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Transgenic Medaka Identify Embryonic Periods Sensitive to Disruption of Sex Determination

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Abstract: Gonadal development in medaka (*Oryzias latipes*) is dependent on the synergy between estrogens and androgens. Disruption of steroid hormone levels can lead to ovo-testis. To determine the sensitive windows for hormonally induced sex reversal in medaka, we developed a novel 42sp50-GFP_ChgH-GFP transgenic medaka line, allowing the identification of female gonadal tissue by fluorescence present in developing oocytes. Germinal transgenesis resulted in a stable line exhibiting a strong green fluorescent protein signal constitutively in the ovaries and in the liver in response to estrogens. The sensitivity of this line to disruption of sex determination following 16-d chronic exposures was in the nanograms per liter range. To identify the developmental period sensitive to exogenous agents, fry were exposed to 24-h pulses of high concentrations of 17β-estradiol (E2) or 5α-dihydrotestosterone (DHT) at various time points between days postfertilization (dpf) 0 and 12. Evaluation of phenotype followed by genotyping at 16 dpf revealed sensitivity to E2 between 1 and 8 dpf as well as 2 periods of susceptibility to DHT between 0 and 1 dpf and 4 and 8 dpf. No phenotypic sex reversal was detected after exposure to DHT or E2 on 11 or 12 dpf. The observed effects persisted to at least 24 dpf. The identified sensitive embryonic time periods for disruption of sex determination will aid future research on sex determination and the development of screening assays using early embryonic life stages. *Environ Toxicol Chem* 2020;39:842–851. © 2020 SETAC

Keywords: Reproductive toxicity; Endocrine disruptors; Androgens; Estrogenic compounds; Intersex; Medaka

INTRODUCTION

Numerous studies have reported the presence of abnormal gonadal morphology in wild fish (Kavanagh et al. 2004; Hinck et al. 2009; Tetreault et al. 2011). The most commonly observed change in gonad structure is the formation of ovo-testis, defined as a gonad containing both testicular and ovarian tissue (O'Toole 2003). Both laboratory and field studies have demonstrated that this condition can be induced by exposure to endocrine-disrupting chemicals (EDCs; Kavanagh et al. 2004). Interestingly, the elucidation of genetic sex determinants for several fish species (*Oryzias latipes* [Matsuda et al. 2002], *Odontesthes hatcheri* [Hattori et al. 2012], *Takifugu rubripes* [Kamiya et al. 2012], *Oryzias luzonensis* [Myosho et al. 2012], *Oncorhynchus mykiss* [Yano et al. 2012]) has revealed that ovo-testis can be induced in fish possessing male or female genotypes (Urushitani et al. 2007; Kobayashi et al. 2011). A challenge remains in linking ovo-testis to genetic sex for zebrafish (*Danio rerio*) because, although the genetic sex determinant has been identified, it has been lost in laboratory strains (Wilson et al. 2014).

Several case studies on wild teleost fish document the presence of oocytes within the testes of genetic males in addition to feminization of the reproductive tract, elevated levels of hormones characteristic for females, and abnormal levels of plasma vitellogenin (Pollock et al. 2010; Sanchez et al. 2011; Bahamonde et al. 2015). Analogous studies of masculinized female fish, very often exposed to pulp and paper mill effluents, show equivalent effects. The genetically female fish exhibited ovo-testis, male secondary sex characteristics such as elongated dorsal fins, and increased concentrations of hormones typical of males (Bortone and Cody 1999; Parks et al. 2001).

In addition to the clear links with EDCs, environmental factors such as high temperature and hypoxia have been linked to the intersex condition (Uchida et al. 2004; Sato et al. 2005; Cheung et al. 2014). These environmental stressors have also been demonstrated to act through

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alterations in sex steroid signaling (Kitano et al. 2012; Thomas and Rahman 2012).

Under the current definitions of an EDC given by the European Union, the Environment Agency, and the World Health Organization's International Programme on Chemical Safety, it is required that the substance provokes an adverse physiological effect secondary to a modification of endocrine signaling (Zoeller et al. 2014). It is the confirmation of an adverse physiological effect, such as histological aberrations of the gonads, which increases the time required for the exposure period of most assays and therefore their cost. Despite the identification of a number of biomarkers capable of rapidly identifying changes in endocrine signaling, confirmation of adverse physiological effects often requires raising the animals to adult life stages and even breeding them to obtain the next generation. Several fish models have been developed that allow the detection of phenotypic sex during embryonic development by the measure of fluorescence in the gonads (Kinoshita et al. 2009; Zhao et al. 2014), potentially facilitating the screening of substances provoking intersex formation. With this in mind, our aim was to develop a model capable of screening potential endocrine disruptors in a reduced time frame, confirming or denying the ability of the given chemical to alter endocrine signaling and to induce physiological changes.

The first objective of the present study was to perform germinal transgenesis to develop a double transgenic 42sp50-GFP_ChgH-GFP medaka line harboring biomarkers for both estrogen axis signaling (choriogenin H–green fluorescent protein [ChgH-GFP]; Kurauchi et al. 2005; Scholz et al. 2005; Yu et al. 2006) and the presence of developing oocytes (42sp50-GFP; Kinoshita et al. 2009). To validate this newly developed model, the next objective was to determine the sensitivity of this line to a chronic exposure with a range of concentrations of a model estrogen and a model androgen, focusing solely on expression of the *42sp50-gfp* transgene in the ovaries, leaving the validation of the sensitivity of the *chgh-gfp* transgene for future studies. The final objective was to investigate sensitive periods for hormonally induced sex reversal in early medaka embryonic development by acute 24-h exposure to high doses of a model estrogen and a model androgen. We believe that the discovery of the most sensitive windows for hormonally induced sex reversal in medaka will help shorten the exposure time and thus greatly facilitate the protocol necessary for screening endocrine-active chemicals acting on the reproductive axes.

