ADVANCED FLUORESCENCE IN SITU HYBRIDIZATION TO LOCALIZE AND QUANTIFY GENE EXPRESSION IN JAPANESE MEDAKA (ORYZIAS LATIPES) EXPOSED TO ENDOCRINE-DISRUPTING COMPOUNDS


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Abstract—In an earlier study, we described the development of fluorescence in situ hybridization (FISH) using confocal microscopy to localize and quantify gene expression in fish. Here, we report the results of FISH application to investigate effects of model endocrine-disrupting chemicals (EDCs), 17α-ethinylestradiol (EE2) and 17β-trenbolone (TB), on expressions of EDC-responsive genes in Japanese medaka (Oryzias latipes) at the cellular/tissue level paired with histological observation. Gene expressions of vitellogenin-II (Vit-II), androgen receptor (AR), and cytochrome P450 gonadal aromatase (CYP19a) were determined after exposure to 5, 50, or 500 ng/L of EE2 or 50, 500, or 5,000 ng/L of TB for 7 d. Exposure to the greatest concentration of EE2 or TB significantly reduced fecundity and caused histological alterations in gonads. 17α-Ethinylestradiol induced Vit-II expression in both male gonads and liver relative to controls and resulted in greater intensity of hematoxylin staining in hepatocytes, which was significantly correlated with Vit-II induction in liver. When exposed to EE2 at less than 50 ng/L, CYP19a expression associated with early stage oocytes was greater than that in controls. However, at 500 ng/L, this trend was reversed. The greater Vit-II expression in testis from all EE2 groups, and the lesser expression of CYP19a in ovaries from the 500 ng/L group, likely is related to changes in the number of cells in which these genes are predominantly expressed rather than to an increase in expression per cell. 17β-Trenbolone significantly induced AR expression in ovaries but did not alter AR expression in female liver. It was concluded that FISH combined with histology enables advanced elucidation of molecular effects of chemicals by associating changes in gene expression with certain tissues and/or cell types and allows these changes to be related to histological effects.

Keywords—Fluorescence in situ hybridization, Genomics, Histology, Fish

INTRODUCTION

To identify the molecular mechanisms of toxic action by a chemical, it is necessary to detect and quantify the expression of mRNAs that encode for proteins involved in key processes of the pathway of interest. One technique to quantify changes in gene expression associated with certain tissues or cells, or to map such changes throughout an organism, is fluorescence in situ hybridization (FISH) [1]. This technique allows direct visualization of specific mRNA sequences in tissues, individual cells, and/or subcellular structures. The advantage of FISH compared to conventional molecular techniques is that it combines molecular biology with histology to evaluate gene expression associated with specific cell types in a tissue [1,2]. Measuring spatial and temporal changes in gene expression as a consequence of chemical exposure can provide information concerning regulation of genes as a function of cell type and/or tissue. Localization of specific genes at the tissue or cellular level also can help to further our understanding of gene expression patterns in context with pathologies as determined by parallel histology.

In a previous study, a FISH protocol was optimized and validated to detect spatial expression of mRNA in whole-mount sections of Japanese medaka (Oryzias latipes) [1]. Here, we report the results of a study in which the optimized FISH method was used to evaluate effects of two model endocrine-disrupting compounds, the synthetic estrogen 17α-ethinylestradiol (EE2) and the synthetic androgen 17β-trenbolone (TB), by measuring the expression of three selected genes responsive to endocrine-disrupting compounds: Vitellogenin-II (Vit-II) was chosen as a model gene because of its strict control by estrogens like EE2, androgen receptor (AR) because of its great affinity to androgens like TB, and cytochrome P450 gonadal aromatase (CYP19a) because of its possible changes in gene expression caused by EE2 or TB. Briefly, EE2 is a synthetic analogue to the endogenous estrogen (17β-estradiol [E2]), is a strong estrogen receptor (ER) agonist [3], and represents one of the most potent xenoestrogens known to be present in the aquatic environment [4]. Trenbolone is the product of the hydrolysis of trenbolone acetate, a synthetic androgen that is a mammalian AR agonist and that is used as a growth promoter for cattle in the United States [5]. 17β-
Trenbolone has the potential to adversely affect aquatic organisms because of its relatively long half-life in water and soil [6], and it has been reported to cause disorders in reproductive endocrine functions in fish, including masculinization of females [7–9]. Several studies have researched the molecular interactions of EE$_2$ and TB with the endocrine system and their subsequent biochemical and pathological changes in fish, but little is known about the spatial regulation of the genes of interest after exposure to these compounds.

The objective of the present study was to investigate the short-term effects of EE$_2$ and TB on physiological, histological, and molecular endpoints in Japanese medaka. Specifically, we applied a recently optimized and validated FISH method to elucidate the effects of EE$_2$ and TB on the tissue- and cell-specific expression of CYP19a, Vit-II, and AR mRNA in whole-mount sections of medaka. Furthermore, changes in target gene expression were compared with histological responses to further our understanding of the molecular mechanisms of action by EE$_2$ and TB.

