FISH FULL LIFE-CYCLE TESTING FOR ANDROGEN METHYLTESTOSTERONE ON MEDAKA (ORYZIAS LATIPES)

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Abstract—We studied the chronic effects of methyltestosterone (MT) on reproductive status of medaka (Oryzias latipes) over two generations under continuous exposure to verify the applicability of the fish full life-cycle test (FFLC) for this androgen with this species. The exposure of parental (F0) medaka to MT was begun on embryos within 12 h postfertilization and continued for up to 101 d; assessment endpoints included embryological development, hatching, posthatch survival, growth, sexual differentiation, reproduction, and hepatic vitellogenin (VTG) levels under flow-through exposure to MT at each mean measured concentration of 0.35, 1.09, 3.29, 9.98, and 27.75 ng/L. Eggs (F0) spawned from the F0 fish at 98, 99, and 100 d posthatch were examined for hatchability, survival after hatching, growth, sexual differentiation, and hepatic VTG level until 60 d posthatch. In the FFLC with medaka, MT induced masculinization of both secondary sex characteristics and gonads. We observed that all F0 fish in the 27.75-ng/L treatment group showed male secondary sex characteristics in which no fish with ovary could be discerned. Several fish with ovaries in F0 and F1 generations treated with 9.98 ng/L showed male secondary sex characteristics. We also observed swollen abdomens in the F0 and F1 female fish in the 9.98-ng/L treatment group. These swollen abdomens were induced by enlarged ovaries and were accompanied with declined fecundity and fertility in the F0 generation. These results indicate that MT reduces the reproductive potential of medaka and that the FFLC with this species is applicable to the evaluation of androgens.

Keywords—Methyltestosterone Medaka Endocrine disrupter Androgen Full life-cycle test

INTRODUCTION

Concern over the potential impact of natural steroid hormones and chemicals mimicking the effects of hormones on aquatic organisms has been heightened in recent years after demonstration that these chemicals can adversely affect sexual development and reproduction in wildlife [1,2]. In aquatic environments, many reports have been made of chemicals regarding their estrogen-related properties in male fish receiving effluents from sewage treatment works [3–8]. Although masculinization has been reported in some fish species exposed to effluents from pulp and paper mills [9,10] and the morphological alteration of fish was associated with androgen receptor agonist activity [11], less concern has been paid to toxicants with androgenic activities. However, no sufficient in vivo test methods exist for screening and testing for endocrine-disrupting chemicals (EDCs), including androgens.

These concerns have led the Organization for Economic Cooperation and Development (OECD) Expert Consultation on endocrine-disrupter testing in fish to develop a three-tiered testing scheme and to propose a series of in vivo screening and testing methods for each tier [12]. A fish full life-cycle (FFLC) test in which fish are exposed to the test substance throughout their lives including their next generation has been proposed as a confirmatory test at tier 3 because EDCs can profoundly disturb the early development period (especially during sex differentiation) and subsequent reproductive output [1]. Moreover, this test is considered useful for evaluating transgenerational toxicity of EDCs because concern exists that exposure of parental fish to EDCs may have enhanced effects on the next generation. The OECD recommended development of test protocols for fathead minnow, zebrafish (Zebrafish danio), and medaka (Oryzias latipes) [12].

Medaka is an attractive model organism for evaluating life-cycle toxicity in both parental and progeny generations because of its short maturation time—within six to eight weeks after hatching [13]. Therefore, we have already conducted the FFLC on medaka for 4-nonylphenol [14] and 4-tert-pentylphenol [15] to verify its applicability for weak estrogens. In addition, this species is a differentiated gonochorist [16,17], and their gonads and external appearance have been functionally sex reversed from female to male via androgen exposure [18–20].

This study was performed to verify the applicability of the FFLC to an androgen, methyltestosterone, in medaka. Methyltestosterone (MT) has been recommended by the OECD Expert Consultation on endocrine-disrupter testing in fish as one of the reference compounds for developmental studies of the test protocols [12]. We exposed medaka continuously to MT over two generations and examined embryological development, hatching success, posthatch survival, growth, sexual differentiation, hepatic VTG induction, and reproduction.