MATERIALS AND METHODS

Reagents

Medaka medium contained 4.02 mM KCl, 1.62 mM $MgSO_4 \cdot 7H_2O$, 1.36 mM $CaCl_2 \cdot 2H_2O$, 0.17 M NaCl, and 62.5 μM methylene blue. The pH was adjusted to between 7.2 and 8.0. Ethyl 3-aminobenzoate methanesulfonate salt (MS222) was prepared at 1 g/L, the pH was adjusted to between 7.5 and 8.0, and it was diluted as necessary in medaka medium. All test substances were purchased from Sigma-Aldrich: 17β-estradiol (E2; Chemical Abstracts Service [CAS] no. 50-28-2, ≥98% purity) and 5α-androstan-17β-ol-3-one (5α-dihydrotestosterone [DHT]; CAS no. 521-18-6, ≥97.5% purity), and dimethyl sulfoxide (DMSO; CAS no. 67-68-5). Romeiod (iodophor solution 0.5% active iodine) was purchased from Cofa and diluted in medaka medium to a final concentration of 2%. SYBR® Safe DNA Gel Stain (Invitrogen), Pure Link Genomic DNA Mini Kits (Invitrogen), Taq polymerase (Thermofisher), and Tris–borate–ethylenediaminetetraacetic acid (TBE) buffer (Thermo Scientific) were used for genotyping.

Genetic construct

A genetic construct was synthesized (GeneCust) comprising 1283 bp of the medaka *42sp50* gene immediately upstream from the translation start site, followed by the coding sequence of *gfp* and terminated by 1641 bp of the medaka *42sp50* gene immediately downstream from the stop codon. This construct was described by Kinoshita et al. (2009). In addition, XhoI sites were synthesized on both sides of the construct and used to insert it into a plasmid containing an additional, previously described cassette comprising a portion of the medaka *chgh* gene driving expression of the *gfp* coding sequence. A single plasmidic clone was selected containing both genetic cassettes facing in opposite directions to limit interactions between the 2 cassettes (Figure 1A).

Germ line transgenesis

Germ line transgenesis was carried out according to a previously described protocol in the absence of meganuclease by injecting a linearized construct into fertilized one-cell stage Carbio red-orange strain medaka eggs (Kinoshita et al. 1996).

Exposure studies

All stocks were prepared in DMSO. For each developmental time point to be studied, 42sp50-GFP_ChgH-GFP medaka eggs were collected within 2 h of fertilization, disinfected with 2% romeiod for 10 min, and divided into Petri dishes containing 100 mL of medaka medium, with one Petri dish per exposure group. The groups of eggs were then randomly assigned exposure conditions. Medium was changed 3 times per week, and dead eggs were removed every 24 h.

All exposure studies were carried out by adding E2 or DHT stock solutions in DMSO to medaka medium. Final concentrations of DMSO were either 0.05 or 0.1%, and in all cases the solvent control group was exposed to the same concentration of DMSO as the test groups. All fry were of the F3 to F5 generations and were exposed in 100-mL Petri dishes. After hatch, transgenic fry were identified by a basal GFP signal in the gills and retained. Nontransgenic fry were excluded from the experiment. Hatched fry were fed with TetraMin baby (Tetra). Exposure studies were repeated, and the results were pooled as necessary to obtain a minimum of 50 transgenic fry per group.

