sex in this species is determined earlier (between 27–30 dpf based on histological observation: Nakamura and Nagahama, 1985, 1989).

Differential expression of the ovarian aromatase gene between females and temperature-induced sex-reversed (i.e., XX) males during sexual differentiation was also proposed in the Japanese flounder, *P. olivaceus* (Kitano et al., 1999). There were no differences in the expression of the aromatase gene between indifferent gonads of females and sex-reversed males. However, "after the initiation of sex differentiation," the level of aromatase gene expression increased rapidly in female flounder gonads.

In rainbow trout, aromatase gene expression was observed at 55 dpf (= 20 dph) two weeks before the first occurrence of the early oocyte meiosis (70 dpf = 35 dph) in the developing ovary (Guiguen et al., 1999). Aromatase expression in female gonads was at least a few hundred times higher than in male gonads during the period studied (55–127 dpf at 10°C: 550–1,270 degree days) in their experiment. However, the previously proposed sex differentiation period in this species (45–55 dpf at 8–10°C: 360–440 or 450–550 degree days) (Takashima et al., 1980; van den Hurk and Slof, 1981) was earlier than the period studied by Guiguen et al. (1999).

These authors' findings appear to suggest that ovarian aromatase plays an important role in maintaining or accelerating ovarian differentiation after the initial sex differentiation. If differential aromatase gene expression in the presumptive gonadal area is to be an initiating factor in sexual differentiation, it should take place before evidence of gonadal differentiation can be seen. One should also answer the question: how can a short immersion treatment by steroids in the very early stages (around hatching or first feeding) cause permanent sex change when the gonadal tissues are not discernible and when no steroid producing cells can be identified in the presumptive gonadal area?

The most distinctive sexual dimorphism of ovarian aromatase gene expression in the present study occurred from 11–27 dpf, compared to 27–30 dpf for the first appearance of steroid producing cells in the gonads (Nakamura and Nagahama, 1985, 1989). It can be suggested that the observed down-regulation of the ovarian aromatase gene in males is a critical step in the determination of phenotypic sex in this species. Results from AI treatment also support this suggestion. When fish fry were fed with AI treated food for the first week of exogenous feeding (11–17 dpf) (Kwon et al., 2000a), or immersed once in a solution containing AI for 3 hr at 13 dpf (Kwon, 2000), the percentage of males was significantly increased. Feeding with AI during the period 18–24 dpf also resulted in some masculinising effect, but later than 24 dpf, longer AI treatment was required to achieve a masculinising effect (Kwon et al., 2000a). Kitano et al. (2000) showed that treatment of genetically female *P. olivaceous* fry with AI or 17α-methyltestosterone (MT) resulted in masculinization and concomitant down-regulation of ovarian aromatase gene expression. This suggests that both natural male differentiation and artifical sex reversal induced by AI or MT are associated with down-regulation of the ovarian aromatase gene in fish.

The expression of the ovarian aromatase gene in females after this proposed critical period for gonadal differentiation (27–43 dpf) was also significantly higher than in males. This is consistent with the previous findings mentioned above, supporting the idea that aromatase may be a maintaining or accelerating factor for ovarian differentiation.

The sex of the fish in the sampled groups was confirmed by sexing the remaining fish in each group at the end of the experiment. The genetic male group (XY) was 100% male and the genetic female group was 84.4% female. Therefore, the dimorphic expression of ovarian aromatase gene from the examined groups can be considered as a true reflection of the ontogenic genetic difference between female (XX) and male (XY) fish. This supports the idea that sex is determined genetically in this species, although sex determining genes have not been identified yet. The present findings clearly suggest the involvement of the ovarian aromatase gene in the determination of phenotypic sex. The next step should be towards identifying the factors that regulate expression of this gene during ontogeny.

The decisive aromatisation site for sex differentiation still remains unknown. The initiation of both brain and ovarian aromatase gene expression was at around hatching (3–4 dpf in this species) in this study. Since the first sign of gonadal differentiation appears more than 20 days later (27–30 dpf) (Nakamura and Nagahama, 1985, 1989), the early aromatisation site seems unlikely to be the gonads or presumptive gonadal area. Based on the results on tissue specific expression in adults, the aromatisation site at this stage could be some other tissue, such as the brain, eye, kidney, or spleen. Among these tissues, only the brain consistently expressed both brain type and ovarian type

aromatase genes in this study. The brain has been suggested as the primordial site of aromatase expression in mammals together with the gonads (Simpson et al., 1994). Furthermore, the central nervous system in vertebrates is fully developed by the time 10–15% of embryonic life has passed (Laming, 1981). Thus, the brain emerges as a promising candidate tissue for early aromatase expression and activity. In lower vertebrates non-gonadal tissues may be responsible for early aromatase gene expression that might direct further sexual differentiation of other tissues including the gonads. In reptiles, steroidogenesis occurs in the adrenals, the mesonephros, and the liver, but little in the genital ridge and the gonads of very early embryos (White and Thomas, 1992). Jeyasuria and Place (1998) suspected the brain as a primary aromatisation site for sex determination in reptiles. They found the presence of aromatase mRNA in the brain prior to its presence in the presumptive ovary. Further studies for localisation of aromatase gene expression using in situ hybridization techniques during ontogeny would reveal the exact aromatisation site(s) during the early stages.

Unlike ovarian aromatase mRNA, brain aromatase mRNA was expressed highly both in males and females through the developmental stages without showing clear sexual dimorphism. Different patterns of tissue specific expression of brain and ovarian aromatase mRNA in this study implied possible functional differences between these two aromatases, although Tchoudakova and Callard (1998) did not find any functional difference between brain and ovarian aromatase genes in terms of catalysing androgens into oestrogens. In addition to the questions about the roles of the two different types of aromatases, there also remains the question of where the expression of these genes takes place during sexual differentiation. Whole body homogenates were used for this study, which did not allow the location of aromatase expression to be addressed, although the study on adult fish did reveal several tissues where aromatase was expressed.

In conclusion, the findings from this study clearly suggest that ovarian aromatase plays a decisive role in sex differentiation in tilapia *O. niloticus*, and that this decisive role is achieved by down-regulation of this gene in males at a time when expression continues to increase in females. The critical period for the influence of aromatase during sexual differentiation in this species appears to lie between 11 and 27 dpf. The ovarian aromatase gene also appears to have a maintaining or accelerating effect on ovarian differentiation. Additionally, the decisive aromatisation may take place in non-gonadal tissues such as the brain but this needs to be further clarified.

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