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Methyltestosterone Efficiently Induces Male Development in the Self-Fertilizing Hermaphrodite Fish, *Kryptolebias marmoratus*

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Summary: A hermaphrodite fish, *Kryptolebias marmoratus*, is the only known vertebrate that reproduces by self-fertilization. In nature, males have been rarely observed. Low-temperature treatment during late embryonic stages is known to induce males but its efficacy is variable. Here we report that 17 α -methyltestosterone (MT) treatment of the embryos converted most of the fish to males. We examined a time course of this male induction with histological and marker gene expression analyses. Oogenesis started in the gonads of the control embryo at hatching; spermatogenesis did not start until two months after hatching. In the MT-treated fish, oogenesis started initially as in the control but stopped completely within one month after hatching. Instead, spermatogonial proliferation started earlier than in the control fish and progressed to full spermatogenesis. Expression profiles of the sex-specific marker genes corresponded well with histological observations. From one month after hatching, expression of an oocyte-specific marker, *fig α* , and a testicular somatic cell marker, *dmrt1*, started to increase in the control and in the MT-treated fish, respectively. *genesis* 44:495–503, 2006. © 2006 Wiley-Liss, Inc.

Key words: sex differentiation; *fig α* ; *dmrt1*; sex change

INTRODUCTION

The mangrove killifish, *Kryptolebias marmoratus* (formerly known as *Rivulus marmoratus*, see Vermeulen and Hrbek, 2005), is the only vertebrate species known to reproduce by self-fertilization (Harrington, 1961). Its bilobed gonad consists mainly of ovarian tissue with small amount of testicular tissue present at the most dorsal position (Harrington, 1975). In nature, males (with only testicular tissue) are rare and have only been found abundantly in Twin Cays, Belize (Turner et al., 1992a), where probably outcrossing between hermaphrodites

and males happens (Lubinski et al., 1995, Mackiewicz et al., 2006). Other than this location, natural populations are composed of homozygous clones presumably because of repetitive self-fertilization (Harrington and Kallman, 1968; Kallman and Harrington, 1964; Turner et al., 1990, 1992b; Vrijenhoek, 1985). General biology of the fish is well described by B.J. Turner at <http://www.biol.vt.edu/faculty/turner/rivmar/>.

We have two main reasons to study *K. marmoratus*. First is our interest in vertebrate sex differentiation. All individuals of known vertebrate species, except this fish and the belted sandfish (Cheek et al., 2000), have either testes or ovaries at one particular developmental stage. Even many teleost species that undergo sex change in their life cycle do not have functional gonads of both sexes at a time; Oogenesis and spermatogenesis never take place simultaneously (see Devlin and Nagahama, 2002). There must be mutually exclusive hormonal environment for either testes or ovaries. Therefore, research on *K. marmoratus* gonads should provide us opportunities to look into this antagonistic effect between male and female gonads. The other reason is a potential of this fish as an alternative model vertebrate for mutant hunting. A self-fertilizing vertebrate would be particularly useful in making mutants for various biological studies (cf. *Caenorhabditis elegans*, see <http://elegans.swmed.edu/>; *Arabidopsis*, see <http://www.arabidopsis.org/>). However, in *K. marmoratus*, crossing in laboratories has not been reported except in a rare occasion

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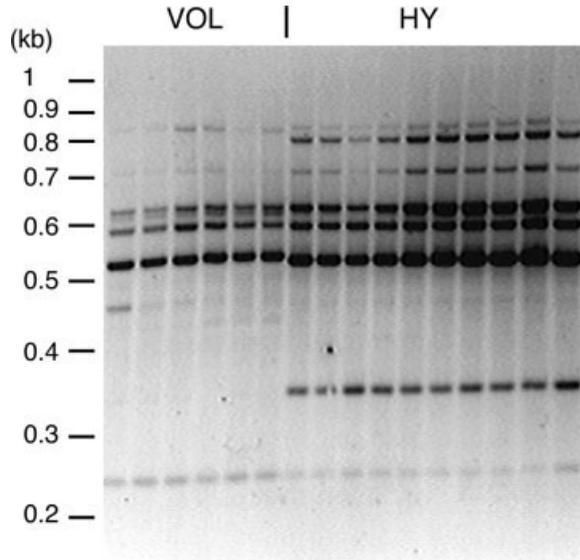


FIG. 1. A representative RAPD analysis with a primer, 5'-AAGAGCCCGT-3', showing homogeneity among the strain of *K. marmoratus* and heterogeneity between the strains. Reversed image of ethidium bromide staining is shown. Several bands are specific either to VOL or to HY individual DNAs.

(Harrington and Kallman, 1968). To overcome this disadvantage, we have been trying to induce pure males and females for possible genetic crosses. Low-temperature during embryogenesis is known to induce males but its efficiency varies according to experimental conditions (Harrington, 1967, 1968). Exogenous androgen treatment has been well documented to induce males in many teleost species (see Devlin and Nagahama, 2002). Therefore, in our study, we hypothesized that embryos treated with MT would develop as males. Here we report in this species as well that most of the embryos immersed in diluted MT solution developed into pure males. We compared MT-induced testis development with normal ovotestis development by histological and marker gene expression analyses. MT apparently inhibited active oogenesis and induced spermatogenesis.

