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Aromatase Plays a Key Role During Normal and Temperature-Induced Sex Differentiation of Tilapia *Oreochromis niloticus*

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ABSTRACT In the tilapia *Oreochromis niloticus*, sex is determined genetically (GSD), by temperature (TSD) or by temperature/genotype interactions. Functional masculinization can be achieved by applying high rearing temperatures during a critical period of sex differentiation. Estrogens play an important role in female differentiation of non-mammalian vertebrates. The involvement of aromatase, was assessed during the natural (genetic all-females and all-males at 27°C) and temperature-induced sex differentiation of tilapia (genetic all-females at 35°C). Gonads were dissected between 486–702 degree × days. Aromatase gene expression was analyzed by virtual northern and semi-quantitative RT-PCR revealing a strong expression during normal ovarian differentiation concomitant with high levels (465±137 fg/g) of oestradiol-17β (E2-17ß). This was encountered in gonads after the onset of ovarian differentiation (proliferation of both stromal and germ cells prior to ovarian meiosis). Genetic males exhibited lower levels of aromatase gene expression and E2-17ß quantities (71±23 fg/g). Aromatase enzyme activity in fry heads established a sexual dimorphism in the brain, with high activity in females (377.9 pmol/head/hr) and low activity in males (221.53 pmol/head/hr). Temperature induced the masculinization of genetic females to a different degree in each progeny, but in all cases repression of aromatase expression was encountered. Genetic males at 35°C also exhibited a repression of aromatase expression. Aromatase brain activity decreased by nearly three-fold in the temperature-masculinized females with also a reduction observed in genetic males at 35°C. This suggests that aromatase repression is required in the gonad (and perhaps in the brain) in order to drive differentiation towards testis development. *Mol. Reprod. Dev. 59: 265–276, 2001.* © 2001 Wiley-Liss, Inc.

Key Words: sex differentiation; fish; tilapia; estrogens; aromatase; gene expression

INTRODUCTION

Sex determination is considered to be essentially a decision between two alternate pathways, which ultimately leads to the development of either testes or ovaries. In many gonochoristic fish, sex is determined genetically (GSD), but the process appears to be very malleable since several factors, such as environment and exogenous hormones, can override GSD modifying the sex during differentiation (Baroiller et al., 1999). The tilapia fish *Oreochromis niloticus* displays predominantly a GSD with a male heterogamety (XY/XX) (Mair et al., 1991). Nevertheless, elevated temperatures (34–35°C) may induce male phenotypes when applied at a critical thermosensitive period of gonadal differentiation (Baroiller et al., 1995a). Progeny testing of offspring has demonstrated a genetic component in this thermosensitivity and consequently, sex in *O. niloticus* appears to be governed by temperature. However, it is also governed by a temperature/genotype interaction (Baroiller et al., 1995b; Baroiller and Clota, 1998).

Sex inversion can be readily performed with sex hormone treatments in most non-mammalian vertebrates (Hunter and Donaldson, 1983; Clinton and Haines, 1999; Pieau et al., 1999; Wallace et al., 1999). In fish, estrogens administered to undifferentiated larvae induce functional female phenotypes in genetic males (Hunter and Donaldson, 1983). Oestrogens are synthesized from androgens (either testosterone or androstenodione) through a reaction catalyzed by the cytochrome P450 aromatase. In tilapia, an elevated activity of this enzyme has been shown (Guiguen et al., 1999), and a positive staining was detected by using aromatase antibodies in the gonads assumed to be females (Nakamura et al., 1998). The use of aromatase inhibitors which block oestrogen synthesis, caused partial or complete inhibition of ovarian differentiation

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and resulted in a masculinization in a number of fish (Piferrer et al., 1994; Guiguen et al., 1999; Kwon et al., 2000). Furthermore, high expression of aromatase mRNA has been established in the developing ovary of rainbow trout but not in the testis (Guiguen et al., 1999). Together, these studies indicate that oestrogen levels in the developing gonad play a critical role in sex differentiation of fish, with high oestrogen synthesis promoting ovarian development.

At this stage it is difficult to generalize on the array of mechanisms underlying sex determination in non-mammalian vertebrates, particularly in fish. Despite this, it is plausible that temperature sex determination (TSD) follows, at some stage, a similar pathway to that of "normal" genetic sex differentiation. In reptiles displaying a TSD, such as turtles and crocodiles, oestrogen treatment actually mimics the effects obtained when eggs are incubated at female-producing temperatures (Crews et al., 1991; Dorizzi et al., 1991; Lance and Bogart, 1992). Moreover, a synergistic effect of feminising temperatures and oestradiol-17β was found (Wibbels et al., 1991; Bergeron et al., 1999). Like in fish, aromatase activity in several TSD reptiles was elevated during ovarian development (Desvages and Pieau, 1992; Smith and Joss, 1994), and aromatase inhibitors were capable of masculinizing gonads at female-producing temperatures (Dorizzi et al., 1994; Wibbels and Crews, 1994; Richard-Mercier et al., 1995). Analysis of aromatase mRNAs performed in terrapin turtle, detected levels initially in the brain which were comparable in both females and males. Subsequently, aromatase expression decreased in female brains whereas expression levels rose in ovaries (Jeyasuria and Place, 1998). Whether aromatase transcription in the brain affects oestradiol-17β levels in the gonad or vice versa is unclear. But expression profiles together with sex-reversal procedures reflect that in TSD species, oestrogens are important for female development and aromatase appears to be a critical step in this differentiating pathway.

Besides the intrinsic biological knowledge, the understanding of tilapia TSD mechanism is primarily based on the practical application of temperature as a means of controlling sex in the commercial production of tilapia. There is a several-fold benefit in generating all-male offspring because males have a better growth-rate than females and it would avoid the overcrowding due to the high reproductive performances of females. Impediments are slowly emerging on the usage of hormones as sex-control techniques, basically for health and ecological reasons, emphasising the importance of using temperature. In the current study we have investigated the role of aromatase in the normal and temperature-induced sex differentiation of tilapia. For this, gene expression and oestradiol-17β levels were analyzed in the gonad and enzyme activity measured in the brain. These analysis were correlated to a precise histological description of gonad differentiation.

MATERIALS AND METHODS

Animal Sampling and Treatments

Tilapia *Oreochromis niloticus* were from the Bouaké strain (Ivory Coast). Several progenies of genetic all-females displaying different degrees of thermosensitivity were produced, in view of the genotype-temperature interactions found in tilapia (Baroiller et al., 1995a, b) by mating XX males with different classic females (XX). The method of producing the XX males has been previously described (Baroiller, 1996). They were sired from a single androgen sex-inversed genetic female (XX) and masculinized by a 11β-OH androstenedione treatment. We also used genetic all-male populations sired from an oestrogen sex-inversed genetic male (XY) mated with a YY male (kindly provided by G.C. Mair). Production of YY males has been previously reported (Baroiller and Jalabert, 1989). Fish were fertilized at the CIRAD Centre (Montpellier, France) and at 9 days post-fertilization (PF), fry were transferred to the Aquaculture INRA facilities (Rennes, France) where treatment was applied.