MATERIALS AND METHODS

Test fish

Medaka (native orange-red strain in Japan) have been bred for several years in our laboratory. As described previously [14,15], about 60 medaka pairs (body wt about 300 mg; total length ~30 mm) were kept for at least two weeks in 1-L flow-through chambers with dechlorinated tap water (pH 7.4–7.6; hardness 44–60 mg CaCO3/L) at 24 ± 1°C. During mating, the fish were placed under a summer photoperiod (16:8 h light:dark) and fed exclusively with Artemia nauplii (<24 h after

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hatching) twice a day. The eggs spawned from each female were carefully collected within a few hours after fertilization and then exposed to MT.

Test chemical

Methyltestosterone (97% pure) was obtained from Wako Pure Chemical (Tokyo, Japan). An aqueous stock solution of MT was prepared as follows: MT was dissolved in acetone to prepare a 1,000-mg/L solution; 0.5 ml of this solution was added to a 500-ml Erlenmeyer flask (Iwaki Glass, Chiba, Japan), and the solvent was then evaporated to dryness under a stream of nitrogen. After evaporation, 500 ml of dechlorinated tap water were added to the flask and stirred for about 24 h by using a magnetic stirrer to dissolve the MT in the water. An appropriate volume of this aqueous solution was diluted with dechlorinated tap water to prepare a MT stock solution of 800 ng/L.

Exposure design

Details of the exposure design have been described previously [14,15]. Nominal treatment concentrations of MT at 0.31, 0.98, 3.13, 10, and 32 ng/L were selected for this study on the basis of the reported reference [21]. The controls received dechlorinated tap water alone. The photoperiod was 16:8 h light:dark. Water temperature was maintained at 24 ± 1°C, except for the reproductive phase at 28 ± 1°C. Residual food and feces in the test chambers were removed at least five times a week.

Biological protocols

Embryological phase. Details have been described previously [15].

Larval–juvenile phase. Details have been described previously [15] except for the day of fish removal. At 61 d posthatch, 20 individuals from each treatment group were randomly removed, and the external secondary sex characteristics, growth, and gonadal histology were observed or measured.

Reproductive phase. As described in our previous study [14,15], six mating pairs from the control group and each of the treatment groups except for the 32-ng/L group were selected at 70 d posthatch for the examination of fecundity and fertility. No pairs from the 32-ng/L treatment group could be selected because the sex ratio was highly skewed to males in this group. Each pair was assigned to a test chamber and exposed to MT (0.31–10 ng/L) until 101 d posthatch. The water temperature was raised from 24 to 28°C to stimulate spawning. We examined fecundity (the number of eggs spawned from each female) and fertility (the percentage of fertilized eggs in total spawned eggs) microscopically over 30 consecutive days (71–100 d posthatch). The day after the end of the reproductive phase (101 d posthatch), all the examined pairs were euthanized. After measurement of body weights, the gonads and livers were removed and weighed. The gonadosomatic index (GSI: 100 × gonad wt/body wt) was calculated. The livers were stored at −70°C until the VTG assay.

F1 embryo phase. Details have been described previously [15].

F1 larval–juvenile phase. Details have been described previously [15] except for the day of fish removal. At 60 d posthatch, the external secondary sex characteristics, growth, gonadal histology, and VTG concentration were measured.

Determination of hepatic vitellogenin concentration

Livers were individually homogenized in 150 μl of ice-cold enzyme-linked immunosorbent assay (ELISA) buffer (10 mM phosphate-buffered saline, pH 7.1, containing 0.05% v/v Tween-20 [Cayman Chemical, Ann Arbor, MI, USA] and 1 mg/ml albumin from bovine serum), using a glass, handheld homogenizer on ice. The homogenized samples were centrifuged at 13,000 g for 10 min at 5°C, and the supernatants were collected as described previously [22]. Hepatic VTG concentrations were measured with a medaka vitellogenin ELISA assay kit (TransGenic, Kumamoto, Japan). Before running in the VTG ELISA, the supernatants were accordingly diluted to get them on the linear part of the standard curve. The determination limits of hepatic VTG were 0.62 ng/mg liver for F0 fish and 0.41 ng/mg liver for F1 fish and were calculated by the detection limit of the standard curve in ELISA buffer and average liver weights in each F0 or F1 fish.