RESULTS

Higashiyama Strain as a Homogenous Population

Initially, we needed to verify our animals as a distinct clonal population because we could not trace back their lineages. Simple RAPD analyses were applied to our Higashiyama (HY) strain and several other strains. With 6 different primers (each 10 bases long), HY and other strains showed identical amplification patterns within the strains. There were several distinct bands among the strains (see Fig. 1). Data from other primers and for other strains, SSHRL and BEL, are not shown here. These results established HY and other strains examined in this study as clonal populations.



FIG. 2. A 6-month-old control hermaphrodite (upper) and a 17-month-old methyltestosterone treated male (lower) of self-fertilizing hermaphrodite fish, *Kryptolebias marmoratus*.

Histological Observations of Gonads After MT Treatment

At our laboratory, embryos usually hatch at 14 days after fertilization (daf). From a preliminary experiment, we know that almost 100% of late stage embryos (12 daf) treated with MT (0.005–0.05 $\mu\text{g/ml}$) developed into pure males. The efficacy of this treatment was far higher than that of low-temperature (20°C) treatment in our hands (data not shown). Therefore, in the current experiment, we treated embryos at 12 daf with 0.025 $\mu\text{g/ml}$ MT for 10 days. We followed the development of the gonad with histology and with sex-specific marker gene expression. Two spontaneous males were observed out of 55 control fishes and two hermaphrodites were found among 63 MT-treated fishes. These four samples were omitted from the following data. A 6-month-old control hermaphrodite and an MT-treated male are shown in Fig. 2. The MT-induced males have body color pattern distinct from the hermaphrodites. The differences include black pigmentation at the ventral side of the caudal fin, less prominent eye-like spot at the dorsal side near the caudal fin, and yellow-brown spots along the body.

We sampled 6–11 individuals of either the control or the MT-treated fish at several developmental stages. The whole gonads were serially sectioned and all of them were observed. At 12 daf, just before the MT treatment, a pair of gonads was found suspended from the dorsal coelomic wall (Fig. 3a). The germ cells are still at mitotic phase and their average number was 56 (Fig. 4a). Somatic cells surrounding germ cells are inconspicuous. We did not find any apparent morphological differences

