# A Model Fish System to Test Chemical Effects on Sexual Differentiation and Development

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## ABSTRACT

A model system was characterized which may be used as an in vivo screen for effects of chemicals or environmental mixtures on sexual differentiation and development of reproductive organs and gametes. We evaluated the effects of a model environmental estrogen, ethinyl estradiol ($EE_2$), on the d-rR strain of medaka, *Oryzias latipes*, using a nano-injection exposure. Gonad histopathology indicated that a single injection of 0.5-2.5 ng $EE_2$/egg can cause phenotypic sex-reversal of genetic males to females. Sex-reversals could be detected as early as seven days post-hatch. Sex-reversed males had female-typical duct development and the secondary sex characteristics we measured were generally consistent with phenotype, with the exception of a few $EE_2$-exposed XX and XY females which possessed ambiguous anal fins. Using discriminant analysis, we determined that the presence or absence of the secondary sex characteristic, a dorsal fin notch, was a very reliable indicator of gonadal sex. No instances of gonadal intersexes were observed. Ethinyl estradiol also appeared to reduce growth but not condition (weight at a given length) and exposed XX females appeared to have a higher incidence of atretic follicles relative to controls. Our results suggest that estrogenic chemicals may influence sexual differentiation and development and that the medaka model is well suited to assessing these effects. (The complete manuscript of this work was submitted to the journal Aquatic Toxicology, December, 1998).

## INTRODUCTION

This study was part of an on-going characterization of the d-rR medaka in which we are evaluating the effects of known estrogenic, androgenic, anti-estrogenic, and anti-androgenic chemicals on sexual differentiation and development.

The recent focus on endocrine disrupting chemicals (EDC) has led to extensive efforts to develop screening assays that detect exposure or demonstrate effects of this group of chemicals on wildlife species (Ankley and others, 1998). However, to date, no test has been accepted that screens chemicals for effects on the primary germ cells (PGC) with subsequent alteration of sexual differentiation of reproductive organs and gametes. Such information is particularly important for those species (fishes, amphibians, birds) for which chemically- or environmentally-induced sex-reversal has been documented.

The d-rR strain of medaka, *Oryzias latipes*, is an ideal organism with which to observe the effects of chemicals on sexual development and differentiation. The males of this strain possess a sex-linked color gene for xanthathin deposition whereas the females are deficient in this gene (Hyodo-Taguchi and Egami, 1989). The orange-red males and white females produced can be distinguished as early as two days post-hatch.

Based on extensive study of the effects of natural and synthetic steroids on fish sexual development, Yamamoto (1962) concluded that sexual differentiation requires that the PGCs be directed towards spermatogenesis or oogenesis by exposure to an endogenous inductor substance, possibly an androgenic or estrogenic hormone, during a particular period in development. Therefore, EDCs which are transferred to the oocyte during gametogenesis, and are available to the developing embryo prior to hatch or sexual differentiation, can pose a risk to the embryo.

In this model, embryos of the d-rR strain of medaka were exposed to a model EDC by nano-injection. Directly exposing the embryos closely simulates maternal transfer of contaminants and is applicable for testing those EDCs which bioaccumulate and are lipophilic.

The objectives of this report are to characterize the response of the d-rR medaka to a single exposure of ethinyl estradiol ($EE_2$), a model environmental estrogen which is found in sewage effluents. Effects on sexual differentiation are assessed by comparing genetic sex to phenotypic sex of newly-hatched and adult fish. Effects on sexual development are assessed through evaluation of the gonadosomatic index, gonad and gonadal duct histopathology, and secondary sex characteristics.

## METHODS

### Test Organism

The d-rR strain of medaka was a generous gift of Dr. Akihiro Shima, University of Tokyo, Japan. In this strain, either (r) or (R) genes are carried by the X or Y chromosome, respectively. Crossovers or genetic imbalances occur at a rate of about 0.005-0.5%, making color a reliable marker of genetic sex (Hishida, 1965). In addition to the genetic marker, there is distinct sexual dimorphism in the medaka. Males are typified by a dorsal fin notch and a convex anal fin with a serrated margin. Females lack the dorsal notch and have a more concave anal fin with a smooth margin.

### Preparation

Eggs were collected in the morning soon after fertilization the day before exposure. Embryos were embedded in a solid 1% agarose matrix which had been set-up in square 36-grid petri dishes. The agarose matrix provided the necessary stability for injecting the embryos. The dish was then filled with well water and stored in a cool place (~20°C) to slow embryo development until injection (prior to completion of the gastrula stage).