**MATERIALS AND METHODS**

**Test chemicals**

In the present research, EE$_2$ (purity, >98%; Chemical Abstracts Service [CAS] no. 57-63-6; 17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol; Sigma-Aldrich) and TB (purity, >95%; CAS no. 10161-33-8; 17β-hydroxyestra-4,9,11-trien-3-one; Sigma-Aldrich) were used. Dimethyl sulfoxide (DMSO) was used as a carrier solvent to deliver EE$_2$ or TB in water at a final concentration of 0.01% (v/v).

**Culture of Japanese medaka**

Wild-type Japanese medaka were obtained from a population cultured by the U.S. Environmental Protection Agency Mid-Continent Ecology Division. Medaka were held in flow-through systems under conditions that facilitated breeding (23–24°C, 16:8-h light:dark photoperiod). Medaka were fed Aquatox® flake food (Aquatic Ecosystems) once daily and brine shrimp (Artemia sp.) twice daily ad libitum. All procedures used during all phases of the present study were in accordance with protocols approved by the Michigan State University Institutional Animal Care and Use Committee.

**Chemical exposures**

Before initiation of the exposure experiments, 12- to 14-week-old medaka were placed into 10-L tanks with 6 L of carbon-filtered tap water and acclimated for 12 d under the same conditions as in the subsequent exposures. Each treatment group consisted of triplicate tanks, and each tank contained five male and five female medaka. After the acclimation period, medaka were exposed to 5, 50, or 500 ng/L of EE$_2$ or 50, 500, or 5,000 ng/L of TB. Carbon-filtered tap water containing DMSO at a final concentration of 1:10,000 (v/v) served as a vehicle control. Every day during the exposure phase of the present study, half the water in each tank (3 L) was replaced with fresh carbon-filtered water dosed with the appropriate amount of EE$_2$ (5 mg/L in DMSO) or TB (50 mg/L in DMSO) stock. Dimethyl sulfoxide has been widely applied to deliver synthetic hormones in studies of endocrine disruption. Although DMSO is considered to have the potential to act as a radical scavenger, it rarely has been reported to affect gene expression in many in vivo or in vitro studies [10–13]. In an in vitro study, DMSO at 0.1% (v/v) caused a time-dependent increase of vitellogenin mRNA expression in salmon hepatocytes [14]. However, considering that the responses observed in the solvent controls of the present study were in accordance with previous reports of gene expressions observed in nonexposed animals and that the concentration of DMSO used in the present study was an order of magnitude less than that for which effects have been observed previously [14], it can be assumed that the concentration of DMSO used here did not affect responses of the endpoints measured here.

Eggs produced during the previous 24 h were counted before each replacement of water. Medaka were fed Aquatox flake food and brine shrimp once daily, and the tanks were kept at 24°C and a 16:8-h light:dark photoperiod. Water-quality parameters (temperature, pH, hardness, dissolved oxygen, ammonia nitrogen, and nitrate nitrogen) were measured daily, and values were within a normal range for water quality. After exposure for 7 d, medaka were killed in a 120 mg/L solution of Tricaine S (Western Chemical) and mass determined. Liver and gonads designated for determining effects on hepatic and gonadal growth were collected and mass determined. Liver somatic index (LSI) and gonadosomatic index (GSI) were calculated as follows:

\[
\text{LSI} = \frac{\text{Liver wt}/\text{body wt without viscera}}{100} \quad (1)
\]

\[
\text{GSI} = \frac{\text{Gonad wt}/\text{body wt without viscera}}{100} \quad (2)
\]

Medaka designated for analysis by FISH were fixed as described below.

**Analysis by FISH**

**Preparation of sections.** Sections were prepared for FISH in accordance with the methods described by Park et al. [1]. Briefly, fish were dissected to remove fins, tail, skull roof, otoliths, and opercula. The body cavity was opened to improve the penetration of fixative (80% Histochoice MB [EMS], 2% paraformaldehyde, and 0.05% glutaraldehyde) for better internal organ fixation. Medaka were then immersed in the fixative and allowed to fix overnight at room temperature. Fixed medaka were washed with methanol, dehydrated through a graded methanol series, and then cleared in chloroform at 4°C. Fixed and cleared samples were infiltrated with melted ParaPlast Plus paraffin (McCormick Scientific), and the resulting paraffin blocks were stored under RNase-free conditions at 4°C until sectioning.

Whole medaka were sectioned longitudinally with a rotary AO-820 microtome (American Optical) under RNase-free conditions using Absolute Ethanol (Fisher Scientific) and RNase-Zap (Sigma-Aldrich). Serial sections were cut at a thickness of 7 μm and placed on SuperFrost® Plus slides (Erie Scientific). Slides were stored in RNase-free containers at room temperature until used for FISH.

**Fluorescence-labeled riboprobe synthesis.** All procedures to synthesize fluorescence-labeled riboprobes were adapted from the methods described by Park et al. [1] with minor modifications. To synthesize the riboprobes, reverse-transcribed first-strand cDNA was used as a template in a conventional polymerase chain reaction (PCR) with corresponding primers to amplify PCR products of CYP19a, Vit-II, and AR (Table 1). Probes for FISH were designed using Beacon Designer 2 (PREMIER Biosoft) to have lengths of approximately 500 bp. Probe length was chosen based on a review of Wilkinson [2], who reported that probes either too short (<100 bp) or too long (>1,000 bp) may give weaker hits.
signals, possibly because of either low specificity to target transcript or low penetration efficiency into tissue, respectively. The sequence of each riboprobe to detect corresponding target mRNA was compared with all sequences of known genes in Japanese medaka, and no sequence homogeneity was found except for the target gene of interest.