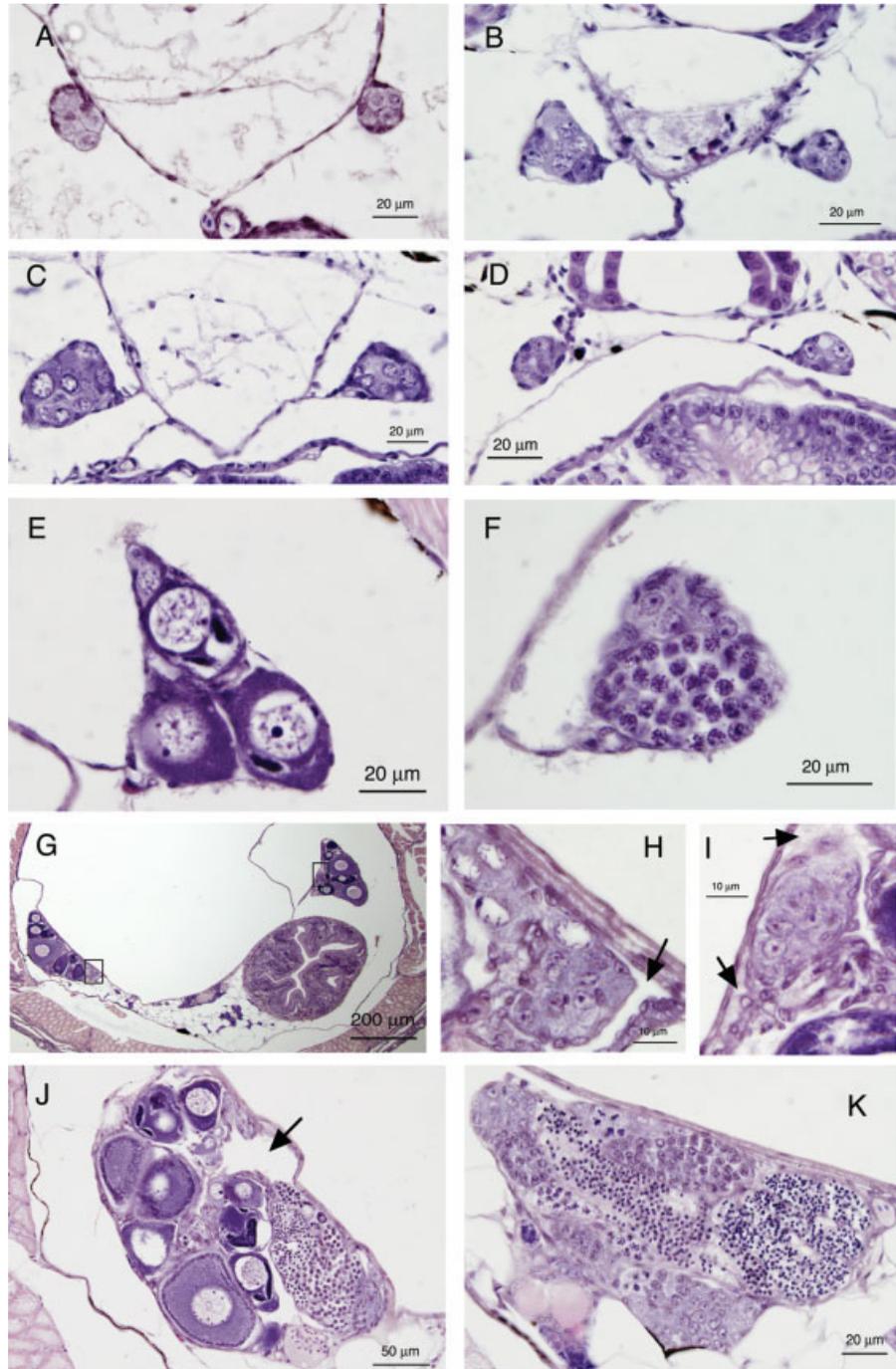


FIG. 3. Gonadal development in the control (b,c,e,g,h,i,j) and in the MT-treated fish (d,f,k). Cross sections are shown. Left gonads are shown at the left in the panels. (a) 12 daf just before the MT treatment. (b) 22 daf (just after the treatment) showing oocytes at the meiotic prophase (zygotene-leptotene). (c,d) 29 daf. No oocytes are shown in this section from the MT-treated fish (d). (g,h,i,j,k) 82 daf. Panel G shows ovotestes where spermatocytes had not appeared yet. Presumptive testicular locations (squares in panel g) contained cluster of the spermatogonia (h,i). Panel j shows ovotestis already containing many spermatogenic cysts. Ovarian cavities have formed by this stage (arrows in panel h,i,j). Panel k shows typical tubular testis of the MT-treated fish.

between the right and the left gonads or along the anterior-posterior axis throughout this study. Just after the treatment (22 daf), both control and MT-treated samples contained many germ cells that had already entered meiotic prophase (mostly at zygotene and leptotene, Figs. 3b and 4b). Because adjacent germ cells were not completely synchronous (as in spermatogenesis) and because of the size of the germ cell (not smaller than gonia), we concluded they entered oogenesis, not spermatogenesis. In the normal development, almost all indi-

viduals had germ cells that had entered oogenesis (meiotic prophase) at hatching (14 daf, data not shown). The clear differences appeared one week later (29 daf). The control gonads contained further developed oocytes (most advanced at pachytene) (Fig. 3c). In contrast, we observed fewer oocytes in the MT-treated gonads (Fig. 4b). Some of the sections contained only germ cells at pre-meiotic stages (Fig. 3d). At 39 daf, the percentage of the sections containing oocytes decreased to 4% in the MT-treated gonads when compared with almost 100% in

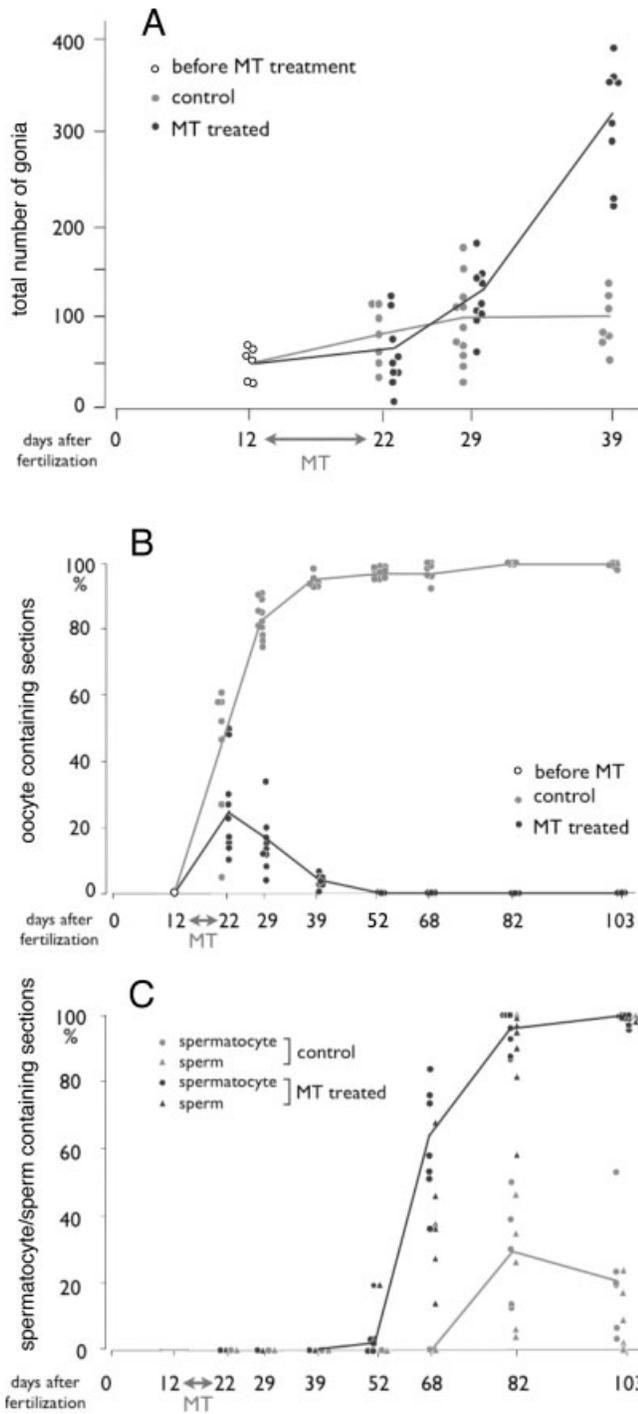


FIG. 4. Semiquantitative analyses of the gonadal development in the control and the MT-treated fish. Total numbers of germ cells before meiosis were counted from serial sections (a). Numbers from the left and the right gonads were summed. Each circle represents a number from single individual. The lines connect the means. Panels b and c show percentages of the sections containing oocytes and spermatocytes/sperms, respectively. In panel c, the means for spermatocytes are connected by the lines.