### Embryo Injection

Embryos were exposed following a slightly modified nano-injection procedure described by Walker and others (1996). Immediately prior to injection, dead embryos or embryos that had completed epiboly were replaced and the well water covering the embryos was exchanged for a sterile saline solution. Aluminosilicate capillary tubes were pulled on a pipette puller to make needles with a 5-10 µ OD tip when beveled at a 15°. Needles were filled either using a vacuum or a pipettor. Triolein was used as the carrier solvent and was filter-sterilized before use. Carrier solvent, steroid working solutions, and filled needles were stored at 4°C under nitrogen. Eggs were injected with five concentrations (0, 0.005, 0.05, 0.5, 2.5, or 5.0 ng/egg) of $EE_2$ dissolved in the carrier triolein or triolein alone. Injections of material were made directly into the oil globule using a picoinjector and micro-manipulator. The injected embryos were left in the agarose at room temperature until the following day.

### Embryo Incubation and Larval Growout

Approximately 24 h after injection, the embryos were inspected for mortalities; survivors were maintained in 27°C well water in 150-ml glass side-arm test tubes. Embryos were rolled in the water during incubation by a gentle stream of air. Upon hatch, the medaka larvae were held in flow-through containers suspended in stainless steel raceways supplied with a constant flow of well-water until they reached maturity.

### Fish Characterization and Procedures

Fish were identified as genetic males (XY, orange-red) or genetic females (XX, white), and then euthanized at 5 mm total length (TL) or as adults. Morphological observations and measurements were made on a total of 145 adult fish averaging $30 \pm 2$ mm TL and $0.26 \pm 0.06$ g body weight (WT). Larvae, whole adults, and mature gonads were preserved in Bouin's solution. Fish of 5 mm TL were used to assess the volume of the developing gonad and the number

of PGCs. In medaka, as for many teleosts, gametogenesis (germ cell division and differentiation) in females begins weeks before that in males, making PGC number and gonad volume useful indicators of male or female phenotypic sex. Adults were measured (TL) and weighed (blotted dry), and were used to assess growth, gonadosomatic index (GSI), sex-reversal, gonad and gonadal duct histopathology, and development of secondary sex characteristics.

Volume of the early gonad was calculated using image analysis (Optimas®) by digitizing the area of serial cross-sections of the gonad of the 5 mm larvae, and computing a volume. The number of PGCs in each cross-section were also recorded by enumerating observable PGC nuclei. Histological analysis followed standard procedures for processing, dewaxing, hydration, and dehydration. All sections were cut at 7µ and stained using a modified Masson trichrome stain.

## RESULTS

### Growth and Survival

Survival from embryo to adult was greater than 60% for all but the 5.0 ng $EE_2$/egg dose. Only a 2% survival was observed at 5.0 ng $EE_2$/egg.

A general linear models analysis of the TL and body WT data indicated that neither weight nor length differed significantly within treatment group among adult males, females, and sex-reversed males ($p$>0.09). However, males tended to be heavier than females at all doses, whereas sex-reversed males were intermediate between these; both TL and body WT correlated negatively and significantly with exposure to $EE_2$ for males, females, and sex-reversed males.

### Gonad Size

Nearly all the female fish examined were in the pre- to early vitellogenic stage (Iwamatsu and others, 1988). The GSI for XX females was approximately ten times that of XY males but was the same as that for XY females ($p$<0.0001; Figure 1). The uninjected and triolein controls did not differ. While there was no significant effect of $EE_2$ on GSI of those fish that were not sex-reversed ($p$>0.05), ovarian weights correlated negatively and significantly with increasing dose of $EE_2$.

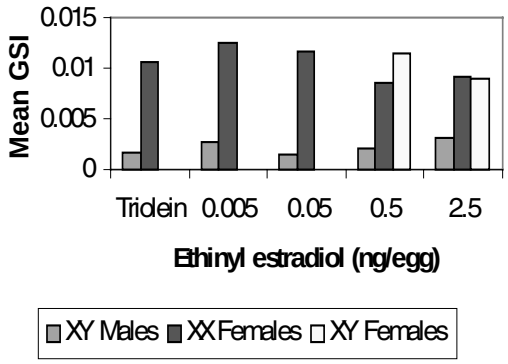


**Figure 1**. Gonadosomatic index (GSI; gonad weight/(body weight less gonad weight)) for XY males, XX females, and XY females (mean ± SD). The GSI for XX females was approximately ten times that of XY males, but was the same as that for XY females ($p$<0.0001). There was no significant effect of $EE_2$ on GSI of those fish that were not sex-reversed ($p$>0.05).