Determination of MT concentration in the test solution

Sample preparation. The concentration of MT in each test solution was measured at least every two weeks during the exposure. Equal volumes of test solutions were collected from the four test chambers of each treatment group and pooled. One liter of the pooled solutions was applied individually to preconditioned MegaBond ElutC18 solid-phase extraction cartridges (Varian Sample Preparation, Harbor City, CA, USA). Each cartridge was rinsed with 5 ml of water:methanol (5:2, v/v). The MT was eluted with 5 ml of ethylacetate:methanol (5:1, v/v), and the eluate was dried under a stream of nitrogen at 40°C. The residue was dissolved in 1 ml of methanol, and then the mixture was shaken for 30 s and centrifuged for 2 min at 3,000 rpm. The solvent was evaporated under a stream of nitrogen at 40°C. The residue was redissolved in 1 ml of an acetonitrile:methanol:water:formic acid (100:300:200:1, v/v/v/v) solution containing 100 ng/ml of testosterone as an internal standard and then shaken for 30 s and centrifuged (3,000 rpm for 2 min).

Liquid chromatography–mass spectrometry/mass spectrometry conditions. The solution was analyzed by high-performance liquid chromatography with a Hewlett-Packard HP-1100 (Palo Alto, CA, USA) equipped with a column packed with octadecylsilylated silica gels (length 150 mm; i.d. 2.1 mm; particle size 5 μm; Chemicals Evaluation and Research Institute, Tokyo, Japan) at 40°C. A 20-μl aliquot of each sample was injected into the chromatograph and eluted in an isocratic mode at a flow rate of 0.2 ml/min in a mobile phase of acetonitrile:methanol:water:formic acid (100:300:200:1, v/v/v/v) solution containing 10 μM of MT as an internal standard. The ion source was 100°C, and that for desolvation was 350°C. Ions of MT were monitored from 303 to 109 m/z, and the internal standard was monitored from 289 to 109 m/z.

Statistical analysis

All statistical analyses were performed by using SPSS Base 10.0J software (SPSS, Tokyo, Japan). The experimental data for the F0 and F1 generations, except for the data on hepatic VTG concentrations and sex ratios, were checked for homo-
geneity of variance across treatments by using Levene’s test. When the assumptions were met, the data were subjected to one-way analysis of variance followed by Dunnett’s multiple comparison test [23]. When no homogeneity was observed, the nonparametric Kruskal–Wallis test was used, followed by the Mann–Whitney U test with Bonferroni’s adjustment [24]. The data on hatchability, cumulative mortality, and fertility were stabilized for variance by applying arcsine transformation before the statistical analysis [25]. The data on sex ratios, as determined by the external secondary sex characteristics and gonadal histology, were assessed by chi-square analysis. The data on hepatic VTG concentrations were assessed by the Mann–Whitney U test with Bonferroni’s adjustment. Since data on VTG concentrations lower than the determination limit were nonnumeric, these were set at the same rank of half the determination limit (0.31 ng/mg liver for F0 fish and 0.20 ng/mg liver for F1 fish) for the analysis [26]. Differences were considered to be significant at $p \leq 0.05$; however, Bonferroni’s $p$ value was used in nonparametric tests.

**RESULTS**

**Concentration of MT in the test solution**

The means of the measured MT concentrations in the test solutions during the exposure period were 0.35 (114% nominal, 8.7% coefficient of variation [CV]), 1.09 (116% nominal, 9.8% CV), 3.29 (105% nominal, 12.4% CV), 9.98 (99.8% nominal, 10.6% CV), and 27.75 (86.6% nominal, 13.9% CV) ng/L. These values indicate that the nominal concentrations of MT remained consistent throughout the exposure period. The concentration of MT in the control treatment was below the determination limit (0.100 ng/L) in all analyses. The following MT concentrations are expressed as average values of each measured concentration.

**F0 generation**

**Mortality, abnormal behavior, and appearance.** The hatchability of all treatment groups and the controls was ≥90% (Table 1). The time to hatch was about 10 d in all groups (Table 1). No statistically significant differences were observed between these treatment groups and the controls in these parameters.

