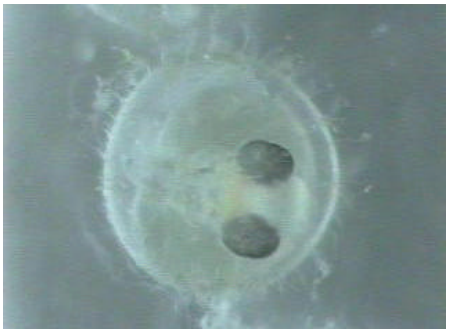
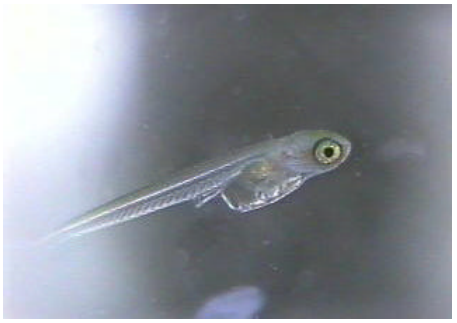
# *M E D A K A*

***Oryzias latipes***

***Development of Test Methods and Suitability of Medaka as Test Organism for Detection of Endocrine Disrupting Chemicals***





**February 2003**

**Ministry of the Environment, Japan**
**Chemicals Evaluation and Research Institute, Japan**

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***Development of Test Methods and Suitability of Medaka as Test Organism for Detection of Endocrine Disrupting Chemicals***

**Ministry of the Environment, Japan**
**Chemicals Evaluation and Research Institute, Japan**

**Ministry of the Environment, Japan**

Environmental Health and Safety Division, Environmental Health Department

Address : 1-2-2 Kasumigaseki, Chiyoda-ku, Tokyo

Tel: +81-3-3580-3596

e-mail ehs@env.go.jp

**Chemicals Evaluation and Research Institute, Japan**

Headquarters

Address: 1-4-25 Koraku, Bunkyo-ku, Tokyo

Tel: +81-3-5804-6131

Chemical Biotesting Departure, Kurume Laboratory

Address: 19-14 Chuo-machi, Kurume, Fukuoka

Tel: +81-942-34-1500

## *Contents*

## *Preface*

The Ministry of the Environment (MOE) released a document entitled "Strategic Programs on Environmental Endocrine Disrupters (SPEED'98)" in May, 1998 and listed 67 substances as "chemicals suspected of having an endocrine disrupting effect". The priority list was revised in November, 2000, due to deletion of several substances, styrene dimer, styrene trimer and n-butylbenzene of which estimated risks were low. Thus 65 substances are listed at present.

Following this initial review program, a research project was launched in 1998. The purpose of the project was to develop screening tests and evaluate the potential endocrine disruptive effects of the 65 substances listed as those of concern. Since 1998, MOE has progressed with several activities focusing on research with **medaka** (***Orizias latipes***).

Under the current endocrine disruptor (ED) research project, the Fish Working Group for Development of Screening Test and Testing was established in 1999. The aim of the Fish Working Group is to develop test methods with **medaka** and submit draft protocols to OECD by following the approaches and concepts of the OECD framework for EDs.

Three laboratories in Japan, including the Chemicals Evaluation and Research Institute (CERI), National Institute for Environmental Studies (NIES) and Institute of Environmental Ecology, METOCEAN ENVIRONMENT INC have been conducting medaka testing with EDs under a grant from the MOE Japan. Many tests, such as vitellogenin assay, partial life-cycle test and full life-cycle test have been conducted. This **MEDAKA** report describes the development of test methods using **medaka** to detect EDs. These tests have been conducted following the current MOE's approach, however further work is needed to decide on a final format for the testing and assessment scheme for EDs effects in fish.

In December, 2002, several data sets describing results from a vitellogenin assay, fish partial life-cycle test and full life-cycle test with chemicals such as phenolic compounds were submitted to the OECD secretariat .

February 25, 2003

Taisen Iguchi

## Members of the Fish Working Group

**Taisen Iguchi : Chairman (Fish)**
Center for Integrative Bioscience, Okazaki National Research Institutes

**Koji Arizono (Fish)**
Faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto

**Minoru Uchiyama (Amphibian)**
Regulatory biological, Department of Biology, Faculty of Sciences, Toyama University

**Yuji Oshima (Fish)**
Fisheries Environmental Science, Faculty of Agriculture, Kyushu University

**Yuta Ohnishi (Fish)**
Institute of Environmental Ecology, METOCEAN ENVIRONMENT INC

**Norihisa Tatarazako (Fish)**
National Institute for Environmental Studies (NIES)

**Yoshitaka Nagahama (Fish)**
Laboratory of Rreproductive Biology, National Institute for Basic Biology, Okazaki National Research Institutes

**Masayuki Yasuno (Fish)**
Department of Environmental Science, University of Shiga Prefecture

**Yuko Wakamatsu (Fish)**
Bioscience Center, Nagoya University

**Masaru Wada (Birds)**
Tokyo Medical and Dental University

**Ministry of the Environment (Sponsor)**
Environmental Health and Safety Division, Environmental Health Department

**Chemicals Evaluation and Research Institute, Japan (Secretariat)**
Chemicals Assessment Center, Headquarters

***Summary***

The purpose of this report is to provide scientific background on the **medaka** bioassay used for the screening of EDs. The results from our project will contribute to the development of fish full life-cycle test (FLC) as well as help the modification of fish early life-stage toxicity test for endocrine disrupting properties. The studies on our project also provide information for toxicity mechanisms of EDs in fish.

In recent years, a global concern has been raised about the potential impact of natural steroid hormones and other chemicals mimicking the effects of hormones in aquatic ecosystems, particularly because of the possibility of adverse effects on sexual differentiation and reproduction in wildlife. However, none of the existing methods for *in vivo* test were sufficient for screening and testing for the endocrine disruption of these substances. Therefore, we commenced, in 1998, a series of studies to develop suitable test methods with fish.

Initially, it was important to establish what kind of biological effects was observed in response to exposure to EDs as well as to determine the changes in sensitivity during various stages of the fish life-cycle. Further, we had to identify what kind of chemicals had biological effects of this nature. FLC is a test method covering all stage of life cycle. Then, we confirmed usefulness of **medaka** as a test model in FLC using reference chemicals.

In the early stage of our project, frameworks of several test methods were developed. The fish partial life stage toxicity test (PLC) is a test focused on a sexually critical developmental-stage. A test for a steroidgenically active stage was termed the reproduction test (REP). In addition, a very sensitive to biological effects and more cost-effective screening method has been developed. Vitellogenin (VTG) assay is a test to detect vitellogenin induction in liver as an effect of EDs. The new **medaka** strains, such as FLF and d-rR, in which sex can be distinguished in a simple manner as observing leucophores or body color have been used for the development of these tests.

**Medaka** is an ideal bioassay model for research and testing in toxicology. It has been widely used for studies on the effects of radiation, carcinogens and

chemicals and has been recommended as a test species in many existing standard eco-toxicological guidelines, such as OECD test guidelines. Furthermore, **medaka** is an attractive model organism for evaluating life-cycle toxicity in both parental and progeny generations because of its short maturation time-within six to eight weeks after hatching. This species can spawn daily under summer photoperiod, temperature conditions with proper food regime. The first chapter on this report describes suitability of medaka as test organ to detect the effect of EDs.

*In vitro* studies also have been developed in order to screen many of the chemicals of concern. To date, **medaka** estrogen receptor (*me*ERα and *me*ERβ) binding assay, *me*ERα and *me*ERβ reporter gene assay and **medaka** androgen receptor (*me*AR) reporter gene assay have been developed. The second chapter describes the reporter binding assay and receptor gene assay.

The current testing strategy by the Ministry of the Environment, Japan, requires evaluating the estrogenic or androgenic activity of chemicals (see attached Testing Scheme). In screening, VTG assay is conducted to detect estrogenic activity of suspected EDs. Then, PLC is performed to elucidate their effects on the sexual differentiation of **medaka,** because this test had higher sensitivity for several estrogens than the REP that sexually matured and constantly spawning fish are exposed to chemicals during the reproductive phase.

In the PLC, fish are exposed to chemicals from an embryo stage to almost matured adult stage. In the short term PLC, fish are exposed to chemicals during embryo stage to early-matured adult stage. The parameters investigated in the tests are hatching success (including next generation), survival, growth, reproduction, gonado-somatic index (GSI), hepato-somatic index (HSI), secondary sex characteristic (sec. SEX), gonadal histology, VTG concentration in liver.

If adverse effects were not observed in the PLC, the FLC is not necessary. If adverse effects were observed in the PLC, FLC would then be conducted.

As a result of our activities to date, the VTG assay had been conducted with 20 chemicals[*1] and the REP has been performed with nonylphenol and bisphenol A. The PLC was done with 12 chemicals[*2] and FLC was performed

with 4 chemicals[*3] respectively. Some of OECD reference chemicals such as 17β-estradiol, ethynylestradiol and methyl testosterone were tested. We also prepared protocols for the PLC and FLC that were submitted to the OECD secretariat in December, 2002 (Appendix Ⅰ-Ⅰ and Ⅱ).

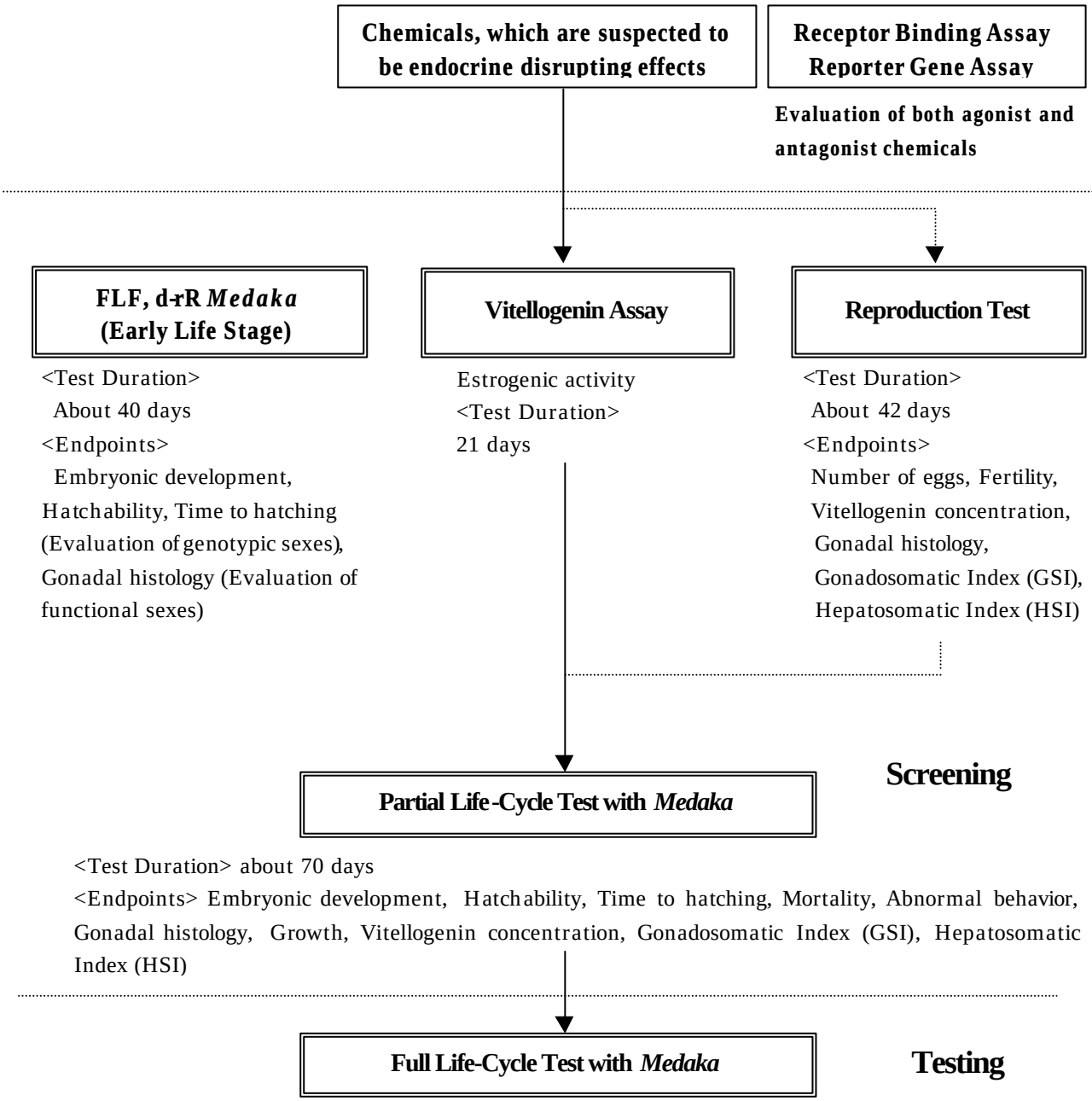
An important study had been developed on the mechanism of sexual differentiation in **medaka** by information from basic and scientific data. In order to make clear the effects of EDs on the mechanism of sexual differentiation **medaka**, some studies have been conducted with utilizing genetic technology as follows. (1) Cloning a series of genes associated with the regulation of sex differentiation in **medaka**. (2) Preparing of a gene, which appears in a period of differentiation of sex. (3) Development of the DNA chip containing a series of gene associated with the sexual differentiation. A series of genes associated with regulating the sexual differentiation have been cloned and prepared to develop a DNA macro array. The sex-determination gene of **medaka**, DMY (Y-specific DM-domain gene) has been first found in non-mammalian vertebrates. The results of these studies with utilizing the gene technology will be useful to make clear the effect of EDs on the mechanism of the sexual differentiation in **medaka**. The last chapter describes genetic approaches to understanding the basic mechanisms of DES actions on sexual differentiation in medaka.

※1 Tributyltin, 4-octylphenol, nonylphenol, di-n-butylphtalate, octachlorostyrene, benzophenone, di-cyclohexylphtalate, di-(2-ethylhexyl)phtalate, butylbenzylphtalate, di-ethylphthalate, di-(2-ethylhexyl)-adipate and triphenyltin are selected in 2000. Pentachlorophenol, amitrol, bisphenol A, 2,4-dichlorophenol, 4-nitrotoluene, di-pentylphtalate, di-hexylphtalate and di-propylphtalare are selected in 2001.

※2 Tributyltin, 4-octylphenol, nonylphenol, di-n-butylphtalate, octachlorostyrene, benzophenone, di-cyclohexylphtalate, di-(2-ethylhexyl)phtalate, butylbenzylphtalate, di-ethylphtalate, di-(2-ethylhexyl)-adipate and triphenyltin

※3 Tributyltin, 4-octylphenol, nonylphenol and di-n-butylphtalate

## Testing Scheme in Evaluation of the Endocrine Disrupting Activities in Fish



# *Chapter 1*
# *Suitability of Medaka Fish as a Test Organism and New Medaka Strains*

**Yuko WAKAMATSU and Kenjiro OZATO**

***Bioscience Center, Nagoya University***

***Furo-cho, Chikusa-ku, Nagoya, Japan***

## 1. Suitability of medaka fish as a test organism

Medaka has been used as a model in various fields of environmental research for the past several decades, namely, radiation biology in the 1970s, carcinogenesis in the 1980s, and toxicology in the 1990s. More recently, about half the number of publications dealing with medaka is on toxicology (1, 2). Thus, it is apparent that medaka has a high potential as a model fish for use in the testing of environmental chemicals. In particular, medaka is useful for studying the effects of endocrine-disrupting substances on reproduction, because of its characteristic reproductive system in which the sex is determined by XY chromosomes. Several medaka stocks developed for reproduction research are powerful tools for investigating the effects of those substances. Here, we will review the potential of medaka as a model fish based on its biological characteristics, resources and strains for use in endocrine-disrupting substance testing. This review is based on our two papers published in ENVIRONMENTAL SCIENCES (3, 4).

### 1-1. Medaka

Medaka (*Oryzias latipes*) is a small, egg-laying freshwater teleost that is widely used as a laboratory fish (5). Its oogenesis, fertilization, and embryonic development were extensively studied (6, 7). Medaka, typically 3 cm long, is among the smallest vertebrates known. The generation time is short, 2 to 3 months, comparable to that of zebrafish and mice. Spawning is daily and year-round under artificial conditions, and the timing of spawning can be controlled by regulating light conditions during a 24-hr period. In addition, the transparency of the eggs is a distinct advantage for embryonic observation and manipulation. Its chromosome number is 48. It is the only fish species with more than ten inbred strains established (8), and about 100 natural mutant strains collected and maintained (9). Physical maps (10) and BAC libraries (11) are available for use in gene analysis. A large-scale mutagenesis using medaka has been started and is progressing rapidly (12). Its small genome size, about 800Mb, which is one-half that of zebrafish and one-fourth that of mammals, enables sequencing of the whole genome. The whole-genome shotgun sequencing has been started very recently (13). The databases are available at the Medakafish Homepage of medaka biology (1) and at the Medaka ToxiNet of toxicology in medaka (2). The biological characteristics and resources of medaka are summarized in Tables 1 and 2, respectively.

Table 1 Biological characteristics of medaka and zebrafish.

| | Medaka | Zebrafish |
|---|---|---|
| Scientific name | *Oryzias latipes* | *Brachydanio rerio* |
| Growth | | |
| Body length | 3 cm | 4 cm |
| Generation time | 2-3 months | 2-3 months |
| Life span | 2 years | 2 years |
| Development | | |
| Spawning cycle | Daily | Twice a week |
| Brood size | 20-30 | 200-300 |
| Egg size | 1 mm | 1 mm |
| Transparency of eggs | High | High |
| Hardness of chorion | Hard | Soft |
| Days to hatching | 7-10 days | 2-3 days |
| Sex determination | | |
| Sex chromosome | XY | Unknown |
| Y chromosome | Identified | Unknown |
| Male-determining gene | *Dmy* | Unknown |
| Breeding temperature | 26℃ | 26℃ |
| Inhabitable temperature | 0-40℃ | 20-30℃ |
| Chromosomes | | |
| Chromosome number | 48 | 50 |
| Genome size | 800Mb | 1,600Mb |

Table 2. Biological resources of medaka and zebrafish.

| | Medaka | Zebrafish |
|---|---|---|
| Scientific name | *Oryzias latipes* | *Brachydanio rerio* |
| Strains | | |
| Inbred | 13 | |
| Natural mutants | 120 | |
| Sex reversal mutants | Several | |
| Strains with sex markers | Several | |
| See-through | Some | |
| Wild populations | 100 | |
| Genome-wide mutagenesis | Primary step | Extensive |
| Transgenic | Available | Available |
| ES cells | Ongoing | Ongoing |
| Nuclear transplantation | Ongoing | Ongoing |
| Genome | | |
| BAC library | Available | Available |
| Physical map | 1,760 | 15,353 |
| EST cloned | 44,531 | 214,214 |
| Genes cloned | 4 | 50 |
| Genome project ranking | 41st | 12th |

### 1-2. Sex Determination and Differentiation in Medaka

Sex in medaka is genetically determined by XY chromosomes, i.e., the female and male sexes are determined by XX and XY chromosomes, respectively (14). The first experimental sex reversal in vertebrates following exposure to hormones was demonstrated in medaka (15). Since then, many studies on sex determination and differentiation have been conducted using medaka as the model fish (16). Genes for xanthophores (14) and leucophores (17) are located on the sex chromosomes. Some PCR markers have been developed to identify sex chromosomes (18). Using these markers, the Y chromosome is identified microscopically by the FISH technique (19). Very recently, the male-determining gene (*Dmy*) has been identified on the Y chromosome and cloned (20, 21). Thus, medaka is an ideal model for determining the effects of endocrine-disrupting substances on fish reproduction.