### Gonad and Duct Histopathology

Histological examination of the gonads of the adults indicated that an injection of 0.5 or 2.5 ng $EE_2$/egg could cause complete sex-reversal in genetic males. The most effective concentration for sex reversal was 2.5 ng/egg. One anomalous sex-reversed female was observed at the 0.005 ng/egg concentration. No intersexes were observed. Degenerating or atretic oocytes were observed in almost all the ovaries of the 0.5 and 2.5 ng/egg dosed fish; few were present in the two lowest doses, and nearly none were observed in the triolein or uninjected controls. Individuals at the highest dosages of $EE_2$ typically exhibited very small ovaries bearing a few late vitellogenic or mature oocytes, the remainder being previtellogenic. Both XX and XY ovaries displayed the described characteristics.

In contrast, the morphology of the testes appeared normal in nearly all specimens, with most having sperm in the efferent ducts. However, of six XY males treated with 0.5 ng $EE_2$/egg only two individuals had testes

resembling the controls, with all stages of germ cells present and sperm in the ducts. The remaining individuals had very few or no sperm in their efferent ducts. One individual receiving this dosage appeared sterile, its testis was devoid of germ cells. The testes of individuals exposed to 0.05 ng $EE_2$/egg generally appeared less mature (most germ cells at early stages of spermatogenesis) and with less sperm in their ducts than did those of the lowest dose or control groups.

Sex-reversed males had typical female ducts, with separate openings for the genital and urinary tracts. Ethinyl estradiol-exposed males which were not sex-reversed possessed normal male morphologies, with the fusion of the urinary and gonadal ducts within the body cavity occurring just proximal to forming a single urogenital opening. Ducts in all but the sex-reversed fish seemed unaffected by the $EE_2$ exposure.

## Secondary Sex Characteristics

Our objective was to discriminate between genetic males and females in order to identify sex-reversals by their misclassification according to several phenotypic attributes. We conducted a stepwise discriminant analysis based on an overall sample of 145 fish described by 9 attributes (anal fin convexity, anal fin serration, dorsal fin notch, abdominal distention, all scored presence/absence; urogenital papilla [UGP] size, scored incrementally from 1 to 4; anal fin length, anal fin width, TL, all measured in mm; body WT measured in grams). This analysis identified the 11 sex-reversals that occurred. The phenotypic attributes which together made significant contributions to the classification included dorsal fin notch, abdominal distention, and TL.

Our attempt to identify which of the external attributes were of most use in predicting the correct genetic sex also proved successful. A repeat of the analysis above, using phenotypic sex as the correct group designator, correctly assigned our fish to functional gender using only a single variable, dorsal fin notch The remaining attributes, while informative, were insufficiently diagnostic. A serrated and convex anal fin was generally consistent with male phenotype although some feminized males and sex-stable females displayed them. Sex-stable males bore longer and wider anal fins than did fish which were either genotypically or phenotypically females. Anal fin growth in length and width appeared to be retarded in feminized males such that overall shape was more similar to XX females. The UGP of sex-reversed males were more female than male-like with respect to size. Whether the abdomen was distended or not was dependent on the stage of vitellogenesis in females. Except for the vitellogenic females, abdomen for both male and female phenotypes was not distended.

## Primary Germ Cells

Only data for 2.5 ng $EE_2$/egg dose were assessed since the greatest percent of sex-reversals occurred at this dose. As expected, gonadal volumes and number of PGCs were greater in uninjected or triolein control females than control males, indicating that sexual differentiation had occurred in the 5 mm larvae (Table 1).

**Table 1.** Mean volume of gonads and number of PGCs in medaka larvae injected as embryos with ethinyl estradiol (n=5). Treatments sharing a letter are not significantly different ($p$>0.05).

| | Genetic ♀ (XX) | | Genetic ♂ (XY) | |
|---|---|---|---|---|
| Dose ng/egg) | Vol. ($x10^{-5}$) $mm^3$±SD | Num. PGCs± SD | Vol. ($x10^{-5}$) $mm^3$±SD | Num. PGCs± SD |
| Con. | 17.2±3[a] | 212±49d | 5.3±.3[c] | 40±4[f] |
| Triolein | 12.6± 3.6[ab] | 129±29e | 4.5±1.6[c] | 55±28[f] |
| 2.5 | 10.7±2.4[b] | 97±19e | 12.6±4.2[ab] | 132±27[e] |

Gonad volumes and PGC numbers for $EE_2$-exposed females were also larger than those for control males and not significantly different from values for triolein-exposed females. Males exposed to 2.5 ng $EE_2$/egg had gonad volumes and PGC numbers similar to both triolein-control and $EE_2$-exposed females, indicating that sex-reversal had occurred in these exposed males.

## DISCUSSION

Our studies demonstrate that a single injection of $EE_2$ into medaka embryos prior to the period of sexual differentiation can result in feminization of males. To our knowledge, the only estrogens which have been injected into fish embryos are estrone (Gresik and Hamilton, 1977) and $EE_2$ (this study).