Cumulative mortalities at 61 d posthatch were 12.1 to 22.5% in all treatment groups, but no statistically significant differences were observed between these treatment groups and the controls (Table 2). Although no abnormal behavior was observed in any treatment and control groups, five female fish in the 9.98-ng/L treatment group showed swollen abdomens after 55 d posthatch.

**Growth, external secondary sex characteristics, and gonadal histology.** No significant differences were observed in either mean total length or body weight of the F0 fish at 61 d posthatch in all treatment groups (Table 2).

From our observation of external sex characteristics at 61 d posthatch, all fish demonstrated male characteristics in the 27.75-ng/L treatment group, and the sex ratio, as estimated from the external sex characteristics, was significantly skewed toward male compared with those of the controls ($p < 0.001$) (Table 2). Sex ratios (male:female) in the 9.98-ng/L treatment group also skewed toward male (14:6), although no statistically significant differences were observed (Table 2). Sex ratios (male:female) in the control and the ≥3.29-ng/L treatment groups were in a range of 12:8 to 9:11, and no significant differences were found between these treatment groups and the control group (Table 2).

Gonadal histology showed that no fish with ovaries could be discerned in the 27.75-ng/L treatment group, and three of 20 fish in this group had testis-ova gonads, composed of both testicular germ cells and oocytes (Table 2). Thus, the sex ratios determined by histological examination were significantly skewed toward male at 27.75 ng/L ($p < 0.001$) compared with those of the controls but not in the ≥9.98-ng/L treatment groups (Table 2). Figure 1A shows a typical section of the gonad of a control male fish. In the testis-ova specimens at 27.75 ng/L, a few oocytes were observed within the testicular tissues, in which spermatocytes and spermatids could adequately be differentiated, indicating active spermatogenesis.

### Table 1. Hatchability and time to hatch in fertilized eggs of the F0 generation

<table>
<thead>
<tr>
<th>Methyltestosterone conc. a (ng/L)</th>
<th>Hatchability b (%)</th>
<th>Time to hatch b (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>96.7 ± 6.7</td>
<td>10.2 ± 0.03</td>
</tr>
<tr>
<td>0.35</td>
<td>96.7 ± 3.9</td>
<td>10.1 ± 0.14</td>
</tr>
<tr>
<td>1.09</td>
<td>96.7 ± 3.9</td>
<td>10.2 ± 0.07</td>
</tr>
<tr>
<td>3.29</td>
<td>93.3 ± 7.7</td>
<td>10.1 ± 0.04</td>
</tr>
<tr>
<td>9.98</td>
<td>98.3 ± 3.3</td>
<td>10.1 ± 0.21</td>
</tr>
<tr>
<td>27.75</td>
<td>98.3 ± 3.3</td>
<td>10.1 ± 0.14</td>
</tr>
</tbody>
</table>

a Mean measured concentration.
b Data expressed as mean ± standard deviation ($n = 4$).

### Table 2. Cumulative mortality, growth, and sex ratios as estimated by gross examination of secondary sex characteristics and by gonadal histology at 61 d posthatch in F0 medaka

<table>
<thead>
<tr>
<th>Methyltestosterone conc. a (ng/L)</th>
<th>Mortality b (%)</th>
<th>n</th>
<th>Growth</th>
<th>Sex ratio (♂ : ♀)</th>
<th>No. of fish</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TL c (mm)</td>
<td>BW c (mg)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>19.1</td>
<td>20</td>
<td>29.5 ± 1.3</td>
<td>250 ± 42</td>
<td>10:10</td>
</tr>
<tr>
<td>0.35</td>
<td>21.1</td>
<td>20</td>
<td>30.5 ± 2.1</td>
<td>296 ± 80</td>
<td>9:11</td>
</tr>
<tr>
<td>1.09</td>
<td>22.5</td>
<td>20</td>
<td>30.3 ± 1.8</td>
<td>298 ± 49</td>
<td>12:8</td>
</tr>
<tr>
<td>3.29</td>
<td>13.6</td>
<td>20</td>
<td>30.2 ± 1.5</td>
<td>293 ± 68</td>
<td>12:8</td>
</tr>
<tr>
<td>9.98</td>
<td>18.7</td>
<td>20</td>
<td>29.9 ± 1.9</td>
<td>292 ± 57</td>
<td>14:6</td>
</tr>
<tr>
<td>27.75</td>
<td>12.1</td>
<td>20</td>
<td>29.5 ± 2.0</td>
<td>265 ± 50</td>
<td>20:0 d</td>
</tr>
</tbody>
</table>

