FEMINIZATION OF FEMALE LEUKOPHORE-FREE STRAIN OF JAPANESE MEDAKA (ORYZIAS LATIPES) EXPOSED TO 17ß-ESTRADIOL

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Abstract—The recently developed female leukophore-free (FLFII) strain of Japanese medaka (Oryzias latipes) carries DNA markers for the identification of genotypic sex. Information regarding genotypic sex is useful for tests in which endocrine-disrupting compounds may masculinize or feminize fish. In the present study, methods were developed to automate DNA extraction and profiling for rapid determination of genotypic sex. Adequate amounts of DNA were isolated by robotic extraction procedures from the caudal fin. New primers were developed to include an 18-base pair segment that is in the X chromosome of female medaka but is absent in the Y chromosome of male medaka. Automated profiling methods with 96-well plates permitted analysis of the genomic sex of medaka at rates of up to 500 fish/d. We investigated the sensitivity of the FLFII strain to the feminizing effects of the potent estrogen 17ß-estradiol (E2), and we compared this sensitivity to that of a wild strain that has been used widely in the study of endocrine-disrupting compounds. All FLFII medaka exposed to 1 µg/L of E2 (n = 50) had the female gonadal phenotype (i.e., ovaries), and all but one wild-strain medaka exposed to 1 µg/L of E2 (i.e., 47 of 48 fish) had the female gonadal phenotype, indicating that the FLFII and wild strains have approximately equal sensitivities to the feminizing effects of E2. Analysis of the genotype of FLFII medaka confirmed that 100% of fish with the male genotype had been feminized to the female gonadal phenotype. The FLFII strain is an excellent teleost model for detecting feminization or masculinization of fish, and automated methods can be used for rapid analysis of the genotypic sex of FLFII medaka.

Keywords—Medaka Estradiol DNA profiling Female leukophore free Oryzias latipes

INTRODUCTION

Japanese medaka (Oryzias latipes) are well suited for the screening of endocrine-disrupting compounds. Medaka reach sexual maturity within three to four months posthatch, and their small size circumvents many of the logistical problems associated with the use of larger fish. A continuous supply of eggs can be maintained throughout the year, thus ensuring year-round availability. Medaka have been used to evaluate biological responses in fish exposed to endocrine-disrupting substances, including estrogens [1–4], androgens [4], and anti-androgens [5]. Exposure of medaka to test compounds results in either complete sex reversal (i.e., feminization or masculinization) or induction of intersex of the gonads and/or secondary sex characteristics. Complete sex reversal in exposed medaka has been detected by changes in the ratios of males to females, a method with limited sensitivity because of the natural variations in sex ratios among small sample populations. Among intersex medaka, it is difficult to determine definitively whether female medaka have been partially masculinized or male medaka have been partially feminized. Clearly, these studies of alterations to the sex phenotype would benefit from a marker of genotypic sex.

In medaka, sex is genetically determined by XX chromosomes in females and XY chromosomes in males. Various closed colonies of medaka, such as the d-rR strain developed by Yamamoto [6] have sex-linked color or pigmentation markers of genotypic sex. However, the utility of these markers has been limited for sex identification. Since the expression of color can be subtle in some fish (particularly when young) and, thus, are subject to interpretation. Some medaka strains have a male-specific sex-linked DNA marker (SL1) that is located close to the male-determining region on the Y chromosome [7]. Wakamatsu et al. [8] recently introduced the female leukophore-free (FLFII) strain of medaka that carries three markers of genotypic sex: Two markers for body color, and one for male- and female-specific DNA markers. In this strain, the genotypic sex can be screened at the early embryonic stages (i.e., 2-d postfertilization) by the presence (male) or absence (female) of pigmented leukophores and at later juvenile stages through the presence of orange-red xanthophores and the definitive confirmation by polymerase chain reaction (PCR) analysis of DNA markers. The error rate in determination of genetic sex using the presence of pigmented leukophores in embryos or the orange-red xanthophores in juveniles is approximately 1.4% and 0%, respectively. The error rate associated with the PCR (SL1) marker is also 0% [8].