The method used to clone the PCR product into pGEM T-Easy vector (Promega) has been described previously [1]. To synthesize the sense probes, their corresponding cloned plasmids were digested with SaI (Invitrogen) for CYP19α and Vit-II and with SpeI (New England Biolabs) for AR. For antisense probes, cloned plasmids were digested with NcoI (Invitrogen) for CYP19α and Vit-II and with SaI (New England Biolabs) for AR. We confirmed the digestion with electrophoresis on agarose gel by observing a single band with the size of the plasmid plus the inserted PCR product (data not shown). Sense and antisense riboprobes were synthesized using in vitro transcription and labeled with fluorescence dye (Alexa Fluor 488; Molecular Probes) as described by Park et al. [1].

**FISH procedures.** The FISH procedures and washing steps were in accordance with the methods described by Park et al. [1], with minor modification to improve probe specificity to the mRNA sequences of interest. To reduce autofluorescence signal originating from either the tissues or the fixative, slides were treated with 10 mg/ml of sodium borohydride (Sigma-Aldrich) in PBS [1]. Microtome sections of medaka were hybridized with the riboprobe (1.5 ng/µl for AR and Vit-II and 2 ng/µl for CYP19α) at 43°C for 17 h. To evaluate the binding specificity of the antisense probe, a subset of sections that received an equal amount of sense probe was analyzed during each hybridization experiment. Expression of Vit-II was measured in male and female medaka exposed to EE2. However, AR expression was measured only in females exposed to TB, both because AR is activated by the binding of androgens and because the expected low basal expression of this gene in females likely allows distinct changes to be observed after TB exposure. Gonadal aromatase mRNA was measured only in female medaka exposed to either EE2 or TB to assess changes in its expression as a consequence of exposure.

**Confocal laser scanning microscopy image analysis**

Distribution of the fluorescent probes bound to sections were identified and quantified by use of confocal fluorescence microscopy (Laser Scanning Microscopy 510 Meta system; Carl Zeiss) as described by Park et al. [1]. Briefly, to account for background autofluorescence because of tissues and/or components of the hybridization procedure, individual spectral components associated with autofluorescence, background, and Alexa Fluor 488 dye were separated using the confocal system. Autofluorescence and background spectral components were obtained from the section of each tissue (ovary, testes, and liver) on the slide hybridized without probe. The specific spectrum of Alexa Fluor 488 dye was obtained directly from dye reagent. Once defined, the number of significant and independent sources of the specific spectral components using confocal laser scanning microscopy was then subjected to linear spectral unmixing to separate the individual components and to remove autofluorescence signal in the recorded images and so obtain the specific fluorescence signal of Alexa Fluor 488 dye (for details, see Park et al. [1]). To avoid the alteration of autofluorescence spectral shape by photobleaching that can result from consecutive laser scans, each set of FISH experiments had at least one FISH section with sense probe as a control for antisense probe specificity and one FISH section without probe for the separation of spectral components.

Images of the tissues were collected with a Zeiss EC Plan NEOFLUAR at ×10 magnification (Carl Zeiss). Gene expression in the ovary was quantified in three randomly selected areas in each section hybridized with the antisense probe specific to the gene of interest. Expression of CYP19α was then quantified in three early stage oocytes (diameter, <100 µm) selected randomly in each area, because the fluorescent signal was strongest and most consistent in this cell type. These oocytes were classified as being previtellogenic fluorescent signal was strongest and most consistent in this cell type. These oocytes were classified as being previtellogenic.

**Table 1. Probes with primers, GenBank accession numbers, amplicon sizes, and cycling conditions for conventional polymerase chain reaction**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Primers (sense/antisense, 5’−3’)</th>
<th>GenBank accession no.</th>
<th>Amplicon size</th>
<th>Denaturing</th>
<th>Annealing</th>
<th>Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP19α</td>
<td>CCTGTTAATGGTCTGGTAGTAC/</td>
<td>D82968</td>
<td>496</td>
<td>94/45</td>
<td>55/30</td>
<td>72/90</td>
</tr>
<tr>
<td></td>
<td>GAAGGCGCTGTGAGGATCT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AR</td>
<td>GTGCGGACAAAGAGGACTG/CCATCTAAAGCGAACCACATC</td>
<td>AB07399</td>
<td>461</td>
<td>94/45</td>
<td>55/30</td>
<td>72/90</td>
</tr>
<tr>
<td>Vit-II</td>
<td>CACATCCATGCAGAATTCCATCTC/</td>
<td>AB074891</td>
<td>480</td>
<td>94/45</td>
<td>55/30</td>
<td>72/90</td>
</tr>
<tr>
<td></td>
<td>TGACCTACTCCATTTGGGAAAG</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*AR = androgen receptor; CYP19α = cytochrome P450 gonadal aromatase; Vit-II = vitellogenin II.*
mounting medium (EMS) and allowed to dry. Images of the tissues on each slide were recorded using a Nikon® Eclipse TE300 microscope with image software (SPOT; Diagnostic Instrument). The intensity of staining of hepatocytes with hematoxylin, a measure of the amount of genetic material present in the tissue, was determined with image-analysis software (Image J 1.38X; National Institutes of Health). Briefly, digitized images of livers were segmented to obtain the purple color on the image by setting the Hue histogram to from 212 to 255, which represents the nucleotide in the cell stained with hematoxylin. Purple-stained spots of more than 400 pixels in size were enumerated.