a Mean measured concentration.
b Cumulative mortality at 61 d posthatch.
c Data expressed as mean ± standard deviation. TL = total length; BW = body weight.
d Significantly different from the control ($p < 0.001$).
Fish full life-cycle testing for methyltestosterone

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Fig. 1. Longitudinal sections of testis in the control (A) and testis-ova in the 27.75-ng/L (B) treatment group of the F0 generation medaka at 61 d posthatch. Each bar in A and B shows 400- and 200-μm length, respectively. (A) Normal genesis of spermatozoa (Sz). (B) A few oocytes (O) appear within the testicular tissue, but numerous Sz are still present in a compacted mass in this section.

Reproduction. The mean fecundity and fertility of paired medaka during the reproductive phase from 71 to 100 d posthatch slightly decreased by MT exposure at 9.98 ng/L, although no statistically significant difference was determined (Fig. 2). In two of the six pairs exposed to 9.98 ng/L MT, fecundity and fertility declined from the beginning of the reproductive phase. Finally, the fecundity (and fertility) of these two pairs during the reproductive phase was low compared to the other four groups. They had on average 168 eggs (18% fertility) and 477 eggs (28% fertility), respectively, compared to those of the other four pairs, which ranged from 721 eggs (90% fertility) to 1,110 eggs (98% fertility). Females in these two pairs showed swollen abdomens during the reproductive trial, and the swelling of the abdomens increased with time. Although one of these two pairs spawned every day, the other paused egg production three times during the reproductive phase, and then the female died at 91 d posthatch after abdominal rupture and release of a large amount of eggs. One of six females in the 0.35-ng/L treatment group died at 88 d posthatch. However, the females did not show any symptoms before death.

Gonadosomatic index. No statistically significant difference in male and female GSI was determined in any group treated with MT at ≤9.98 ng/L at 101 d posthatch, compared with the controls (Fig. 3). However, one of five female fish in the 9.98-ng/L group showed a high GSI level of 29%, whereas those of the other four females in this group ranged from 7.7 to 8.8%, which were almost equal to those of the controls, resulting in the mean female GSI of 12.3% in this group. This female fish with enlarged ovaries showed a swollen abdomen and subsequent decreased egg production and fertility.

Hepatic vitellogenin concentration. The hepatic VTG levels of male medaka at the day after the end of the reproductive phase increased in all groups treated with MT at 0.35 to 9.98 ng/L, although no statistically significant difference was determined (Fig. 4). The VTG levels in female medaka were not affected by exposure to MT at ≤9.98 ng/L (Fig. 4).

F1 generation

Mortality, abnormal behavior, and appearance. No embryological abnormalities or hatching failures of fertilized eggs (F1 embryos) were observed in any of the MT treatments of ≤9.98 ng/L (Table 3). The mean hatchabilities in all treatments were >80%, and the time to hatch in most embryos was about 9 d in every treatment, with no significant differences between the treatment groups and the controls (Table 3). Although the cumulative mortality in the 0.35-ng/L treatment group at 60
d posthatch was 33.3%, the highest value among all F1 treatments at ≤9.98 ng/L, no significant difference in the mortality was observed compared with that (11.7%) in the controls (Table 4). In the 9.98-ng/L treatment group, one of 30 female fish showed a swollen abdomen after 58 d posthatch.

Growth, external secondary sex characteristics, gonadal histology, and hepatic VTG concentration. The body weights and mean total lengths of F1 juveniles at 60 d posthatch were not affected by MT treatments at ≤9.98 ng/L (Table 4).