## 2. Strains

### 2-1. Orange-red strain

The medaka with the wild-type body color has four main types of pigment cells in the skin, namely, melanophores, xanthophores, leucophores and iridophores (22, 23). Xanthophores and leucophores show sex-linked pigmentation. The strain with an orange-red body is commercially available and widely used in research and testing of environmental chemicals, because it is the easiest strain to maintain under laboratory conditions. The body color is due to the wild-type phenotype of orange-red pigmentation in xanthophores, determined by the sex-linked *r* locus ($X^RX^R$ for females and $X^RY^R$ for males) (14), and the recessive phenotype of black pigmentation in melanophores, determined by the *b* locus (24). Most studies of endocrine-disrupting substances in medaka were conducted using this strain (25). However, this orange-red strain lacks markers for identifying the genotypic sex at the embryonic or early stages of its life cycle. Thus, the occurrence of sex reversal, which is used as an endpoint of exposure experiments, is evaluated from the deviation in the phenotypic sex ratio of experimental fish. If genotypic sex identification is possible, the sex reversal evaluation becomes more reliable. Furthermore, we can use the female or male populations distinguished from each other at the embryonic or larval stage before experiments and thus reduce the number of fish required.

### 2-2. Strains with body-color and PCR sex markers

#### 2-2-1. d-rR strain with *r* marker

This strain was developed by Yamamoto (15). The d-rR stock has a genotypic sex

marker of the orange-red body color, in which females ($X^rX^r$) exhibit a recessive phenotype for *r* expressing the white body color and males ($X^rY^R$) exhibit a wild-type phenotype expressing the orange-red body color (Fig. 1). The marker can be observed at the later stages of embryonic development under a microscope and two to three weeks after hatching by the naked eye. The frequency of recombination between the *r* locus and the sex-determinant (*SD*) locus by crossing over of the X and Y chromosomes is 0.2 %, that is, an error of 0.2% is unavoidable in sex identification based on this color marker. d-rR has been used in studies of fish reproduction (16) and recently, in the testing of endocrine disrupters (26, 27, 28). A similar strain, s-rR, is also used in substance exposure experiments (29, 30).

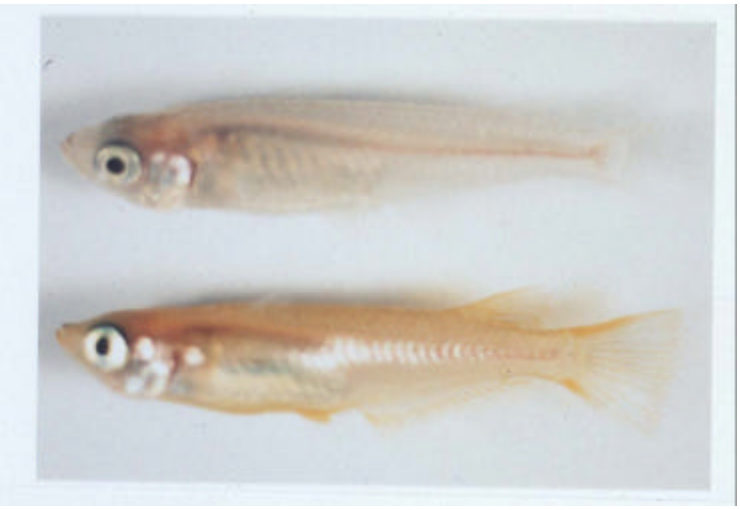


Figure 1.

d-rR strain. The female (upper) has a white body ($X^rX^r$) and the male (bottom) has an orange-red body ($X^rY^R$) (4).

### 2-2-2. FLF stock with *lf* and *SL1* markers

The *lf* (leucophore free) locus is sex-linked and determines leucophore expression in the skin (17). In the wild-type strain, leucophores first appear in two-day-old embryos in both females ($X^{LF}X^{LF}$) and males ($X^{LF}Y^{LF}$) (31). The lf strain is homozygous recessive at the *lf* locus and has no visible leucophores in the skin throughout life (9).

In addition to color markers, some PCR markers that are linked to sex chromosomes were reported (18, 19). One of these markers, sex-linked 1 (*SL1*), is closely linked to the *SD* locus. In some medaka stocks, the molecular sizes of PCR products of *SL1* are different between the X and Y chromosomes, resulting in detection of only one band in samples from females whereas two bands in those from males in PCR analyses.

The lf stock and a wild-type stock (Nagoya) were crossed to generate the FLF stock. In this stock, females ($X^{lf}X^{lf}$) exhibit the recessive phenotype for *lf*, that is, having no visible leucophores in the skin, and their samples show one band in PCR analysis. On the other hand, males ($X^{lf}Y^{LF}$) exhibit the wild-type phenotype, that is, having leucophores and their samples show two bands. The skin of the FLF stock is dark due to the wild-type nature of the autosomal *b* locus (24).

In males of the FLF stock, leucophores appear first on the ventral side of the midbrain of stage-26 embryos two days after fertilization and then along the dorsal and ventral midlines at later stages five days after fertilization. No visible leucophores are observed throughout the embryonic stages in females. The pattern of leucophore expression in males is the same as that in the wild-type strain (17). Leucophores appear reddish brown under transmitted light and rather difficult to distinguish from differentiating melanophores which also appear dark in color. Under reflective light, leucophores appear yellow to white. Under a fluorescence stereoscopic microscope, leucophores are easily identified by their intense yellow autofluorescence.

The frequency of recombination between the *lf* and *SD* loci by crossing over of the X and Y chromosomes is 4.2% and an error at this rate is unavoidable in the sex identification based on the leucophore marker. This error can be reduced to 0 %, if necessary, by utilizing the *SL1* marker, because there is no recombination between the *SL1* and *SD* loci (18, 19). Thus, the genotypic sex can be identified first by the leucophore marker at early embryonic stages and then the error of identification can be reduced by utilizing the *SL1* marker. The FLF stock has been used for testing of endocrine-disrupting substances (32).

The Qurt stock carrying the genotypic sex marker of leucophore is similar to the FLF stock, although it does not carry a PCR marker (17).

### 2-2-3. FLFII stock with *lf*, *r* and *SL1* markers

The Hd-rR.$Y^{HNI}$ strain (8) has *r* and *SL1* markers for identifying the genotypic sex. The FLF stock was crossed with this strain to generate the FLFII stock (3). In the case of the FLFII stock, the females show the recessive phenotype of $X^{lf,r}/X^{lf,r}$ and the males show the wild-type phenotype of $X^{lf,r}/Y^{LF,R}$ for both the *lf* and *r* characteristics. Leucophores appear in males in the same spatiotemporal manner as that in the wild-type strain, whereas there are no leucophores in females. In embryos, leucophores are more easily observed in this stock than in FLF because of the absence of melanophores in the skin. Xanthophore pigmentation is microscopically observed in the skin of male embryos at late embryonic stages, but not in that of female embryos. The orange-red pigmentation in the skin is easily observed by the naked eye in the male fry around two to three weeks after hatching (Figs. 2 and 3).

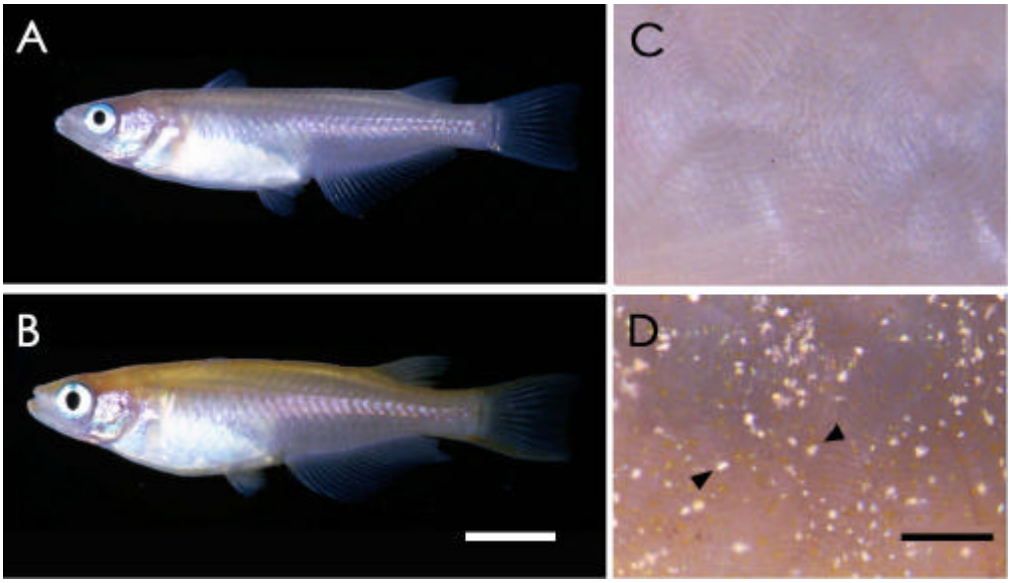



Figure 2.

Body colors in adult fish of the FLFII stock. A: a female showing a white body. B: a male showing an orange-red body. C: the skin of a female exhibiting no visible leucophores. D: the skin of a male exhibiting many leucophores (arrowheads). The magnification is the same for pairs A and B, and C and D. The bars in B and D represent 5 mm and 300 μm, respectively (3).

The frequency of recombination between the *lf* locus and the SD locus by crossing over of the X and Y chromosomes is 1.4 % in this stock, which is apparently lower than that in the FLF stock. No recombinant for the *r* locus and the *SD* locus is obtained. For the *SL1* marker, one band is detected in all samples from females examined whereas two bands are detected in all samples from males examined (Fig. 4).

In the FLFII stock, the genotypic sex can be screened at early embryonic stages based on the presence of leucophores, confirmed at larval stages by the presence of xanthophores, and finally reconfirmed by presence of the SL1 marker, eliminating errors due to the use of color markers (3). Furthermore, the FLFII stock may be superior to the FLF stock because of its lower frequency of recombination between the *lf* and *SD* loci.

Recently, a candidate male-determining gene, *Dmy*, has been isolated from the *SD* locus on the Y chromosome in medaka (20, 21). *Dmy* could be a tool for identifying the genotypic sex of wild-type populations or laboratory strains of medaka with or without the known genotypic sex markers such as genes for color and *SL1*. Further studies of *Dmy* are required to determine its applicability as a genotypic sex marker for general use in medaka.

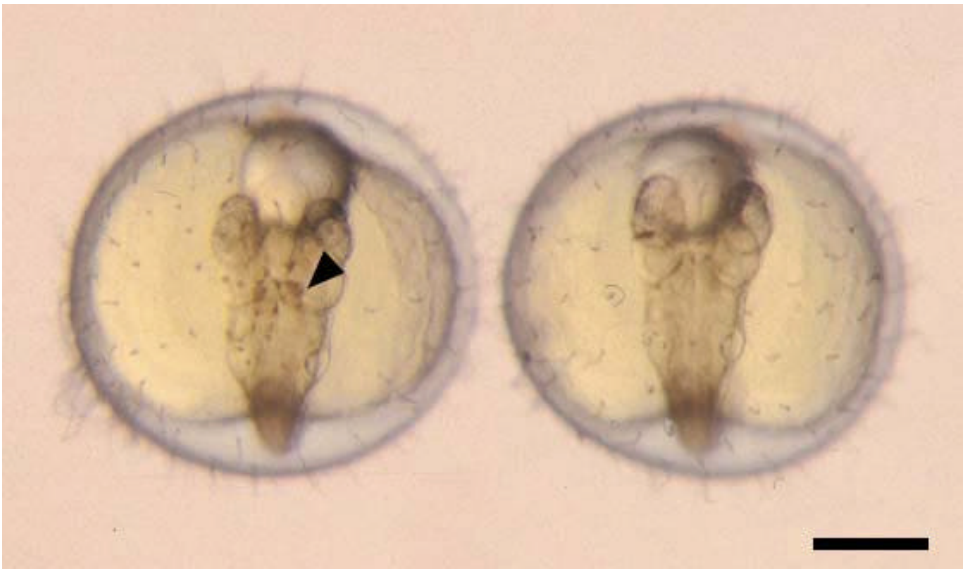


Figure 3.

First appearance of leucophores in embryos of the FLFII stock at stage 26 (two days after fertilization). Leucophores are observed as reddish-brown cells (arrowhead) on the ventral side of the midbrain in a male embryo (left) under transmitted light. A female embryo (right) at the same developmental stage shows no leucophores. The bar represents 300 μm (3).


Figure 4.

PCR analysis of *SL1* from the FLFII stock. Each lane contains the sample from each fish. Lane 1; sample from a control male of the Hd-rR.$Y^{HNI}$ stock exhibiting two bands corresponding to $SL1^a$ and $SL1^n$. Lane 2; sample from a control female of the Hd-rR.$Y^{HNI}$ stock exhibiting one band corresponding to $SL1^a$. Lanes 3-7; samples from males of the FLFII stock exhibiting two bands corresponding to $SL1^a$ and $SL1^n$. Lanes 8-12; samples from females of the FLFII stock exhibiting one band corresponding to $SL1^a$. Lane 13; sample from a male recombinant for *lf* and *SD* loci exhibiting two bands corresponding to $SL1^a$ and $SL1^n$. Lanes 14 and 15; samples from female recombinants for *lf* and *SD* loci exhibiting one band corresponding to $SL1^a$ (3).

### 2-2-4. Stability of newly established stocks

Both of the FLF and FLFII stocks are healthy and easy to breed. They mature approximately two months after hatching and show no abnormalities in morphology and behavior. Their reproductive activity is normal, that is, adult females spawn 20 to 30 eggs per day everyday under controlled temperature and lighting conditions. These stocks are stably maintained in our laboratory at Nagoya University. Sex markers for seven medaka strains or stocks are summarized in Table 3.

Table 3 Medaka stocks and genetic sex markers

| Strains | *lf* | *r* | *SL1* |
|---|---|---|---|
| Orange-red | - | - | - |
| d-rR | - | H14 days 0.2% | - |
| Hd-rR | - | H14 days ? | - |
| Hd-rR.$Y^{HNI}$ | - | H14 days ? | 0% |
| Qurt | E2 days 2.2% | - | ? |
| FLF | E2 days 4.2% | - | 0% |
| FLFII | E2 days 1.4% | H14 days 0% | 0% |

Hd-rR: inbred strain of d-rR (8).

E2 days: embryos two days after fertilization.

H14 days: fry 14 days after hatching.

%: percentage of recombination frequency.

## 3. Medaka model for testing based on bioimaging

### 3-1. See-through medaka

The bodies of most vertebrates are opaque, and thus internal organs are not visible from the outside. This makes noninvasive studies of internal organs difficult in vertebrate models. The opacity of the fish body is mainly due to the existence of pigment cells in the skin, peritoneum, and some other tissues. In the see-through medaka , most of the pigments are genetically removed by crossing selected color mutants (33). In this stock, the main internal organs, namely, the heart, spleen, blood vessels, liver, gut, gonads, kidney, brain, spinal cord, lens, air bladder, and gills, in a living adult fish are visible to the naked eye or can be visualized under a simple stereoscopic microscope (Fig. 5).

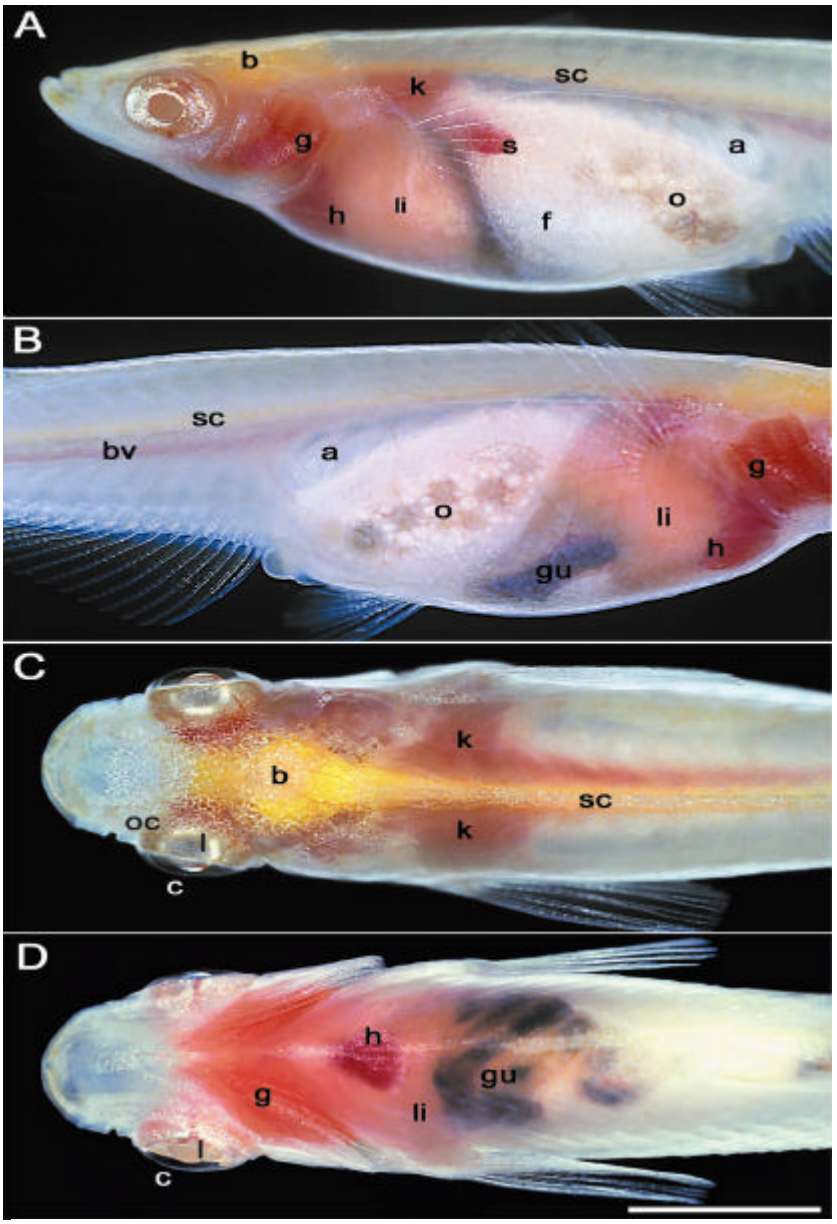



Figure 5.