Progenies were divided into two groups and exposed to either 27°C (control) or 35°C (masculinizing treatment) temperatures. Treatments were applied at 10 days PF [= 270 degree × days (DD)] and lasted for a period of 30 days. Sex ratios were determined by microscopic analysis (× 100 magnification) of gonadal squash after 90 days PF (= 2430 DD) on part of the populations (n = 100). Undifferentiated gonads were dissected under microscope between 486–702 DD, corresponding to 16–22 days PF for the 35°C fish and 18–26 days PF for the 27°C. On account of the limited material obtainable during these precocious stages, pool of gonads were selected according to population. For the RNA extraction, 400–690 undifferentiated gonads were dissected, frozen immediately in liquid nitrogen and stored at −80°C till RNA extraction. For the oestradiol-17β radio-immunoassays (RIA) analysis, two pools of 1,100 and 672 gonads were sampled (18–26 days PF) from both genetic all-females and genetic all-males reared at the standard 27°C. For analysis of brain aromatase activity, 175–178 heads were respectively sampled from genetic all-females and all-males reared at either 27°C or 35°C temperature. In addition, aromatase activity were measured on gonads of adult tilapia reared for 2 months at either 27°C or 35°C.

Histological Procedures

Five fry from several broods were collected every 2–3 days from 9–65 days PF and thereafter till 90 days PF collected every 5–7 days. Weight and total body length were measured. Horizontal serial sections (4 μm) were fixed in Bouin Holland fluid of whole fry at early stages (from 9–45 days PF) and subsequently (from 45–90 days PF) on either trunk or gonads. We performed a thorough examination of gonads before differentiation which were entirely cut into serial sections. Sections were stained with Regaud's hematoxylin, Orange G and aniline blue. In addition, sections of 1 μm belonging

to differential stages were processed for light microscopy after staining with toluidine. For electron microscopy, gonads between 40–60 days PF were fixed in 2.5% glutaldehyde cacodylate buffer at 4°C for 3 hr and then post fixed in the same buffer supplemented with 1% osmium tetraoxyde for 1 hr. All pieces were dehydrated in acetone and embedded in epon-araldite. Ultrathin sections were double stained with uranyl acetate and lead citrate and examined with a Hitachi HU-12 electron microscope.

Histochemical detection of 3β-hydroxy-5-steroid dehydrogenase (3βHSD) was performed on whole gonads of 27 day PF fry and on anterior, middle and posterior sections of gonads belonging to 90 days PF fry. The testes or ovaries were removed, frozen for 2 h in a medium containing 4 ml 0.1 M PBS, 4 ml 9 mM NAD, 1 ml aqueous tetranitro-tetrazolium blue and 1 ml DMSO (all products from Sigma). Incubation was carried out at 37°C for 15 min (27 days PF) or 40 min (90 days PF), in the same medium supplemented with 1 mg of 5β-androstan-3β-ol-17-one (Sigma)/ml of DMSO. Simultaneously, control gonads were incubated with the same substrates but without NAD. The tissues were then washed with the buffer, fixed with 20% ethanol and observed under a light microscope.

RNA Extractions

Total RNA was extracted from the various pools of frozen gonads by the TRIzol method (GibcoBRL), using 1 ml of reagent. Tissue was lysed at 65°C by successive aspirations in a 26-gauge needle. Chloroform (200 μl) was then added at room temperature and well mixed. Samples were subsequently centrifuged at 14,000*g* for 10 min. The aqueous phase containing the RNA was carefully removed, followed by RNA precipitation in an equivalent volume of isopropanol. The RNA was washed in 80% ethanol and resuspended in 25 μl of DEPC treated water. RNA was quantified by spectrophotometry and RNA integrity was then analyzed on an agarose gel.

Cloning of Aromatase and GAPDH cDNA

A tilapia aromatase cDNA fragment of 1,300 bp was obtained by PCR amplification from a juvenile tilapia ovary. Primers were designed according to the published *O. niloticus* sequence (Chang et al., 1997) and were the following: forward 5′-GACCTCAAATGCCATCCAATCG-3′, and reverse 5′-GACAGCAGTGTCACCAAAATGG-3′. For the normalization of cDNA loadings, a 620 bp cDNA coding for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also obtained. It was generated by PCR using primers derived from sequence comparisons: forward 5′-TAYATGGTNTAYATGTTYAA-3′ and reverse 5′-CAGATCCACCACTGACACATC-3′ coding for YMVYMFK and DVSVVDL, respectively. The cDNAs were cloned into pCRII vector (Invitrogen) and sequenced entirely.

Semi-Quantitative RT-PCR

One microgram of total RNA from each group were first DNase I treated (Gibco-BRL) and reverse transcribed with 1 μl (1,000 U/μl) of MoMLV (Promega) in 20 μl volume containing [4 μl of 5 × RT buffer; 1.6 μl 250 μM dNTP; 2 μl of 1 μg/μl oligo $(dT)_{12\text{-}18}$ (Pharmacia) primer, 0.4 μl DEPC-H_2O] at 37°C for 1 hr. Each reaction was then diluted to 100 μl and 1 μl used as template for PCR amplification. Internal primers from both the aromatase and GAPDH cDNAs were designed using forward primers containing putative intron-exon boundaries to avoid genomic amplification. The aromatase primers were: forward 5′-CGCTCATACTAAGCAGATCTTC-3′, reverse 5′-AACCGAACGGCTGAAAGTAGCG-3′. The GAPDH primers were: forward TCTGTCTTCCAGTGTATGAAGC, reverse ATGCCATGCCTGTCAGCTTACC. The RT-PCR reaction was performed in duplicates for 25 cycles, once the linear range of amplification had been established. PCR reaction contained 200 μM of each dNTP (Boehringer, Indianapolis), 2 mM $MgCl_2$ and 30 pM of each primer with 0.1 μl of 5U/μl Perkin-Elmer taq polymerase in a total volume of 12.5 μl. PCR conditions were 94°C for 30 sec, annealing for 1 min at 60°C for aromatase and 64°C for GAPDH, and an extension at 72°C for 1 min. One μl of the PCR product was subsequently dotted onto a nylon membrane, denatured for 3 min (0.5 M NaOH, 1.5 M NaCl), neutralized for 5 min (0.5 M Tris-HCl pH 7, 1.5 M NaCl), and then cross-linked under UV light. The cDNA probes were [α-^{32}P]-dCTP labelled using the Multiprime DNA labelling system (Amersham). Following pre-hybridization, membranes were hybridized with the corresponding probe overnight at 42°C in a solution containing 6 × SSC, 30% formamide, 5 × Denhart's, 0.5% SDS, and 20 μg/ml denatured calf thymus DNA. Washings were carried out at 65°C under stringent conditions (2 × SSC/0.1% SDS, 1 × SSC/0.1% SDS, followed by 0.2 × SSC/0.1% SDS). Amounts were quantified by Instant Imager analysis (Packard) and expressed as relative ratio of aromatase vs. GAPDH.