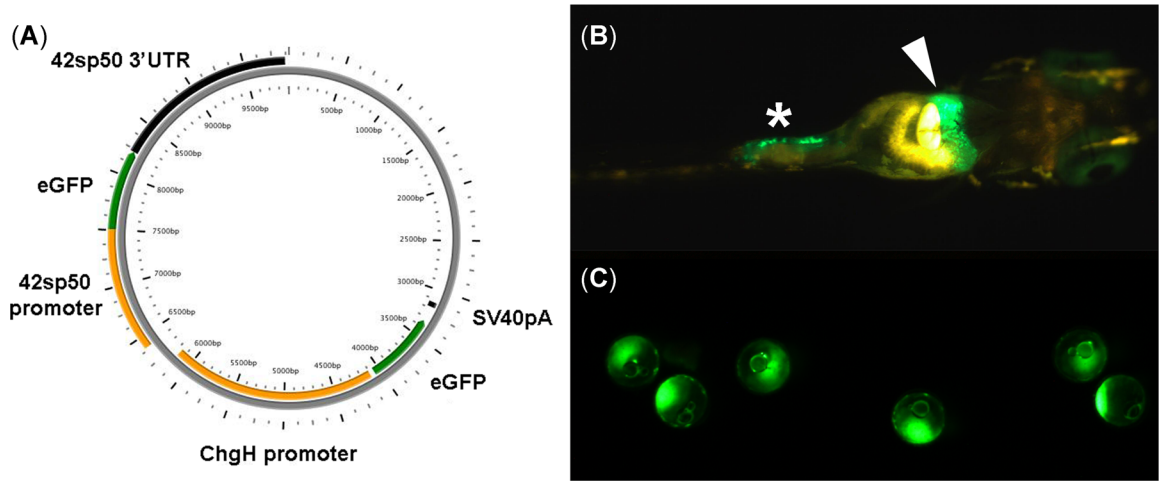

FIGURE 1: (**A**) The ChgH-GFP_42sp50-GFP bicistronic plasmid. (**B**) A phenotypically female fry of the 42sp50-GFP_ChgH-GFP line expressing fluorescence in the ovary (*) and liver (arrowhead) following exposure to 17α-ethinyl-estradiol. The yellow color indicates autofluorescence of the embryo attributable to the use of long-pass green fluorescent protein (GFP) filters. (**C**) Newly fertilized eggs from a transgenic female expressing GFP. ChgH = choriogenin H; eGFP = enhanced GFP; SV40 = simian virus 40; UTR = untranslated region.

For the 16-d chronic studies, fry were exposed to E2, DHT, or solvent alone from fertilization until day postfertilization (dpf) 16. Exposure solutions were renewed 3 times per week.

For the 24-h acute studies to determine developmental windows of sensitivity, fry were exposed to E2, DHT, or solvent alone at the developmental day of interest. Precisely 24 h postexposure the fry were rinsed 3 times with fresh medaka medium and placed into clean medium. The clean medium was renewed 3 times per week. All procedures and animal use were evaluated and validated by the local ethics committee (no. 51) and the institutional review board of Laboratoire WatchFrog.

Image capture

Six days after hatching, fry were anesthetized with 200 mg/L MS222 and manually positioned to expose the ventral region. A Leica MZ10F stereomicroscope (Leica Microsystems) equipped with ET-GFP long-pass filters (excitation 480/40, emission 510LP; Leica Microsystems) and a 200-W fluorescence source (Prior Scientific) was used to image the fry. Images of the genital region of the fry were taken with an exposure time of 0.75 s at ×8 magnification using an Infinity 1-3C camera (Lumenera). Each fry was then euthanized with MS222, rinsed with fresh medaka medium, and placed separately into 1.5-mL tubes. After the removal of all liquid, the tubes were immediately frozen at –80 °C for subsequent genotyping.

Genotyping

A subset of fry from each exposure group was genotyped for the presence of *dmy*, indicating the presence of a Y chromosome. Extraction and purification of genomic DNA were carried out using the Pure Link Genomic DNA Mini Kit (Invitrogen) according to the provided protocol. Amplification by polymerse chain reaction (PCR) of a region of both the *dmrt1* and *dmy* genes was carried out using a single primer pair, as described (Patil and Hinze 2008). The PCR amplification was carried out using a BioRad MyCycler with a program consisting of one cycle of 94 °C for 9 min, followed by 42 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min and terminating with one cycle of 72 °C for 5 min. The DNA bands were then visualized by gel electrophoresis using 0.5 × TBE and 1% agarose stained with SYBR Safe DNA Gel Stain (Invitrogen).

Statistical analysis

The percentage of fry in each group displaying gonadal fluorescence was compared to the percentage observed in the corresponding control group. The comparison was carried out using the binomial ratio (phenotypic sex), with the probability of success set to the ratio observed in the control group. All data were Bonferroni-corrected to increase the threshold for statistical significance based on the number of statistical tests carried out. Results which were significantly different from the null hypothesis are indicated: ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$.

RESULTS AND DISCUSSION

The 42sp50-GFP_ChgH-GFP transgenic line

Germ line transgenesis generated one stable line. As with the previously described *42sp50-GFP* line, fluorescence was first visible in the gonadal region of certain fry 1 or 2 d post-hatch (dph). Developing oocytes became highly fluorescent at approximately 5 or 6 dph (Figure 1B). Phenotypically male fry display a complete absence of green fluorescence in the gonads (Kinoshita et al. 2009; Kanamori and Toyama 2013). Because of the extremely strong fluorescence in the vitellus of the eggs of this line (Figure 1C), eleuthero-embryos from crosses with transgenic females also present strong fluorescence in their maternal reserves of vitellus. As this passes through their

gastrointestinal tract, it continues to fluoresce, confounding correct identification of fluorescence in the gonads. Therefore, all experiments were carried out by crossing heterozygotic transgenic males with wild-type (orange-red strain) females.

Ectopic fluorescence, possibly attributable to the genomic integration site of the transgenes as observed by Kurauchi et al. (2008), was observable in the gills of males and females. This faint fluorescence allowed transgenic and nontransgenic fry to be discriminated shortly after hatching.

As reported in a number of publications (Kurauchi et al. 2008; Spirhanzlova et al. 2015), the ChgH-GFP transgene was expressed in the liver in response to E2 and 17α-ethinylestradiol (EE2). During an embryonic chronic exposure to E2, GFP was first visible in the liver of unhatched embryos at 6 dpf (data not shown).

Sensitivity of sex determination to exogenous hormones

To determine the sensitivity of this transgenic model to disruption of sex determination by a model estrogen, embryos were exposed from fertilization to 16 dpf to a range of concentrations of E2. The sex ratio was skewed toward female when embryos were treated with 500 ng/L or higher of E2 (Figure 2A). The lowest observed effect concentration for E2 fits well with previous, longer studies. It has previously been demonstrated in a 21-d Organisation for Economic Co-operation and Development (OECD) guideline 229 exposure of medaka that fecundity was reduced at 488 but not at 261 ng/L EE2, equivalent in terms of potency to approximately 976 and 522 ng/L of E2, respectively (Seki et al. 2002). Testis-ova and vitellogenin induction were observed at 63.9 ng/L EE2 (equivalent to approximately 128 ng/L E2) but did not affect fecundity. This suggests that this model may be capable of predicting the results of longer studies such as OECD test guidelines 229 or 230.