Adult STIII fish. The left (A) and right (B) sides of a female body . The dorsal (C) and ventral (D) views of a male body. a, air bladder; b, brain; bv, blood vessels; c, conjunctiva; f, fat tissue; g, gill; gu, gut; h, heart; k, kidney; l, lens; li, liver; o, ovary; oc, optic cup; s, spleen; sc, spinal cord. The dark color of the gut is due to ingested feed. The bar represents 4 mm (33).

### 3-2. *GFP* transgenic see-through medaka

A transgenic see-through medaka carries the *GFP* gene fused to the promoter region of the medaka *vasa* gene that exhibits a germline-specific expression. In the *GFP* transgenic see-through medaka, the expression of *GFP* is evident in the gonad not only at embryonic stages, but also at postembryonic stages through the transparent body wall and peritoneum (33). The testis and ovary can be clearly observed under excitation light (Fig. 6). The fluorescent tag is effective in visualizing the internal organs and affords a unique opportunity to monitor in vivo expressions of tissue-specific genes during all stages of the life cycle of the see-through medaka. Using these transgenic see-through medaka, we can evaluate the effects of endocrine-disrupting substances as changes in the fluorescence image. Research on the applicability of *GFP*-transgenic see-through medaka in in vivo testing is in progress (34, 35).

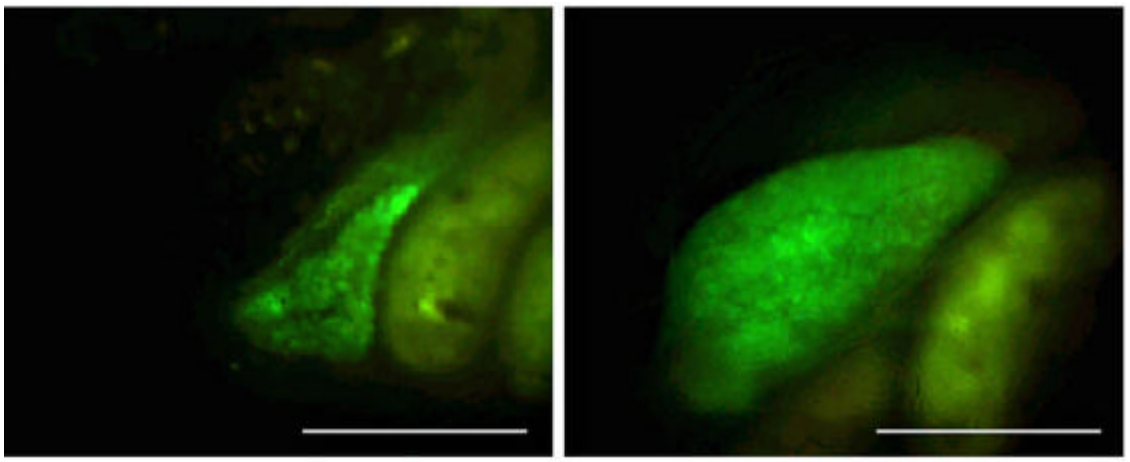


Figure 6.

The testis (left) and ovary (right) of a young adult of the transgenic see-through stock expressing the *olvas-GFP* gene (STII-Y1). This stock carries the leucophore marker (Arrowhead). The fish are oriented with its head on the right side. The bar represents 1 mm.(Unpublished data)

Acknowledgements

The Figure 6 is a photograph of the *olvas-GFP* transgenic see-through medaka generated by Wakamatsu, Y., Pristyazhnyuk, S., Kinoshita, M., Tanaka, M., and Ozato, K.

## 4. Conclusions

Medaka is a useful model for testing endocrine-disrupting substances by virtue of its biological characteristics. In particular, medaka is advantageous for determining the effects of these substances on reproduction, because the molecular and cellular mechanisms of sex determination and differentiation have been extensively studied in this species. The male-determining gene, first discovered in nonmammalian species, is a key tool for future toxicogenomics. Medaka stocks whose sex markers had been identified make testing reliable. The see-through medaka enables visualization of the effects of chemicals in living fish from the embryonic to the adult stage.

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# *Chapter 2*
# *Receptor Binding Assay and Reporter Gene Assay of Medaka*

**Makoto NAKAI**

***Chemical Evaluation and Research Institute, Japan***

***1600 Shimotakano, Sugito-machi, Kitakatsushika-gun, Saitama, Japan***

## 1. Principle of the assay

### 1-1. Receptor binding assay

Figure 1 shows the principle of receptor binding assay. Some of procedure for receptor binding assay is as follows.

#### 1-1-1. Procedure

(1) Sample solution (10 μl) and 5 nM [2,4,6,7,16,17-$^3$H] 17β-estradiol (10 μl) were dissolved in Tris-HCl (pH 7.4, 70 μl) containing 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10% glycerol, 10 mg/ml γ-globulin, 0.5 mM phenylmethylsulfonyl fluoride, and 0.2 mM leupeptin.
(2) A solution (10 μl) of recombinant estrogen receptor ligand binding domain fused with glutathione *S*-transferase expressed in *E. coli* was added to the solution.
(3) Incubated for 1 h at 25°C.
(4) When test chemicals possessed receptor-binding ability, they competed with [$^3$H] 17β-estradiol for the ligand binding domain of the receptor.
(5) In order to remove free radioligand, 100 μl of dextran-coated charcoal (DCC, 0.2% activated charcoal and 0.02% dextran in PBS (pH 7.4)) was added and incubated for 10 min at 4°C.
(6) After centrifugation or filtration, radioactivity in supernatant was measured using liquid scintillation counter.

The percent ratio ($B/B_0$ (%)) of standard ligand ([$^3$H] 17β-estradiol) bound to the receptor was represented as the formula below.

$$B/B_0\ (\%) = \frac{(X - NSB)}{(Y - NSB)} \times 100$$

where,

X: amount of standard ligand bound to the receptor in the presence of test chemical
Y: amount of standard ligand bound to the receptor in the absence of test chemical
NSB: amount of standard ligand bound to the receptor nonspecifically

### 1-1-2. Data analysis

Data were analyzed by using the computer program GraphPad Prism® and $IC_{50}$ value of each chemical was calculated. The binding abilities of test chemicals to the receptor were evaluated by relative binding affinity (RBA), ratio of $IC_{50}$ values to estradiol.

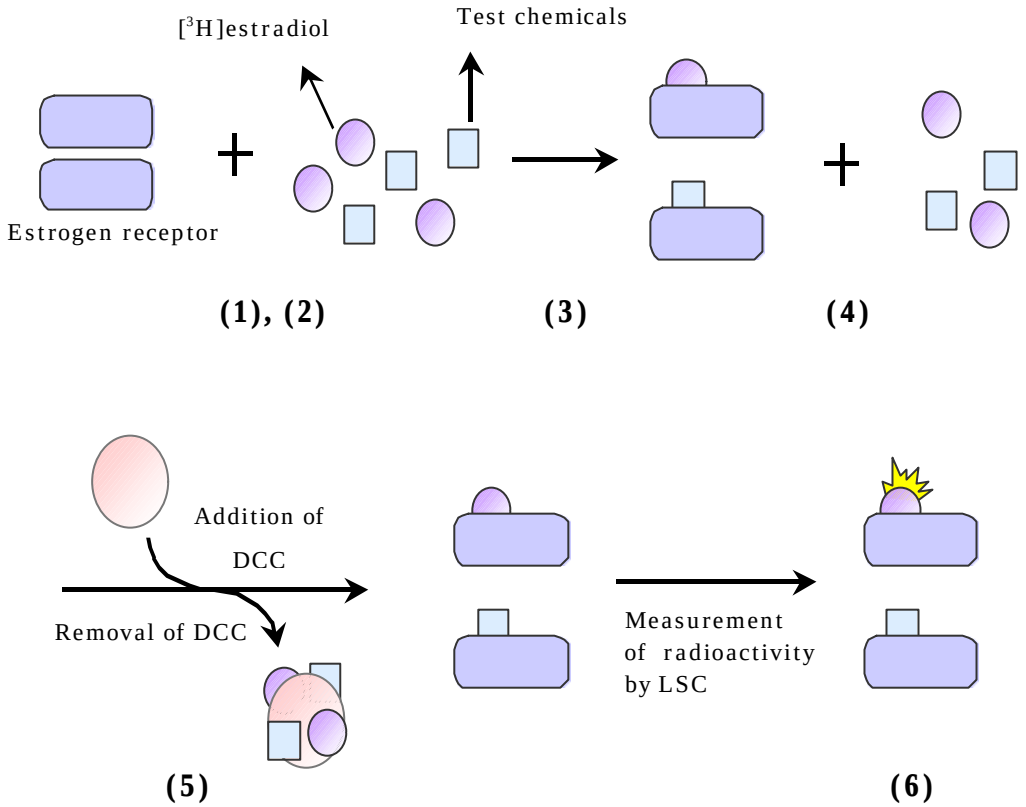


Fig. 1. Principle of receptor binding assay

### 1-2. Reporter gene assay

Figure 2 shows the principle of reporter gene assay. For procedure of the reporter gene assay, it takes about three days. Each procedure is as follows. This procedure was used in common for both ER and AR.

Day 1

(1) Cells were co-transfected with both receptor expression and reporter plasmids (3 μg each) in serum-free medium.
(2) After incubation for 4.5 h, the serum-free medium was replaced with serum-containing medium.

Day 2

(3) Cells were harvested and sample solution (1 x $10^{-5}$～1 x $10^{-11}$ M) was exposed to the cells in 96 well plate.

(4) When test chemicals induced transcriptional activation mediated by binding to hormone receptor, firefly luciferase was produced according to their estrogenicities in the cells.

Day 3

(5) The medium was wasted and cells were lysed and enzymatic activity of luciferase was measured.

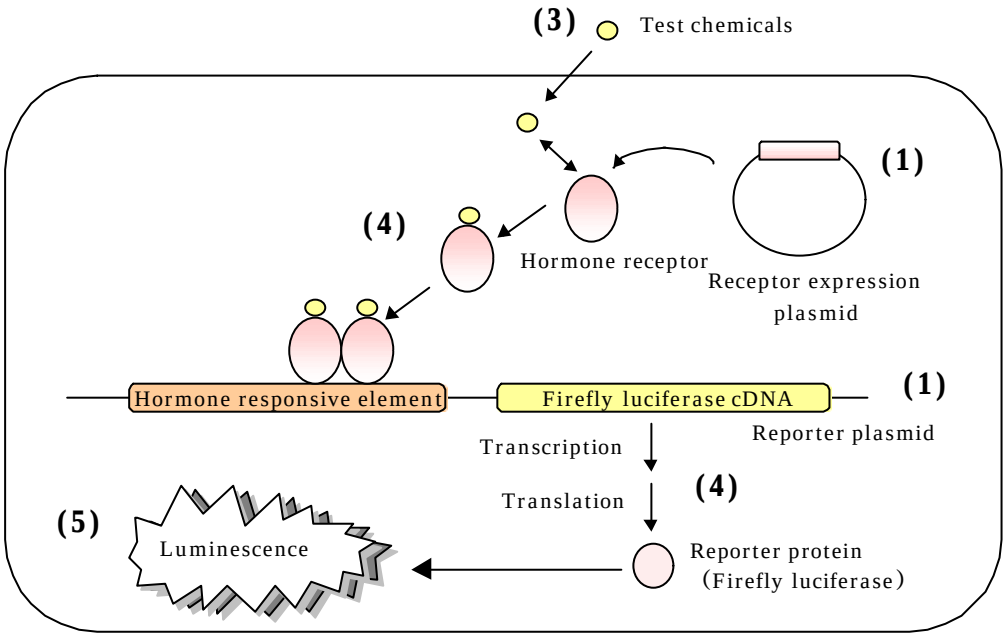


Fig. 2. Principle of reporter gene assay

## 2. Medaka estrogen receptor (meER a and meER b) binding assay and reporter gene assay

### 2-1. Objective

To evaluate the binding affinities and estrogenic activities of endocrine-disrupting chemicals to medaka estrogen receptor, receptor-binding assay and reporter gene assay were carried out.

## 2-2. Principle

Nuclear hormone receptors function as ligand-induced transcriptional factors. It is thought that exhibition of most endocrine-disrupting effects of chemicals, for instance, modulation of gene expression, are mediated by binding to hormone receptors, especially estrogen receptor, and it is very important to demonstrate the binding and gene transactivational potencies of endocrine disrupting chemicals. Receptor binding assay is a simple and convenient method to elucidate the binding properties of many chemicals. In this assay, binding affinities of several endocrine-disrupting chemicals to recombinant Medaka estrogen receptors α and β expressed in *E. coli* are measured. Reporter gene assay shows hormonal activities mediated by binding to target hormone receptors. Results are presented by dose-responsive sigmoid curves in both assays and estrogenicities of chemicals can be analyzed quantitatively comparing to standard ligands, i.e., endogenous hormones.

## 2-3. Test method

The procedures of receptor binding assay and reporter gene assay were described in 1-1 and 1-2, respectively.

## 2-4. Results and discussion

We have been demonstrated receptor binding and reporter gene assay of following twelve substances to medaka estrogen receptors α and β. The relative binding affinities and reporter gene transactivational activities of test chemicals were summarized in Tables 1 and 2, respectively. When compared receptor binding assay to reporter gene assay, former was more sensitive and quantitative than latter assay. Tributyltin chloride and triphenyltin chloride seemed to bind to both receptors strongly, however, they have strong protein denaturation property. Then, we examined GST activity of fusion protein (ER α) using 1-chloro-2,4-dinitrobenzene as a substrate. The GST activity was decreased in a dosedependent manner and it was suggested that the apparent binding affinity of each organo-tin compound for both receptors was caused by their denaturative characteristics.

Table 1 Relative binding affinities of chemicals to Medaka ERs α and β

| Chemical | Relative binding affinity (%) | |
|---|---|---|
| | ER α | ER β |
| 17β-estradiol | 100 | 100 |
| dibutyl phthalate | 0.023 | 0.0063 |
| dicyclohexyl phthalate | 0.045 | 0.016 |
| di-2-ethylhexyl phthalate | 0.79 | 0.80 |
| 4-*t*-octylphenol | 16 | 0.83 |
| nonylphenol | 8.1 | 0.83 |
| benzophenone | 0.02 | not determined |
| octachlorostyrene | 0.023 | 0.021 |
| tributyltin chloride | 0.10 | 0.19 |
| triphenyltin chloride | 0.24 | 0.29 |
| butylbenzyl phthalate | 0.23 | 0.057 |
| diethyl phthalate | 0.012 | 0.0024 |
| di-2-ethykhexyl adipate | 0.014 | 0.0040 |

Table 2 Gene transactivational activities of chemicals mediated by Medaka ERs α and β

| Chemical | Relative potency (%) | |
|---|---|---|
| | ER α | ER β |
| 17β-estradiol | 100 | 100 |
| dibutyl phthalate | not determined | negative |
| dicyclohexyl phthalate | negative | negative |
| di-2-ethylhexyl phthalate | negative | negative |
| 4-*t*-octylphenol | 1.3 | negative |
| nonylphenol | 0.35 | negative |
| benzophenone | negative | negative |
| octachlorostyrene | negative | negative |
| tributyltin chloride | negative | negative |
| triphenyltin chloride | negative | negative |
| butylbenzyl phthalate | not determined | not determined |
| diethyl phthalate | negative | negative |
| di-2-ethykhexyl adipate | negative | negative |

### 3 Medaka androgen receptor (meAR) reporter gene assay

Similarly, reporter gene assay using medaka AR was carried out (Table 3). All chemical substances tested in this study had no gene transcriptional activity mediated by binding to medaka AR.

Table 3 Gene transactivational activities of Chemicals mediated by Medaka AR

| Chemical | Relative potemcy(%) |
|---|---|
| 5α-dihydrotestosterone | 100 |
| dibutyl phthalate | negative |
| dicyclohexyl phthalate | negative |
| di-2-ethylhexyl phthalate | negative |
| 4-*t*-octylphenol | negative |
| nonylphenol | negative |
| benzophenone | negative |
| octachlorostyrene | negative |
| tributyltin chloride | negative |
| triphenyltin chloride | negative |
| butylbenzyl phthalate | negative |
| diethyl phthalate | negative |
| di-2-ethykhexyl adipate | negative |

### 4. Future work

As shown in Table 1, 4-*t*-octylphenol and nonylphenol bound to medaka ER α at 1/6 and 1/12 of E2, respectively. On the other hand, they bound to human ER α at approximately 1/3,000 of $E_2$. These results suggest there are species differences in receptor binding characteristics and show importance of the differences in sensitivities to chemicals among diverse species to assess the endocrine disrupting effects of chemicals to ecosystem. Consequently, we should clarify these issues by receptor binding assay using various fish estrogen receptors.

# *Chapter 3*
# *Medaka Screening and Definitive Test for Endocrine Disrupting Chemicals*

**Masanori SEKI, Tatsuo ABE, Masanobu MAEDA**
*Chemical Evaluation and Research Institute, Japan*
*19-14 Chuo-machi, Kurume, Fukuoka, Japan*

**Norihisa TATARAZAKO**
*National Institute for Environmental Studies*
*16-2, Onogawa, Tsukuba-shi, Ibaraki, Japan*

**Yuta OHNISHI**
*METOCEAN ENVIRONMENT INC*
*1334-5, Riemon, Ooigawa-cho, Shida-gunn, Shizuoka, Japan*

## *Part1* Validation with Reference Chemicals

Several works have been performed to verify the applicability of each test using mainly some reference chemicals recommended by the OECD Expert Consultation on testing in fish [1].

### 1. Materials and Methods

Medaka (*Oryzias latipes*) were originally purchased from a local fish farm in Kumamoto, Japan, and a breeding stock of medaka has been maintained in our laboratory. Female Leucofore Free strain (FLF) medaka were supplied from Bioscience Center of Nagoya University and has been maintained in our laboratory.

The following chemicals recommended by OECD [1] were tested as reference chemicals; ethynylestaradiol ($EE_2$, Wako Pure Chemical Industries Ltd., Tokyo, Japan), methyltestosterone (MT, Wako Pure Chemical Industries Ltd., Tokyo, Japan) and 4-*tert*-pentylphenol (PP, Tokyo Chemical Industry, Tokyo, Japan). $EE_2$ and MT were used for vitellogenin (VTG) assay and reproduction test (REP), and PP was used for fish partial life-cycle test (PLC) and fill life-cycle test (FLC). In short term PLC, 4-*tert*-octhylphenol (OP, Wako Pure Chemical Industries Ltd., Tokyo, Japan) was used as a screening chemical. 4-Nonylphenol (NP, Kanto Chemical Co., Inc., Tokyo, Japan) was used for all test types (FLC, PLC, short term PLC, REP, VTG assay) for hazard assessment (see chapter 3-2). Stock solutions were prepared by dissolving into dilution water or solvents (dimethyl sulfoxide or ethanol). The prepared stock solutions were delivered to each test chamber with a continuous-flow mini-diluter system modified from Benoit et al. [2].

The experimental conditions used in each test are briefly shown in Table 1 and the details of those are described in [3] for FLC, [4] for PLC and [5] for REP. Measurement of hepatic VTG is described in [6]. The draft protocols for FLC and PLC have been prepared (http://www.env.go.jp/en/topic/edcs/approach/2002.html) and submitted to the OECD.