Virtual Northern Blots

Due to the reduced amount of tissue available during differentiation (first meiosis occurring in 96 mg fry), classical Northern blots have been substituted with a Virtual northern technique, generated using cDNAs amplified by PCR. Because full-length cDNAs can be achieved, analysis of the expression can be performed of the original mRNA profiles (Endege et al., 1999; Franz et al., 1999). Total RNA (1 μg) belonging to each group was simultaneously reverse transcribed using 200 units of Superscript II (Gibco-BRL). The reaction was performed in a 10 μl volume using a modified oligo dT (CDS) and a 5′ SMART-II oligonucleotide containing a G sequence, by following the SMART PCR cDNA synthesis kit procedure (Clontech, CA). The PCR reaction was performed in a total volume of 100 μl using 2 μl of undiluted first strand cDNA as template

and the PCR primer (which contains a sequence from both CDS and SMART-II primers). PCR conditions were 95°C for 1 min followed by 18 cycles at 95°C for 15 sec, and extension at 68°C for 5 min. Testing of PCR products to optimise PCR cycles was initially performed using 16, 17, 18, and 19 PCR cycles, and then established at 18 cycles. Volumes of 4, 6, and 8 μl of the PCR products from each sample were then loaded onto a 1% TBE/agarose gel. The gel was denatured (2×7 min), neutralized (2×7 min), and then transferred overnight to a nylon membrane (Hybond-N, Amersham) using $20 \times$ SSC. DNA was fixed by baking the membrane at 80°C for 2 hr. The cDNA probe was [α-^{32}P]-dCTP labelled and hybridization performed as mentioned above. The membrane was first probed with aromatase cDNA, then dehybridized at 95°C in 0.1% SDS for 15 min and re-hybridized with tilapia GAPDH under the same conditions as above.

Brain Aromatase Activity

Temperature effect on the enzyme as well as optimal assay incubating temperature was initially tested in gonads of adult tilapia reared at 27°C or 35°C for a period of 2 months. The gonads from each group were sampled and then homogenized with a mixer in 1 ml/g tissue of homogenizing buffer (H buffer) consisting of 20 mM phosphate buffer solution pH 7.55 (0.25 M NaCl, 0.25 M sucrose and 1 mM PMSF). Homogenate was centrifuged at 800*g* (2°C) for 20 min, the supernatant collected and then re-centrifuged at 10,000*g* (2°C) for 20 min. Activity assay was performed following the procedure of Fostier (1995), based on the release of tritiated water from the conversion of 1β ^{3}H-androstenedione to estrogens. Incubations were performed at 27°C or 35°C for 1 hr, vortexing every 15 min. For the analysis of brain aromatase activities, 175–178 heads were sampled from genetic all-females and all-males treated at 27°C or 35°C, before and during sex differentiation. Heads were homogenized using an ultraturax in a 10 ml volume of H buffer. Volume was then increased to 20 ml and homogenate centrifuged at 800*g* and at 10,000*g* as mentioned above. The resulting supernatant was finally ultracentrifuged at 195,000*g* (2°C) for 2 hr. The microsomes were then resuspended in 1 ml incubating buffer of 20 mM PBS pH 7.55 (0.21 M NaCl, 0.15 M KCl, 0.25 M sucrose, 5 mM DTT), and stocked overnight at −20°C. Assay was performed in duplicate using 150 μl of microsomes/tube with incubations done under agitation at 27°C for 3 hr.

Gonadal Oestradiol Radioimmunoassay

Separate analysis of two pools of 672 and 1,100 frozen gonads from both genetic all-females and all-males were used for the RIA. They were homogenized in a glass douce with 1 ml ethanol/water (50:50), washed with 3 ml 100% ethanol and then centrifuged at 4,000*g* for 15 min. The supernatant (S1) was put aside, and the pellet resuspended in 1 ml ethanol/water (80:20). It was re-centrifuged at 4,000*g* for 15 min, the supernatant collected and added to S1. The pool of supernatants were completely evaporated after three successive extractions performed with 5 ml dichloromethane. The dry residue was resuspended in 2×100 μl 100% ethanol and frozen until the assay was performed. The resulting organic phase was evaporated at 40°C and the dry residue dissolved in phosphate buffer (0.01M, pH 7.5) containing 0.1% gelatin. Radioimmunoassay (RIA) for oestradiol-17β was performed according to Fostier and Jalabert (1986). Statistical differences for RIA was analyzed using Student's *t*-test.

RESULTS

Normal Tilapia Sex Differentiation

Histological analysis of morphogenesis. Histologic gonadal sex differentiation has been previously studied, although there is no general consensus concerning the different features and on the timing of this differentiation. Here we describe the gonadal ontogenesis by light and ultrastructural approach to characterize the differentiation period more precisely, in a mixed sex population which was reared at the standard temperature of 27°C. These features could then be used in correlation with other experiments.

Fry hatched at 4 days PF. By the ninth day PF, they had (or almost) completed vesicle reabsorption. These fish (8.94±0.6 mm; 10.89±0.9 mg), contained a pair of tubular gonads which appeared closely attached to the dorsal peritoneal wall of the coelomic cavity by a mesogonium. The gonads were sexually undifferentiated with an average of 48 (38–58) primordial germ cells/gonad scattered within the stroma. The primordial germ cells (PGC) could be easily characterized due to their large round or oval shape (17 μm), bright cytoplasm and large nucleus (12 μm). Morphological changes remained discrete until 20 days PF. The gonads were mainly composed of somatic cells, with only a slight and very gradual increase taking place in the number of both stroma and germ cells.