Statistics
Statistical analyses were conducted using SAS® (SAS Institute). Data sets were tested for normality using the Shapiro–Wilks test and were log-transformed if necessary to achieve normality. Statistical differences between treatment groups were determined using one-way analysis of variance followed by the Student–Newman–Keuls test for multiple comparisons. For comparison of means of two groups, the Student’s t test was applied. The relationship between the degree of staining of hepatocytes with hematoxylin and the expression of Vit-II as measured by FISH was investigated by use of nonparametric two-tailed Spearman rank correlation. The criterion for significance in all statistical tests was \( p < 0.05 \).

RESULTS

Biological indices and fecundity
The wet weight and length of medaka exposed to either EE\(_2\) or TB were not significantly different from those of control medaka (data not shown). In female medaka, EE\(_2\) did not significantly affect LSI or GSI, whereas in male medaka, exposure to all concentrations of EE\(_2\) resulted in statistically greater LSIs compared with those of the controls (Supporting Information, Fig. S1A; http://dx.doi.org/10.1897/08-574.S1). Trenbolone caused a statistically significant increase in LSI at 50 ng/L in females, whereas the concentration required to cause a statistically significant increase in GSI was 500 ng/L. For males, the only statistically significant effect was observed for LSI after exposure to 500 ng/L of TB (Supporting Information, Fig. S1B; http://dx.doi.org/10.1897/08-574.S1). Production of eggs was 48% less in medaka exposed to 5,000 ng/L of EE\(_2\) compared with that in the controls. However, this difference was not statistically significant (Supporting Information, Fig. S2A; http://dx.doi.org/10.1897/08-574.S1). Cumulative egg production was 60 and 79% less in medaka exposed to 500 or 5,000 ng/L of TB, respectively (Supporting Information, Fig. S2B; http://dx.doi.org/10.1897/08-574.S1). Egg production almost completely ceased after the second day of exposure to 5,000 ng/L of TB.

Histology
Both testes and ovaries of control medaka exhibited the complete gonadal differentiation and maturation characteristic for this species (Fig. 1A and B). Testicular tissue was well developed, and all types of germ cells were present with spermatogonia and spermatocytes located in the periphery and spermatids and spermatозоа in the center of the testis. All stages of oocyte maturation were present in the ovary, including primary oocytes, previtellogenic oocytes, vitellogenic oocytes, mature oocytes that had a clearly expressed yolk globule, and visible corpora lutea.

Exposure to EE\(_2\) caused changes in both the ovary and testes. All types of germ cells were present in testes of males exposed to 500 ng/L of EE\(_2\), but initial signs of degeneration of spermatids and spermatozoa and a greater proportion of connective tissue also were observed (Fig. 1C). Testicular ovarian follicles (perinucleus stage) were observed in one testis section of one male medaka exposed to 500 ng/L of EE\(_2\) (figure not shown). Exposure of females to EE\(_2\) resulted in alterations of the composition of cells with different maturation stages in the ovary. Fewer mature oocytes, more atretic oocytes, and larger volume of somatic stromal tissue were observed (Fig. 1D). Exposure to TB resulted in several effects on the ovary but only a few effects on the testis. The only effects of TB on the histology of testes were observed in medaka exposed to 5,000 ng/L. All types of germ cells were present in the testes of medaka exposed to this concentration, but an accelerated development of spermatids was observed such that fewer spermatogonia were observed compared to the controls (Fig. 1E). Exposure of females to TB at 5,000 ng/L resulted in the predominant cell type being mature oocytes, with fewer previtellogenic oocytes present when compared to controls (Fig. 1F). Most of the mature oocytes of females from this group exhibited signs of disrupted yolk accumulation and increased yolk vacuolization.

Exposure to EE\(_2\) caused changes in the histology of livers of males. Liver tissue of control males was stained mainly with eosin, which stains extracellular or intracellular proteins in the cytoplasm of hepatocytes (Fig. 2A). After exposure to 500 ng/L of EE\(_2\), a shift occurred in the staining of hepatocytes in the livers of males toward predominantly hematoxylin, which is an indicator of the presence of nucleic acids (Fig. 2B). The number of stains with hematoxylin in the livers of males exposed to 500 ng/L of EE\(_2\) (Fig. 2B*) was significantly greater than that in the livers of unexposed males (Fig. 2A*). No statistically significant difference in numbers of hematoxylin stains between livers of males exposed to EE\(_2\) and control females was found (Fig. 2D).