### 2. Results and discussion

#### 2-1. VTG assay and REP

The design of VTG assay is almost the same as REP; therefore, we show the results of the latter. REP using $EE_2$ has already been reported by us [5] (*see Appendix II- I*). We concluded that REP with medaka is applicable to the evaluation of estrogens from the observation of the estrogenic activity of $EE_2$ in medaka, as shown by induction of sex differentiation and VTG, and also its effects on reproductive potential.

Table 1. Experimental conditions used in each test

| Item | Type of in vivo test | | | | |
|---|---|---|---|---|---|
| | VTG assay | REP | PLC | FLC | Short term PLC |
| Species | Medaka (*Oryzias latipes*) | Medaka (*Oryzias latipes*) | Medaka (*Oryzias latipes*) | Medaka (*Oryzias latipes*) | FLF medaka (*Oryzias latipes*) |
| Age of test organisms at beginning of test | Reproductively matured fish | Reproductively matured fish | Fertilized egg | Fertilized egg | Fertilized egg |
| Duration | 21 days | 21 days | 70 days | F0; 110 days<br>F1; 70 days | 45 days |
| Test type | Flow-through | Flow-through | Flow-through | Flow-through | Flow-through |
| Dilution water | Dechlorinated tap water | Dechlorinated tap water | Dechlorinated tap water | Dechlorinated tap water | Dechlorinated tap water |
| Water temperature | 24±2℃ | 24±2℃ | 24±2℃ | 24±2℃<br>(28 ± 2 ℃ in reproductive phase) | 24±2℃ |
| Illumination quality | Fluorescent bulbs | Fluorescent bulbs | Fluorescent bulbs | Fluorescent bulbs | Fluorescent bulbs |
| Photoperiod | 16-h light, 8-h dark | 16-h light, 8-h dark | 16-h light, 8-h dark | 16-h light, 8-h dark | 16-h light, 8-h dark |
| Test chamber size | Round glass jar (diameter 15 cm, depth 17.5 cm) | Round glass jar (diameter 15 cm, depth 17.5 cm) | Round glass jar (diameter 15 cm, depth 17.5 cm) | Round glass jar (diameter 15 cm, depth 17.5 cm) | Round glass jar (diameter 15 cm, depth 17.5 cm) |
| Test solution volume | 1.8 L | 1.8 L | 1.8 L | 1.8 L | 1.8 L |
| Volume exchanges of test solutions | 16 daily | 16 daily | 16 daily | 16 daily | 16 daily |
| No. of vessels per level | 2 | 6 | 4 | 4 | 4 |
| No. of fish per level | 10 male | 12 (6 male and 6 female) | 60 | 60 | 60 |
| No. of treatment | 5 (plus controls) | 5 (plus controls) | 5 (plus controls) | 5 (plus controls) | 5 (plus controls) |
| Dilution factor | <3.2 | <3.2 | <3.2 | <3.2 | <3.2 |
| Feeding regime | *Artemia* nauplii twice daily (*ad libitum*) | *Artemia* nauplii twice daily (*ad libitum*) | *Artemia* nauplii twice daily (*ad libitum*) | *Artemia* nauplii twice daily (*ad libitum*) | *Artemia* nauplii twice daily (*ad libitum*) |
| Pre-exposure period | None | 21 days | None | None | None |
| Biological endpoints | survival, behavior, HSI, VTG | survival, behavior, sec. SEX, fecundity, fertility, GSI, HSI, VTG, gonadal histology | survival, behavior, sec. SEX, growth, GSI, HSI, VTG, gonadal histology | survival, behavior, sec. SEX, growth, fecundity, fertility, GSI, HSI, VTG, gonadal histology | survival, behavior, growth, GSI, HSI, VTG, gonadal histology |

VTG assay; Vitellogenin assay, REP; Reproduction test, PLC; Partial life cycle test, FLC; Full life cycle test, short term PLC; Short term partial life cycle test, sec. SEX; secondary sex characteristics, VTG; Vitellogenin, GSI; Gonad-somatic index, HSI; Hepato-somatic index

Although there have been no reports on the reproductive effects of $EE_2$ exposure in fish other than medaka, there are a few studies on reproduction in fish exposed to 17ß-estradiol ($E_2$). Kang et al. [7] reported that the exposure of medaka to 463 ng/L $E_2$ for three weeks decreased fecundity, but that the exposure to 227 ng/L had no effect. When fathead minnows were exposed to $E_2$ for 19 d, the $E_2$ concentrations expected to cause 50% and 10% inhibition of egg production were 120 and 6.6 ng/L, respectively [8]. Therefore, medaka may be less sensitive than fathead minnow with regard to fecundity. There are several studies on VTG induction in fish exposed to $EE_2$. Although in our study [5] the lowest-observed-effect-concentration (LOEC) of $EE_2$ for VTG induction in medaka was 63.9 ng/L, Purdom et al. [9] found that a 10-d immersion exposure of male rainbow trout to $EE_2$ caused VTG induction at concentrations ranging from 0.1 to 10 ng/L. Furthermore, Jobling et al. [10] reported that exposure of adult rainbow trout to 2 ng/L $EE_2$ for three weeks caused significant induction of VTG. Länge et al. [11] reported that the LOEC of $EE_2$ for VTG induction over the full life cycle of fathead minnow was 16 ng/L. These differing sensitivities in terms of VTG induction may be caused partly by dissimilarities in the period and fish stage of exposure or by different methods of VTG assessment. Although medaka seems to be less susceptible to $EE_2$ than rainbow trout and fathead minnow in terms of VTG induction, further work is required to determine the relative sensitivity to $EE_2$ among various fish species.

We also have conducted REP with MT (unpublished). In MT exposure, we detected a significant decrease in the fecundity and fertility of medaka. In addition, male secondary sexual characteristics were observed in female medaka exposed to MT. We concluded that REP with medaka is applicable to androgens from the observation of the androgenic activity of MT in medaka, as shown by male secondary sexual characteristics, and its effects on reproductive potential. In other OECD recommended fish, Ankley et al. [12] reported that exposure of the fathead minnow to 200 µg/L of MT (nominal concentration) for 21 d caused significant decrease in fecundity. The authors suggested that MT might inhibit the natural process of endogenous androgens, which probably play a role in final maturation and/or release of eggs in female fish. The authors also reported on the easily discernible alterations in secondary sex characteristics, nuptial tubercles of both sexes, suggesting that nuptial tubercles in this species should be an excellent diagnostic endpoint for androgenic chemicals. These results suggest that both madaka and fathead minnow may be responsive to androgens in expression of the appearance of the secondary sex characteristics.

## 2-2. PLC and FLC

The design of PLC is the same as a part of FLC. FLC using PP has already been reported by us [13] but not published yet. In summary, for all of the endpoints monitored in parent medaka ($F_0$), the LOEC of PP for lethal and sublethal toxicity was 931 µg/L, whereas those for abnormal sex differentiation and VTG induction were 224 and ≦ 51.1 µg/L, respectively, and that for reproductive impairment was 224 µg/L. Typical gonadal sections of testis-ova are shown in *Figure 1*.

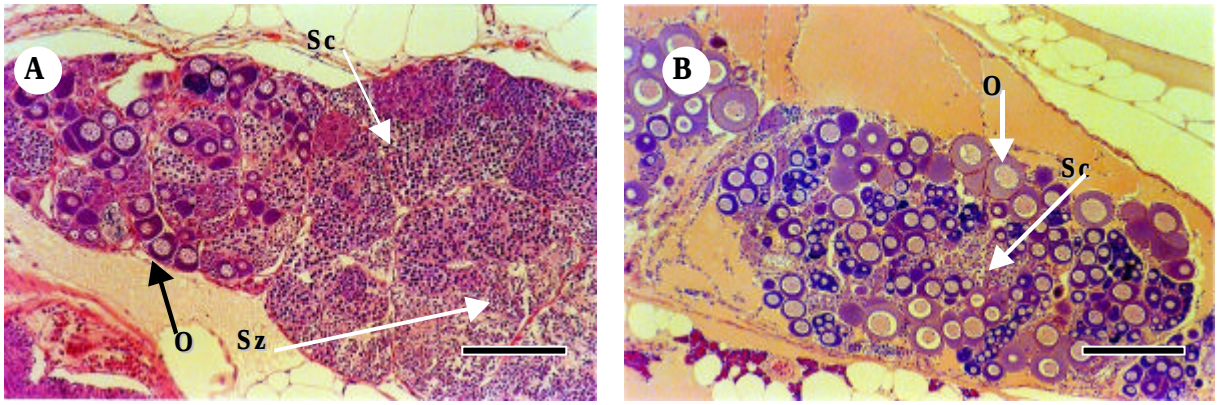


Figure 1. Longitudinal sections of testis-ova in the 224- µg/L (A) and 931-µg/L (B) 4-*tert*-pentylphenol treatment groups of $F_0$ generation medaka at 60-d posthatch. Each bar in (A) and (B) shows 200 and 400 µm length. (A): Oocytes (O) appear in clusters within the testicular tissue. Numerous spermatocytes (Sc) and spermatozoa (Sz) are still present in a compacted mass in this section. (B): A relatively advanced testis-ova. Almost the entire area is composed of oocytes (O), accompanying small testicular tissues interspersed with a few spermatocytes (Sc).

These results indicate that PP was about four times more sensitive for abnormal sex differentiation and reproductive impairment compared with lethal and sublethal toxicity. In the progeny ($F_1$) generation, the LOECs of PP for sublethal toxicity and abnormal sex differentiation, were 224 and ≦ 51.1 µg/L, respectively. We conclude that FLC with medaka is applicable to weak estrogens from the observation of the estrogenic activity of PP in medaka, as shown by abnormal sex differentiation and VTG induction, and its effects on reproductive potential. PP has already been reported to affect the sexual differentiation of fish. Gimeno et al. [14] exposed genetic male common carp (*Cyprinus carpio*) to PP from 50 d posthatch for 90 d at measured concentrations of 36, 90, and 256 µg/L, and then observed the effect on gonadal development. The authors observed not only

the formation of oviducts, a female permanent feature in these genetic male gonads at all tested PP concentrations, but also intersex gonads (testis-ova) as of 90 µg/L, demonstrating the phenotypic feminization of the male gonads by the estrogenic activity of PP. In our study, abnormal sexual differentiation in the $F_0$ generation of medaka was observed at 224 µg/L of PP, approximating to that in common carp, as described above. These results suggest that the effective concentrations of PP on gonadal development in medaka and common carp are similar and that medaka is as useful as other test fish in terms of the detection of abnormal sexual differentiation. In the progeny generation of medaka, we observed growth inhibition and feminization of the gonad at lower concentrations compared with its parent generation. Our previous study [3] showed that the exposure to NP over two generations of medaka induced the formation of hermaphroditic gonads at lower concentrations in the progeny than in the parent generation. We mentioned a possibility of maternal transfer of NP into the $F_1$ embryo as one of the possible explanations for the enhanced response in the progeny. Thus, the FLC for the testing of weak estrogens should be carried out over at least two generations, because some estrogenic chemicals may have adverse effects at lower concentrations in the progeny than in their parent generation. The mechanisms of this enhanced response of progeny to particular estrogens should be investigated by transgenerational exposure.

FLC using MT has been conducted by us, but not published yet [15]. In summary, although no dose-dependent effects of MT were observed on the embryo survival, hatching success, mortality after hatching and growth of the $F_0$ and $F_1$ fish at 60-d posthatch, we observed that all $F_0$ fish in the 27.7-ng/L treatment group showed male secondary sex characteristics, in which no fish with ovary could be discerned. In addition, several fish with ovary showed male secondary sex characteristics in the 9.98 ng/L treatment group in $F_0$ and $F_1$ generations. On the other hand, we observed swollen abdomens in the female fish at MT level of 9.98 ng/L in $F_0$ and $F_1$ fish. These swollen abdomens were induced by enlarged GSI, accompanying the declined fecundity and fertility in $F_0$ generation. These responses were most likely due to inhibition of the natural process of endogenous androgens, which probably play a role in final maturation and/or release of eggs in female fish, as reported by Goetz [16]. In respect to VTG, the levels of $F_0$ male medaka at the day after the end of the reproductive phase increased in all groups treated with MT at 0.348 – 9.98 ng/L, although no statistically significant difference was determined. In addition, VTG levels in the $F_1$ males exposed to 9.98 ng/L of MT were significantly increased compared with those of the controls. On the other hand, although the VTG concentrations of females exposed to $\leqq$ 9.98

ng/L of MT were not affected in $F_0$ generation, these were significantly decreased in the $F_1$ generation. In other OECD recommended fish, Ankley et al. [12] reported on the induction of plasma VTG in adult male and female fathead minnow exposed to MT for 21 d. We presume that some species and/or sexual differences are present with respect to VTG response in fish when exposed to MT. However, further work is needed to determine the applicability of this VTG parameter to andorogens in PLC and FLC. Overall, these results indicate that MT has androgenic effects which reduce the reproductive potential of medaka, and the FLC with medaka is applicable to the evaluation of androgens.

### 2-3. Short term PLC

We have conducted short term PLC with OP (unpublished). Overall, we detected abnormal sex differentiation (testis-ova) in genetically male (XY) medaka at 35-d posthatch while all gonads differentiated into ovary in genetically female (XX) medaka. We have already conducted the medaka PLC, and the abnormal sex differentiation was observed at 11.4 – 94.0 µg/L OP [17]. This result suggests that the effective concentrations of OP on gonadal development are similar between short term PLC and PLC. Consequently, short term PLC can detect an estrogenic activity such as induction of testis-ova, and its sensitivity may be almost the same as the PLC.

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## *Part2* Hazard assessment of tributyltin (TBT) and 4-nonylphenol (NP)

The Fish Working Group in Japan has developed vitellogenin (VTG) assay, and partial life-cycle test (PLC) for screening purposes, and full life-cycle test (FLC) for definitive test with medaka (*Oryzias latipes*), and also conducted hazard assessment of chemicals listed in SPEED '98 using these test methods. The activities of the group were reported at the special session of the Endocrine Disrupter Testing and Assessment (EDTA) in 2002, and the data are shown at the end of this part. In this part, tributyltin (TBT) and 4-nonylphenol (NP) (*see Appendix II-II*) that is a known environmental alkyl phenol are featured.

### 1. 4-Nonylphenol (NP)

#### 1-1. *In vitro* assays

##### 1-1-1. Competitive binding assay to medaka estrogen receptor a (ERa)

Competitive receptor binding assay was performed using medaka (*Oryzias latipes*) and human estrogen receptor α ligand binding domains expressed in E. coli., and the binding affinities of nonylphenol (mixture), 4-*t*-octylphenol, 4-*t*-pentylphenol and 4-*t*-butylphenol to these recombinant receptors were measured. Release of the radiolabelled ligand from medaka ER α depending on the concentrations of 17β-estradiol, nonylphenol, and 4-t-octylphenol were observed (Fig.1). Their relative binding affinities (RBA) to both medaka and human receptors compared with 17β-estradiol was summarized in Table 1. It was found that alkyl phenols with branched alkyl chain bound to medaka ER α according to their chain length and RBA values were about several hundreds times stronger than those to human ER α. Especially, nonylphenol (mixture) and 4-*t*-octylphenol had high receptor binding abilities and their RBA values were about 1/10 and 1/15 of 17β-estradiol, respectively. Other branched alkylphenols tested also showed relatively high receptor binding abilities. The RBA values of 4-*t*-pentylphenol and 4-*t*-butylphenol were 1.1 and 0.15, respectively, and they were hundreds times greater than those to human ERα as in the case with nonylphenol and 4-*t*-octylphenol. On the other hand, linear alkylphenols bound to medaka ER α weakly. Their RBA values were less than 0.1% when compared with 17β-estradiol and almost alike to those to human ERα.

Furthermore, the binding abilities of nonylphenol were examined for medaka ERβ, and ERα from other fish, carp (*Cyprinus carpio*) and mummichog (*Fundulus heteroclitus*) with a same procedure. The RBA values of nonylphenol to medaka ER β and mummichog ER α were 1/110 and 1/200 of 17β-estradiol, respectively.

However, it bound to carp ER α weaker than to other fish ERs (RBA ~ 0.1%). In conclusion, alkylphenols with branched bulky alkyl chains showed relatively high binding affinities to estrogen receptors from fish compared with those to human estrogen receptor.

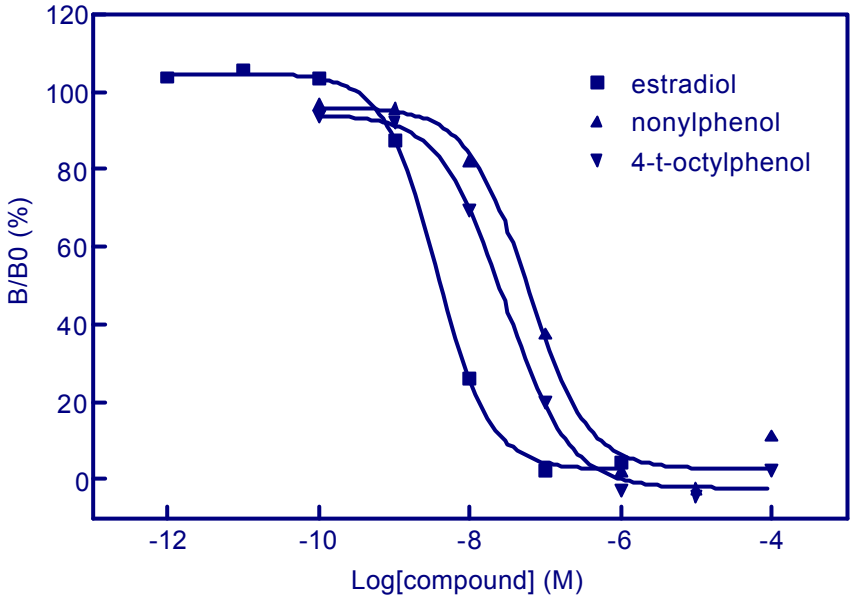


Fig. 1 Dose-response curves of 17β-estradiol and alkylphenols in the radioligand receptor binding assay using [$^3$H]17β-estradiol and medaka ER α expressed in *E. coli*.