At the end of the third week PF (around Day 21 PF), a dimorphism started to be apparent in the fry, with part (14.3±0.9 mm; 42±5.6 mg) exhibiting a rise in the number of germ cells in their gonads. In these fish, germ cells became more numerous reaching a 2.7 fold higher amount (217:79) between Day 25 PF (18± 1.8 mm; 96.5±22.2 mg), and Day 35 PF (26.12±1.1 mm; 350.7±60.6 mg). Between Day 28 PF and 35 PF, these cells were in meiotic prophase, mainly at the leptotene and zygotene stages (Fig. 1A). Simultaneously, a thin groove was observed along the lateral sides of these gonads. This groove developed later at diplotene stages, into a classic ovarian cavity. Therefore, the female gonads presented both a somatic and germinal criteria of differentiation (Fig. 1B), highlighted by the existence of auxocytes.

The gonads of the other fry ($\approx$50% of the individuals) persisted in a quiescent state from a mitotic point of view, with an early and gradual increase in PGC number (Fig. 2A). After 35 days PF, an active proliferation of both germinal and somatic components

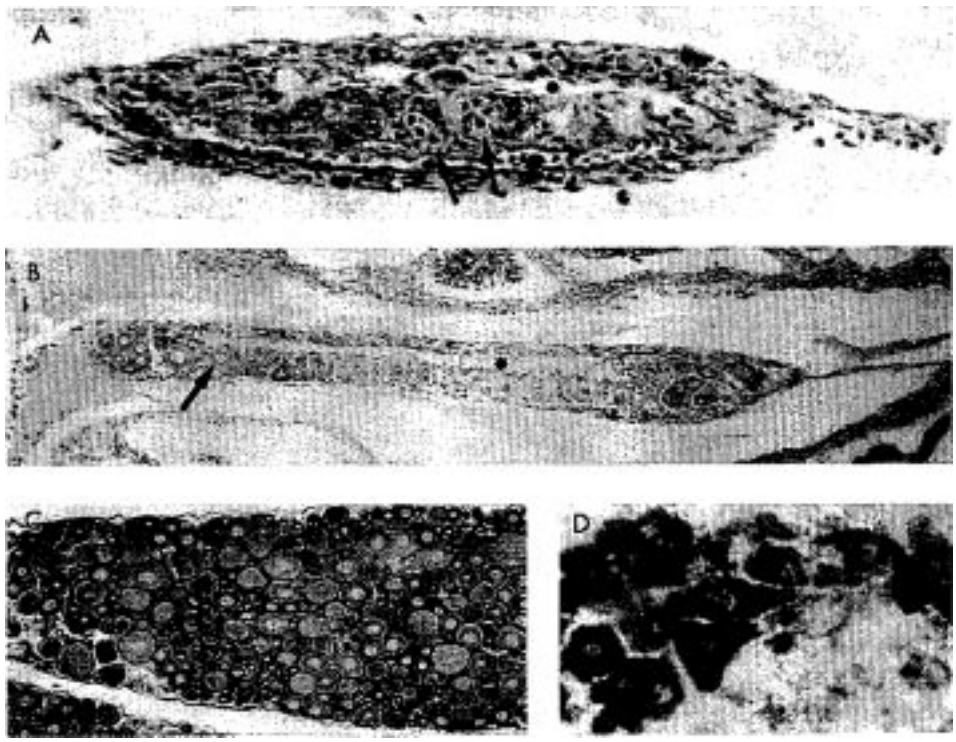

Fig. 1. A: Gonads from tilapia fry of 31 days post-fertilization presenting the meiotic prophase (arrows) and the future ovarian cavity seen with symbol * (magnification: 450×). **B**: Gonads (magnification: 80×) from tilapia fry of 39 days post-fertilization beginning the oocytes previtellogenic stage (arrow) and showing the ovarian cavity (*). **C**: A fish ovary of 90 days post-fertilization showing previtellogenic ovocytes (magnification: 45×). **D**: 3βHSD activity in 90 days post-fertilization detected in large polygonal cells containing an oval or round nucleus (magnification: 450×).

took place. These somatic components diffused inside the gonads to progressively form lobules, leading at 50 days PF (40.2±3.7 mm; 1.02±0.27 g) to the lobular configuration typical of testicular differentiation (Fig. 2B). From 55 days PF onwards, the germ cells which had been in the quiescent state, underwent meiotic prophase (Fig. 2C). During the following 35 days a specific area of the gonad initiated spermatogenesis, which developed progressively throughout the entire testes. At 90 days PF (62.5±13 mm; 5.84±1.49 g) testes were in an active spermatogenesis (Fig. 2D) while in the ovaries, the most advanced oocytes were still in a previtellogenic stage (Fig. 1C). Histological detection of steroidogenic cells using 3βHSD was clearly demonstrated in 90 day PF ovaries (Fig. 1D) and testes but it was also seen in undifferentiated gonads of 27 day PF fry. The enzymatic activity was detected in large polygonal cells containing an oval or round nucleus. These cells appeared in a very restricted longitudinal area. Although the methodology used did not allow a precise localization, these potential steroidogenic cells are hypothesised to be mainly located in the peripheral area of the gonads. From 60 days PF onwards, typical steroid producing cells could be clearly characterized by their large mitochondria with tubular cristae in an electron dense matrix and abundant smooth endoplasmic reticulum (Lofts and Bern, 1972). These steroid producing cells were observed in the stroma of both testes and ovaries and grouped mainly in the vicinity of blood vessels.

Genetic all-females and all-males were sired in order to dissect gonads at precocious stages long before sex can be morphologically or histologically distinguished. The genetic all-females yield a high percentage of fry showing early mitotic activities followed by the first meiosis, typical of ovarian development at 28 days PF (data not shown). Sex ratios of these control progenies reared at 27°C ranged between 91–98% females. For genetic all-males reared at 27°C, an opposite sex ratio with 90–100% males was encountered. Their gonads (data not shown) followed the same pathway and kinetics described earlier for testes development.

Gonadal aromatase expression. Aromatase expression was analyzed in the gonad using RT-PCR and virtual northern. For this study, we first generated a tilapia aromatase cDNA by PCR amplification. The expected product of 1,300 base pair coded for aromatase P450 in view of the elevated homology found for the deduced amino acid sequence, sharing 74.3% homology with the rainbow trout sequence (Tanaka et al., 1992), 80.2% with the medaka sequence (Tanaka et al., 1995), and 97.3% homology with the previously published sequence of *O. niloticus* (Chang et al., 1997). A 615 bp cDNA corresponding to tilapia GAPDH was also amplified by PCR. The deduced amino acid sequence shared 78.5% homology with the human gene (acces-