Gene expression
Vit-II mRNA expression in medaka exposed to EE\(_2\). Expression of Vit-II mRNA in livers and gonads of both male and female medaka was detected by use of optimized FISH (Fig. 3). Exposure to EE\(_2\) resulted in an induction of Vit-II mRNA expression except in the livers of female medaka (Fig. 4). Sections hybridized with sense probe revealed no fluorescent signal (Fig. 3A–D). In ovaries hybridized with Vit-II antisense probe, the fluorescence signal was specific to previtellogenic oocytes and rarely detected in matured oocytes. Although Vit-II expression appeared to be induced in ovaries of medaka exposed to 500 ng/L of EE\(_2\), no statistically significant difference from the fluorescence signal intensity in ovaries of control medaka was observed (Fig. 4B). In testes, the fluorescence signal was specific to the region where spermatogonia prevailed and rarely was observed in the region of matured spermatida. Intensities of fluorescence were in the following order: Spermatogonia \(\gg\) spermatocytes and spermatids \(\gg\) spermatooza (Fig. 3A*). Average fluorescence intensities of the exposed fish were approximately twofold greater than those observed for the controls (Fig. 4A). However, this difference was not statistically significant. The
Vit-II mRNA expression also was detectable in female livers, but no difference was found between livers of females exposed to EE$_2$ and the controls (Fig. 4C). Fluorescence signal in livers of control males rarely was detected. However, once exposed to EE$_2$, the expression of Vit-II was significantly induced in this tissue. Expression of Vit-II in livers of males exposed to 500 ng/L of EE$_2$ was comparable to that observed in livers of females (Fig. 4C). Expression of Vit-II in livers of males as measured by FISH was directly proportional to the number of hematoxylin-stained spots in this tissue ($r^2 = 0.821$, $n = 23$, $p < 0.05$).

Expression of AR mRNA in medaka exposed to TB. Expression of AR mRNA could be detected with the antisense probe in both ovaries and livers of exposed female medaka by use of FISH (Fig. 5A and B). However, no fluorescence signal could be observed in control sections (i.e., hybridized with AR sense probe; figure not shown). In the ovary, AR expression was observed primarily in early stage oocytes, and TB caused a significant dose-dependent up-regulation of this gene. No significant changes in AR expression in liver tissue were observed between any of the TB treatment groups and the controls (Fig. 5C and D). Overall, AR expression in tissues of...
both control and exposed female medaka was low except for \(AR\) gene expression in ovaries of medaka exposed to 5,000 ng/L of TB (Fig. 5C).

Expression of \(CYP19a\) mRNA in medaka exposed to \(EE_2\) or TB. The specificity of hybridization was demonstrated by FISH using sense probe, which resulted in a very weak fluorescence signal (Fig. 6A). Hybridization of longitudinal sections of whole medaka with \(CYP19a\) antisense probe revealed that this gene was predominantly expressed in the ovary. The greatest fluorescence intensity was associated with premature, early stages of oocytes and with previtellogenic oocytes. Expression of \(CYP19a\) was less in vitellogenic oocytes, and mature oocytes expressed little or no \(CYP19a\) (Fig. 6B–D). (D) Also shown is the number of hematoxylin-stained spots greater than 400 pixels in size (mean ± standard error of the mean; arrow in D). Significant differences relative to the control are indicated with an asterisk \((p < 0.05)\). Bar = 50 μm. In color online at http://dx.doi.org/10.1897/08-574.

DISCUSSION

Exposure to \(EE_2\)

Fecundity and histology. Exposure to \(EE_2\) resulted in fewer eggs being produced by Japanese medaka, a result that is consistent with a study by Scholz and Gutzzeit [15], in which exposure of Japanese medaka to 10 or 100 ng/L of \(EE_2\) resulted in reduced egg production and a lesser GSI compared to the controls. Production of fewer eggs by medaka exposed to \(EE_2\) may be explained by impairment of the reproductive system in females or deficient sperm and/or suppression of sexual behavior in males [16]. In the present study, based on histological examination, testes from medaka exposed to

Fig. 2. Hematoxylin- and eosin-stained cross section of liver of Japanese medaka (\(Oryzias latipes\); A–C) and segmented images using Hue histogram (212–255) to present nucleotide stained with hematoxylin (A*–C*): Control (CTR) male liver showing eosinophilia (A and A*), male liver of fish exposed to 500 ng/L of 17α-ethinylestradiol (EE2; B and B*), and CTR female liver showing intense staining with hematoxylin (C and C*). (D) Also shown is the number of hematoxylin-stained spots greater than 400 pixels in size (mean ± standard error of the mean; arrow in D). Significant differences relative to the control are indicated with an asterisk \((p < 0.05)\). Bar = 50 μm. In color online at http://dx.doi.org/10.1897/08-574.
500 ng/L of EE₂ appeared to be normal except for some early signs of spermatozoon degeneration. Given that testicular ovarian follicles were observed in only one male exposed to 500 ng/L of EE₂ and considering the limited number of replicates and sections investigated in the present study, it is unclear whether this phenomenon can be attributed to the exposure to EE₂ or can be considered a natural occurrence, as has been reported for the Japanese medaka [17]. A more thorough histological investigation (screening of all histological testicular sections) would be required to address this uncertainty, and this was beyond the scope of the present study. In contrast, histological examination of ovaries of female medaka exposed to EE₂ revealed fewer oocytes and atrophy of the ovary, which indicates that the reduced fecundity observed at this stage was caused by impaired oocyte development. However, the duration of exposure was only 7 d, and it cannot be excluded that the degenerative changes observed in the testis would have progressed over time and, thus, could impact fecundity because of effects in the males as well as those observed for the females.