This experiment was repeated with a model androgen. Embryos were exposed from fertilization to 16 dpf to a range of concentrations of DHT. All concentrations tested from 500 ng/L to 1 µg/L were effective at skewing the phenotypic sex ratio toward male (Figure 2B). To our knowledge, this is the first study to address the effects of DHT on medaka, although a number of studies have investigated the effects of the synthetic analogue mDHT on medaka sex determination (Iwamatsu et al. 2006; Kobayashi et al. 2011).

Developmental sensitivity to disruption of sex determination

A previous study in medaka tested a number of developmental time points for sensitivity to E2- and testosterone-induced intersex (Koger et al. 2000). Koger et al. (2000) tested 6-d exposures commencing at 0 dpf and 0, 7, or 21 dph, with histological examination of the gonads at 5 mo of age, and found that the highest sensitivity was at 0 dph. In our study, 0 dph, defined as the day that >50% of the fry had hatched, corresponded to 9 dpf for all 3 of the replicate experiments which were pooled to give the unexposed control group (Table 1). We therefore decided to carry out a systematic screening of developmental sensitivity to waterborne agents activating the estrogen receptor and androgen receptor from 0 to 12 dpf (or 3 dph). Our rationale was that a 1-d, rather than a 6-d, protocol and a greater number of developmental time points would allow the developmental dynamics of susceptibility to hormonally induced disruption of sex determination to be determined with greater precision.

Effects of medaka medium and DMSO

A preliminary verification of the sex ratio in untreated embryos raised in the same manner as the treated fry demonstrated that 53% of the fry developed as phenotypic females and 47% as phenotypic males (dotted line Figure 2). No disagreement between phenotypic and genotypic sex was observed (Table 1).

To aid the solubility and dispersion of test compounds, DMSO was chosen as the solvent to be used in all exposure groups. Low concentrations of DMSO have been shown not to interfere with the sexual differentiation of fish (Hutchinson et al. 2006).

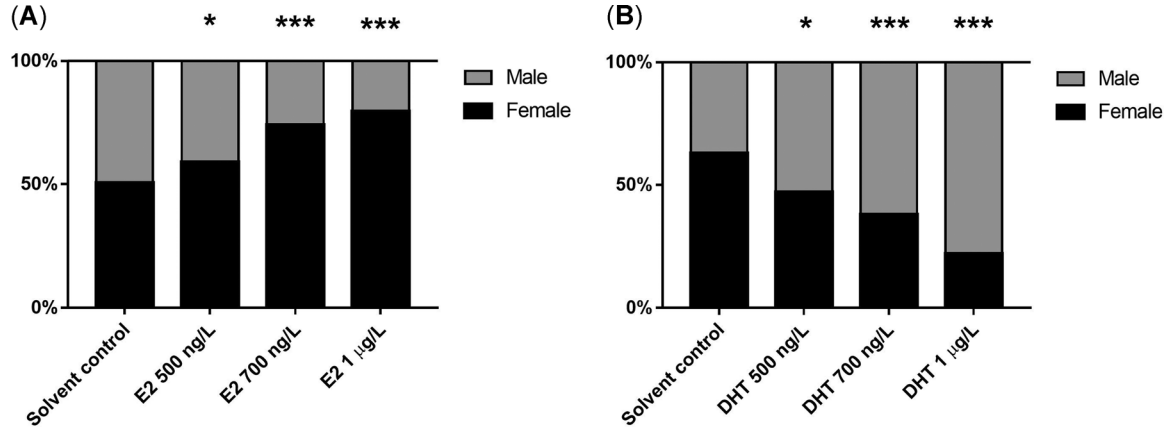

FIGURE 2: Sensitivity of the model to perturbation of sex differentiation by estradiol (**A**) or dihydrotestosterone (**B**) in 16-d exposures. Exposures were carried out from fertilization to 16 d postfertilization. $n \geq 50$ per exposure condition. DHT = dihydrotestosterone; E2 = estradiol.

TABLE 1: Genotyping results of a subset of fry from the water control group[a]

	XY	XX
Male phenotype	16 100%[b]	0 0%
Female phenotype	0 0%	15 100%
n	31	

[a]Phenotype was determined by the presence or absence of fluorescence in the gonads.
[b]Percentages indicate relative numbers of fry developing with a normal concordance between phenotypic and genotypic sex.

All exposures were carried out in the presence of either 0.05 or 0.1% DMSO, with solvent control groups exposed to the same concentration of DMSO as the steroid-exposed groups. A 24-h acute exposure to the higher concentration of DMSO (0.1%) did not induce a significant difference in the phenotypic sex ratio compared to nonexposed medaka at any developmental time point (Figure 3); therefore, genotyping of fry from this experiment was not carried out.

Effects of E2

To determine the relative sensitivity of different periods of embryonic development to E2, acute (24-h) exposures were carried out at different developmental time points (Figure 4). The concentration of E2 selected for the acute exposures was that determined by Kobayashi et al. (2011) as the minimum dose inducing a maximum effect.