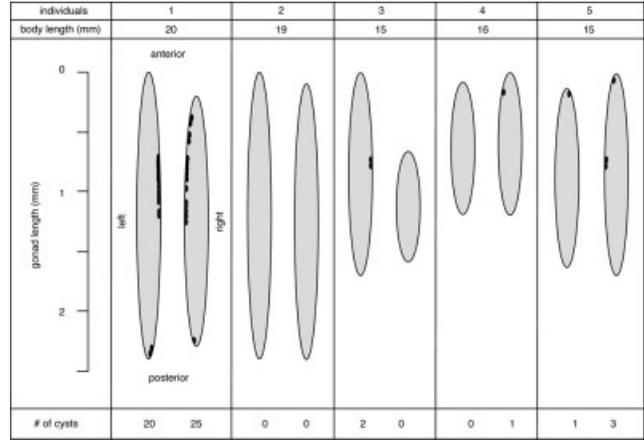


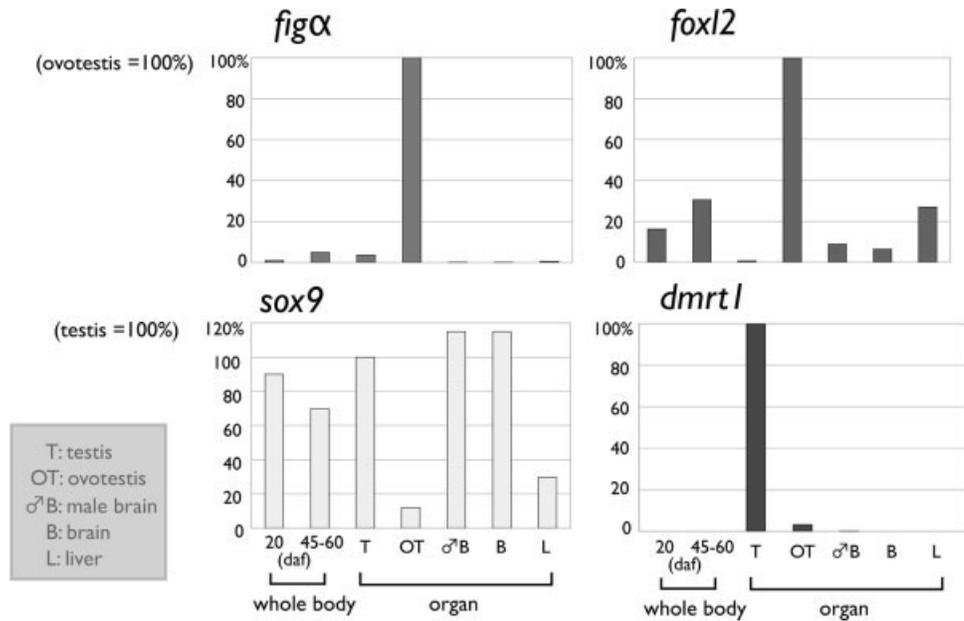
FIG. 5. Locations of spermatogenic cysts are diagrammatically presented for five representative hermaphrodites at 2 months after hatching. The figures are reconstructed from serial sections. Each black dot represents individual spermatogenic cyst.

the control gonads (Fig. 4b). Instead, gonial proliferation was initiated (Fig. 4a), followed by entry into meiosis (spermatogenesis) at 52 daf (Fig. 3f). Oogenesis continued in the control fish with oocyte size increasing and most advancing to the diplotene stage of meiotic prophase (Fig. 3e). At 68 daf, some MT-treated individuals contained sperms in their gonads (Fig. 4c). At 82 daf, the testes of many MT-treated fish showed typical tubular structure of the adult testes; gonidia reside at the periphery and spermatogenesis proceed in the thin Sertoli cell walled cysts toward the center where sperm duct is formed (Fig. 3k). At this stage, spermatogenesis also started in the control to form ovotestes. In some individuals, spermatogenic cysts appeared at the most dorsal side of the gonad where thin connective tissue sheets from the coelomic epithelium (called mesogonadium) are connected to the gonad (Fig. 3j). This area is also at the base of the dorsal wall of the ovarian cavity, which was formed between 68 and 82 daf. Other control fish had as yet spermatogenic cysts. With close inspection, the portion of these gonads where testicular tissue would be formed already contained several clustered gonidia, which were presumably spermatogonia (Fig. 3g,h,i). For 5 control fish at this stage, the position of each spermatogenic cyst along the body axis is described in Fig. 4. The number of spermatogenic cysts varied from 0–45 per each individual. As already noted, there was no particular tendency of the cyst positions between the right and the left or along the anterior to the posterior axis of the gonads. We allowed about 20 fish in the control and MT treatment group to grow to sexual maturity. Approximately 3–4 months after hatching, most hermaphrodites (control fish) started producing fertilized eggs (data not shown).