No significant differences were found in the sex ratios as estimated from the appearance of secondary sex characteristics at 60 d posthatch in F1 fish exposed to MT at ≤9.98 ng/L (Table 4). No abnormal sexual differentiation in the gonads of the F1 fish at 60 d posthatch was observed in any of the groups treated with MT at ≤9.98 ng/L (Table 4). However, in the 9.98-ng/L treatment group, one of 14 fish with ovaries showed typical male secondary sex characteristics as described previously.

The VTG levels in the F1 males exposed to MT at 9.98 ng/L were significantly increased compared with those of the controls (p = 0.036), whereas those of the F1 females in all MT treatment groups at ≤9.98 ng/L were significantly decreased compared with those of the controls (p = 0.016 at 0.35, 1.09, and 3.29 ng/L; p = 0.036 at 9.98 ng/L) (Fig. 5).

**DISCUSSION**

Our study clearly demonstrates that the FFLC with medaka is available to detect androgenic effects of MT as follows. We observed the masculinized appearance of secondary sex characteristics. In the 27.75-ng/L treatment, all the F0 fish at 61 d posthatch showed male secondary sex characteristics. In addition, we observed that fish with ovaries showed male secondary sex characteristics in the 9.98-ng/L treatment group at 61 d posthatch in F0 and also at 60 d posthatch in F1 generations. Particularly, these fish clearly had papillary processes on the anal fin. These findings suggest that the masculinization of the secondary sex characteristics probably has been caused by the androgenic activity of MT in genetically female fish. It has been widely accepted that androgens control the expression of male secondary sexual characteristics in fish [27]. In medaka, Asahina et al. [20] reported that various androgens affect the formation of papillary processes on the anal fin rays.

**Table 3. Hatchability and time to hatch in F1 embryos collected in the last 3 d of the reproductive phase of the F0 generation**

<table>
<thead>
<tr>
<th>Methyltestosterone conc.* (ng/L)</th>
<th>Hatchability (%) of F1 embryos (days to hatch)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>From F0 at 98 d posthatch</td>
</tr>
<tr>
<td></td>
<td>n^p</td>
</tr>
<tr>
<td>Control</td>
<td>149</td>
</tr>
<tr>
<td>0.35</td>
<td>148</td>
</tr>
<tr>
<td>1.09</td>
<td>136</td>
</tr>
<tr>
<td>3.29</td>
<td>138</td>
</tr>
<tr>
<td>9.98</td>
<td>102</td>
</tr>
</tbody>
</table>

*Data expressed as mean ± standard deviation (n = 6; except ^n = 5, ^n = 4).

^Mean measured concentration.

^Number of fertilized eggs.
in the female medaka. In other OECD-recommended fish, fathead minnow, Ankley et al. [28] reported that exposure of the adult fish to MT for 21 d caused easily discernible alterations in secondary sex characteristics (nuptial tubercles in both sexes). The authors suggested that nuptial tubercles in this species should be an excellent diagnostic endpoint for androgenic chemicals. In addition, some reports have shown that exposure to estrogen agonists decreases the expression of nuptial tubercles in male fathead minnow [29,30] and also the number of papillary processes on the anal fin in medaka [15]. These results suggest that expression of the appearance of the secondary sex characteristics may be differentially responsive to androgens and estrogens in both medaka and fathead minnow and may be useful parameters for these chemicals.

We also detected an androgenic effect of MT in the form of masculinized gonads. Gonadal histology in the F₀ generation at 60 d posthatch showed that no fish with ovaries could be discerned in the 27.75-ng/L treatment group. It has been known in aquaculture that exposure to androgens at selected developmental stages leads to the development of phenotypic male populations [13]. In medaka, Yamamoto [16] reported that functional sex reversals of genetic females to males were successfully induced by administration of MT at adequate levels. Therefore, the gonadal masculinization of the F₀ female medaka in the 27.75-ng/L treatment group was probably induced by the androgenic activity of MT. Overall, we confirmed the androgenic effects of MT on the sex differentiation of medaka, as shown by the masculinized appearance of secondary sex characteristics and gonads. The results indicate that the FFLC with medaka is applicable to the study of androgens.