Table 1 $IC_{50}$ values and relative binding affinities (%) of alkylphenols to medaka and human estrogen receptors α ligand binding domain

| Chemical substances | Medaka *1 | | Human*2 | |
|---|---|---|---|---|
| | $IC_{50}$ values (M) | Relative binding affinity(%) | $IC_{50}$ values (M) | Relative binding affinity (%) |
| Estradiol | $4.8 \times 10^{-9}$ | 100 | $2.1 \times 10^{-9}$ | 100 |
| Nonylphenol(mixture) | $7.9 \times 10^{-8}$ | 8.1 | $3.4 \times 10^{-6}$ | 0.061 |
| 4-*t*-Octylphenol | $3.2 \times 10^{-8}$ | 16 | $6.6 \times 10^{-6}$ | 0.032 |
| 4-*t*-Pentylphenol | $3.9 \times 10^{-7}$ | 1.1 | $4.1 \times 10^{-5}$ | 0.0051 |
| 4-*t*-Butylphenol | $3.0 \times 10^{-6}$ | 0.15 | $1.6 \times 10^{-4}$ | 0.0013 |
| 4-*n*-Nonylphenol | $1.1 \times 10^{-6}$ | 0.038 | $4.2 \times 10^{-6}$ | 0.050 |
| 4-*n*-Octylphenol | $5.3 \times 10^{-6}$ | 0.077 | $1.1 \times 10^{-5}$ | 0.020 |
| 4-*n*-Pentylphenol | $5.5 \times 10^{-6}$ | 0.084 | — | — |
| 4-*n*-Butylphenol | $6.5 \times 10^{-6}$ | 0.066 | $8.8 \times 10^{-5}$ | 0.0024 |

*1: Measured four times for nonylphenol (mixture) and 4-*t*-octylphenol, and three times for other chemical substances.

*2: Measured three times for all chemical substances.

### 1-1-2. Reporter gene assay

Transcriptional activities of alkylphenols mediated by medaka ERα were measured using HeLa cells transiently co-transfected with both receptor expression and reporter (firefly luciferase) vectors. It was found that transactivation function presented by EC50 value of nonylphenol was three hundred times weaker than 17β-estradiol (Fig. 2).

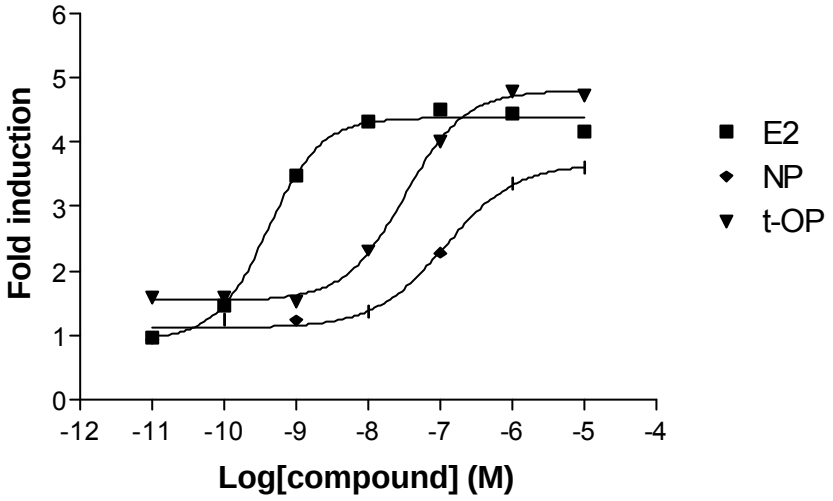


Fig. 2 Reporter gene transactivation assay using HeLa cells co-transfected with medaka estrogen receptor expression and reporter vectors.

## 1-2. *In vivo* studies using medaka

### 1-2-1. Screening

***Medaka vitellogenin assay***

The estrogenic potency of nonylphenol (mixture; NP) and 4-*t*-octylphenol (4-*t*-OP) was evaluated using in vivo vitellogenin (precursor of egg yolk protein) synthesis in medaka. About 3-month-old medaka (respectively 8 females and males / treatment) were exposed to 5 test concentrations of each substance (NP; 7.40, 12.8, 22.5, 56.2 and 118 μg/L, 4-t-OP; 12.7, 27.8, 64.1, 129 and 296 μg/L as mean measured concentrations) under flow-through conditions for 21 days. 17β-estradiol (E2; 100ng/L) was tested as positive control. Daily observation was made to examine mortality and abnormal behavior and appearance during the exposure period. At the end of exposure, the livers of exposed fish were removed, and vitellogenin concentration in each liver was measured.

In either NP or 4-*t*-OP study, any death or particular symptom was not observed through the exposure period. The hepatic vitellogenin concentrations in males were increased in a concentration-dependent manner, and a statistically significant induction was observed at ≥ 22.5 μg/L for NP study and ≥ 64.1 μg/L for 4-t-OP study (Fig.3).

These results suggested that both NP and 4-*t*-OP could cause vitellogenin synthesis in the livers of male medaka through their estrogenic activities.

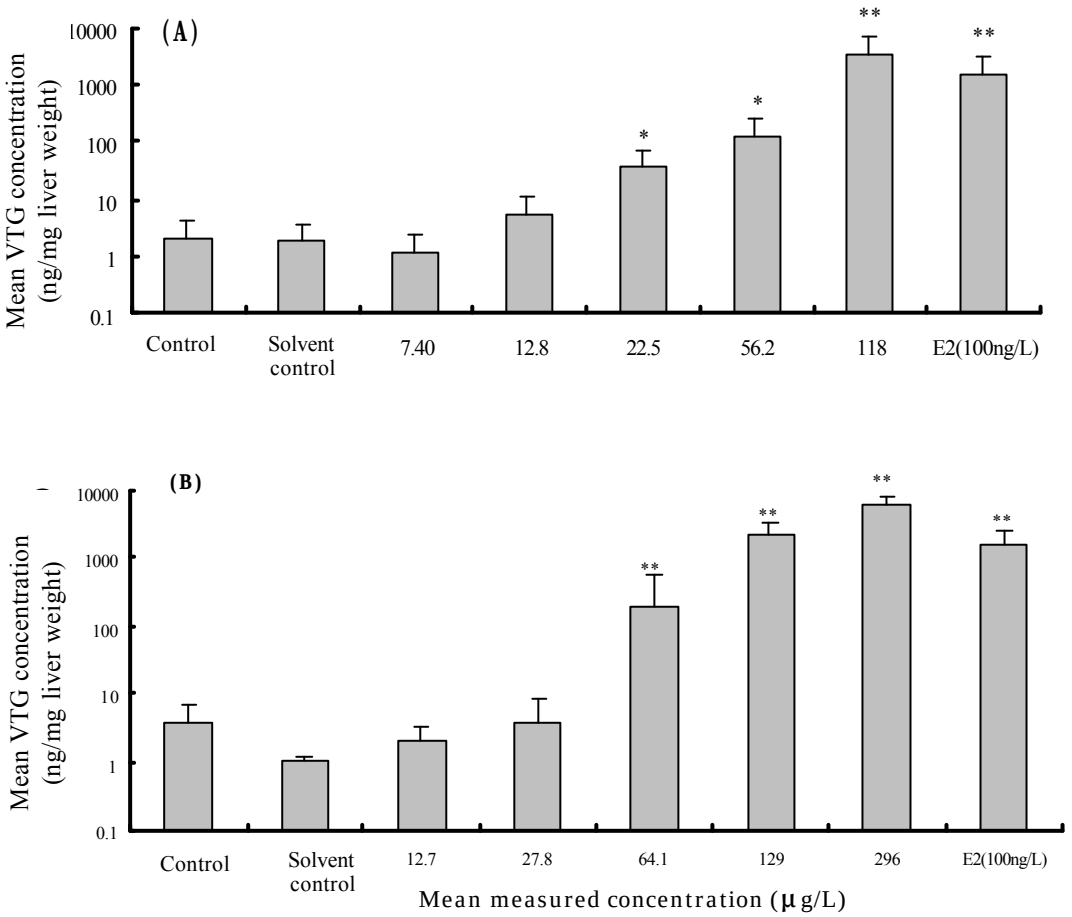


Fig. 3 Vitellogenin (VTG) concentrations in the livers of male medaka (*Oryzias latipes*) in NP study (A) and 4-*t*-OP study (B).Data is shown as mean± standard deviation. * and ** denote significant differences at $p < 0.05$ and $p < 0.01$, respectively.

***Medaka partial life test***

This test was performed to assess endocrine disrupting effects of nonylphenol (mixture; NP) and 4-*t*-octylphenol (4-*t*-OP) on sex differentiation of medaka. Medaka (60 eggs/treatment) were exposed to 5 test concentrations of each substance (NP; 3.30, 6.08, 11.6, 23.5 and 44.7 μg/L, 4-*t*-OP; 6.94, 11.4, 23.7, 48.1 and 94.0 μg/L as mean measured concentrations) under flow-through conditions from fertilized eggs to 60-day posthatch. During the exposure period, hatching, posthatch mortality, and abnormal behavior and appearance were observed daily. At the end of exposure (at 60-day posthatch), the total length and body weight of all the surviving fish were measured, and the sex of each individual was determined from the appearance of secondary sex characteristics. Furthermore, 20 individuals from each treatment group were randomly sampled, and then their livers and gonads were removed for vitellogenin measurement and gonadal histology.

In either NP or 4-*t*-OP test, any particular effect on hatching of fertilized eggs and posthatch mortality was not observed at the concentrations tested. As for growth of fish at 60-day posthatch in the NP test, however, a significant decrease was observed in both total length and body weight in the 44.7 μg/L treatment, and in body weight in the 23.5 μg/L treatment. This result suggests that NP adversely affects the growth of medaka. In the 4-*t*-OP test, no growth reduction was observed at the concentrations tested. The sex ratio estimated from the appearance of secondary sex characteristics of the surviving fish at 60-day posthatch was significantly skewed toward female at ≥ 23.5 μg/L in NP test and ≥ 48.1 μg/L in 4-*t*-OP test (Table 4 and 5). Furthermore, gonadal histology showed that the fish in ≥ 11.6 μg/L NP treatment groups and ≥ 11.4 μg/L 4-*t*-OP treatment groups had testis-ova as shown by the presence of oocytes in the testis (hereinafter referred to as testis-ova, or, Table 2 and 3). The hepatic vitellogenin concentrations in males exposed to ≥ 11.6 μg/L NP and ≥ 11.4 μg/L 4-*t*-OP were significantly increased(Fig.4).

These results indicate that both NP and 4-*t*-OP exert estrogenic effects on sex differentiation of male medaka, and suggest that the Lowest-Observed-Effect Concentrations (LOECs) of NP and 4-*t*-OP for feminization of the appearance of their secondary sex characteristics were 23.5 μg/L and 48.1 μg/L, respectively, and that the LOECs of them for induction of testis-ova and vitellogenin were 11.6 μg/L and 11.4 μg/L.

Table 2 Sex ratios as determined by gross examination of secondary sex characteristics of medaka (*Oryzias latipes*) at 60-day posthatch in NP test, and by their gonadal histology.

| NP concentration (μg/L) | Secondary sex characteristics N: Number of fish Sex ratio (♂: ♀) | | Gonadal histology N: Number of fish Testis | Ovary | Testis-ova | |
|---|---|---|---|---|---|---|
| Control | 55 | 25 : 30 | 20 | 8 | 12 | 0 |
| Solvent control | 57 | 27 : 30 | 20 | 10 | 10 | 0 |
| 3.30 | 59 | 27 : 32 | 20 | 9 | 11 | 0 |
| 6.08 | 59 | 25 : 34 | 20 | 10 | 10 | 0 |
| 11.6 | 57 | 28 : 29 | 20 | 9 | 7 | 4* |
| 23.5 | 58 | 11 : 47** | 20 | 2 | 9 | 9** |
| 44.7 | 60 | 1 : 59** | 20 | 1 | 15 | 4** |

* and ** denote significant differences at $p < 0.05$ and $p < 0.01$, respectively.

Table 3 Sex ratios as determined by gross examination of secondary sex characteristics of medaka (*Oryzias latipes*) at 60-day posthatch in 4-*t*-OP test, and by their gonadal histology.

| 4-*t*-OP concentration (μg/L) | Secondary sex characteristics N: Number of fish Sex ratio(♂: ♀) | | Gonadal histology N: Number of fish Testis | Ovary | Testis-ova | |
|---|---|---|---|---|---|---|
| Control | 55 | 25 : 30 | 20 | 10 | 10 | 0 |
| Solvent control | 56 | 21 : 35 | 20 | 9 | 11 | 0 |
| 6.94 | 55 | 26 : 29 | 20 | 10 | 10 | 0 |
| 11.4 | 56 | 25 : 31 | 20 | 8 | 11 | 1 |
| 23.7 | 48 | 13 : 35 | 20 | 8 | 10 | 2 |
| 48.1 | 56 | 13 : 43** | 20 | 7 | 10 | 3* |
| 94.0 | 54 | 0 : 54** | 20 | 1 | 15 | 5* |

* and ** denote significant differences at $p < 0.05$ and $p < 0.01$, respectively

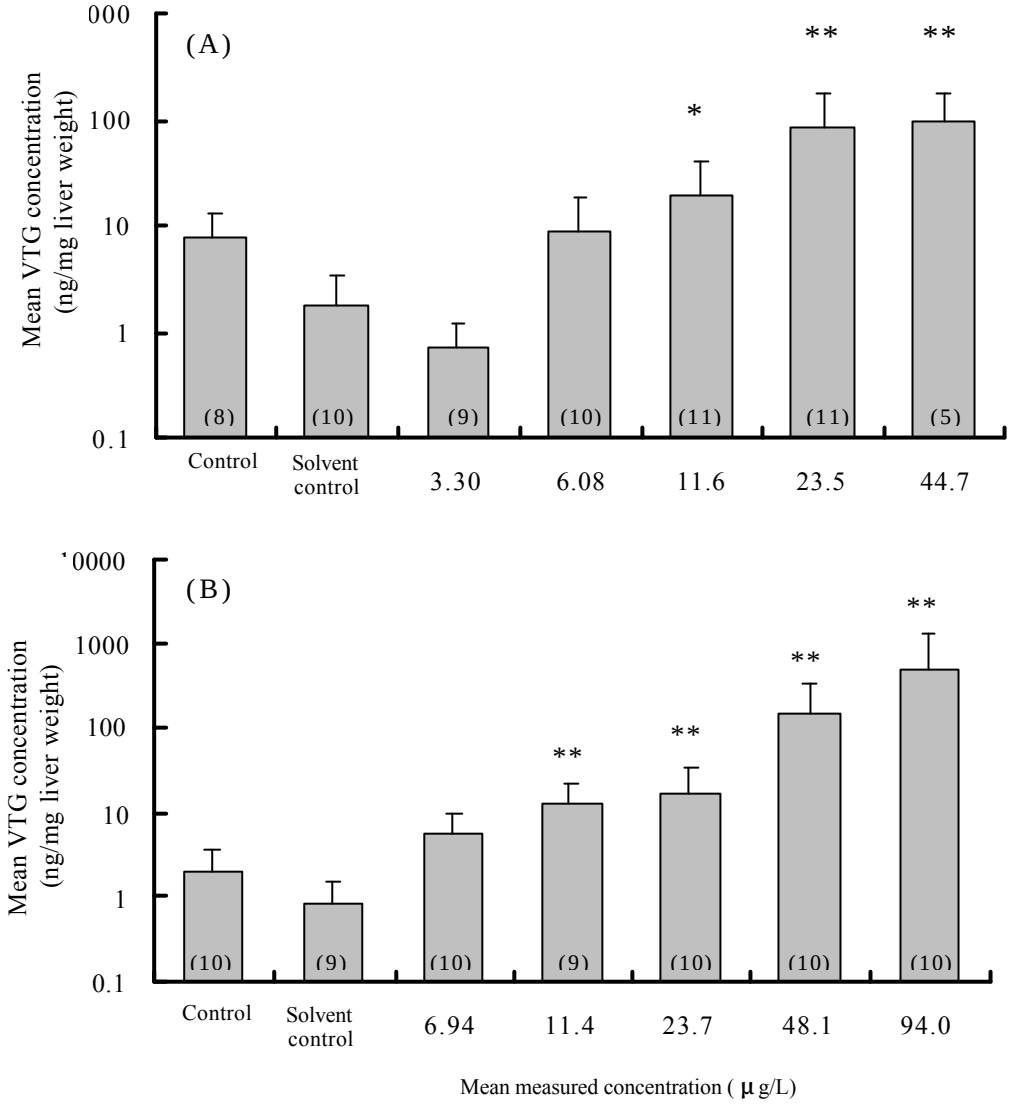


Fig. 4 Vitellogenin (VTG) concentrations in the livers of male medaka (*Oryzias latipes*) at 60-day posthatch in NP test (A) and 4-*t*-OP test (B). Data is shown as mean±standard deviation. Numbers in parentheses indicate the number of fish. * and ** denote significant differences at $p < 0.05$ and $p < 0.01$, respectively.

### 1-2-2. Definitive test (medaka full life-cycle test)

This test was conducted to elucidate chronic toxicity and endocrine disrupting effects of nonylphenol (mixture; NP) on the life cycle of medaka. Medaka (60 eggs/treatment) were exposed to mean measured NP concentrations of 4.2, 8.2, 17.7, 51.5 and 183 μg/L under flow-through conditions from fertilized eggs to

104-day posthatch. During the exposure period, hatching, posthatch mortality and abnormal behavior and appearance were observed daily. At 60-day posthatch, phenotype sex was determined from the appearance of secondary sex characteristics, and histological observation of gonad was made for 20 fish per treatment. Furthermore, at 70-day posthatch, 6 mating pairs in the 2 low treatment (4.2 and 8.2 μg/L) and the controls and solvent controls were selected. No pairs from the 51.5 μg/L treatment and only 3 pairs from the 17.7 μg/L treatment could be selected due to a skewed sex ratio and/or the limited number of surviving fish. The eggs spawned from each female were counted daily and assessed for viability until 104-day posthatch. The fertilized eggs spawned on 102- and 103- day posthatch of the parental generation were also exposed in the same system until 60-day posthatch, and effects were examined.

The 183 μg/L treatment significantly reduced the embryo survival and swim-up success of the $F_0$ fish. The cumulative mortality of the $F_0$ fish from swim-up to 60-day posthatch were significantly increased in the 17.7 and 51.5 μg/L treatments. No concentration-related effect was observed on the growth of fish at 60-day posthatch. However, the sex ratio estimated from the appearance of their secondary sex characteristics was completely skewed toward female in the 51.5 μg/L treatment (Table 4).

Additionally, gonadal histology showed that the fish in 17.7 and 51.5 μg/L treatments had testis-ova (Table 4). The sex ratio of the F0 fish in the 51.5 μg/L treatment was completely skewed toward female, subsequently the mating pairs from ≤ 17.7 μg/L treatments were selected at 70-day posthatch, and their fecundity and fertility were observed daily until 103-day posthatch. Fecundity was unaffected by any of the treatments examined. The mean fertility in the 17.7 μg/L treatment was reduced to 76% of that in the controls, although no statistically significant differences were determined (Fig.5). Overall, these results suggest that the lowest-observed-effect-concentration and no-observed-effect-concentration of NP through the life cycle of the $F_0$ medaka were 17.7 μg/L and 8.2 μg/L, respectively. In the progeny generation ($F_1$), no significant effects were observed on hatching, posthatch mortality, or growth at the concentrations tested (4.2 to 17.7 μg/L). However, induction of testis-ova in the gonads of the $F_1$ fish at 60-day posthatch was observed in both the 8.2 μg/L and 17.7 μg/L (Table 5). This result suggests that NP could have significant effects on reproductive potential of the $F_1$ medaka at lower concentrations than 17.7 μg/L.