Using the FLFII strain of medaka for studies of alterations to the phenotypic sex of fish exposed to endocrine-disrupting substances has obvious advantages. Several questions, however, require investigation before this strain can be widely used. The wild strains of medaka that traditionally have been used for in vivo assays are very sensitive to alterations of gonadal development after exposure to estrogens and androgens [1,2,5]. Studies are required to determine whether the FLFII strain is more or less sensitive to the biological effects of these compounds compared with other, previously investigated strains, such as the wild golden (Carolina Biological Supply, Gladstone, OR, USA). In addition, in vivo assays with
Japanese medaka typically involve large numbers of test organisms—as many as 50 per treatment among five treatments for a single chemical. Rapid, sensitive, and inexpensive techniques are required in the molecular analysis of large numbers of medaka for male- and female-specific DNA markers.

In the present study, we investigated the sensitivity of the FLFII strain to a potent estrogen, 17β-estradiol (E2), and we compared the sensitivity of this new strain to the wild strain (Carolina) of medaka that has been used historically in studies of endocrine-disruptive compounds. Methods were also developed to automate DNA extraction and profiling to facilitate rapid determination of genotypic sex using sex-specific DNA markers.

**MATERIALS AND METHODS**

Genetic sex determination of the FLFII strain using PCR techniques for the sex-linked marker (SL1), as previously described by Matsuda et al. [7], was modified for rapid analysis using an automated methodology for DNA extraction and profiling. Oligonucleotide primers developed by Matsuda et al. [7] were used to amplify and sequence the male- and female-specific regions of the extracted DNA. Sequencing was used to identify sex-specific differences and to characterize a shorter diagnostic region that could be analyzed using an automated DNA sequencer. Method development for the automated PCR techniques was performed with the use of unexposed, adult FLFII medaka. This PCR protocol was later applied to E2-exposed FLFII medaka to demonstrate the utility of this technique. The sensitivity of the FLFII strain to the estrogen (i.e., E2) in terms of alterations to gonad development and secondary sex characteristics was compared to the sensitivity of the widely studied wild golden (Carolina) strain.

**DNA extraction, PCR amplification, and characterization of male-specific fragments**

The DNA was extracted from whole caudal fins using an automated extraction protocol on an integrated robotic arm platform (CRS Robotics, Oakville, ON, Canada), including a multiprobe II liquid handler (Perkin-Elmer Packard Bioscience, Mississauga, ON, Canada) and vacuum manifold, using 96-well tissue-extraction plates (Qiagen, Mississauga, ON, Canada) and vacuum manifold, using an automated methodology for DNA extraction and profiling. Oligonucleotide primers developed by Matsuda et al. [7] were used to amplify and sequence the male- and female-specific regions of the extracted DNA. Sequencing was used to identify sex-specific differences and to characterize a shorter diagnostic region that could be analyzed using an automated DNA sequencer. Method development for the automated PCR techniques was performed with the use of unexposed, adult FLFII medaka. This PCR protocol was later applied to E2-exposed FLFII medaka to demonstrate the utility of this technique. The sensitivity of the FLFII strain to the estrogen (i.e., E2) in terms of alterations to gonad development and secondary sex characteristics was compared to the sensitivity of the widely studied wild golden (Carolina) strain.

PCR amplification conditions of X chromosome- and Y chromosome–specific fragments using internal primers with fluorescence (FAM) tags (ph 05.5_6-FAM and ph 05.5_9) were as described above. Control male and female samples were amplified using 10 ng of DNA template. Amplified products were run on an automated ABI 377 DNA sequencer using GeneScan® software (Applied Biosystems).

**Feminization of medaka from E2 exposure**

Medaka strains and husbandry. A breeding colony of the FLFII strain was established at Trent University (Peterborough, ON, Canada) approximately one year before the present study. The colony was developed from eggs supplied by Y. Wakamatsu from a colony established at Nagoya University (Japan). Further details concerning the FLFII strain and its development are described by Wakamatsu et al. [8].

Trent University has held a breeding colony of the golden wild strain (originating from Carolina Biological Supply) since 1991, with new members of the wild strain being introduced into the broodstock every three to four years in an attempt to maintain genetic variability. Fish of both strains were reared under identical husbandry conditions, which were within the ranges outlined by Kirchen and West [11]. Fish were fed ad libitum a mixture of live artemia (San Francisco Bay variety; Brine Shrimp Direct, Ogden, UT, USA) and commercial flake food (Total Color; Wardley, Secaucus, NJ, USA) two to three times per day. Water temperature was maintained at a range of 25 ± 2°C, and the photoperiod was 16:8: light:dark. The pH of the water ranged from 7.4 to 7.8. Alkalinity was 60 to 80 mg/L of CaCO3, and hardness was 80 to 100 mg/L of CaCO3. Fertilized eggs were collected from these broodstocks and raised to hatch as described previously [1]. Newly hatched medaka embryos were collected and placed in glass containers for exposure to the test chemical.