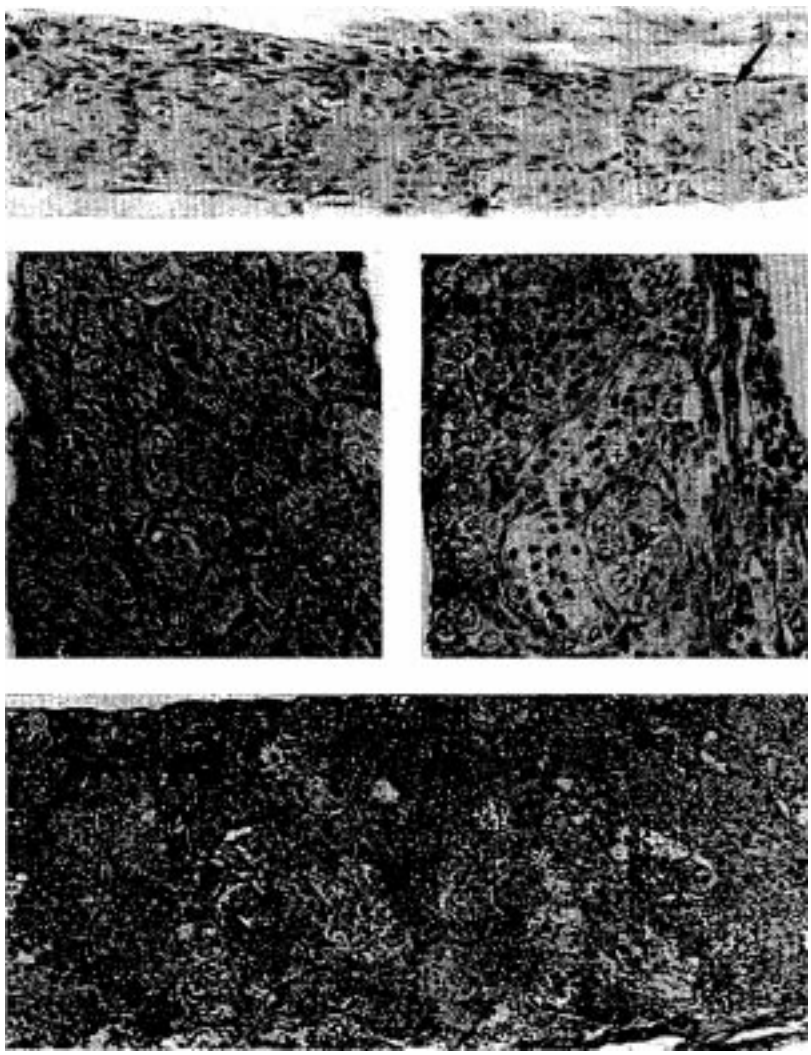

Fig. 2. A: Nonmeiotic gonads from tilapia fry of 35 days post-fertilization presenting a gradual increase in both germinal (gonial cells = arrow) and somatic cell number (magnification: 490×). **B**: Fry of 50 days post-fertilization: after the beginning of an active proliferation of both cell types, the somatic cells diffuse inside the gonads to progressively form the lobules indicated by arrows (magnification: 400×). **C**: Gonads (magnification: 410×) from fry of 55 days post-fertilization presenting the meiotic prophase (*) and gonial cells indicated with an arrow. **D**: Fish testes of 90 days post-fertilization showing an active spermatogenesis (magnification: 90×).

sion number J04038), and 90% with the GeneBank sequence of rainbow trout (accession number AF027130).

Qualitative analysis of aromatase gene expression was performed by virtual northern. This technique revealed two different size bands for aromatase (Fig. 3), with a major band corresponding to 1.8 kb and a minor band of about 1.2 kb. A strong expression was detected for all genetic female progenies reared at 27°C, whereas in males, expression was extremely low. Virtual Northern bands showed a good representation of original mRNAs (Endege et al., 1999). In our study, we have detected two bands for aromatase in some samples which might correspond to two different transcripts or alternatively the 1.2 kb band could be a truncated form. Both bands, however, showed a higher expression in females at 27°C.

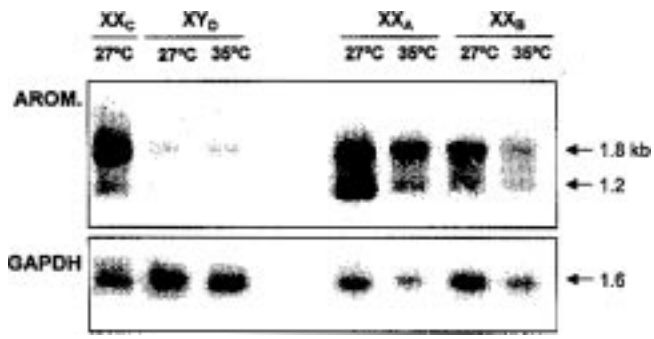

Fig. 3. Virtual northern blot showing aromatase mRNA profiles of tilapia differentiating gonads. A volume of 6 μl of the SMART-PCR amplified products (18 cycles) were loaded/sample onto a 1% TBE/agarose gel. The top panel shows aromatase gene expression and the bottom panel corresponds to the same blot hybridized with a GAPDH cDNA as a control.

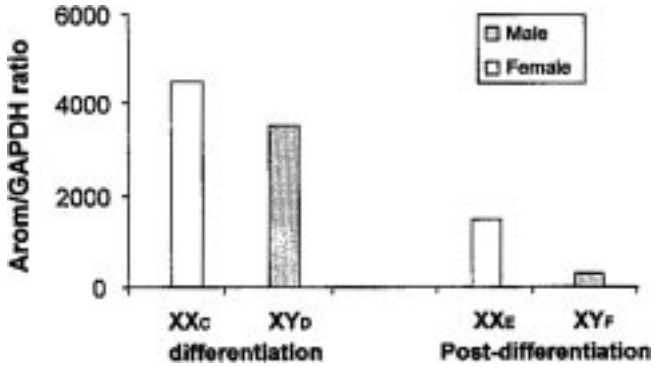

Fig. 4. Relative aromatase expression levels (aromatase:GAPDH ratio) analyzed by semi-quantitative PCR of genetic all-female and all-male gonads dissected during normal sex differentiation (18–26 days post-fertilization) and at a post-differentiated stage. The analysis was performed in duplicate on a pool of 400–690 gonads from both stages.

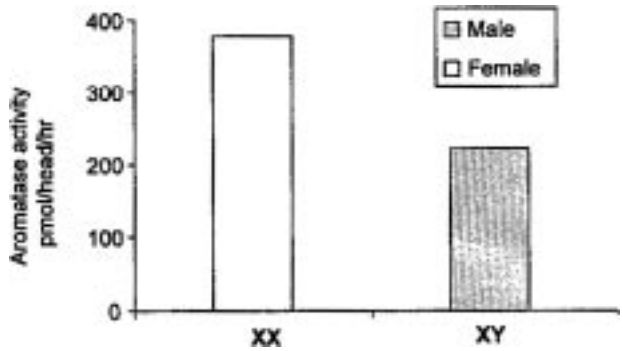

Fig. 6. Brain aromatase activity calculated in the heads of genetic all-females and all-males sampled during normal sex differentiation. Analysis was performed in duplicate on 175–178 heads.