Gene expression profiles. Vitellogenin is under strict control of estrogen and is found only at very low concentrations in male fish under normal physiological circumstances. The FISH method used in the current study allowed the detection of a specific Vit-II antisense signal in both the liver and gonads of Japanese medaka sections. The detection of small amounts of Vit-II expression in the testis or liver of control male medaka indicates that the FISH method represents a sensitive technique to detect minute changes of gene expression as a function of cell/tissue types that cannot be detected when using other tools, such as northern blotting and quantitative real-time PCR (qRT-PCR), as shown for whole-tissue homogenates of control male zebrafish (Danio rerio) [10,18]. In general, a gender-specific difference was found in the sensitivity of the response to EE₂ exposure as measured by Vit-II expression in the liver and gonads, with males being more sensitive than females.

As also demonstrated by a number of different studies, exposure to EE₂ caused greater expression of Vit-II in both testis and liver of male medaka compared to unexposed fish. Up-regulation of Vit-II in testis of males exposed to EE₂ is in accordance with findings in another oviparous fish, the zebrafish [10,18]. Similarly, xenoestrogens up-regulated expression of vitellogenin mRNA in testes of sea bream (Diplodus sargus) [19] and resulted in elevated concentrations of vitellogenin in testis of rainbow trout (Oncorhyncus mykiss) [20]. Vitellogenin transcription is activated through binding of estrogen to the ER. One possibility that would explain the up-regulation of vitellogenin mRNA expression in the testis observed during the present study would be the up-regulation of ER by EE₂. In a parallel study analyzing fish from the same experiment by means of qRT-PCR, an up-regulation of ER gene expression was observed in the liver, but not in the testis, after exposure to the two greatest concentrations [21]. However, significant up-regulation of transcript or protein levels of ERs after estrogen treatment has been reported in other studies with testes of sea bream [19], medaka [22], and goldfish (Carassius auratus) [23]. This would indicate the presence of functional ER in the testes of those fish species. Also, the finding that Vit-II mRNA expression was observed in liver of males treated with EE₂ is consistent with the results of previous studies, in which exogenous estrogen induced either vitellogenin mRNA [10,11,18,20,24] or protein [11,25,26] in male livers of teleost fish species.

Fig. 3. In situ hybridization of vitellogenin-II (Vit-II) mRNA with fluorescently labeled sense (A–D) and antisense probe (A*–D*) in the gonads (upper panels) and liver (lower panels) of Japanese medaka (Oryzias latipes). The four left and right panels are male and female, respectively. Expression of Vit-II mRNA was detected in the testes (A*) exposed to 17α-ethinylestradiol (EE₂) and control ovary (B*) of fish, especially strongly in the region of spermatogonia in testes and the primary stage of oocytes in ovary, respectively. Very weak fluorescence signal was detected in the section hybridized with sense probe (A–D). The Vit-II expression in the male liver (C*) of Japanese medaka exposed to EE₂ (500 ng/L) was as high as that in the section of female liver (D*). Display channel was set to green for antisense probe labeled with Alexa Fluor 488 (Molecular Probes) and to red for autofluorescence. Bar = 200 μm. In color online at http://dx.doi.org/10.1897/08-574.
The FISH method applied in the present study had a distinct advantage over other conventional techniques commonly used to assess changes in gene expression, such as qRT-PCR and northern blotting, because it allowed localization of the expression of specific genes within the whole organism, tissue, or cell. In the testis, fluorescence specific to Vit-II was localized in spermatogonia, which are located in the peripheral region of the testis. It has been reported that Japanese medaka exposed to exogenous estrogen accumulated vitellogenin protein as measured by immunohistochemistry in the cytoplasm of spermatocytes in the seminiferous tubule but not in spermatogonia [26]. The fact that fluorescence of the Vit-II probe was rarely detected in the spermatocytes of testis after treatment of male medaka with EE$_2$ in the present study (Fig. 3A*) implies that synthesis and accumulation of vitellogenin in the testis can occur at different locations. The increase of Vit-II expression in testes exposed to EE$_2$ likely is caused by a shift in cell type composition, resulting in an increase in number of spermatogonia, in which Vit-II mRNA is primarily transcribed. The expression of Vit-II in spermatogonia may be explained by the less differentiated nature of these cells. In fact, spermatogonia of adult trout that were transplanted into newly hatched female trout developed into fully functional eggs when those fish grew into adulthood [27]. This demonstrates that less differentiated cell types, such as spermatogonia, possess a high level of sexual bipotency, which would explain why under estrogen exposure these cells would express vitellogenin genes, as shown for previtellogenic oocytes in the present study (see discussion of subsequent sections). Whereas Vit-II mRNA expression as determined by FISH was detected throughout the entire liver section, there appeared to be a tendency toward slightly greater gene abundance in the outer layer of the liver. This result suggests that the surface regions are primarily involved with vitellogenesis, which confirms previous reports in zebrafish [18].