Conditions of exposure. Two treatments were established for both the FLFII and wild medaka strains. In one treatment, medaka were exposed to E2, whereas the second treatment was a control in which fish were exposed to a low concentration (100 μL/L) of acetone (distilled in glass grade) used as the carrier solvent in the E2 treatment. The test compound, E2 (catalog no. E-8875; lot no. 17H0190; purity, ≥98%; Sigma, Oakville, ON, Canada) was dissolved in acetone and added to dechlorinated municipal tap water in an E2/facetone solution (100 μL of E2/facetone stock per liter of water) to produce the nominal E2 concentrations of 1 μg/L. This nominal level of E2 was selected on the basis of previous observations of concentrations that feminized male medaka [1]. Exposures were conducted under static conditions, with 100% of the exposure water being renewed three times per week. Previous analytical confirmations within our laboratory concerning the mean exposure concentration of E2 indicated that the measured mean percentage concentration of nominal was approximately 43% during the 48-h static exposure period between the renewal of test solutions [1]. Exposure to E2 began within 2 d after hatch and continued for approximately 100 d. The tank sizes and water volumes were increased to 1, 2, and then 10 L of dechlorinated tap water as the fish grew, reaching a final density of approximately 25 adult fish in 10 L of water. Fish were killed at the end of the exposure period by an overdose of tricaine methane sulfonate (MS-222; Fisher Scientific, Toronto, ON, Canada) and then measured for wet weight and body length. The caudal fins of killed fish were removed and stored frozen for the determination of genetic sex. Statistical differ-
ences between the acetone and $E_2$ treatments regarding final fish lengths and weights were determined by the Mann–Whitney U test ($p < 0.05$) for the wild strain and by the unpaired t test ($p < 0.05$) for the FLFII strain. Statistical differences in the condition factor (mg/mm$^3$) of the medaka strains were analyzed using a two-tailed, unpaired t test ($p < 0.05$).

Secondary sex characteristics. The secondary sex characteristics were assessed on freshly killed fish with the aid of a dissecting microscope using the criteria described by Metcalfe et al. [12]. Anal and dorsal fins as well as the urogenital pore were examined, and results were recorded for the expression of either male or female characteristics. Statistical comparisons of the prevalence of mixed secondary sex characteristics were performed with Fisher’s exact test ($p < 0.05$, two-tailed).

Histological evaluation. Medaka carcasses were placed in tissue capsules and fixed in Bouin’s fixative in preparation for a histological examination of gonadal tissue. Medaka carcasses were dehydrated in a graded alcohol series and embedded in paraffin. Sagittal sections (thickness, 5–7 μm) were taken in a step-section manner (6–12 sections/fish), mounted on microscope slides, and stained with hematoxylin and eosin using standard staining techniques.

Gonadal tissues were histologically assessed for the stages of gametogenesis and the morphological appearance of the gonad plus the presence of oocytes within the testicular tissue of phenotypic males (i.e., testis–ova). Fish exhibiting testis–ova were classified as intersex fish. The stages of oogenesis in female medaka were classified as previtellogenic, vitellogenic, and postvitellogenic according to criteria developed by Iwamatsu et al. [13]. The stages of spermatogenesis were primarily based on criteria developed by Grier [14].

Genotypic sex was determined by the automated PCR method described above. These results were compared with the phenotypic sex as determined from the gonadal tissue and secondary sex characteristics.

RESULTS

Automation of DNA extraction and profiling

Extraction of DNA from the caudal fin using robotic techniques produced an average of 21.9 ng/μl of DNA (range, 8–45 ng/μl; n = 21). The PCR amplification using sex-specific ph 05.5F and 05.5R primers produced two distinct bands (a fragment at ~1,300 bp and a fragment at ~1,700 bp) in males (XY), whereas females (XX) produced only one band (a fragment at ~1,700 bp). A clear distinction between males (two bands) and females (one band) was evident based on gender-specific banding patterns.