Relative amounts of aromatase expression analyzed by semi-quantitative RT-PCR also revealed high levels in all genetic females with lower levels in males, reared at 27°C (Fig. 4). A mark decrease in level of expression was observed for both sexes at a post-differentiated stage, with nearly a 3.5-fold decrease found in males when compared to females.

Oestradiol-17β radioimmunoassay. Measurements of oestradiol-17β were performed directly on two different pool of gonads, from both genetic females and genetic males. In genetic females high concentrations were measured, with a mean of 465±137 fg/gonad (Fig. 5). The number was much lower (71±23 fg/g) in genetic males but were still in the sensitive range of the assay.

Brain aromatase activity. The aromatase enzyme purified from tilapia treated to different temperatures (27°C or 35°C) may present activities which could be modified further by the in vitro incubating temperature of the assay. Therefore, we first examined direct effect of the temperature treatment and what the optimal in vitro temperature was for each enzyme, using the gonads of adult tilapia reared at either 27°C or 35°C. Incubation temperature was assigned at 27°C, though no large variation was found between both temperatures. By homogenizing the heads of tilapia we calculated the total brain aromatase activity before and during gonadal differentiation. High aromatase activity levels were measured for genetic females (377.9 pmol/head/hr) wheras, activity was reduced approximately by half in male brains (221.5 pmol/head/hr) (Fig. 6).

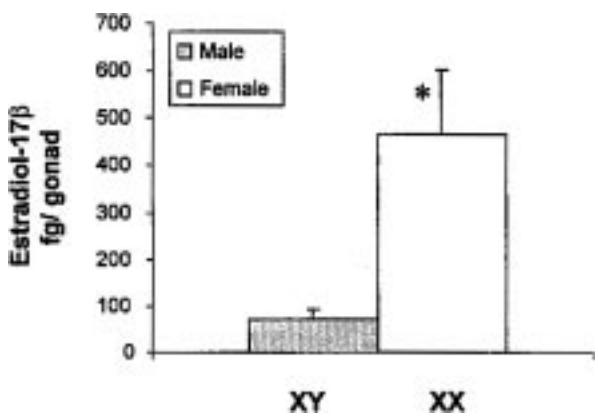

Fig. 5. Oestradiol-17β concentrations measured in gonads from genetic all-female and all-male tilapia during normal sex differentiation. * represents significant difference (*$P < 0.05$).

Temperature Induced Masculinization

Elevated temperatures of 35°C had masculinizing effects on the sexual differentiation of gonads from genetic all-female populations (data not shown). High temperature increased the proportion of males to 86 and 33% for progenies A and B, respectively. However, the degree of masculinization varied, with progeny A showing different features of intersexuality. Some individuals had a normal male gonad while the opposite gonad was sterile (20%); others (54.3%) had male gonads enlosing areas devoid of germ cells; or containing oocytes dispersed among lobules (11.4%). In contrast, the B progeny presented testis with the lobular structure typical of testes.

Gonadal aromatase expression. A strong detection of the major 1.8 kb band corresponding to aromatase mRNA was observed for progenies A and B reared at 27°C (Fig. 3). Expression was considerably reduced when these progenies were treated to 35°C. When genetic males were also treated to the 35°C temperature, no signal was observed. Semi-quantitative RT-PCR also revealed a lower expression of aromatase in genetic female progenies A and B when exposed to a 35°C masculinizing treatment compared to their controls (Fig. 7A). The differences between controls and treated groups were more pronounced in the B progeny. In the negative controls corresponding to genetic all-males, a 35°C treatment also caused a reduction of aromatase expression (Fig. 7B).

Brain aromatase activity. Aromatase activity corresponding to the brain of genetic females decreased up to almost three-fold (125.2 pmol/head/hr) when they

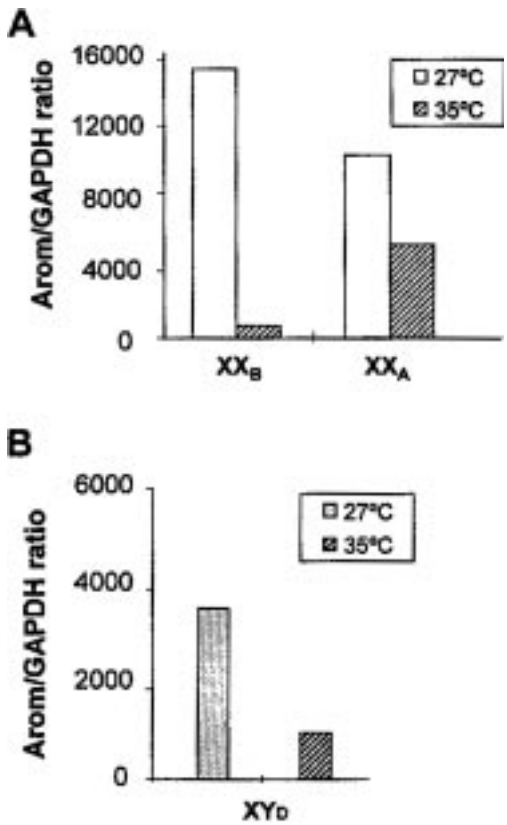

Fig. 7. Relative aromatase expression levels (aromatase:GAPDH ratio) analyzed by semi-quantitative PCR. **A**: Expression levels from two different genetic all-female progenies reared at the standard 27°C temperature and at the 35°C masculinizating temperature, during the early morphological sex differentiation. **B**: Levels of aromatase gene expression of genetic all-male gonads (considered as controls) reared at the standard 27°C temperature of normal sex differentiation and the same progeny treated to the 35°C treatment, during sex differentiation. Analysis were performed in duplicate on different pools of 400–690 gonads from each group.

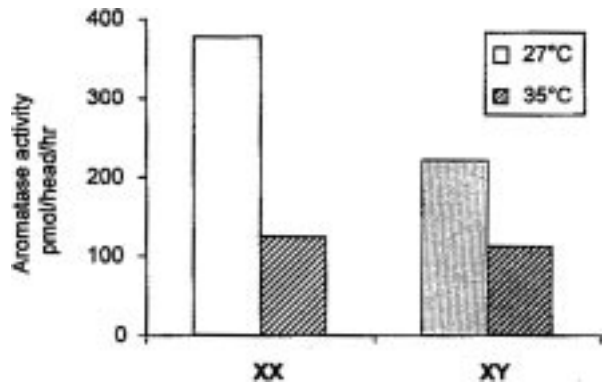

Fig. 8. Brain aromatase activity calculated in the heads of genetic all-females and all-males sampled during normal sex differentiation performed at 27°C and the same progenies treated at the 35°C masculinizating temperature applied during sex differentiation. Analysis was performed in duplicate on 175–178 heads.

were exposed to a 35°C treatment (Fig. 8). In the genetic males reared at the standard 27°C, levels were lower than females but high temperatures of 35°C induced a further reduction of activity to amounts (113.2 pmol/head/hr) that were slightly lower than those found for masculinized females.