Traditionally, the ovary of teleost fish has been regarded as the destination of vitellogenin, with the liver believed to be the primary place for synthesis of vitellogenin [28]. As a consequence, most efforts to date have focused on the deposition and accumulation of vitellogenin in oocytes, but information regarding the synthesis or gene expression of vitellogenin in the gonads is rare. Based on the optimized FISH method, Vit-II mRNA expression was detected in the protoplasm of previtellogenic oocytes in both control and EE$_2$-exposed fish, with the expression in ovaries of EE$_2$-treated medaka being greater than that in control fish. This is consistent with the findings of studies with spotted ray (Torpedo marmorata) that revealed active synthesis of vitellogenin in granulosa cells associated with both previtellogenic and vitellogenic oocytes. This also demonstrates that these

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**Fig. 4.** Fluorescence intensity of vitellogenin-II (Vit-II) mRNA in testes (A), ovary (B), and liver (C) of Japanese medaka (Oryzias latipes) exposed to 17α-ethinylestradiol (EE$_2$). Each bar represents the mean ± standard error of the mean. Significant differences relative to the control are indicated with an asterisk (p < 0.05). CTR = control.
cells seem to play a role in vitellogenesis in the ovary [29]. Vitellogenin mRNA in ovary of zebrafish as measured by use of in situ hybridization (ISH) was reported to be slightly greater after exposure to E$_2$ [18]. However, those authors stated that vitellogenin mRNA was expressed in adipose tissues of ovary, not in oocytes. In the present study, fluorescence of the Vit-II mRNA probe was localized in the cytoplasm of previtellogenic oocytes. The relevance of vitellogenesis in ovaries of fish is unclear, but it may be an additional source of vitellogenin when hepatic vitellogenesis is disrupted. Another possibility is that ovarian vitellogenesis could have a supporting function in context with maturation of the previtellogenic oocyte.

In a previous study, it had been proposed that hematoxylin-and-eosin staining can be used to detect changes in the mRNA content of cells or tissues [24]. The significantly greater number of spots stained with hematoxylin in the liver of males exposed to EE$_2$ is an indication that there was more genetic material, such as mRNA, in the hepatocytes, and this was assumed to be related to the greater Vit-II mRNA production observed with FISH. The greater number of cells stained with hematoxylin in livers was consistent with similar results in zebrafish [24]. The intensity of staining with hematoxylin is a function of the presence of greater amounts of genetic material in this tissue. The greater staining observed in livers is in accordance with the significant Vit-II gene expression as determined by FISH, suggesting that the greater incidence of hematoxylin-stained nucleic acids could be a potential indicator of estrogen stimulation of male fish [24]. This hypothesis was confirmed in the present study by the strong correlation between the increase in Vit-II mRNA and the intensity of hematoxylin staining ($r^2 = 0.821$, $p < 0.05$).

The key enzyme responsible for the conversion of androgens to estrogens is aromatase. The products of this reaction, specifically E$_2$, are critical in ovarian development, reproductive function, and sexual differentiation. Therefore, disruption of either activity or production of this enzyme could alter developmental or reproductive processes of an organism. As observed in a previous study [1], CYP19a mRNA expression as measured by FISH was most prominent in the protoplasm of early stage oocytes, which is consistent with findings in other teleost species, such as killifish (Fundulus heteroclitus) [30], zebrafish [31], and Atlantic croaker (Micropogonias undulates) [32]. Expression of CYP19a mRNA was directly proportional to EE$_2$ concentrations except for the greatest concentration, in which it was less than the controls. There have been studies showing that EE$_2$ at relatively low concentrations increases expression of CYP19a in ovary. In fathead minnows (Pimphales promelas), exposure to 10 ng/L of EE$_2$ caused up-regulation of CYP19a expression [33], and exposure to 100 ng/L of EE$_2$ resulted in up-regulation of CYP19a expression in Japanese medaka [15]. Other studies have found that exposure to relatively high concentrations of estrogens resulted in down-regulation of CYP19a expression in adult zebrafish (5 μg/L of

![Fig. 5. Expression of androgen receptor (AR) mRNA in the ovary and liver of female Japanese medaka (Oryzias latipes). Androgen receptor mRNA expression was detected, but at low levels, in the ovary (A) and liver (B) exposed to 17β-trenbolone (TB). Display channel was set to green for antisense probe labeled with Alexa Fluor 488 (Molecular Probes) and to red for autofluorescence. Fluorescence intensity of AR in ovary (C) and liver (D) of medaka exposed to TB also is shown. Each bar represents the mean ± standard error of the mean. Significant differences relative to the control are indicated with an asterisk ($p < 0.05$). Bar = 100 μm. In color online at http://dx.doi.org/10.1897/08-574.]
EE2 [34], juvenile zebrafish (−30 µg/L of EE2 [35]), and embryonic zebrafish (−270 µg/L of E2 [36]). The mechanism of EE2 interaction with CYP19a expression (induction at low doses and reduction at high dose) is unknown. However, the down-regulation of CYP19a expression in ovary at the greatest concentration of EE2 can be explained by histological alterations because of degenerated ovaries, with fewer pre-vitellogenic oocytes where CYP19a mRNAs are primarily transcribed. Moreover, it has been suggested that the down-regulation of CYP19a mRNA by EE2 exposure is not controlled by transcription because of the lack of the estrogen-response element on its 5'-flanking region but that this inhibiting effect is more likely the result of a direct effect of EE2 on gametogenesis in teleost zebrafish [35,37].