Isolation of the male-specific fragment, DNA sequencing, and primer design

Male-specific bands were isolated by taking gel plugs of the 1,300-bp fragment from an agarose gel and resuspending in Tris-EDTA buffer. Amplification from one of the four gel plugs was successful in isolating only the male fragment, with no evidence of the female X chromosome. Four female samples were cleaned for the approximately 1,700-bp fragment and then sequenced to represent the X chromosome sequence for comparison. Alignment of the Y and X chromosome fragments identified an 18-bp indel segment that is present in the X chromosome but is absent in the Y chromosome (Fig. 1). Two additional indels were identified that generated a net 16-bp size difference between the Y chromosome–specific and X chromosome–specific fragments. Based on the sequence differences, internal primers were designed to amplify a smaller fragment that would include the indel sites. Male versus female classification was based on this 16-bp difference between the X chromosome– and Y chromosome–amplified regions and the amplification of two fragments and one fragment, respectively. The banding pattern is similar to the original pattern amplified using the original ph 05.5 primer set. However, the fragments are smaller and, therefore, can be run on an automated sequencer using fluorescently labeled primers. The internal primer sequences for the smaller fragments are as follows:

ph 05.5INT 6 FAM:5’ GGC TTA ATT TTC TCC TAT GG 3’
ph 05.5INT 2:5’ GTC AAA TGA ACC ACA TGC TC 3’

Validation of internal primer sequences

Testing and validation of the internal primer sequences were performed on male and female samples. An automated sequencer was used to identify the fluorescently labeled primers. The results were visualized as peaks corresponding to the sizes of the fragments amplified from the X and Y chromosomes. The X band is slightly larger than the Y band, which is consistent with the indel polymorphism observed between the two amplified fragments. The male sample generated two peaks, one of each size (Fig. 2), whereas the female sample generated only a single peak.

Responses of medaka to $E_2$ exposure

Growth and mortality. Survival at the end of the 100-d exposure period within the acetone-treated fish was 73% in the wild strain and 95% in the FLFII strain. In the $E_2$ treatments, survival was 83% in the wild strain and 81% in the FLFII strain. All treatments exceeded the 70% survival criteria that we normally apply to this test protocol and, therefore, were considered to be valid. Growth of the FLFII strain was similar to that of the wild strain. In the control treatment, body length was $23 \pm 2$ (mean ± standard deviation) and $24 \pm 2$ mm in the FLFII and wild strain, respectively. Likewise, weights were $123 \pm 20$ and $112 \pm 28$ mg in the FLFII and wild strain, respectively. When exposed to $E_2$, growth was significantly suppressed in the wild strain (Mann–Whitney U test; weight, $Z = -8.10, p < 0.0001$; length, $Z = -0.47, p < 0.0001$) and also in the FLFII strain (weight, $F_{1,98} = 350, \ldots$
to their corresponding control treatments. In E2 treatments, body lengths were 17 ± 2 mm in the wild strain, and body weights were 54 ± 17 and 67 ± 14 mg for the FLFII and wild strains, respectively. Overall, the condition factor of the FLFII strain (acetone control, 0.0104 ± 0.00157; E2, 0.0110 ± 0.00187) was moderately but significantly greater (acetone control, $p < 0.001$; length, $F_{1,98} = 378$, $p < 0.0001$) when compared to their corresponding control treatments. In E2 treatments, body lengths were 17 ± 2 mm in the FLFII strain and 19 ± 2 mm in the wild strain, and body weights were 54 ± 17 and 67 ± 14 mg for the FLFII and wild strains, respectively. Overall, the condition factor of the FLFII strain (acetone control, 0.0104 ± 0.00157; E2, 0.0110 ± 0.00187) was moderately but significantly greater (acetone control, $F_{1,115} = 129$, $p < 0.0001$; E2, $F_{1,137} = 11.3$, $p = 0.001$) than that found in the wild strain ($p < 0.0001$; length, $F_{1,98} = 378$, $p < 0.0001$) when compared to their corresponding control treatments. In E2 treatments, body lengths were 17 ± 2 mm in the FLFII strain and 19 ± 2 mm in the wild strain, and body weights were 54 ± 17 and 67 ± 14 mg for the FLFII and wild strains, respectively. Overall, the condition factor of the FLFII strain (acetone control, 0.0104 ± 0.00157; E2, 0.0110 ± 0.00187) was moderately but significantly greater ($p < 0.001$; Fisher’s exact test, two-tailed).