DISCUSSION

The Y-linked gene SRY, is considered to be the triggering factor of male sex determination, inducing testis development in mammals (Sinclair et al., 1990; Koopman et al., 1991), although no SRY equivalent has been found in other vertebrates. Lower vertebrates, such as fish present an array of sex determining and differentiating mechanisms. Some of the genes implicated in mammalian sex differentiation have conserved roles in nonmammalian vertebrates but on the whole the genes operating during the normal and temperature-dependent sex differentiation processes of lower vertebrates and their action, are still largely unknown. It is not clear whether these processes are intertwined, sharing some of the regulatory elements acting along the cascade of differentiation. Here we show that aromatase has an important role in the cascade process exhibiting a sexual dimorphism, with a decrease or repression evidenced in the developing gonad and brain, of both natural tilapia males and temperature-induced phenotypic males.

In teleosts there is a lack of indisputable criteria of precocious differentiation. Their great plasticity in reproductive strategies added to the fact that the chronology of when the features appear depends on rearing conditions or genetic strains, makes gonadogenesis in fish more complex to understand. Coinciding with the majority of authors, our study recognizes that the first histological indication of ovarian differentiation is the initiation of meiotic activity shortly after an active proliferation of germ cells (brown trout: Ashby, 1957; rainbow trout: Van den Hurk and Slof, 1981; medaka: Yoshikawa and Oguri, 1981; nile tilapia: Nakamura and Nagahama, 1985). However, from a molecular point of view, ovarian differentiation is in fact initiated earlier. In our opinion gonadogenesis in tilapia should be distinguished in four broad periods. An early undifferentiated stage starting with the formation of gonadal primordia (Nakamura et al., 1998) in which the gonad can be considered clearly bipotent as demonstrated by the effects of exogenous hormone or temperature treatments inducing sex inversion. The second period starts around day 10 post-fertilization (PF) when gonads are undifferentiated but contain some somatic components and few primordial germ cells. The gonad bipotentiality persists at the beginning of this second period, since sex inversion can be induced by exogenous treatments until day 14 PF. Gonads begin to be differentiated when the morphological features consisting in an increase in somatic cells followed by multiplication of germ cells, are apparent in ovaries and distinguished from testis (19–28 day PF). The onset of this differentiation (or morphological differentiation) was esti-

mated to be around day 18 or 19 PF. The fourth stage which we consider as a late ovarian differentiation (the histological differentiation) is characterized by oocyte meiosis simultaneous to the development of the ovarian cavity. Features which started to occur in our case around day 28 PF, a period slightly earlier than the one defined by Nakamura and Nagahama (1985). The developing testis, on the contrary, present a late appearance of the first meiotic prophase (onset around day 55 PF in our case) in future spermatocytes (Kanamori et al., 1985; Nakamura et al., 1998). The initiation of a lobular configuration appears to be the most indisputable somatic criteria characteristic of male differentiation (Nakamura et al., 1998). The period defined as stage two persists in testis until around day 35 PF. A time lag of 3 weeks established in this study and over a month by Nakamura and Nagahama (1989) has been reported for the occurrence of the first meiotic events between female and male gonads of the same species.

Using genetic populations of all-females and all-males we were able to dissect gonads at precocious stages from 18–26 days PF. Our study revealed at this stage, a sexual dimorphism of P450 aromatase gene expression (CYP19) with higher levels found in female gonads compared to males. These fish were reared at the standard temperature of 27°C and showed a normal gonadogenesis. The gonads from genetic females were either just preceding or initiating ovarian differentiation, estimated previously on a mixed sex population. Therefore, we detected an elevated aromatase expression in females at an early stage of differentiation prior to ovarian meiosis, with expression decreasing later at a post-differentiating stage. Aromatase gene expression was also detected in the gonads from the all-male tilapia but mRNA abundance was considerably lower than those found in females. At these stages the testis appear in a quiescent undifferentiated state with few somatic cells and a reduced number of germ cells. Our expression patterns contrast with those found during the sex differentiation of rainbow trout where aromatase mRNAs were hardly detected in males while relative abundance was shown by RT-PCR, to be nearly exponential in females (Guiguen et al., 1999). Chicken embryos also exhibited a female specific pattern of aromatase expression at the initiation of ovarian development (Yoshida et al., 1996; Smith et al., 1997). Most likely in tilapia, the bipotentiality of the gonad is sustained for a longer length of time during the differentiating cascade. Because aromatase was detected in both sexes at early stages, a certain bipotentiality must also exist in the activation of the aromatase CYP19 although the gene's role in the undifferentiated testis is unknown.

We evidenced a correlation between the high aromatase expression detected in genetic females with elevated levels of oestradiol-17β, measured in gonads belonging to the same stage of ovarian differentiation. In contrast, reduced amounts of oestradiol-17β were exhibited by males but were consistent with the low levels of aromatase mRNAs. The oestradiol-17β levels of males could be the result of a down-regulation brought about by a repression of aromatase transcription and/or a post-translational regulation decreasing the enzyme activity. Alternatively, it could be simply due to the fact that few steroid producing cells are present at this stage of testis differentiation. Our study establishes that ovaries at an early stage of differentiation, as well as, undifferentiated testis, are steroidogenically active. Few studies have dealt with the steroidogenic potentialities during gonadogenesis (Baroiller et al., 1999). Nearly all steroid assays have been performed on whole-body homogenates because of the reduced fish size. In tilapia, a peak of E2 levels was found only around the histological ovarian differentiation, while testosterone was either detected after testis differentiation or before (at 22 days PF) in gonads of mixed sex fish associated with 11-ketotestosterone (Hines et al., 1998; Nakamura et al., 1998). The appearance of steroid producing cells has been studied by histochemistry detection of various steroidogenic enzymes and indicate that they are present in histologically undifferentiated rainbow trout (Van den Hurk et al., 1982). Putative steroidogenic cells were also found in tilapia at the onset of ovarian meiosis around 27–30 days PF (Nakamura and Nagahama, 1985), although they could only be ultrastructurally identified once differentiation had occurred. Based on the ultrastructural criteria described in adults (Lofts and Bern, 1972) and in tilapia larvae (Nakamura and Nagahama, 1985; 1989), we have also distinguished the steroid producing cells by histological detection using 3β-HSD and found a similar localization. They were distinguished in the differentiating ovary around day 27 PF when the number of stromal and germ cells started to increase. The steroid producing cells were also found in the undifferentiated testis.