In the present study, we confirmed that treatment with this concentration of E2 caused a statistically significant increase in oocyte-bearing fry at 16 dpf compared to the solvent control group when the 24-h treatment was carried out at 0 to 8 dpf but not at 11 or 12 dpf (Figure 4). The greatest percentage of phenotypic females was obtained when the treatment was carried out at 1, 2, 4, or 5 dpf (3 dpf was not tested). Following

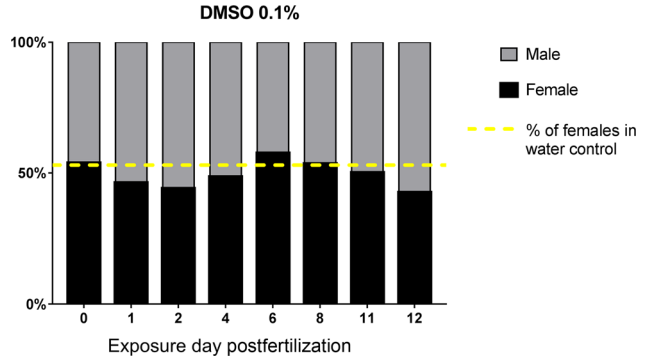

FIGURE 3: Percentage of fry exhibiting gonadal fluorescence at day postfertilization (dpf) 16. The fry were treated with a 24-h pulse of dimethyl sulfoxide (0.1%) at different developmental time points (*x*-axis). Following this treatment, they were returned to clean media. The percentage of untreated control fry displaying green fluorescent protein–positive cells in their gonads at 16 dpf is shown by the yellow dotted line. $n \geq 50$ per exposure condition. None of the exposure conditions were statistically different from the water control. DMSO = dimethyl sulfoxide.

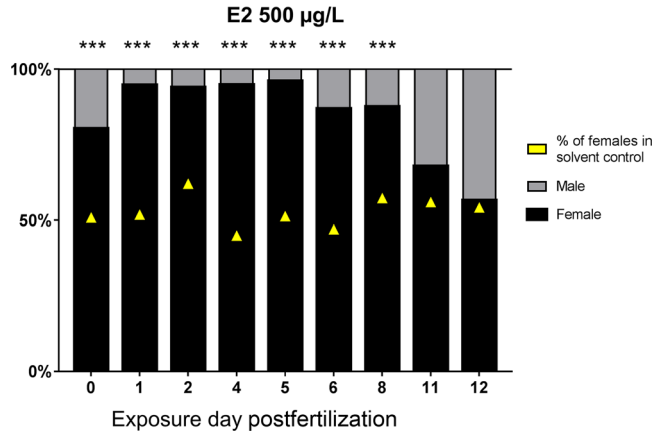

FIGURE 4: Percentage of fry exhibiting gonadal fluorescence at day postfertilization (dpf) 16. The fry were treated with a 24-h pulse of estradiol (500 µg/L) at different developmental time points (*x*-axis). Following this treatment, they were returned to clean media. The percentage of fry displaying green fluorescent protein–positive cells in their gonads at 16 dpf following 24-h treatment with a solvent control solution at matching developmental time points is shown in yellow. $n \geq 50$ per exposure condition. E2 = estradiol.

5 dpf, the effect diminished in an age-dependent manner until 12 dpf. We therefore confirm that a 24-h treatment of medaka eggs with 500 µg/L of E2 immediately after fertilization results in a high incidence of XY females and a skewed phenotypic sex ratio toward female.

Genotyping of a random set of fry from some of the exposure time points indicated that a large number (80%) of XY embryos treated at 0 dpf developed oocytes (Table 2). This developmental period matches the period of somatic reprogramming in medaka with not yet determined cell fate; it is thus possible that this effect is caused by prolonged retention of E2 in the yolk (Wang and Bhandari 2019a). This peaked at 94% at 5 dpf and then dropped to 79% at 6 dpf, falling to 6% at 12 dpf. Paradoxical effects on sexual development were observed for most of the E2-exposed groups, with 6 to 22% of XX embryos failing to exhibit oocytes. This may be attributable to stress-induced inhibition of aromatase expression as a result of the experimental conditions, as reported (Hayashi et al. 2010).

Effects of DHT

To determine the relative sensitivity of different developmental time points to a model androgen, a high concentration of DHT was used to perform 24-h acute exposures of developing medaka embryos. The concentration of DHT chosen for the first series of experiments (2.5 µg/L) was the same as the concentration of methyl-DHT previously identified as the minimum concentration producing maximum effect on sex ratio (Kobayashi et al. 2011).

Our decision to use the natural hormone DHT rather than its synthetic analogue methyl-DHT allows the effects of DHT at 0 dpf (present study) to be compared with those published for methyl-DHT (Kobayashi et al. 2011). However, the percentage of fry treated with this concentration of DHT at 0 dpf and exhibiting

TABLE 2: Genotyping results of a subset of fry exposed to estradiol 500 μg/L[a]

	Exposure day postfertilization									
	0		4		5		6		12	
	XY	XX	XY	XX	XY	XX	XY	XX	XY	XX
Male phenotype	4	4	1	3	1	1	4	0	16	2
	20%[b]	22%	9%	14%	6%	6%	21%	0%	94%	11%
Female phenotype	16	14	10	19	15	15	15	13	1	16
	80%	78%	91%	86%	94%	94%	79%	100%	6%	89%
n	38		33		32		32		35	

[a]Phenotype was determined by the presence or absence of fluorescence in the gonads.
[b]Percentages indicate relative numbers of fry developing with a normal concordance between phenotypic and genotypic sex.