Expression of Sex-Specific Marker Genes

In addition to the histological observations, we described the expression of vertebrate marker genes for

FIG. 6. Relative amount of gonadal marker gene expression by quantitative PCR. Total RNA extracted from whole bodies of single individuals at 20 or at 45–60 daf, testes of a MT induced adult male, ovotestes of a control adult hermaphrodite, brain from a MT induced adult male, and liver from a control adult hermaphrodite. For *figα* and *foxl2*, cDNA from the ovotestes of an adult hermaphrodite was serially diluted to make a standard curve. For *sox9* and *dmrt1*, cDNA from the testes of a MT induced adult male was used as a standard curve.



either ovaries (*figα* and *foxl2*) or testes (*sox9* and *dmrt1*). Initial quantitative RT-PCR experiments showed higher expressions of *figα* and *foxl2* in the ovotestes than in the testes and vice versa for *sox9* and *dmrt1* (see Fig. 5). We found that *foxl2* was also expressed in the liver and *sox9* in the brains. Therefore, we chose *figα* and *dmrt1* for further analyses. Before doing the time course experiments on quantitative RT-PCR, we examined their cell type specific expression in the gonads of the adults (4 months after hatching) by section in situ hybridization (see Fig. 6). *figα*, as in the case for other vertebrate species, is an oocyte-specific marker (cf. Kanamori, 2000 for medaka; Liang et al., 1997 for mouse). The strong signals are present from smallest oocyte to the larger ones (Fig. 7a). Testicular portion of the ovotestes or the testes are weakly stained (Fig. 7b and c). We found that the *dmrt1* expression is similar to those in other vertebrate species (cf. Kobayashi et al., 2004 for medaka; Raymond et al., 1999 for mouse and chicken); the gene is expressed strongly in the testicular somatic cells either in the ovotestes (Fig. 7d and e) or in the testes (Fig. 7f and g). The signals are present in the somatic cells adjacent to the germ cells (Sertoli cells) and in the sperm duct cells in the testes. The oocytes were stained weakly (Fig. 7d and e). Either *figα* or *dmrt1* sense probes gave no significant signals (data not shown).

The relative levels of *figα* and *dmrt1* mRNA were analyzed by quantitative RT-PCR with cDNA templates made from total RNAs extracted from each individual fish (see Fig. 8). *figα*, an oocyte-specific marker, began to be expressed higher in the control from 39 daf and its expression level further increased as development proceeded. Correspondingly, in the MT-treated fish, *dmrt1* expression became higher from 39 daf onward. The initiation of gonial proliferation was observed at 39 daf, interestingly. These quantitative PCR data support the histological observations described in the previous section.

DISCUSSION

The strains of *K. marmoratus* used in this or in the previous studies are, in most cases, different clones of the same species (see Fig. 1), between which exist rather extensive genetic diversities at nucleotide level (Turner et al., 1990, 1992b). Therefore, we need to point out that there could be strain-specific differences in the results reported here or in other studies on this species.

The MT treatment, applied from later stages of embryogenesis, efficiently converts hermaphrodites into pure males. The rate of male induction was about 97% and was higher than those reported for the low-temperature treatment (Harrington, 1967, 1968). The timing and duration of the MT treatment necessary for the male induction was not examined in this study but the timing are apparently overlapping that of first morphological sex differentiation of the germ cells, namely at hatching when many germ cells enter oogenesis (Figs. 3 and 4). Although its mechanism of action is unknown, MT has been known to induce female to male sex change in many teleost species and its effective period also overlap the start of oogenesis in other species (see Devlin and Nagahama, 2002). The histological studies of the processes described in the previous studies closely resemble that of *K. marmoratus* presented here. Namely, after oogenesis ceases, gonial proliferation is initiated and then followed by meiosis (spermatogenesis). In zebrafish, elimination of growing oocytes triggered testicular transformation (Uchida et al., 2002), suggesting maintenance of ovarian identities by the presence of oocytes. Once *K. marmoratus* developed into males by the MT treatment, they did not re-differentiate into hermaphrodite, at least within 103 daf. In later stages of life cycle, some hermaphrodites develop into males (called secondary males) according to Harrington (1971).