Exposure to TB

Fecundity and histology. Effects of TB on reproductive and related functions were more pronounced than those observed for EE2, with the two greatest concentrations completely inhibiting egg production shortly after initiation of the exposure experiments. Similar results also have been observed in other teleost species, such as channel catfish (Ictalurus punctatus) [7] and fathead minnow [8]. Histological observations of the gonads revealed that the lesser fecundity was caused by impairment of gonadal development in both males and females. Males exhibited an increase in the proportion of spermatozoa and stimulated spermatogenesis, and females showed impairments of vitellogenesis and oocyte development. Disruption of vitellogenin accumulation in the ovary most likely resulted from TB causing lesser plasma vitellogenin concentrations, as has been demonstrated for fathead minnow [8]. Other studies also have found histological changes in the testis similar to those observed in the present study [8,9].

Gene expression profiles. Androgen receptor is a nuclear receptor that is activated by the binding of androgens. It functions as a transcription factor to regulate androgen-specific gene expression. Thus, binding of AR with xenosteroids could interfere with processes such as normal male or female gonadal development. The optimized FISH method revealed that expression of AR mRNA was induced in a dose-dependent manner in the ovary, but no significant changes were observed in the liver. This observation is in agreement with those of a previous study in which AR mRNA expression in liver as measured by means of qRT-PCR was not altered [21]. In the present study, a dose-dependent increase in AR mRNA abundance was observed in the ovary, but no comparable effects were observed when AR was measured using qRT-PCR [21]. The reasons for this difference are not clear. However, in some studies, exposure to TB resulted in inductions of AR mRNA levels in the anal fin of female mosquitofish (Gambusia affinis affinis) [12] and in bovine satellite cell cultures at lower concentration (0.001 nM) but not...
at greater concentrations (0.01–10 nM) \[38\]. Therefore, those results confirm that TB has the potential to up-regulate AR expression in medaka.

17β-Trenbolone is a nonaromatizable androgen, so it is not likely to affect CYP19 expression in fish. In the present study, there appeared to be a trend toward increased abundances of CYP19a mRNA in fish from all TB treatment groups when compared to the controls, although these differences were not significantly different. This trend also was observed in a parallel study using qRT-PCR analysis, which also showed a clear, dose-dependent increase of CYP19a expression in fish from the same experiment \[21\]. Given that TB is not a substrate for aromatization, the increase of CYP19a expression in ovary could be explained by TB-induced AR expression, possibly leading to an increase of ARs that can bind the AR-binding element in the promoter region of CYP19a to activate the transcription in teleost fish, including medaka \[39\]. Similarly, induction of CYP19 expression by TB exposure was reported in H295R human adrenocortical carcinoma cell line \[13\]. Also, induction of CYP19 expression in ovaries exposed to TB might be a compensatory response to the decreased levels of plasma estrogen caused by this chemical \[8,40\]. A comparable up-regulation of ovarian CYP19a expression also has been observed after the exposure of medaka to an aromatase inhibitor, fadrozole, which suggests a similar compensatory mechanism in response to decreased endogenous estrogen \[1\]. However, the mode of action of TB on CYP19 expression still needs to be investigated by assessing the levels of endogenous proteins.

**Application of the FISH method**

The optimized FISH method used in the present study was able to detect and quantify changes in Vit-II, AR, and CYP19a mRNA transcripts in tissues of Japanese medaka exposed to two common endocrine disruptors, the xenoestrogen EE2 and the androgen TB. The primary strengths of the FISH approach were its ability to localize changes in the expression of target genes at the cellular and/or tissue level and to directly integrate effects at the level of gene expression with traditional histological analysis. The latter may be of particular interest with regard to predicting potential pathologies based on changes in the expression of specific genes in certain tissues. The elucidation of relationships between tissue- and gene-related effects is of great relevance with respect to the characterization of chemical effects. Furthermore, FISH also is a powerful method that can be used to further our understanding of basic biological processes, as demonstrated in the present study.

Developing a reliable quantification method for the detection of fluorescence signal in medaka tissues was challenging because of the semiquantitative nature of this method and the variability observed for some of the measured signals. Thus, additional work is needed to improve the quantification of changes in gene expression with these methods and to improve the statistical power of this technology. Regardless of these rather minor uncertainties, the method used in the present study to (semi)quantify fluorescence signals using both individual spectral components with a confocal microscope system and reduction in autofluorescence could be shown to reliably detect mRNA expression in tissues. The FISH methodology used in the present study may have the potential to become a milestone in fluorescence-based studies, especially for the development of methodologies enabling the simultaneous detection of multiple fluorescence signals in whole-mount sections. Such technologies could provide an understanding of complex molecular mechanisms involving multiple key genes in response to exposure to endocrine-disrupting compounds. Finally, the method established here seems to represent a valuable tool to aid in linking effects at the molecular level to pathologies.

**SUPPORTING INFORMATION**

**Fig. S1.** Liver somatic index (LSI) and gonadosomatic index (Oryzias latipes) exposed to 17α-ethinylestradiol (EE2; A) or 17β-trenbolone (TB; B). Values are presented as the mean ± standard error of the mean. Significant differences relative to the control are indicated with an asterisk \((p < 0.05, n = 4–6)\).

**Fig. S2.** Cumulative numbers of fertilized eggs spawned by female Japanese medaka (Oryzias latipes) exposed to 17α-ethinylestradiol (EE2; A) or 17β-trenbolone (TB; B). Each treatment consisted of triplicate tanks, and each tank contained six pairs of medaka. Significant differences relative to the control are indicated with an asterisk \((p < 0.05)\).

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