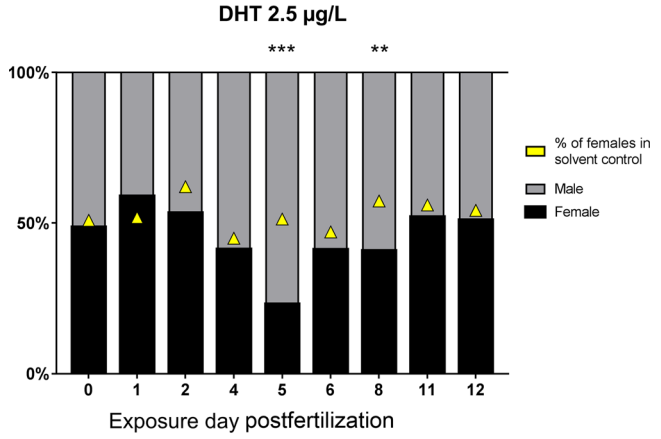

FIGURE 5: Percentage of fry exhibiting gonadal fluorescence at day postfertilization (dpf) 16. The fry were treated with a 24-h pulse of dihydrotestosterone (2.5 μg/L) at different developmental time points (*x*-axis). Following this treatment, they were returned to clean media. The percentage of fry displaying green fluorescent protein–positive cells in their gonads at 16 dpf following 24-h treatment with a solvent control solution at matching developmental time points is shown in yellow. $n \geq 50$ per exposure condition. DHT = dihydrotestosterone.

oocytes at 16 dpf was not statistically different from that of the solvent control group (Figure 5). Genotyping showed that despite the near identical sex ratio to the control group, 30% of XX fish exposed to DHT at 0 dpf failed to display oocytes (Table 3). This effect was masked in the phenotypic ratio because an equivalent number of fish (39%) displayed oocytes despite possessing a Y chromosome. Kobayashi et al. (2011) described this as paradoxical sex reversal, which they did not observe with 2.5 μg/L of methyl-DHT but they did observe with higher concentrations of the same molecule.

Statistically significant decreases in the number of fry displaying oocytes were, however, observed following treatment with DHT at 5 or 8 dpf (Figure 5). Genotyping of a subset of fry confirmed an increased number of XX males over solvent control values at 0, 4, and 5 dpf (24, 11, and 11% increases, respectively) but not at 6 or 12 dpf (Table 3).

A higher concentration of DHT was used in the second set of exposures. This concentration, 500 μg/L, was chosen based on a range-finder experiment (Supplemental Data, Figure S1) with a 24-h acute exposure to the hormone. The aim of this second set of exposures with DHT was to induce a higher level of disruption of the sexual axes than that observed with the lower DHT concentration used in the first series of experiments. It was hoped that this would facilitate identification of the developmental time points most sensitive to disruption of sex determination by a model androgen.

This higher concentration of DHT caused a significant reduction in the number of fry displaying ovaries at 16 dpf for all developmental time points tested from 0 to 8 dpf except 2 dpf (Figure 6). Interestingly, as with the E2 treatment, fry were insensitive to disruption of sexual phenotype when exposed at 11 to 12 dpf. There appear to be 2 windows showing the greatest sensitivity to this molecule, from 0 to 1 dpf and from 6 to 8 dpf. Intriguingly, the period of relative insensitivity between 2 and 4 dpf may coincide with the cessation of maternal expression of the androgen receptor and the beginning of zygotic expression. Interestingly, these 2 developmental

TABLE 3: Genotyping results of a subset of fry exposed to dihydrotestosterone 2.5 μg/L[a]

	Exposure day postfertilization									
	0		4		5		6		12	
	XY	XX	XY	XX	XY	XX	XY	XX	XY	XX
Male phenotype	11	6	20	1	21	2	18	1	18	0
	61%[b]	30%	87%	11%	100%	17%	82%	10%	90%	0%
Female phenotype	7	14	3	8	0	10	4	9	2	14
	39%	70%	13%	89%	0%	83%	18%	90%	10%	100%
n	38		32		33		32		34	

[a]Phenotype was determined by the presence or absence of fluorescence in the gonads.
[b]Percentages indicate relative numbers of fry developing with a normal concordance between phenotypic and genotypic sex.

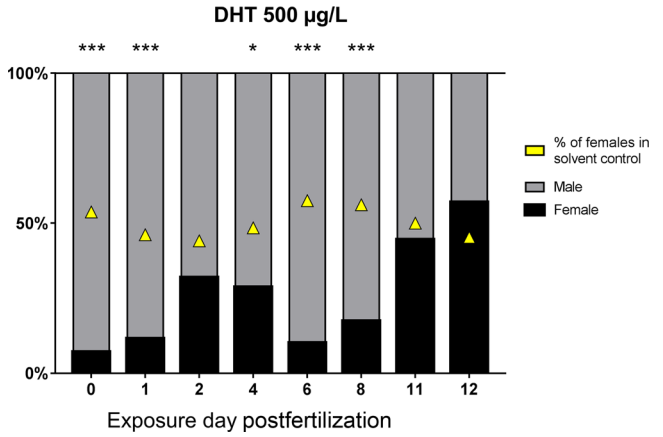

FIGURE 6: Percentage of fry exhibiting gonadal fluorescence at day postfertilization (dpf) 16. The fry were treated with a 24-h pulse of dihydrotestosterone (500 µg/L) at different developmental time points (*x*-axis). Following this treatment, they were returned to clean media. The percentage of fry displaying green fluorescent protein–positive cells in their gonads at 16 dpf following 24-h treatment with a solvent control solution at matching developmental time points is shown in yellow. $n \geq 50$ per exposure condition. DHT = dihydrotestosterone.

windows match the window of epigenetic reprogramming in medaka somatic cells, which might also suggest an explanation for why these 2 windows are sensitive to environmental factors (Wang and Bhandari 2019b).