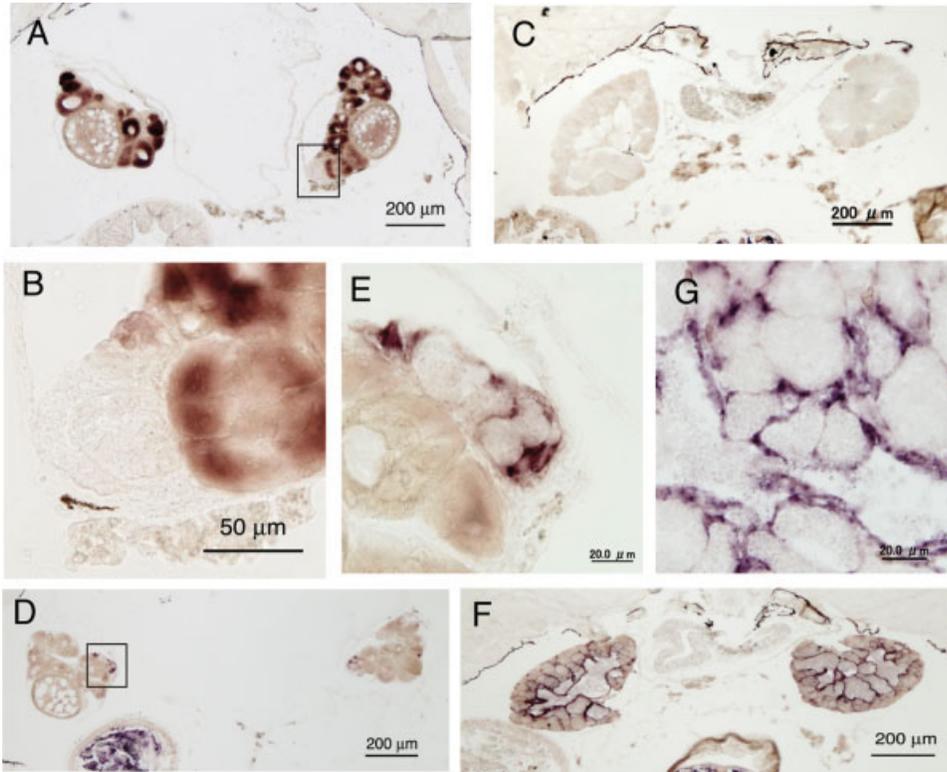
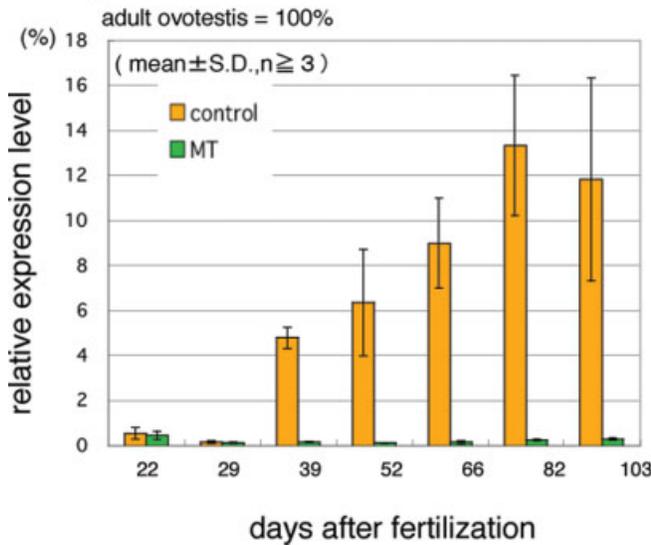


FIG. 7. In situ hybridization of *figα* (a,b,c) and *dmrt1* (d,e,f,g) on gonadal sections prepared from fish at 4 months after hatching. Cross sections are shown. Left gonads are shown at the left in the panels. *figα* is expressed predominantly in the growing oocytes (a,b). The testicular portion of the ovotestes and the testes showed weak signals. *Dmrt1* were detected strongly in the somatic cells of the testicular portion of the ovotestes and also weakly in the oocytes. In the testes, most somatic cells, including Sertoli cells and epithelial cells of a sperm duct expressed *dmrt1* strongly.

A: *figα*



B: *dmrt1*

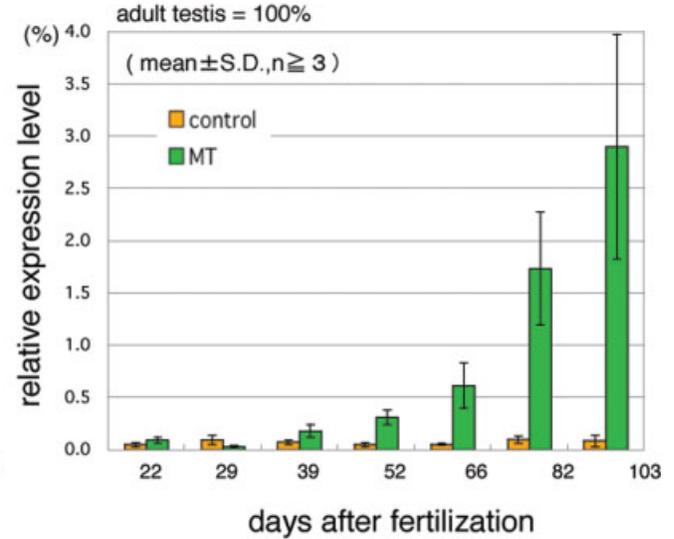


FIG. 8. Expression changes of *figα* and *dmrt1* during gonadal development examined by quantitative PCR. Total RNAs were extracted from whole bodies of single individuals at various stages and were converted to cDNA individually. Finally, analyzed in quantitative PCR individually. Standard curves were prepared as in Fig. 5.