We demonstrated that $EE_2$-induced sex-reversal coincides with sexual differentiation and can be identified as early as a few days post-hatch. In medaka, as in many teleosts, female and male phenotypes are first differentiated based on the activity of their PGCs within 24 h after hatch. Those PGCs which enter mitosis first are identified as oogonia, while they are refered to as spermatogonia in those larvae in which no division is taking place. Up until this point of differentiation, the male and female PGCs are morphologically the same and are considered to be bipotent with respect to their fate as future sperm or ova (Reinboth, 1982). Our observations of gonad size and PGC number indicate that sex-reversal has already occurred by the time the medaka larvae reached 5 mm in length (about six days post-hatch). The injected $EE_2$ was present for at least eight days before and four days after hatch, at which time the yolk and oil droplet were completely absorbed.

The relative potencies of $EE_2$ compared with other estrogens in effecting sex-reversal, either by nano-injection or via the diet, corresponds with their respective binding affinities for the estrogen receptor. This suggests that sex-reversal is induced through the estrogen receptor.

Given that only a single white (XX) male was found in the lowest treatment, we believe that this individual was more likely to have been one of the normal exceptions to the white female ($X^rX^r$) and orange-red male ($X^rY^R$) color pattern which is expected to occur at low frequencies in the d-rR strain population.

It should be noted that in our study, the gonads of treated fish displayed varying degrees of pathology. Atretic oocytes were much more common in the ovaries of XX and XY $EE_2$-treated fish. Atresia is believed to be uncommon in physiologically healthy females (Wallace and Selman, 1981). Factors such as environmental stress, poor nutrition, or stressors that could decrease gonadotropin levels (e.g. anti-gonadotropic substances), are believed to be the major causes leading to atresia (Saidapur, 1978). The high incidence of atresia we observed in $EE_2$-exposed XX and XY females suggests that embryonic exposure to $EE_2$ may have disrupted the development of gonadotropin-producing cells, or in some other way impaired the release or uptake of gonadotropins.

Development of male or female secondary sex characteristics is influenced by the type of gonad present. Generally, these characteristics are male-positive, in that testosterone production by the gonad results in dimorphisms between males and females (Okada and Yamashita, 1944). Estrogen does not induce development of secondary sexual characteristics, except for the UGP. Thus, a sex-reversed fish would be expected to display secondary sexual characteristics which would match its gonadal phenotype and not its genotype. Although the secondary sex characteristics we measured (size and shape of anal fin, dorsal fin notch, UGP, and size of abdomen) generally followed the phenotype of the gonad independent of the genetic sex, we also found the anal fin was slightly more ambiguous among a few individual XY females and $EE_2$-exposed XX females.

There were lethal and growth-related effects from exposure to $EE_2$ in addition to effects on sexual differentiation of the gonad and consequent development of secondary sex characteristics. Embryo mortality was 91% at the 5.0 ng $EE_2$/egg dose with only two females surviving. We found no reports of $EE_2$-induced fish mortality in the literature. However, $E_2$, estrone, and estriol are all reportedly toxic when administered via diet at high concentrations (Yamamoto, 1961, 1965; Yamamoto and Matsuda, 1963). Control XX females tended to be less heavy (although not significantly so) than XY males and the males, which were sex-reversed were smaller than the XY males but larger than XX females. It also appears that $EE_2$ had a suppressive effect on growth, but not condition (the TL-body WT relationship). The length and weight of medaka exposed, from hatch, to estrone in their food showed a similar negative influence, and unexposed control males were larger than XX females (Fineman and others, 1974a 1974b).

However, since the duration, timing of exposure, and route of administration of steroid were quite different in the latter experiments relative to our studies, direct comparisons must be made cautiously.

## CONCLUSIONS

Our results suggest that estrogenic chemicals may influence sexual development. However, the normal process of sexual differentiation in fishes is not yet completely understood. Thus, the specific mechanism through which exposure to exogenous steroids during the period of sexual differentiation may result in sex-reversal or impaired sexual development is unknown. The medaka model presented here is well suited to testing chemicals for their effects on sexual differentiation and development. The same endpoints could be evaluated with water bath exposures of embryos, alone or in combination with egg injection, to further simulate exposure scenarios for both bioaccumulative and water soluble chemicals. Our studies have demonstrated the benefit of using the d-rR strain for testing for effects of chemicals on sexual differentiation. Utilizing the sex-linked color gene marker and gender-specific characteristics of the PGCs allows detection of an effect as early as two weeks after exposure. Furthermore, growing the fish to maturity (approximately three months) allows the evaluation of additional endpoints for assessment of effects on sexual development.

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