Koger et al. (2000) found the greatest sensitivity to DHT to be with a 6-d exposure from 0 to 6 dph (corresponding approximately to 9–15 dpf). Because we observed no effect at 11 or 12 dpf, this suggests that, in addition to the 2 periods of heightened sensitivity to DHT observed between 0 to 1 and 5 to 8 dpf, it is possible that a third period of sensitivity lies between 12 and 15 dpf (corresponding to 3–6 dph). It remains to be shown whether the 42sp50-GFP_ChgH-GFP model is sensitive to disruption of sex determination when short-term exposures are carried out at developmental stages beyond 12 dpf. Performing exposures after hatch (9 dpf) would allow molecules to be tested without consideration for their ability to pass the chorion and would allow the model to be used for screening of pure chemicals at high concentrations in a short-term assay.

Genotyping confirmed the observed result with high numbers of XX males from 0 to 6 dpf (76–86%) dropping to 0% at 12 dpf (Table 4). At 2 time points XY females were observed, which may have been attributable to the paradoxical effects of this molecule that have been observed (Kobayashi et al. 2011). However, the numbers of XY females (15% at 4 dpf and 5% at 6 dpf) were relatively low compared to the numbers of XX males.

Timing and persistence of the observed response

We were intrigued by the lack of effect observed when fry were exposed to either E2 (500 µg/L) or DHT (2.5 or 500 µg/L) at 11 or 12 dpf. In particular, we were interested in confirming whether the lack of effect was attributable to a resistance to disruption of sex determination at this period of development or whether the delay between the treatment at 11 or 12 dpf and the readout of the experiment at 16 dpf was insufficient.

To test these 2 possibilities, acute (24-h) exposures were repeated at 6 and 12 dpf with 500 µg/L of either DHT or E2 or solvent alone—6 dpf was chosen because a strong effect on sex determination was observed with both molecules at this period of development, and 12 dpf was selected because no effect was observed with either molecule. In both experiments readout of the same group of fry was carried out at 16 dpf as in previous experiments as well as at 20, 22, and 24 dpf (Figure 7).

The results show that there is no difference in effect when the readout is carried out at 16, 20, 22, or 24 dpf (Figure 7). This very clearly demonstrates that the lack of effect when fry are exposed at 12 dpf is attributable to insensitivity to these 2 molecules at this period of development and not to an insufficient time between the exposure and the readout. The present study also indicates that, at least up to 24 dpf, the observed disruption of sex determination is stable.

CONCLUSIONS

The perfect concordance between genotypic sex and phenotypic sex for the unexposed control group demonstrates the ability of this 42sp50-GFP_ChgH-GFP line to accurately identify the phenotypic sex of fry at 16 dpf (Table 1).

When fry were exposed from fertilization to 16 dpf, the lowest-observed-effect concentration for disruption of sex

TABLE 4: Genotyping results of a subset of fry exposed to dihydrotestosterone 500 µg/L[a]

	Exposure day postfertilization							
	0		4		6		12	
	XY	XX	XY	XX	XY	XX	XY	XX
Male	15	16	11	13	19	12	17	0
phenotype	100%[b]	84%	85%	76%	95%	86%	100%	0%
Female	0	3	2	4	1	2	0	14
phenotype	0%	16%	15%	24%	5%	14%	0%	100%
n	34		30		34		31	

[a]Phenotype was determined by the presence or absence of fluorescence in the gonads.
[b]Percentages indicate relative numbers of fry developing with a normal concordance between phenotypic and genotypic sex.