Table 4 Sex ratios as determined by gross examination of secondary sex

characteristics of the $F_0$ medaka (*Oryzias latipes*) at 60-day posthatch and by their gonadal histology.

| NP concentration (μg/L) | *N*：Number of fish | Sex ratio (♂:♀) | Gonadal histology *N*：Number of fish | | |
|---|---|---|---|---|---|
| | | | Testis | Ovary | Testis-ova |
| Control | 20 | 9 : 11 | 9 | 11 | 0 |
| Solvent control | 20 | 8 : 12 | 8 | 12 | 0 |
| 4.2 | 20 | 12 : 8 | 12 | 8 | 0 |
| 8.2 | 20 | 13 : 7 | 14 | 6 | 0 |
| 17.7 | 20 | 9 : 11 | 5 | 11 | 4 |
| 51.5 a | 20 | 0 : 20 | 0 | 12 | 8 |

a: The sex ratio obtained from gonadal histology differed significantly from that of the solvent control at $p < 0.001$.

Table 5 Sex ratios as determined by gross examination of secondary sex characteristics of the $F_1$ medaka (*Oryzias latipes*) at 60-day posthatch and by their gonadal histology.

| NP concentration (μg/L) | *N*：Number of fish | Sex ratio (♂:♀) | Gonadal histology *N*：Number of fish | | | |
|---|---|---|---|---|---|---|
| | | | Testis | Ovary | Testis-ova | |
| Control | 59 | 28 : 31 | 20 | 7 | 13 | 0 |
| Solvent control | 54 | 26 : 28 | 20 | 11 | 9 | 0 |
| 4.2 | 54 | 25 : 29 | 20 | 9 | 11 | 0 |
| 8.2 | 49 | 24 : 25 | 20 | 10 | 8 | 2 |
| 17.7a | 28 | 9 : 19 | 20 | 4 | 11 | 5 |

a: The sex ratio obtained from gonadal histology differed significantly at $p < 0.001$.

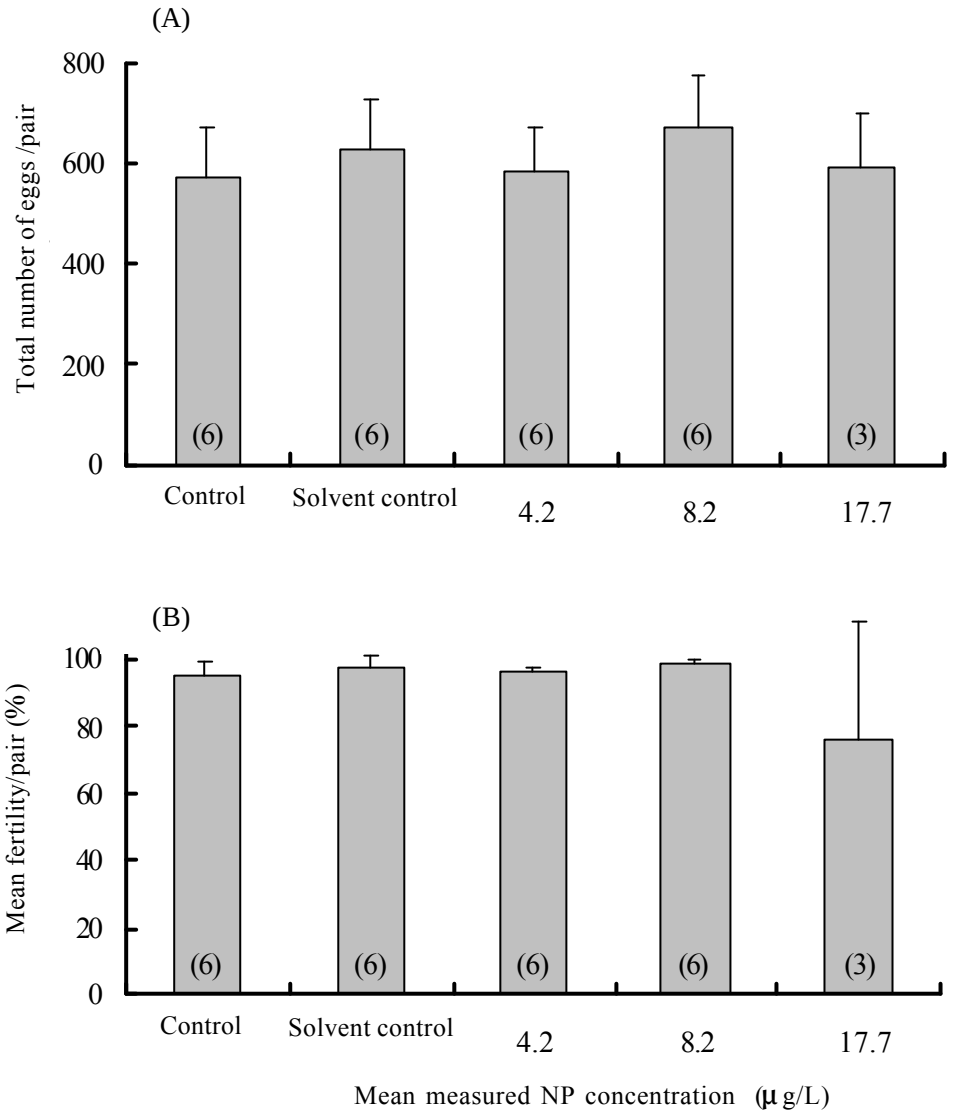


Fig. 5 Total number of eggs spawned by each pair from 71- to 104-day psothatch (A), and mean fertility per pair (B). Data is shown as mean ±standard deviation. The number of pairs in each treatment was indicated on each bar.

**1-3. Hazard assessment of 4-Nonylphenol on fish**

Within the literature information of 1972-2000 obtained through TOXLINE, etc., in-water nonylphenol concentrations suspected to have endocrine disrupting effects on fish in the reported test results, whose reliability was confirmed, were 1.6 μg/L where abnormality in testis tissue of fathead minnow was observed in electron microscopic

examination, 10 μg/L where vitellogenin mRNA was induced in liver of juvenile rainbow trout, 20.3 μg/L where vitellogenin was produced in plasma of mature male rainbow trout (the threshold value was estimated at 10 μg/L in the report), etc.

Within the *in-vitro* test results, nonylphenol showed the relative strength of binding to estrogen receptor at 1/10, as compared with $E_2$, in medaka receptor binding assay, and at 1/200 in mummichog receptor binding assay, and the transcription activating power at a several-hundredth, as compared with $E_2$, in medaka reporter gene assay. Although, it was reported that nonylphenol's binding affinity was in the range of 1/2,000 to 1/3,000, as compared with $E_2$, in the test of binding to estrogenic receptor using Atlantic croaker, but the test concerned examined responses not in the receptor alone, but also in the cell from which its cytosol was extracted along with its surrounding cells. So the reported data is deemed lacking in reliability, as compared with MOE's test series examining genuine binding to receptor.

Within the screening results, in male medaka vitellogenin assay, significant production of vitellogenin was observed at concentration of 22.5 μg/L in water (NB.:not observed at ≦12.8 μg/L), and in medaka partial life cycle test, feminization of males in secondary sexual character at concentration of 23.5 μg/L in water, and appearance of testis-ovas and production of vitellogenin at concentration of 11.6 μg/L in water were observed significantly (NB.:not observed at ≦6.08 μg/L).

Further, in medaka full life cycle test, abnormality in sex differentiation of males, decrease in fertilization rate, etc. were observed at concentration of 17.7 μg/L in water, and testis-ovas not observed in the first generation were observed in the second generation at 8.2 μg/L (NB.: not observed at 4.2 μg/L).

As for nonylphenol, it has been reported in the past that vitellogenin was induced at low concentrations, indicating suspected endocrine disrupting activity to fish. However, there are many unknown points concerning vitellogenin, including the fact that while it is deemed peculiar to female, it was also observed in male fish not exposed to nonylphenol. As a result, vitellogenin was only used as a biomarker in screening techniques, and could not become an index to judge the existence or extent of endocrine disrupting activities. Under these circumstances, it may be safely said that this is the first evidence in the world to show, with regard to the suspected endocrine disrupting effects of nonylphenol, that morphogenetic abnormality such as testis-ova was observed at low concentrations in the test using medaka, sex ratio of which hardly changes despite environmental change. In supporting this, it was proved in *in-vitro* tests that nonylphenol has strong binding affinity to estrogen receptor and strong estrogenic effects on fish, though varied widely by fish species. As mentioned above, it was strongly supposed that nonylphenol has strong endocrine disrupting effects on fish.

Typical gonadal sections of testis and testis-ova are shown in Fig.6. Some of sections regarding normal testis and ovary development and secondary sexual characteristics from embryo to almost matured adult stage will be available from:
http://www.nies.go.jp/edc/edcdb/imdex_e.html

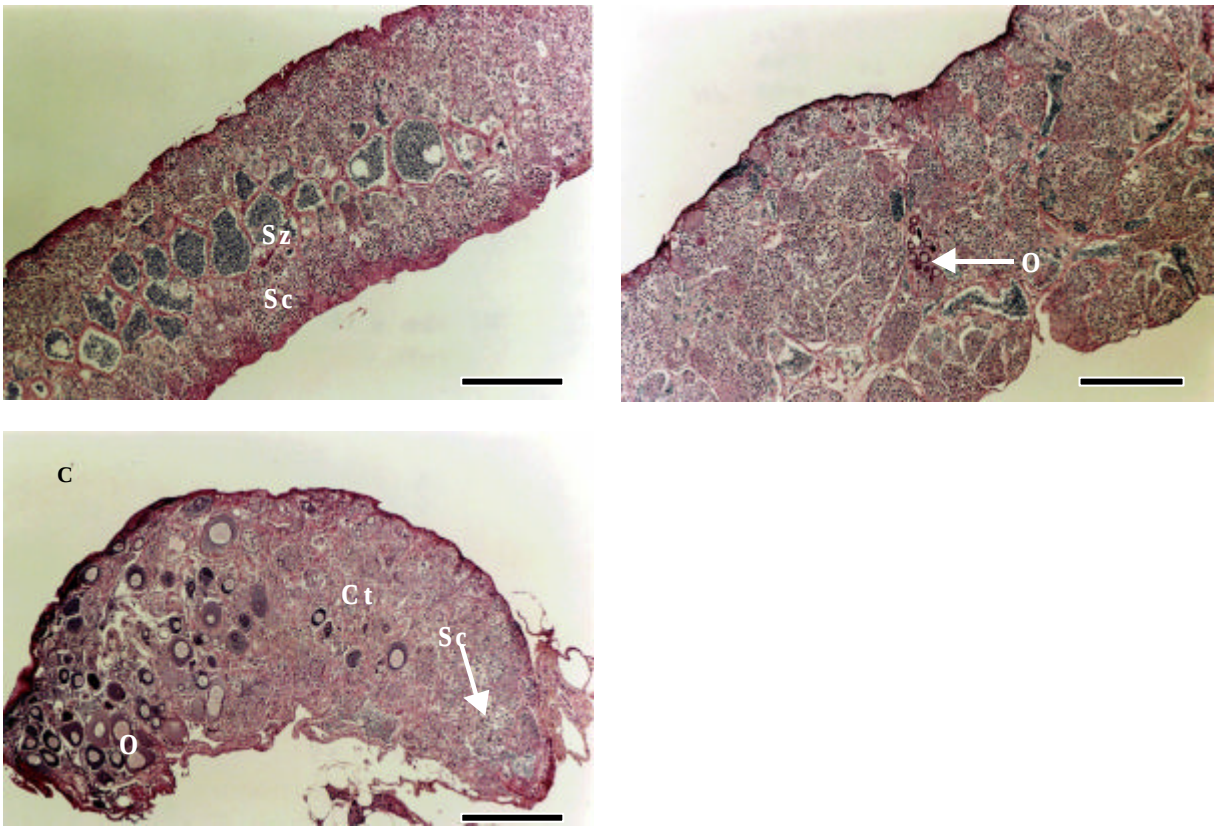


Figure 6. Gonadal sections (4 µm) from medaka at the end of exposure (60 d posthatch) in the partial life-cycle test (PLC) of NP treatment, stained with hematoxylin and eosin. Each bar shows 400µm length. (A) Testis of a control male in the NP exposure experiment, showing normal spermatogenesis: spermatocytes and spermatozoa. (B) Testis-ova of a male exposed to 23.5 µg/L NP. Oocytes appear in clusters within the testicular tissue. (C) More progressed testis-ova exposed to 44.7 µg/L NP. More than half of the area is composed of oocytes, accompanying abnormal connective tissue and small testicular tissues interspersed with a few spermatocytes.
Ct, connective tissue; O, oocytes; Sc, spermatocytes; Sz, spermatozoa.

## 2. Tributyltin

### 2-1. *In vitro* assays

#### 2-1-1. Competitive binding assay to medaka estrogen receptor

The binding affinities of TBTCl to medaka (*Oryzias latipes*) and human estrogen receptors were measured by competitive binding assay using the ligand binding domain of these estrogen receptors(α) fused with glutathione S-transferase (GST), expressed in E. coli, and radiolabelled estradiol as a ligand. At TBTCl concentration of more than 10-6 M, the release of the ligand from estrogen receptor depending on the concentration of TBTCl was observed with both receptors. There is not any difference in release curves between medaka and human estrogen receptors. These results, however, do not indicate that TBTCl replace the ligand specifically bound to estrogen receptor because there is the possibility that the ligand could be released by denaturing of estrogen receptor with TBTCl due to its strong protein denaturing ability. Then the denaturing of estrogen receptor was measured as a function of TBTCl by monitoring the enzyme activity of GST fused to the receptor, which is expected to be denatured with TBTCl in the same manner as the receptors. The similar relationship between the enzymatic activity and TBTCl concentration as the release of ligand was observed though the decreases of the enzyme activity begin at slight lower concentration than the release of ligand. These results strongly suggest that the release of ligand from estrogen receptors is caused by denaturing of the receptors by TBTCl（Fig7 and Table6）.

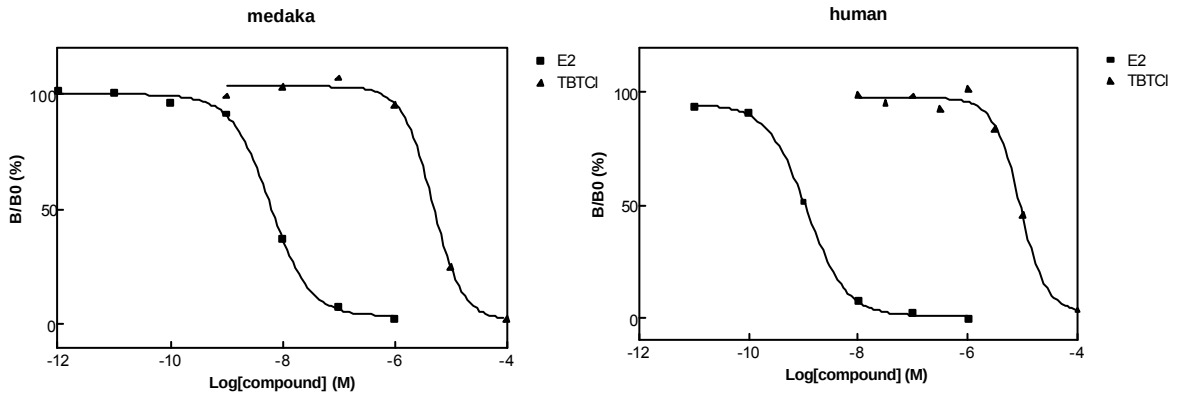


Fig. 7 Elimination of [$^3$H]-estradiol from estrogen receptor by estradiol and TBTCl (left: medaka; right: human

Table6. Relative binding strength of TBTCl obtained from [3H]-estradiol elimination curves

| Species | Relative binding strength (%) | |
|---|---|---|
| | Estradiol | TBTCl |
| Medaka | 100 | 0.13 |
| Human | 100 | 0.013 |

**2-1-2. Reporter gene assay**

The expression and reporter plasmids were transiently co-transfected to HeLa cell exposed with TBTCl, and after incubation overnight the luciferase activity induced by transcriptional activation by TBTCl bound estrogen receptor was measured as a function of TBTCl concentration. No enhancement of the luciferase activity was observed over $10^{-12}$ to $10^{-3}$ M of TBTCl, but the activity decreased from the basal value at more than10nM. The decreasing curve of luciferase activity from basal activity is similar to decreasing one of GST activity, which is the indication of denaturing of estrogen receptor. These results supported the finding that TBTCl denature proteins in cell including estrogen receptor and consequently inhibit of cell activity at these concentrations.

In conclusion, no data indicating that TBTCl bind specifically to estrogen receptor is obtained from competitive binding and reporter gene assays.

**2-2. *In vivo* studies using medaka**

**2-2-1. Medaka vitellogenin assay**

This study was conducted to assess the effects of tributyltin chloride (TBTCl) on vitellogenin (precursor of egg yoke protein) synthesis in medaka. About 3-month-old medaka (respectively 10 females and males/treatment) were exposed to TBTCl at the concentrations of 117, 269, 606, 1,640 and 4,000ng/L (mean measured concentrations) under flow-through conditions for 21days. 17 β-estradiol (E2, 100ng/L) was tested as positive control. Daily observation was made to examine mortality and abnormal behavior and appearance during the exposure period. At the end of exposure, the livers of fish were removed, and vitellogenin concentration in each liver was measured.

Neither death nor particular symptom was observed during the exposure period. At the end of exposure, the hepatosomatic index (HSI) of male fish exposed to ≥ 269 ng/L was significantly higher than that in the controls. As for vitellogenin

concentration, however, no statistically significant change was observed in both males and females in any treatment, as compared with that in the controls (Fig.8).