We have found a similar profile of high aromatase transcripts with oestradiol-17β levels in females indicating that synthesis of oestradiol-17β was already taking place when stromal and germ cells were proliferating in the ovary. In males despite levels being low, both aromatase gene expression and oestradiol-17β could be detected in testis which were still undifferentiated. Thus the regulatory factor which activates aromatase must act earlier. An ontogeny study of aromatase expression should be performed to determine when aromatase transcription is turned-on and whether the low aromatase gene expression which we have found in males is an aromatase repression. Sex inversion treatments in tilapia (hormonal or temperature) must be initially applied during a narrow window estimated between 12 and 14 days PF (Contreras-Sanchez et al., 2000). After this period treatments are not effective and do not cause sex reversal. Therefore, triggering of sex differentiation may be taking place before or perhaps around day 12 or 14 PF. We determined that initiation of gonial multiplication occurs at ≈day 19 PF which must be in fact a period when sex differentiation is already proceeding. Based

on our studies it does not seem likely that aromatase is the trigger of sex differentiation but operates slightly further-downstream. However, elevated levels of aromatase gene expression appear to be necessary to promote ovarian development.

A key question concerning temperature sex determination is whether it acts on the same signals or at other regulatory levels. Moreover, does the masculinization of tilapia induced by temperature also affect aromatase? In the current study, temperature caused masculinization of genetic female progenies, although to a different degree. In these phenotypic males we determined that temperature induces a repression of aromatase expression. Temperature also affected the aromatase expression of genetic males by lowering the expression levels even further. Similar to what occurs in tilapia, a suppression of aromatase expression has recently been shown after the onset of sex differentiation in the high temperature masculinization of the Japanese flounder *Paralichthys olivaceus* (Kitano et al., 1999). In this species suppression of aromatase was suggested in view of the gradual decrease exhibited in phenotypic males. Whole body oestradiol-17β levels were measured in this study and only seen to be significant in females when aromatase expression became female-specific. Temperature dependent sex determination is largely exhibited by reptiles, with egg incubating temperatures inducing either testis or ovary differentiation. At female-producing temperatures, aromatase activity increases (Desvages and Pieau, 1992; Smith and Joss, 1994) and inhibition of ovarian development can be induced using aromatase inhibitors (Wibbels and Crews, 1994; Richard-Mercier et al., 1995). In the terrapin turtle, mRNA abundance rose exponentially during the temperature-sensitive period in developing ovaries but remained basal at male producing temperatures (Jeyasuria and Place, 1998). Bogart (1987) based a theory of sex differentiation on androgen to oestrogen levels and hypothesized that a Y linked repressor of aromatase acted when males were the heterogametic sex. In our study, aromatase expression appears to be repressed by temperature during masculinization. The low levels of aromatase expression during natural male differentiation also agrees with a repression hypothesis. A candidate repressor gene lying up-stream of the differentiating pathway could be Sox9. It has been implicated in the testis development of mouse embryos (da Silva et al., 1996) and has been shown to be male-specific in chicken embryos (Oreal et al., 1998; Smith et al., 1999). It is also testis specific in the embryos of the American alligator, a reptile displaying TSD (Western et al., 1999). Regarding aromatase gene activation, it could be caused by the action of one or more regulatory factors but a putative gene could be SF1. It has been shown to regulate expression of steroidogenic enzymes. In mammals, it is down-regulated in female embryos (Koopman, 1999) while in chicken, expression is activated during ovarian differentiation (Smith et al., 1999). Both Sox9 (Takamatsu et al., 1997) and SF1 or related genes (Ito et al., 1998) have been identified in fish although no study has been performed regarding sex differentiation.

As the brain is a site where environment effects are largely integrated, we were also interested in analyzing whether temperature exerts an effect on the brain aromatase during the sex differentiating process of tilapia. The central nervous system in mammals is a target of gonadal steroids. A sexual differentiation is hypothesized to occur in mammalian brain during prenatal stages brought about by gonadal hormones, modulating amongst other functions the masculine behaviour of adults (Cooke et al., 1998). Perinatal exposure to androgens showed that the sex differentiation of the brain is mediated by higher aromatase activity and partly regulated by local oestrogen synthesis (Roselli and Klosterman, 1998). Interestingly, when aromatase activity increased in the preoptic area after estradiol treatment, no change in mRNA abundance occurred implying that increased activity can be modulated in some cases by a post-translational mechanism (Abdelgadir et al., 1994). We have evaluated in the brain the potentiality of oestradiol-17β synthesis by measuring aromatase enzyme activity during the period when morphological differentiation of ovaries is initiated (18–26 PF). Higher activity was estimated in female brain compared to males during the natural sex differentiation of tilapia (at 27°C). When these same genetic progenies were treated at elevated temperatures, enzyme activity was greatly reduced in both groups. We have estimated total aromatase activity, but this activity may in fact originate from one or two aromatase enzymes. Recently two aromatase cDNA variants (a brain and an ovarian type) were identified in the brain of goldfish (Tchouda-Kova and Callard, 1998). Jeyasuria and Place (1998) have detected in the brain an aromatase expression preceding that of the gonad during the TSD of the terrapin turtle. Levels were detected in both the female and male brain but rose exponentially in males. Oestradiol administration caused a reduction in brain transcripts, and the authors suggested that a negative feed-back was exerted by oestradiol-17β on the brain. No study has been performed in fish brain regarding aromatase mRNAs during TSD. Aromatase gene expression should be compared in both gonads and brain of tilapia in order to verify whether the decrease in aromatase activity is mediated by a repression of mRNAs. Our observations imply that an early sexual differentiation occurs in the tilapia brain but whether this is mediated or not by gonadal control is unknown. If we consider that the brain is a target for gonadal steroids, the brain could be an interesting and easier parameter to indirectly define the stages of gonadal differentiation which are rather hard to monitor due to the limited size of fish larvae.

This study establishes that high aromatase expression is associated with elevated oestradiol-17β levels in the gonad during female differentiation at a stage when the onset of ovarian differentiation occurs. Aromatase

may be responsible for driving ovarian differentiation. Temperature may be following a similar mechanism in the masculinization process of tilapia as that of natural male differentiation, since a decrease of aromatase expression was found in both genetic and phenotypic males. We suggest that a genetic and temperature regulatory factor represses aromatase expression, blocking ovarian differentiation and driving development towards testis.

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