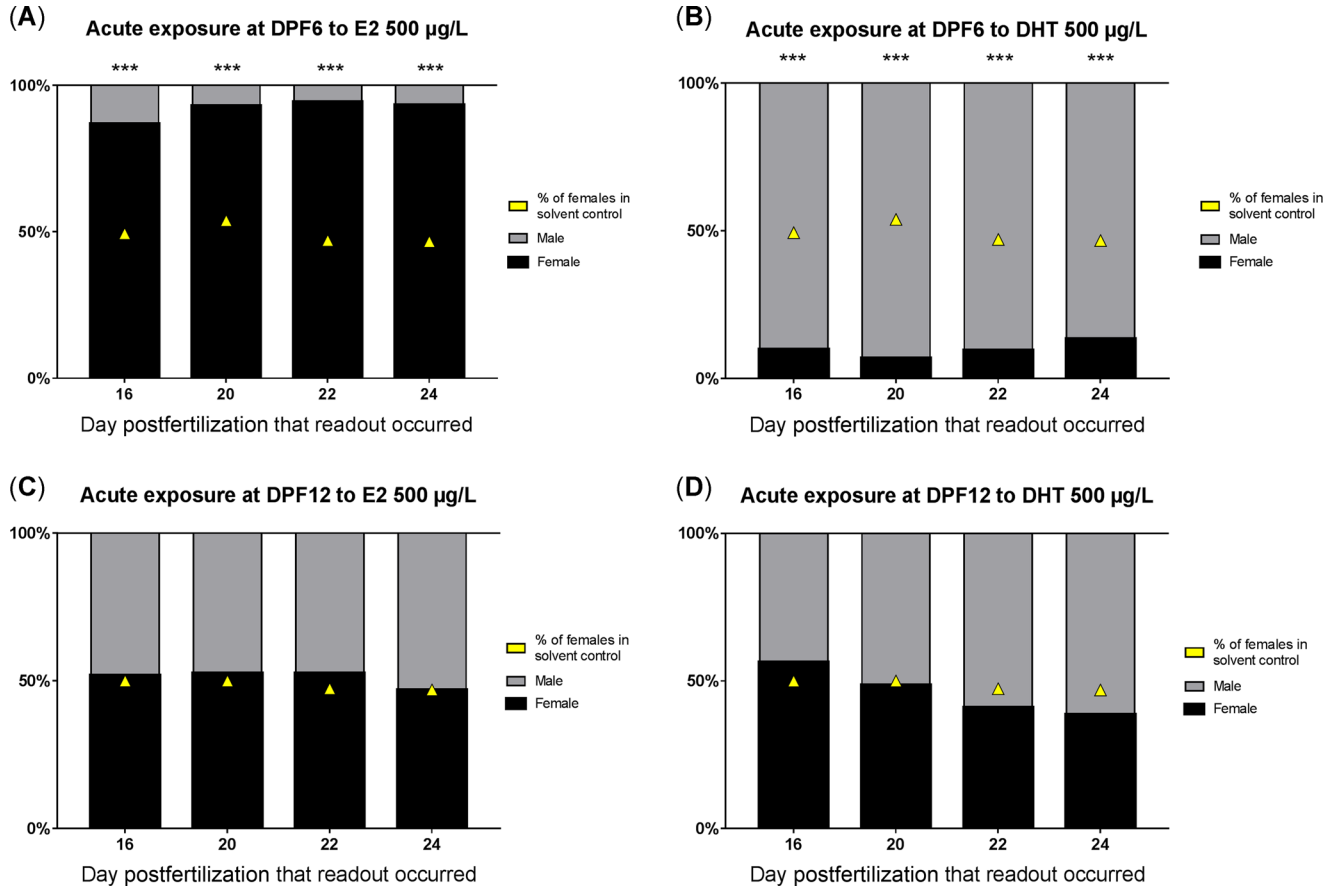

FIGURE 7: Percentage of fry exhibiting gonadal fluorescence at days postfertilization (dpf) 16, 20, 22, and 24 (*x*-axis). The fry were treated with a 24-h pulse of (**A**) estradiol (E2; 500 µg/L) at 6 dpf, (**B**) dihydrotestosterone (DHT; 500 µg/L) at 6 dpf, (**C**) E2 (500 µg/L) at 12 dpf, or (**D**) DHT (500 µg/L) at 12 dpf. Following treatment, they were returned to clean media. The percentage of fry displaying green fluorescent protein–positive cells in their gonads at 16, 20, 22, and 24 dpf following 24-h treatment with a solvent control solution at matching developmental time points is shown in yellow. $n \geq 50$ per exposure condition.

determination was 500 ng/L for both E2 and DHT (Figure 2). Sensitivity to E2 was greatest between 1 and 5 dpf (Figure 4). Exposure studies with DHT showed 2 periods of increased sensitivity between 0 to 1 dpf and 5 to 8 dpf (Figure 6). No effect on phenotypic sex ratio was observed with DHT or E2 at 11 or 12 dpf. We demonstrated that this lack of effect was attributable to the fact that sex determination was stabile to the tested molecules and not to a period of latency longer than the washout period between exposure and readout (Figure 7).

Further development of this model will involve crossing of this line with another line harboring a fluorescent reporter cassette on the Y chromosome to allow fluorescent detection of genetic males, increasing throughput and reducing costs related to genotyping. We then plan to use the ChgH-GFP transgene carried by this line to investigate links between the level of ChgH transcription induced by environmentally relevant chemicals (e.g., environmental estrogens such as bisphenol A or EE2) and disorders of sexual differentiation in fish. The investigation of these links is a timely and important subject in terms of the development of adverse outcome pathways for the identification of endocrine disruptors and the evaluation of their predictability.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.4674.

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Author Contributions—A.J. Tindall and G.F. Lemkine conceived of and supervised the creation of the transgenic line. A. Kanamori gave scientific advice on ovarian markers in medaka and contributed to interpretation of the results. A.J. Tindall carried out all bioinformatics and molecular biology. A. Sébillot performed germinal transgenesis. A.J. Tindall and P. Spirhanzlova designed and supervised the in vivo experiments. P. Spirhanzlova, J. Lallement, P. Trébulle, and A.J. Tindall performed the experiments. A.J. Tindall and P. Spirhanzlova wrote the manuscript.

Disclaimer—The authors declare the following competing financial interests. G.F. Lemkine has financial interests in Laboratoire WatchFrog, which plans to commercialize the test described. A.J. Tindall and G.F. Lemkine are currently employed by Laboratoire WatchFrog. P. Spirhanzlova, P. Trébulle, J. Lallement, and A. Sébillot were employed by Laboratoire WatchFrog while they were involved in the present study. A. Kanamori declares no competing interests.

Data Availability Statement—Data are available upon request from the corresponding author (tindall@watchfrog.fr).

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