**Secondary sex characteristics.** Common patterns of mixed secondary sex characteristics included attributes such as a female-specific anal fin together with a male-specific dorsal fin, or vice versa, within the same individual. None of the control fish from the wild strain exhibited mixed secondary sex characteristics (Table 1). Therefore the morphology of the anal fin was appropriate and consistent with the morphology of the dorsal fin and urogenital pore, providing unambiguous gender identification. In contrast, mixed secondary sex characteristics were found in the control treatment of the FLFII strain, suggesting that the gender specificity of secondary sex characteristics in this new strain may not be as reliable as that in the wild strain. When exposed to E2, the incidence of fish with a mixture of female and male secondary sex characteristics was significantly elevated ($p < 0.05$, Fisher’s exact test, two-tailed) in the wild strain, but not in the FLFII strain (Table 1), when compared to the corresponding control treatment.

**Gonadal tissues.** Testicular development in the control treatments appeared to be normal for males of both the wild and FLFII strains. Ovarian development in the control treatments of the wild strain generally contained oocytes at all stages of oogenesis, including previtellogenic, vitellogenic, and post-vitellogenic stages (stages I–IX as defined by Iwamatsu et al. [13]). In contrast, ovarian development in the FLFII control fish was not as advanced as in the wild strain. Most FLFII ovaries contained oocytes at early vitellogenic stages (stages VI, V, or earlier).

All E2-exposed fish of both strains were phenotypic females except for one phenotypic male that was observed in the wild strain. The testes of the one phenotypic male (wild strain) appeared to be normal except for the absence of spermatozoa. Ovarian development in most females of both strains consisted of oocytes that did not progress beyond the early vitellogenic stage (stages V and VI), similar to what was observed in the control FLFII treatment. Approximately one-quarter of the ovaries also exhibited what appeared to be an eosinophilic fluid interspersed among developing oocytes. In addition, nearly all medaka exhibited histopathological alterations to kidney tissue, such as greatly dilated Bowman’s capsules that were often filled with the same eosinophilic fluid as found in the ovaries.

**Assessment of phenotypic and genotypic sex.** Histological examination of the gonadal tissue revealed that the phenotypic

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Table 1. Assessment of secondary sex characteristics as well as phenotypic and genotypic sex of the wild and female leukophore-free (FLFII) medaka exposed to either 1 μg/L of 17β-estradiol (E2) or the acetone carrier solvent (control)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Nominal exposure concn. (μg/L)</th>
<th>% With mixed secondary sex characteristics</th>
<th>Total (n)</th>
<th>Females (n [%])</th>
<th>Males (n [%])</th>
<th>Without gonadal tissue (n)</th>
<th>Profilled (n)</th>
<th>Females (n [%])</th>
<th>Males (n [%])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild</td>
<td>Control</td>
<td>0</td>
<td>0 (0/50)</td>
<td>50</td>
<td>27 (54%)</td>
<td>23 (46%)</td>
<td>0</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Estradiol</td>
<td>1</td>
<td>14 (7/50)</td>
<td>50</td>
<td>20 (49%)</td>
<td>21 (51%)</td>
<td>9</td>
<td>25^a</td>
<td>10 (40%)</td>
<td>15 (60%)</td>
</tr>
<tr>
<td>FLFII</td>
<td>Control</td>
<td>0</td>
<td>14 (7/50)</td>
<td>50</td>
<td>20 (49%)</td>
<td>21 (51%)</td>
<td>9</td>
<td>25^a</td>
<td>13 (52%)</td>
<td>12 (48%)</td>
</tr>
<tr>
<td></td>
<td>Estradiol</td>
<td>1</td>
<td>30 (15/50)</td>
<td>50</td>
<td>41 (100%)</td>
<td>0</td>
<td>9</td>
<td>25^a</td>
<td>13 (52%)</td>
<td>12 (48%)</td>
</tr>
</tbody>
</table>

^a The percentage of phenotypic females or males determined from the total number of fish with visibly confirmed gonadal tissue.

NA = not analyzed.

^Significantly elevated above acetone control of wild strain ($p < 0.05$, Fisher’s exact test, two-tailed).

^Significantly elevated above response observed in acetone control of wild strain ($p < 0.05$, Fisher’s exact test, two-tailed).