We observed testis-ova gonads in the F₀ fish exposed to 27.75 ng/L of MT. Two explanations for this finding are possible. First, those hermaphroditic gonads were probably induced by the androgenic activity of MT in genetically female fish because all fish showed male secondary characteristics and none of fish with ovaries have been observed in this treatment group. However, a report was made on the paradoxical response observed in fish exposed to MT. Pfiferrer et al. [31] investigated the effects of several androgens, including MT, on sex determination in genetically monosex females of chinook salmon (Oncorhynchus tshawytscha) administered at different doses via immersion. The authors reported that the treatment with MT (3.2–10,000 μg/L) resulted in dose-dependent masculinization at low concentrations (3.2–400 μg/L) but not at higher doses (2,000–10,000 μg/L), whereas such paradoxical effects were not observed in the treatment with 17α-methyltestosterone (MDTH). The authors suggested that the difference in the capacity of these steroids for aromatization is the most likely explanation for these findings because the structural difference between MT and MDTH is the presence of the 5α-reduction in the latter, which confers resistance to aromatase activity. In addition, several studies have reported VTG increase or enhancement of the expression of aromatase genes in fish exposed to MT [28,32–34]. In this study, we suggest that a part of MT might be metabolized to an estrogen by aromatase, resulting in testis-ova gonads in genetically male fish. In a further study, the measurement of aromatase activity and observation of gonadal histology in the fish exposed to MT will allow us to resolve whether this androgen exerts a superficial estrogenic effect on this species.

In our study, MT exerts not only abnormal sex differentiation but also other effects as well. Although no significant effects of MT were observed on embryo survival, hatching success, mortality after hatching, and growth of the F₀ and F₁ fish at 60 d posthatch, we observed swollen abdomens in the F₀ and F₁ female fish at a MT level of 9.98 ng/L. These swollen abdomens enlarged with time during the F₀ reproductive phase, accompanying the declines in fecundity and fertility. In addition, GSI in the female fish with swollen abdomen increased extremely at the day after the end of the F₀ reproductive trial (101 d posthatch). Ankley et al. [28] reported that exposure of the fathead minnow to MT caused cessation in egg production. The authors suggested that MT might inhibit the natural process of endogenous androgens, which probably play a

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**Table 4. Cumulative mortality, growth, and sex ratios as estimated by gross examination of secondary sex characteristics and by gonadal histology at 60 d posthatch in F₁ medaka**

<table>
<thead>
<tr>
<th>Methytestosterone concn. (ng/L)</th>
<th>Mortality (%)</th>
<th>Growth</th>
<th>Sex ratio (♂:♀)</th>
<th>No. of fish with</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.7</td>
<td>53</td>
<td>30.8 ± 1.4</td>
<td>20:33</td>
</tr>
<tr>
<td>0.35</td>
<td>33.3</td>
<td>40</td>
<td>31.0 ± 2.1</td>
<td>21:19</td>
</tr>
<tr>
<td>1.09</td>
<td>3.5</td>
<td>57</td>
<td>30.1 ± 1.9</td>
<td>22:35</td>
</tr>
<tr>
<td>3.29</td>
<td>6.7</td>
<td>56</td>
<td>30.6 ± 1.5</td>
<td>26:30</td>
</tr>
<tr>
<td>9.98</td>
<td>10.0</td>
<td>54</td>
<td>30.2 ± 2.1</td>
<td>24:30</td>
</tr>
</tbody>
</table>

* Data expressed as mean ± standard deviation. TL = total length; BW = body weight.

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**Fig. 5. Hepatic vitellogenin (VTG) concentrations of F₁ medaka at 60 d posthatch.** Values are shown as the mean VTG concentrations in males (open bar) and females (solid bar) (n = 6). Data were expressed as mean ± standard deviation. Significant difference from the control (p = 0.016, except p = 0.036 for the 9.98-ng/L male group). MT = methytestosterone.
role in final maturation and/or release of eggs in female fish [35]. Therefore, in our study, MT might interfere with endogenous androgens and cause swollen abdomens with enlarged ovaries (the females had eggs, but they could not ovulate), resulting in reduced fecundity and fertility. Further work is needed to elucidate the mechanisms of ovary enlargement and reproductive impairment in the fish exposed to MT.