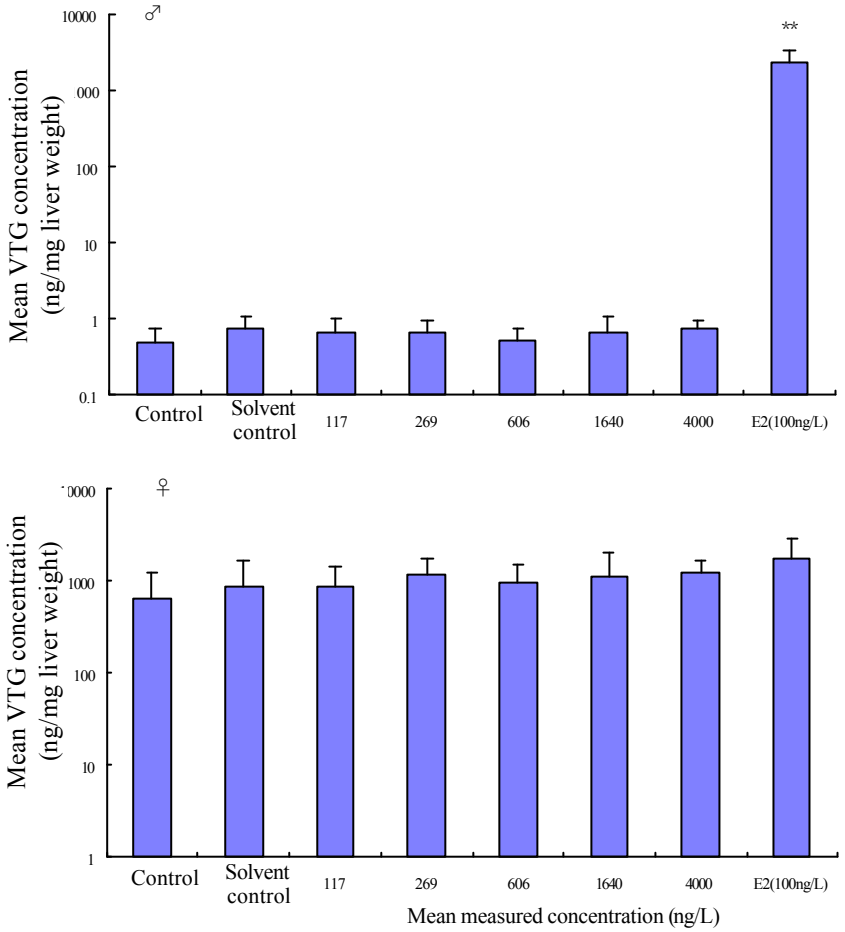


Fig. 8 Vitellogenin (VTG) concentrations in the livers of male and female medaka (*Oryzias latipes*) in the vitellogenin assay. Data is shown as mean ± standard deviation. ** denotes a significant difference at $p < 0.01$.

### 2-2-2. Medaka partial life test

This test was conducted to evaluate endocrine disrupting effects of tributyltin chloride (TBTCl) on sexual differentiation of medaka. Medaka (60 eggs/treatment) was exposed to TBTCl at the concentrations of 20.1, 64.1, 205, 594 and 1,650ng/L (mean measured concentrations) under flow-through conditions from fertilized eggs to 61-day posthatch. No significant effects were observed on hatching of embryos at the concentrations tested. However, posthatch mortality in the highest treatment

(1,650ng/L) increased markedly, and the cumulative mortality at 61-day posthatch was significantly higher than the control mortality. The growth of fish at 61-day posthatch were suppressed with increasing TBTCl concentrations, resulting in significant differences in the total length at 594 and 1,650ng/L, and in the body weight at 1,650ng/L. These results suggested that TBTCl would have lethal toxicity or growth-inhibitory effects on medaka larvae and juveniles at ≥ 594ng/L. Based on an examination of the secondary sex characteristics of the surviving fish at 61-day posthatch, there were no significant differences in the sex ratio in any treatments, although more females than males were identified in the 594ng/L and 1,650ng/L treatments. Gonadal histology showed that histological abnormalities, such as hermaphrodism, were not observed in any treatments (Table 7). The HSI of fish at 61-day posthatch was increased in 594ng/L and 1,650ng/L treatments, suggesting that TBTCl exerts hepatotoxicty at these concentrations. The hepatic vitellogenin concentrations in males at the end of exposure were significantly increased in all treatments relative to the solvent controls, but not to the controls. And there was no clear concentration-response relationship between the vitellogenin concentrations and the TBTCl treatments. It was not possible, therefore, to conclude that there was a clear vitellogenin induction in male medaka (Fig.9). The vitellogenin concentration in females did not show significant differences in any treatments.

As mentioned above, it was suggested that TBTCl would have chronically lethal toxicity or growth inhibitory effects on medaka at ≥ 594ng/L TBTCl. In this test, however, it was not observed that TBTCl affected sexual differentiation of medaka by its endocrine disrupting effects.

Table 7. Sex ratios as determined by gross examination of secondary sex characteristics of medaka (*Oryzias latipes*) at 61- day posthatch and by their gonadal histology.

| TBTCl concentration* (ng/L) | Secondary sex characteristics | | Gonadal histology | | |
|---|---|---|---|---|---|
| | *N* | Sex ratio (♂:♀) | *N* | Number of fish | |
| | | | | Testis | Ovary |
| Control | 50 | 23 : 27 | 20 | 12 | 8 |
| Solvent control | 53 | 28 : 25 | 20 | 14 | 6 |
| 20.1 | 53 | 26 : 27 | 20 | 11 | 9 |
| 64.1 | 50 | 23 : 27 | 20 | 7 | 13 |
| 205 | 51 | 25 : 26 | 20 | 10 | 10 |
| 594 | 53 | 22 : 31 | 20 | 7 | 13 |
| 1,650 | 33 | 13 : 20 | 20 | 8 | 12 |

* Shown in mean measured concentrations.

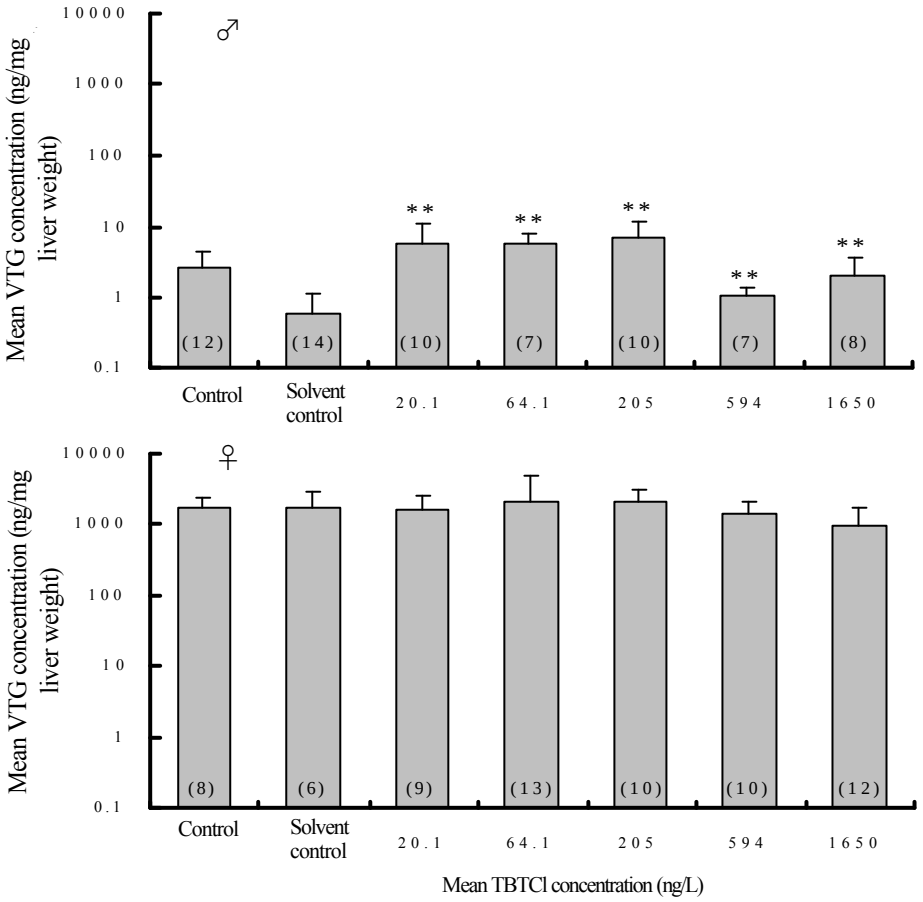


Fig. 9 Vitellogenin (VTG) concentrations in the livers of medaka (*Oryzias latipes*) at 61-day posthatch in each treatment. Data is shown as mean±standard deviation. Numbers in parentheses refer to number of fish. ** denotes a significant difference relative to the solvent controls at $p < 0.01$.

**2-3. Effects on fish**

Of the literature information of 1972-2000 obtained through TOXLINE, etc., no report was obtained on the *in vitro* test on estrogenic or androgenic effects of fish. In animal tests, effects on sheepshead minnow were examined, but in any exposure group, no effect on reproduction was observed [1]. On the other hand, though reliability assessment was not conducted, a report was obtained that TBT had an effect on sex differentiation of flounders, causing sex reversal of hereditary total females [2].

Of MOE's *in vitro* test results, the medaka receptor binding assay showed that the binding was not high because the relative binding strength to estrogen receptor was about 1/1000, as compared with $E_2$. It was also strongly indicated that the binding could possibly be affected by TBTCl's denaturing activity.

Of MOE's screening results, in the male medaka vitellogenin assay, significant change was not observed at any concentration.

Also, in the medaka partial life-cycle test, an increase in female rate was observed at TBTCl concentration of 594ng/L or higher, but without any significant difference. Any physiological abnormality, such as hermaphrodite, was not observed, either. As for male medaka vitellogenin, a statistically significant difference was observed at all TBTCl concentration sectors against auxiliary agent sector, but not against control sector. And the change in vitellogenin concentration did not show any clear dependence on TBTCl concentration. So, it was not concluded that any clear vitellogenin induction occurred in male bodies.

As mentioned above, from the results of MOE's various tests using medaka and from the literature information on which reliability assessment was carried out, any clear result was not obtained that tributyltin (TBT) compounds had endocrine disrupting effect on fish. However, there was a report that sex reversal was caused in flounders[2], though it is not known whether the reversal was due to endocrine disruption or not. Further, it is not known yet whether such a sex reversal was a response peculiar to flounders liable to reverse sex in response to water temperature change or stress, whether it was due to difference in sensitivity among fish species, or whether it was due to difference in exposure methods (water exposure, feed exposure) or resultant exposure concentration, etc. The report also assumed that the masculinization was caused by the TBT activity as aromatase inhibitor, but the world's effort to elucidate the effect on aromatase was just started. So it is necessary to accumulate further scientific data on various fish species, including verification of reproducibility of sex reversal as observed in flounders.

## Reference

1. Manning,C.S., Lytle,T.F.,Walker,W.W. and Lytle,J.S.(1999) Life-cycle toxicity of bis(tributyltin) oxide to the sheepshead minnow(*Cyprinodon variegatus*). Arch.Environ.Contam.Toxicol.,37, 258-266.

2. Shimazaki, Y., Kitano, T., Oshima, Y., Imada, N. and Honjo, T. (2000) Masculinization of flounders by tributyltin, Collected summaries of lectures at the 3rd symposium of Japan Society of Endocrine Disrupter Research, A-3-1, 65.

# Results of Assay and Tests in Evaluation of the Endocrine Disrupting Activities in Fish(*Medaka*)

## 4-*tert*-Octylphenol

### 1. Vitellogenin Assay

Table 1. Results

| Treatment (μg/L) | Mortality (%) | Hepatosomatic index (%) ♂ | Hepatosomatic index (%) ♀ | Vitellogenin (ng/mg liver) ♂ | Vitellogenin (ng/mg liver) ♀ |
|---|---|---|---|---|---|
| Control | 0 | 1.9 ± 1.0 | 4.8 ± 1.0 | 3.6± 3.5 | 1,500 ± 320 |
| Solvent control | 0 | 1.8 ± 0.9 | 4.0 ± 1.1 | 1.4± 1.2 | 1,800 ± 1,300 |
| 12.7 | 0 | 2.0 ± 0.6 | 4.4 ± 0.8 | 1.9± 1.2 | 1,800 ± 540 |
| 27.8 | 0 | 1.8 ± 0.3 | 4.0 ± 0.6 | 3.6± 4.4 | 1,900 ± 510 |
| 64.1 | 0 | 2.2 ± 0.8 | 4.3 ± 1.1 | 190 ± 370** | 1,500 ± 400 |
| 129 | 6.3 | 2.6 ± 0.3 | 3.8 ± 0.9 | 2,300 ± 1,100** | 3,000 ± 2,900 |
| 296 | 0 | 2.8 ± 0.6 | 4.2 ± 0.9 | 6,100 ± 1,800** | 3,300 ± 1,900** |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

### 2. Partial Life-Cycle Test

Table 2-A. Results

| Treatment (μg/L) | Hatchability (%) | Time to hatching (day) | Mortality (%) | Total length (mm) | Body weight (mg) |
|---|---|---|---|---|---|
| Control | 98 ± 3.3 | 9.1 ± 0.3 | 5.0 ± 3.3 | 26.2± 2.1 | 188 ± 35 |
| Solvent control | 98 ± 3.3 | 9.0 ± 0.1 | 5.0 ± 6.4 | 26.2± 2.0 | 157 ± 35 |
| 6.94 | 95 ± 6.4 | 8.9 ± 0.1 | 3.6 ± 4.2 | 26.6± 1.8 | 163 ± 37 |
| 11.4 | 98 ± 3.3 | 9.0 ± 0 | 5.1 ± 6.4 | 26.6± 1.5 | 169 ± 34 |
| 23.7 | 100 ± 0 | 8.9 ± 0.1 | 20 ± 12* | 27.3 1.6** | 187 ± 39※※ |
| 48.1 | 95 ± 6.4 | 9.0 ± 0 | 1.9 ± 3.9 | 26.1± 1.7 | 167 ± 34 |
| 94.0 | 97 ± 6.7 | 9.0 ± 0 | 5.5 ± 7.3 | 25.5± 2.1 | 159 ± 39 |

Table 2-B. Results (continued)

| Treatment (μg/L) | Gonadosomatic index (%) ♂ | Gonadosomatic index (%) ♀ | No. of fish | No. of males with testis-ova/No. of males | Hepatosomatic index (%) ♂ | Hepatosomatic index (%) ♀ | Vitellogenin (ng/mg liver) ♂ | Vitellogenin (ng/mg liver) ♀ |
|---|---|---|---|---|---|---|---|---|
| Control | 0.47 ±0.2 | 1.8 ± 1.8 | 20 | 0/10 | 2.2 ± 0.6 | 3.1 ± 0.5 | 1.9 ± 1.8 | 810± 600 |
| Solvent control | 0.85 ±0.9 | 2.9 ± 2.9 | 20 | 0/9 | 2.1 ± 0.7 | 3.5 ± 0.6 | 0.83 ±0.7 | 810± 770 |
| 6.94 | 0.78 ±0.3 | 4.5 ± 2.9 | 20 | 0/10 | 2.8 ± 0.6* | 3.8 ± 0.8 | 5.4 ± 4.5 | 1,600± 1,000 |
| 11.4 | 0.88 ±0.4 | 4.4 ±3.0 | 20 | 1/9 | 2.7 ± 0.5 | 3.3 ± 0.8 | 13 ± 9.4** | 2,300± 1,700 |
| 23.7 | 0.71 ±0.3 | 3.2 ±3.3 | 20 | 2/10 * | 2.8 ± 0.8* | 4.1 ± 0.7* | 17 ± 19** | 1,700± 1,300 |
| 48.1 | 0.64 ±0.2 | 2.8 ±2.4 | 20 | 3/10 * | 2.4 ± 0.7 | 3.9 ± 0.7 | 140 ± 190** | 3,600± 1,400** |
| 94.0 | 0.39 ±0.4 | 0.60 ±0.5** | 20 | 5/10 ** | 3.3 ± 0.6** | 4.0 ± 0.9 | 500 ± 880** | 4,000± 790** |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)
Statistically significant differences from solvent control group(※※indicates $p<0.01$, ※indicates $p<0.05$)

## 3. Full Life-Cycle Test

Table 3-A. $F_0$ generation

| Treatment (μg/L) | Hatchability (%) | Time to hatching (day) | Mortality (%) | Total length (mm) | Body weight (mg) | No. of fish | No. of males with testis-ova/No. of males |
|---|---|---|---|---|---|---|---|
| Control | 100 | 8.5 ± 0.3 | 1.7 | 30.5 ± 1.3 | 269 ± 32 | 20 | 0/8 |
| Solvent control | 97 ± 3.9 | 8.4 ± 0.2 | 6.7 | 31.6 ± 1.4 | 307 ± 43 | 20 | 0/9 |
| 1.68 | 88 ± 11 | 8.1 ± 0.2 | 8.4 | 31.7 ± 1.8 | 310 ± 62 | 20 | 0/10 |
| 4.27 | 92 ± 8.4 | 8.2 ± 0.4 | 5.8 | 31.5 ± 1.2 | 298 ± 35 | 20 | 0/10 |
| 9.92 | 97 ± 6.7 | 8.4 ± 0.1 | 12 | 32.0 ± 1.2 | 301 ± 42 | 20 | 1/10 |
| 30.4 | 88 ± 6.4 | 8.3 ± 0.4 | 11 | 32.1 ± 1.5 | 322 ± 50 | 20 | 5/7 ** |
| 82.3 | 92 ± 8.4 | 8.2 ± 0.1 | 5.8 | 31.7 ± 1.5 | 310 ± 44 | 20 | 7/8 ** |

Table 3-B. $F_0$ generation (continued)

| Treatment (μg/L) | No. of eggs | Fertility (%) | Gonadosomatic index (%) ♂ | Gonadosomatic index (%) ♀ | Hepatosomatic index (%) ♂ | Hepatosomatic index (%) ♀ | Vitellogenin (ng/mg liver) ♂ | Vitellogenin (ng/mg liver) ♀ |
|---|---|---|---|---|---|---|---|---|
| Control | 653 ± 89 | 97 ± 3.3 | 0.81 ± 0.1 | 7.5 ± 1.5 | 1.7 ± 0.6 | 5.5 ± 1.8 | 10 ± 15 | 1,600 ± 740 |
| Solvent control | 500 ± 170 | 90 ± 15 | 0.65 ± 0.2 | 7.7 ± 1.7 | 1.6 ± 0.4 | 4.1 ± 0.7 | 8.6 ± 8.8 | 1,600 ± 1,300 |
| 1.68 | 659 ± 130 | 97 ± 1.2 | 0.86 ± 0.2 | 7.6 ± 1.5 | 1.4 ± 0.5 | 4.2 ± 1.5 | 8.5 ± 8.8 | 1,700 ± 780 |
| 4.27 | 667 ± 60 | 98 ± 2.1 | 0.98 ± 0.2 | 8.0 ± 0.7 | 1.4 ± 0.2 | 3.8 ± 0.4 | 16 ± 10 | 2,100 ± 1,100 |
| 9.92 | 631 ± 80 | 93 ± 7.4 | 0.93 ± 0.2 | 8.3 ± 1.2 | 1.8 ± 0.8 | 3.5 ± 0.7 | 290 ± 640** | 2,600 ± 2,400 |
| 30.4 | 520 ± 150 | 92 ± 8.0 | 0.92 ± 0.3 | 7.8 ± 1.9 | 1.9 ± 0.1 | 4.5 ± 0.8 | 630 ± 850** | 4,900 ± 2,600** |
| 82.3 | 45 ± 87** | 35 ± 36* | 1.0 ± 0.3 | 8.2 ± 3.8 | 2.6 ± 0.7** | 3.9 ± 0.7 | 2,800 ± 2,800** | 11,000 ± 6,700** |