The overall time-course of the ovotestis development observed here in the control is in good agreement with the previous studies (Cole and Noakes, 1997; Harrington, 1975; Soto et al., 1992). Many germ cells start oogenesis at hatching. Then, at about two months later, meiotic phases of spermatogenesis commence at the

most dorsal part of the gonad, along with continuing oogenesis. Arguably, one of the most interesting questions here is how spermatogenesis starts in this particular and restricted region of the gonad. There are no thick connective tissue separating testicular and ovarian territories; an oocyte and a spermatogenic cyst are some

times separated by only a few somatic cells. We suggest two hypotheses to explain the development and maintenance of these limited testicular tissue regions. (1) A determinant gradient of short distance signals, such as hedgehogs, from near or within these territories determine testicular identity. Similarly, two signal gradients, one from the testicular and the other from the ovarian tissues, can determine a boundary between the ovarian and testicular territories. (2) The cells at this particular region (germ cells or somatic cells or both) are predetermined to form testis, independently of the inductive signals.

In general, vertebrate gonads develop into either ovaries or testes. Both male and female hormonal environments have intrinsically antagonistic effects (see Devlin and Nagahama, 2002). However, in *K. marmoratus*, testicular tissue somehow develops in the presence of ovarian tissue (cf. in natural hermaphrodite development or in secondary male development). There may be some mechanism that enables development of testes within ovarian hormonal environment. Interestingly, Minamoto et al. (2006) found little difference in plasma sex steroid levels and gonadal steroidogenic capacities between hermaphrodites and males of this species. Recent cloning of metabolizing enzymes and receptors for sex steroids in this fish would help elucidate this puzzle (Lee et al., 2006; Orlando et al., 2006; Seo et al., 2006).

Gene expression profiles of several marker genes supported the histological observations in this study. *figa*, encoding a bHLH transcription factor, is expressed in the developing oocytes as reported in other vertebrates (human, Huntriss et al., 2002; medaka, Kanamori, 2000; mouse, Liang et al., 1977; zebrafish, Onichtchouk et al., 2003) suggesting a common role in regulating transcription of batteries of oocyte-specific genes. *Dmrt1* is one of dm-domain containing genes in vertebrates. This DNA binding domain is found in many genes in testis-determining cascades from diverse animals such as *doublesex* in *Drosophila melanogaster* and *mab-3* in *Caenorhabditis elegans* (see Zhu et al., 2000). In vertebrates, *dmrt1* is expressed in somatic cells of testes such as Sertoli cells and is proved to be important in mouse testis formation (Raymond et al., 2000). Its duplicated gene, *dmy*, is the medaka male-determining gene (Matsuda et al., 2002; Nanda et al., 2002). In *K. marmoratus*, *dmrt1* expression coincides with testis formation (gonial proliferation) and its expression is also in the testicular somatic cell. It is, therefore, likely that *dmrt1* expression in the dorsal somatic cells determines testicular tissue regionalization in this fish. The question of how *dmrt1* induction occurs in just these presumptive testicular cells remains to be solved.

There are two phylogenetically distant model species in teleost fish, medaka and zebrafish (see Wittbrodt et al., 2002), as well as fully characterized genomes of *Takifugu* and *Tetraodon*. Still, we advocate *K. marmoratus* as yet another fish model for two reasons: research on sex differentiation and ease in mutagenesis. In addition, we have almost indefinite number of inbred strains in natural populations. Life cycle is short (3–4 months)

and laboratory care is easy. *K. marmoratus* belong to Cyprinodontiformes, which is relatively close to medaka (they belong to the same superorder Atherinomorpha; see Wittbrodt et al., 2002). The gene sequences are similar enough that, practically, there is no problem cloning orthologous *K. marmoratus* genes present in medaka. Making genetic cross systems, either by natural or by artificial means, is helpful for positional cloning. Efficient induction of pure males reported in this study will be useful in the future biological experiments on this wondrous and enigmatic fish.

MATERIALS AND METHODS

Animals

In 2003, about ten adults (G1) of *K. marmoratus* were obtained from Nagoya Higashiyama Zoo (Nagoya, Japan), where 7 generations had passed since they purchased adults from a German dealer. The original place of collection cannot be traced back. The data in the present study were from their G2 and G3 reared at Nagoya University. The individuals at all stages of life cycle were kept under 14L:10D light cycle at 24–27°C except during MT treatments (see below). Multiple adults were kept together in 12.5% artificial seawater (Sealife, MarineTech Inc., Tokyo, Japan) and fed live or frozen red worms (larvae of midges) adequately. Embryos were picked up from the tank bottom daily, cleaned, and kept in dishes containing 50% artificial seawater. They usually hatch at 14 daf. We frequently observed unhatched but fully grownup embryos at 14 daf. Their egg envelopes were manually removed by forceps on 14–15 daf. Hatched fries were kept in 50% artificial seawater and fed with newly hatched brine shrimp until about one month after hatching. Then they were kept under the same condition as the adults. The other lines used in the RAPD analyses were VOL and SSHRL from Florida and BEL from Belize.

RAPD

Genomic DNA was extracted by homogenizing adult tissue (frozen or stored in 100% ethanol) in TNES-urea (Asahida et al., 1996), followed by phenol-chloroform extraction and ethanol precipitation. Ten nanograms of the genomic DNA was amplified with Ready-to-go RAPD analysis kit (GE healthcare) according to the manufacturer's protocol. All 6 primers (decamers) were used. The PCR products were separated overnight at 4°C in 25 cm TAE gel made of 3% Nusieve 3:1 gel (Cambrex, USA) immersed in TAE containing ethidium bromide.