Little is known about the variability of VTG levels in fish during exposure to MT and also the applicability of this parameter to androgens in an FFLC. In the F₀ generation, the hepatic VTG levels of male medaka the day after the end of the reproductive phase increased in all groups treated with MT at 0.35 to 9.98 ng/L, although no statistically significant difference was determined. In addition, VTG levels in the F₁ males exposed to 9.98 ng/L of MT were significantly increased compared with those of the controls, although the levels of VTG in the fish in the 9.98-ng/L group were similar to the levels in control F₀ males. The reason for lower VTG levels overall in the F₁ generation than those in the F₀ generation in males may be caused by the different stages that were subjected to VTG measurement; that is, VTG levels in the F₀ generation were measured in fully mature stage (101 d posthatch), while VTG levels in the F₁ generation were measured in the early mature stage (60 d posthatch). This suggests that endogenous estrogens excreted by females in the F₀ generation may be higher than those in the F₁ generation, resulting in increased background VTG levels in F₀ males. On the other hand, although the VTG concentrations of females exposed to ≤9.98 ng/L of MT were not affected in the F₀ generation, those were significantly decreased in the F₁ generation. It is difficult to speculate on mechanisms for different sensitivities of VTG induction in the F₀ and F₁ generations. Metcalfe et al. [36] reported the potentiation of VTG response in male medaka exposed to DDT by maternal transfer compared with that in control males when all males were subsequently exposed to 17β-estradiol. The authors suggested that prior exposure to DDT could have enhanced the number of estrogen receptors or enhanced the affinity for the estrogen receptor. In the present study, MT might alter estrogenic responses in subsequent generations, resulting in different magnitudes of VTG induction in the F₀ and F₁ generations.

Several studies have reported on VTG responses in fish exposed to MT. Hori et al. [32] reported on the induction of VTG synthesis in juvenile goldfish (Carassius auratus) receiving large oral doses of MT. Ankley et al. [28] reported on the induction of plasma VTG in adult male fathead minnow exposed to MT for 21 d. Both authors suggested that MT was aromatized, resulting in the induction of the VTG synthesis. Actually, Trant et al. [33] reported that MT enhanced the expression of aromatase genes in zebrafish (Danio rerio) fry. Zerulla et al. [34] reported that the treatment of juvenile fathead minnow with MT led to messenger ribonucleic acid (mRNA) expression as well as increase of VTG. In addition, the authors reported that the combined exposure of MT and the aromatase inhibitor fadrozole had no effect on VTG mRNA expression and VTG protein synthesis in the fish. They also suggested that MT might be converted to an estrogen in fish tissues. Therefore, in this study, MT might be metabolized to an estrogen by aromatase, and the metabolite may lead to increase in hepatic VTG levels of male medaka. On the other hand, some studies have reported VTG decreases in female fish exposed to MT. Lazier et al. [37] reported that the treatment of mature female tilapia (Oreochromis niloticus) with MT resulted in a pronounced decline in hepatic VTG mRNA, serum VTG, and serum E₂ levels. In addition, Kitano et al. [38] reported that MT suppressed P450 aromatase gene expression in genetically female larvae of Japanese flounder (Paralichthys olivaceus). However, Ankley et al. [28] reported the induction of plasma VTG in adult female fathead minnow exposed to MT for 21 d. We presume that some species and/or sexual differences are present with respect to VTG response in fish when exposed to MT. However, further work is needed to determine the applicability of this VTG parameter to androgens in the FFLC.

In summary, an FFLC with medaka can detect androgenic effects of MT. We observed that all fish in the 27.75-ng/L treatment group showed male secondary sex characteristics in the F₀ generation, and several fish with ovaries in the F₀ and F₁ generations treated with 9.98 ng/L also showed male secondary sex characteristics. In addition, we found that no fish with ovaries could be discerned in the 27.75-ng/L treatment group in the F₀ generation, in which the sex ratio estimated by gonadal histology was significantly skewed toward males.

We also observed enlarged ovaries in the 9.98-ng/L treatment group of the F₀ generation, which accompanied declines in fecundity and fertility. We conclude that the FFLC with medaka is applicable to the evaluation of androgens based on the observation of masculinized appearance of secondary sex characteristics and gonads, which may ultimately affect reproductive potential.

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