^Selected at random from 50 in each treatment.
The FLFII strain of medaka

**DISCUSSION**

The present study demonstrates a rapid and practical technique for monitoring the genotypic sex of the FLFII medaka strain when used as model organisms in tests with endocrine-disrupting compounds. The quantity of DNA extracted from the caudal fin of the FLFII medaka strain using automated robotic techniques is suitable for PCR analysis. By removing only the caudal fin, the remaining body tissues can be used for evaluation of other relevant tissues, including the blood serum for analysis of vitellogenin induction and the body cavity for histological characterization of the gonads. Automation of PCR analysis allows rapid throughput of a large number of samples. For example, our robotic facilities have the capability to analyze 500 samples within a 24-h period.

The response of the FLFII strain to a potent estrogen (i.e., \(E_2\)) appears to be similar to that of the wild medaka strain in terms of survival, growth, and sensitivity to the feminizing effects of \(E_2\). The FLFII strain, however, appears to have a slightly larger condition factor. The significance of this is unknown, but it likely reflects slight differences in body morphology. Similar effects of \(E_2\) exposure on growth in medaka have been noticed by Patyna et al. [15], who found that prolonged feeding of \(E_2\)-amended food (0.05–0.5 mg/kg) stunted the body length of the medaka but had less impact on body weight, resulting in shorter but heavier fish. In addition, Patyna et al. [15] and Metcalfe et al. [1] noted renal hemorrhaging, edema, congestion, and hydropic degeneration in glomeruli of the kidney in \(E_2\)-exposed medaka, which was similar to the pathology of \(E_2\)-exposed medaka in the present study. Zillioux et al. [16] noticed similar effects in the kidneys of sheepshead minnows chronically exposed to 400 ng/L of 17\(\alpha\)-ethinylestradiol. Metcalfe et al. [1] theorized that the eosinophilic fluid found in the various organs of the medaka exposed to estrogenic compounds may be vitellogenin that accumulated because of inadequate deposition in oocytes.

Gonadal development of control fish from the FLFII strain appeared to be normal, although somewhat delayed, in females compared with development of females from the wild strain. Previous observations (G. Balch, unpublished data) indicate that FLFII females first start to produce eggs a few weeks later than females of the wild strain. The Y chromosome near the sex-determining region of the FLFII strain originated from the...
HNI strain of medaka from northern Japan. The HNI strain is slower to sexually mature compared with the wild strain used in the present study, which originated from a population in southern Japan. The depth of the anal fin in the HNI strain also appears to be shallower than that in the wild strain, making it slightly more difficult to recognize the male phenotype from external sex characteristics.

The effect of E₂ on delayed ovarian development in both the wild and FLFII strains in the present study is similar to the delayed ovarian maturation observed by Patyna et al. [15] in medaka exposed to E₂. The skewed ratio of phenotypic females in relation to males observed by Metcalfe et al. [1] in medaka exposed to 1 µg/L of E₂ was interpreted as being evidence that genotypic males were undergoing complete feminization to phenotypic females. The techniques employed in the present study for the identification of genotypic sex provide definitive evidence that chronic exposure to E₂ at the same concentration can result in complete sex reversal of genotypic males to the female phenotype. The PCR analysis eliminates reliance on ratios of females to males, which can be problematic when sample sizes are small.

Some questions remain concerning the use of mixed secondary sex characteristics in the FLFII strain as an indicator of feminization or masculinization. The assessment of mixed secondary sex characteristics is subjective at best, and it depends on the experience of the assessor. Typically, in our laboratory, the incidence of mixed secondary sex characteristics in control treatments with the wild strain at 100-d posthatch varies between 0% to 4% (G. Balch, unpublished data). However, the higher prevalence of mixed secondary characteristics observed during the present study in unexposed fish from the FLFII strain indicates that the secondary sex characteristics may not be fully developed by 100 d posthatch. Secondary sex characteristics should not be used as an indicator of masculinization or feminization with the FLFII strain until these fish have fully reached sexual maturity, which appears to happen after 100 d posthatch. Further studies are required to determine an appropriate age at which this phenotypic sex identifier can be applied.

Overall, studies of gonadal development with the FLFII medaka strain, when coupled with PCR determination of genotypic sex, show great potential as a model assay system for evaluating responses to endocrine-disrupting compounds. The methods described in the present publication permit rapid genotypic analysis of individual fish while maintaining adequate amounts of tissue for both biochemical and histological surveys. These techniques eliminate much of the subjective interpretation that is required to evaluate skewed sex ratios, intersex, and ambiguous mixed secondary sex characteristics in fish. This model is particularly useful when it is not known if a test compound or mixture is an androgen, estrogen, antagonist, or agonist.

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