MT Treatments

The embryos at 10 daf were treated with Pronase (0.025% in 0.1 M Tris, pH 9.3; Roche diagnostics) (Smithberg, 1966) for two days at 25–27°C to remove chorions. The dechorionated embryos (20–30) were immersed in 200 ml of 0.025 µg/ml MT (TCI, Tokyo, Japan) in 50% artificial seawater at 25°C for 10 days. Small number of

dead individuals (less than 5%) were removed daily. The solutions were not changed during the treatment. The embryos started swimming at about 14 daf, which is normal timing for hatching under these conditions. The young fish after the treatment were washed and transferred to 50% artificial seawater.

Histology

All the samples were fixed overnight in Bouin's solution, embedded in paraffin, serially sectioned at 7 μ m in thickness throughout the gonad, and stained with hematoxylin and eosin. Most of the histological preparation was done by iJARD Ltd. (Hakodate, Japan).

Cloning and Sequencing

Initial genomic clones of *dmrt1* were isolated from a lambda genomic library constructed, which is described previously (Lee et al., 1994), by PCR with degenerate primer pairs after screening with medaka *dmy* probe (kindly provided by Prof. M. Schartl, Wuerzburg, Germany). Then, cDNA fragments of *dmrt1*, *figa*, *sox9*, and *foxl2* were isolated by RT-PCR with various primer pairs (degenerate or gene-specific). Total RNA extracted from ovotestes of a single HY strain adult was used as a template (RNeasy kits, QIAGEN). Except for *sox9*, where we cloned cDNAs from 3 distinct but closely related genes, the other 3 genes are apparently single copy gene as multiple cDNAs cloned were all almost identical in nucleotide sequences. Full-length cDNAs of *figa* and *dmrt1* were identified by a RACE kit (GeneRacer, Invitrogen). Sequencing was done with BigDye terminator v.3.1 and analyzed by an ABI 3100 sequencer (Applied Biosystems). In all cases, at least 3 independent clones were sequenced. GenBank accession numbers for cDNA sequences are as follows: *foxl2*, DQ683738; *sox9a,b,c*, DQ683739-41; *dmrt1*, DQ683742; *figa*, DQ683743. Detailed primer sequences and DNA clones used in the present study are available upon requests.

Quantitative RT-PCR

Total RNA was extracted from the whole body of each individual by RNeasy kits (QIAGEN). Quantity and quality of all the RNA samples were verified by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE) and by denatured formalin agarose gel electrophoresis (Ausubel et al., 1987) before use. Total RNAs (0.5 μ g) were reverse-transcribed with oligo(dT) primer (Promega) with Superscript II (Invitrogen) in 20 μ l reaction. One microliter of the above cDNA products was used for 50 μ l PCR reaction with SYBR green PCR mix on ABI 7700 according to the manufacturer's instructions (Applied Biosystems). Primer pairs used in the present study were made on the separate exons so that only 100–200 bp fragments from cDNAs were amplified except for *foxl2*, intronless gene. Primer pairs for *sox9* identify all three cDNAs. Complimentary DNA, either from ovotestes or from testes (described in the figure), was serially diluted to make standard curves. Quantita-

tive PCR assays were done with duplicated samples and at least twice. All the post-PCR samples were analyzed in agarose gels and single bands of expected sizes were detected.

Section *In Situ* Hybridization

We basically followed methods described in Kobayashi et al. (2000). Samples were fixed for 2 h at RT, followed by overnight at 4°C, in 4% paraformaldehyde (PFA) with constant agitation, dehydrated in ethanol series and stored in 100% methanol at –30°C overnight. They were transferred to xylene three times, embedded in paraffin (paraplast x-tra, Sigma), and sectioned in 7 μ m. After rehydration in an ethanol series, the sections were treated with 10 μ g/ml Proteinase K, 4%PFA, and were acetylated. DIG labeled RNA probes were made with DIG RNA labeling kit (Roche) from a 5'RACE clone of *figa* and a near full-length cDNA clone lacking the 3' end for *dmrt1*. Both antisense and sense probes were made. Hybridization with heat-denatured RNA probes (final conc. 1 μ g/ml) was done in moist chambers with a hybridization buffer (50% formamide, 5 \times SSC, 5 \times Denhart's, 250 μ g/ml torula RNA(Sigma)) at 65°C for 20 h. The slides were washed successively in 5 \times SSC at 65°C for 1 h, 2 \times SSC at 42°C for 20 min, and 0.2 \times SSC at 42°C for 1 h. They were transferred to NT buffer (0.1 M Tris pH 7.5, 0.15 M NaCl), then to 1.5% blocking reagent (Roche)/5% lamb serum (Invitrogen)/NT for 1 h before antiDIG was added at 1:4000 dilution. They were incubated overnight at 4°C, washed three times in NT, transferred to NT with 50 mM MgCl₂ (NTM), and stained in NBT (450 μ g/ml)/BCIP (175 μ g/ml) in NTM overnight at 4°C covered with aluminum foil. Finally, the sections were washed with TE (10 mM Tris pH 8.0, 1 mM EDTA) and 1 \times PBS (Ausubel et al., 1987) successively, and mounted with 60% glycerol.

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