Table 3-C. $F_1$ generation

| Treatment (μg/L) | Hatchability (%) | Time to hatching (day) | Mortality (%) | Total length (mm) | Body weight (mg) | No. of fish | No. of males with testis-ova/No. of males |
|---|---|---|---|---|---|---|---|
| Control | 94 ± 7.6 | 9.7 ± 0.4 | 1. 7 | 28.7 ± 1.6 | 252 ± 45 | 20 | 0/11 |
| Solvent control | 80 ± 29 | 9.4 ± 0.6 | 6.7 | 28.9 ± 1.7 | 253 ± 41 | 20 | 0/12 |
| 1.68 | 90 ± 14 | 9.2 ± 0.4 | 6.7 | 28.2 ± 1.7 | 242 ± 39 | 20 | 0/14 |
| 4.27 | 92 ± 7.8 | 9.4 ± 0.5 | 8.3 | 28.7 ± 1.7 | 243 ± 37 | 20 | 0/11 |
| 9.92 | 96 ± 6.8 | 9.5 ± 0.6 | 0 | 28.3 ± 2.1 | 243 ± 27 | 20 | 0/8 |
| 30.4 | 97 ± 7.4 | 9.5 ± 0.5 | 0 | 28.7 ± 1.1 | 243 ± 30 | 20 | 4/8 ** |
| 82.3 | 51 ± 49 | 9.6 ± 0.3 | 6.1 | 28.8 ± 1.0 | 252 ± 28 | 20 | 10/15 ** |

Table 3-D. $F_1$ generation (continued)

| Treatment (μg/L) | Vitellogenin (ng/mg liver) ♂ | Vitellogenin (ng/mg liver) ♀ |
|---|---|---|
| Control | 3.1 ± 2.6 | 1,700 ± 1,000 |
| Solvent control | 4.3 ± 5.0 | 1,500 ± 1,000 |
| 1.68 | 3.1 ± 4.4 | 990 ± 920 |
| 4.27 | 6.5 ± 19 | 2,300 ± 1,100 |
| 9.92 | 24 ± 22** | 3,200 ± 1,200** |
| 30.4 | 42 ± 29** | 4,300 ± 2,000** |
| 82.3 | 22 ± 22* | 6,200 ± 540** |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

# Di-n-butyl phthalate

## 1. Vitellogenin Assay

Table 1. Results

| Treatment (μg/L) | Mortality (%) | Hepatosomatic index (%) ♂ | Hepatosomatic index (%) ♀ | Vitellogenin (ng/mg liver) ♂ | Vitellogenin (ng/mg liver) ♀ |
|---|---|---|---|---|---|
| Control | 0 | 1.9 ± 0.4 | 4.6 ± 0.9 | 0.5± 0.1 | 1,200 ± 580 |
| Solvent control | 0 | 1.8 ± 0.6 | 4.1 ± 1.2 | 0.8± 0.8 | 1,200 ± 580 |
| 24.4 | 0 | 2.0 ± 0.6 | 4.1 ± 1.2 | 0.5± 0.2 | 950 ± 720 |
| 55.3 | 0 | 2.1 ± 1.0 | 4.4 ± 0.8 | 0.7± 0.3 | 1,200 ± 560 |
| 133 | 0 | 2.3 ± 0.7 | 4.5 ± 1.1 | 0.7± 0.5 | 660 ± 610 |
| 328 | 5 | 2.5 ± 0.6* | 5.6 ± 1.8 | 0.4 ± 0.1 | 790 ± 780 |
| 822 | 0 | 2.8 ± 0.6** | 4.3 ± 0.9 | 0.4± 0.1 | 1,100 ± 880 |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

## 2. Partial Life-Cycle Test

Table 2-A. Results

| Treatment (μg/L) | Hatchability (%) | Time to hatching (day) | Mortality (%) | Total length (mm) | Body weight (mg) |
|---|---|---|---|---|---|
| Control | 97 ± 3.9 | 10 ± 0.1 | 8.6 ± 6.6 | 29.5± 1.4 | 256 ± 42 |
| Solvent control | 97 ± 3.9 | 10 ± 0.2 | 11 ± 4.4 | 29.6± 1.3 | 256 ± 41 |
| 7.09 | 95 ± 6.4 | 9.8 ± 0.1 | 12 ± 8.6 | 29.8± 1.5 | 266 ± 50 |
| 21.9 | 87 ± 14 | 9.8 ± 0.2 | 8.9 ± 10 | 29.5± 1.4 | 259 ± 46 |
| 72.8 | 97 ± 3.9 | 9.9 ± 0.1 | 21 ± 5.1* | 30.1± 1.5 | 269 ± 38 |
| 235 | 100 | 10 ± 0.3 | 48 ± 18* | 29.6± 1.7 | 269 ± 48 |
| 850 | 8.3 ± 10* | 16 ± 0.7 | 100 | — | — |

—indicates 100% mortality

Table 2-B. Results (continued)

| Treatment (μg/L) | Gonadosomatic index (%) ♂ | Gonadosomatic index (%) ♀ | No. of fish | No. of males with testis-ova/No. of males | Hepatosomatic index (%) ♂ | Hepatosomatic index (%) ♀ | Vitellogenin (ng/mg liver) ♂ | Vitellogenin (ng/mg liver) ♀ |
|---|---|---|---|---|---|---|---|---|
| Control | 0.64 ±0.2 | 3.9 ± 2.5 | 20 | 0/10 | 3.4 ± 0.9 | 4.1 ± 0.6 | 2.4 ± 3.8 | 1,200± 950 |
| Solvent control | 0.68 ±0.2 | 2.5 ± 2.7 | 20 | 0/12 | 3.2 ± 1.0 | 5.0 ± 1.4 | 0.83 ± 1.6 | 760± 1,200 |
| 7.09 | 0.61 ±0.2 | 3.9 ± 3.4 | 20 | 1/11 | 3.6 ± 0.9 | 5.6 ± 0.6* | 3.5 ± 3.3 | 1,400± 1,200 |
| 21.9 | 0.63 ±0.3 | 4.5 ±4.0 | 20 | 0/12 | 3.5 ± 1.2 | 4.4 ± 1.0 | 1.2 ± 2.6 | 1,400± 840 |
| 72.8 | 0.73 ±0.3 | 4.6 ± 3.8 | 20 | 2/12 * | 3.2 ± 1.1 | 4.3 ± 0.8 | 4.1 ± 8.0 | 1,200± 1,300 |
| 235 | 0.63 ±0.3 | 2.8 ± 3.6 | 20 | 0/9 | 3.4 ± 0.8 | 4.2 ± 1.0 | 3.7 ± 5.6 | 360± 730 |
| 850 | — | — | — | — | — | — | — | — |

—indicates 100% mortality

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

## 3. Full Life-Cycle Test

Table 3-A. $F_0$ generation

| Treatment (μg/L) | Hatchability (%) | Time to hatching (day) | Mortality (%) | Total length (mm) | Body weight (mg) | No. of fish | No. of males with testis-ova/No. of males |
|---|---|---|---|---|---|---|---|
| Control | 92 ± 8.4 | 9.9 ± 0.3 | 13 | 29.1 ± 1.4 | 236 ± 44 | 20 | 0/8 |
| Solvent control | 95 ± 6.4 | 10 ± 0.4 | 18 | 29.6 ± 1.2 | 245 ± 35 | 20 | 0/9 |
| 2.61(μg/L) | 98 ± 3.3 | 9.8 ± 0.2 | 12 | 28.6 ± 1.5 | 218 ± 39 | 20 | 1/8 |
| 7.52(μg/L) | 97 ± 3.8 | 9.8 ± 0.2 | 16 | 29.3 ± 1.3 | 239 ± 34 | 20 | 0/11 |
| 23.9(μg/L) | 95 ± 6.4 | 10 ± 0.3 | 5.6 | 29.1 ± 1.4 | 233 ± 38 | 20 | 0/6 |
| 74.5(μg/L) | 95 ± 6.4 | 10 ± 0.1 | 24 | 30.1 ± 1.7 | 259 ± 49 | 20 | 1/9 |
| 233(μg/L) | 98 ± 3.3 | 10 ± 0.2 | 15. | 28.6 ± 1.8 | 226 ± 50 | 20 | 2/8 * |

Table 3-B. $F_0$ generation(continued)

| Treatment (μg/L) | No. of eggs | Fertility (%) | Gonadosomatic index (%) | | Hepatosomatic index (%) | | Vitellogenin (ng/mg liver) | |
|---|---|---|---|---|---|---|---|---|
| | | | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ |
| Control | 560 ± 210 | 94 ± 6.5 | 0.78 ± 0.2 | 9.3 ± 1.6 | 1.3 ± 0.4 | 4.2 ± 0.8 | 14.0 ± 30 | 2,000 ± 2,000 |
| Solvent control | 625 ± 130 | 87 ± 26 | 0.89 ± 0.2 | 8.1 ± 0.9 | 1.5 ± 0.3 | 4.2 ± 0.7 | 7.0 ± 6.3 | 1,600 ± 950 |
| 2.61 | 602 ± 110 | 96 ± 5.6 | 0.86 ± 0.2 | 7.5 ± 0.7 | 1.4 ± 0.3 | 4.0 ± 0.7 | 9.9 ± 9.5 | 1,500 ± 890 |
| 7.52 | 668 ± 100 | 94 ± 8.9 | 0.92 ± 0.2 | 8.0 ± 0.9 | 1.4 ± 0.2 | 4.0 ± 0.3 | 15 ± 9.4 | 1,400 ± 330 |
| 23.9 | 543 ± 110 | 94 ± 3.1 | 1.1 ± 0.1* | 9.0 ± 0.5 | 1.3 ± 0.4 | 4.5 ± 0.6 | 8.1 ± 7.4 | 1,800 ± 470 |
| 74.5 | 554 ± 180 | 97 ± 1.6 | 0.92 ± 0.2 | 7.8 ± 1.1 | 1.6 ± 0.4 | 3.8 ± 1.3 | 13 ± 13 | 1,700 ± 520 |
| 233 | 539 ± 240 | 91 ± 11 | 0.97 ± 0.3 | 9.4 ± 2.6 | 1.8 ± 0.2 | 4.4 ± 1.0 | 4.6 ± 4.0 | 2,100 ± 2,200 |

Table 3-C. $F_1$ generation

| Treatment (μg/L) | Hatchability (%) | Time to hatching (day) | Mortality (%) | Total length (mm) | Body weight (mg) | No. of fish | No. of males with testis-ova/No. of males |
|---|---|---|---|---|---|---|---|
| Control | 87 ± 8.9 | 9.4 ± 0.6 | 0 | 30.7 ± 1.2 | 276 ± 39 | 20 | 0/9 |
| Solvent control | 85 ± 11 | 9.4 ± 0.5 | 0 | 30.5 ± 1.4 | 281 ± 39 | 20 | 0/7 |
| 2.61 | 89 ± 8.8 | 9.1 ± 0.6 | 0 | 30.8 ± 1.2 | 274 ± 34 | 20 | 2/10 |
| 7.52 | 94 ± 6.4** | 9.4 ± 0.5 | 1.7 | 31.7 ± 1.1** | 297 ± 41* | 20 | 2/13 * |
| 23.9 | 72 ± 21 | 8.6 ± 1.1 | 1.7 | 30.8 ± 1.3 | 283 ± 33 | 20 | 1/11 |
| 74.5 | 90 ± 12 | 9.8 ± 0.4* | 0 | 30.8 ± 1.3 | 290 ± 31 | 20 | 1/14 |
| 233 | 94 ± 6.6* | 11 ± 1.2** | 3.3 | 30.2 ± 1.2 | 292 ± 39 | 20 | 0/9 |

Table 3-D. $F_1$ generation (continued)

| Treatment (μg/L) | Vitellogenin(ng/mg liver) | |
|---|---|---|
| | ♂ | ♀ |
| Control | 0.8 ± 1.1 | 440 ± 720 |
| Solvent control | ND | 470 ± 1,000 |
| 2.61 | 3.8 ± 5.0* | 1,700 ± 820** |
| 7.52 | 9.1 ± 8.5** | 1,600 ± 1,100* |
| 23.9 | 14 ± 29 | 1,200 ± 580** |
| 74.5 | 3.3 ± 2.7** | 850 ± 790 |
| 233 | 2.5 ± 3.0 | 730 ± 570 |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

# Di-(2-ethylhexyl) phthalate

## 1. Vitellogenin Assay

Table 1. Results

| Treatment (μg/L) | Vitellogenin (ng/mg liver) | | Hepatosomatic index (%) | |
|---|---|---|---|---|
| | 14-d | 21-d | 14-d | 21-d |
| Control | ND | 0.53±0.13 | 1.56±0.27 | 1.44±0.21 |
| Solvent control | 0.55±0.21 | ND | 1.50±0.27 | 1.46±0.24 |
| 19 | 0.62±0.46 | ND | 1.46±0.33 | 1.59±0.19 |
| 43 | ND | ND | 1.74±0.27 | 1.33±0.28 |
| 96 | 0.58±0.31 | ND | 1.60±0.38 | 1.59±0.32 |
| 210 | ND | ND | 1.54±0.18 | 1.44±0.34 |
| 410 | ND | ND | 1.74±0.22 | 1.39±0.31 |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

## 2. Partial Life-Cycle Test

Table 2-A. Results

| Treatment (μg/L) | Hatchability (%) | Time to hatching (day) | Mortality (%) | Total length (mm) | Body weight (mg) |
|---|---|---|---|---|---|
| Control | 93 ± 7.7 | 8.8 ± 0.5 | 0.0 ± 0.0 | 30.3 ± 1.5 | 271 ± 43 |
| Solvent control | 98 ± 3.3 | 9.2 ± 0.2 | 1.9 ± 3.9 | 30.4 ± 1.6 | 275 ± 52 |
| 11.0 | 93 ± 0 | 9.0 ± 0.3 | 1.8 ± 3.6 | 30.7 ± 1.5 | 290 ± 45 |
| 28.4 | 100 ± 0 | 9.1 ± 0.1 | 0.0 ± 0.0 | 30.1 ± 2.0 | 270 ± 58 |
| 73.4 | 95 ± 10 | 9.1 ± 0.1 | 0.0 ± 0.0 | 30.5 ± 1.6 | 263 ± 49 |
| 186 | 95 ± 6.4 | 9.0 ± 0.1 | 1.8 ± 3.6 | 30.2 ± 2.0 | 261 ± 51 |
| 446 | 95 ± 6.8 | 9.0 ± 0.2 | 2.1 ± 4.2 | 30.3 ± 2.0 | 264 ± 48 |

Table 2-B. Results (continued)

| Treatment (μg/L) | Gonadosomatic index (%) | | No. of fish | No. of males with testis-ova / No. of males | Hepatosomatic index (%) | | Vitellogenin (ng/mg liver) | |
|---|---|---|---|---|---|---|---|---|
| | ♂ | ♀ | | | ♂ | ♀ | ♂ | ♀ |
| Control | 0.78 ± 0.21 | 5.0 ± 2.7 | 20 | 0/13 | 2.2 ± 0.7 | 3.7 ± 0.4 | 1.3 ± 1.4 | 1,100± 730 |
| Solvent control | 0.71 ± 0.23 | 6.2 ± 3.6 | 20 | 0/10 | 2.0 ± 0.2 | 3.9 ± 0.4 | 2.8 ± 3.6 | 1,600± 1,000 |
| 11.0 | 0.82 ± 0.27 | 3.8 ± 2.6 | 20 | 0/12 | 1.8 ± 0.6 | 3.9± 1.3 | 2.5 ± 4.1 | 1,100± 890 |
| 28.4 | 0.97 ± 0.40 | 4.3 ± 2.9 | 20 | 0/10 | 1.6 ± 0.7 | 3.5 ± 0.5 | 3.5 ± 5.4 | 1,500± 920 |
| 73.4 | 0.83 ± 0.26 | 5.2 ± 3.4 | 20 | 1/11 | 2.6 ± 0.9 | 3.7 ± 0.4 | 0.4 ± 0.4 | 1,500± 1,100 |
| 186 | 0.76 ± 0.26 | 6.4 ± 3.9 | 20 | 0/10 | 2.3 ± 0.6 | 4.2 ± 0.9 | 0.5 ± 0.5 | 1,700± 1,100 |
| 446 | 0.86 ± 0.37 | 6.0 ± 3.3 | 20 | 0/12 | 2.3 ± 0.7 | 3.9 ± 0.5 | 4.3 ± 9.3 | 1,200± 570 |

Statistically significant defferences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

# Di-cyclohexyl phthalate

## 1. Vitellogenin Assay

Table 1. Results

| Treatment (μg/L) | Vitellogenin (ng/mg liver) | | Hepatosomatic index (%) | |
|---|---|---|---|---|
| | 14-d | 21-d | 14-d | 21-d |
| Control | 0.55±0.21 | ND | 1.55±0.13 | 1.42±0.20 |
| Solvent control | ND | ND | 1.67±0.21 | 1.40±0.26 |
| 18 | ND | ND | 1.58±0.28 | 1.39±0.18 |
| 38 | 0.53±0.13 | ND | 1.56±0.23 | 1.61±0.22* |
| 87 | ND | ND | 1.88±0.37* | 1.56±0.21 |
| 190 | ND | ND | 1.84±0.26* | 1.50±0.20 |
| 390 | ND | ND | 2.04±0.37** | 1.55±0.39 |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

## 2. Partial Life Cycle Test

Table 2-A. Results

| Treatment (μg/L) | Hatchability (%) | Time to hatching (day) | Mortality (%) | Total length (mm) | Body weight (mg) |
|---|---|---|---|---|---|
| Control | 98 ± 3.3 | 9.7 ± 0.2 | 0 ± 0 | 28.0 ± 1.4 | 220 ± 36 |
| Solvent control | 92 ± 13 | 9.2 ± 0.3 | 3.3 ± 6.7 | 27.3 ± 2.8 | 250 ± 50 |
| 0.429 | 100 ± 0 | 9.1 ± 0.1 | 1.8 ± 3.6 | 28.8 ± 1.5** | 225 ± 41※ |
| 1.41 | 93 ± 9.4 | 9.1 ± 0.1 | 7.6 ± 11 | 28.4 ± 2.3 | 241 ± 44 |
| 4.39 | 92 ± 8.4 | 9.1 ± 0.1 | 5.6 ±7.3 | 30.0 ± 1.6** | 250 ± 47 |
| 13.3 | 100 ± 0 | 9.3 ± 0.4 | 0 ± 0 | 29.0 ± 1.7** | 237 ± 45 |
| 35.8 | 90 ± 8.6 | 9.1 ± 0.1 | 13 ± 10 | 29.8 ± 1.8** | 265 ± 48 |

Table 2-B. Results (continued)

| Treatment (μg/L) | Gonadosomatic index (%) | | No. of fish | No. of males with testis-ova / No. of males | Hepatosomatic index (%) | | Vitellogenin (ng/mg liver) | |
|---|---|---|---|---|---|---|---|---|
| | ♂ | ♀ | | | ♂ | ♀ | ♂ | ♀ |
| Control | 0.75 ± 0.2 | 4.3 ± 3.3 | 20 | 0/13 | 2.7 ± 0.7 | 3.6 ± 1.0 | 1.8 ± 2.4 | 1,600± 1,500 |
| Solvent control | 0.74 ± 0.2 | 5.2 ± 3.3 | 20 | 0/12 | 2.5 ± 0.4 | 4.0 ± 0.7 | 2.2 ± 2.4 | 1,800± 1,300 |
| 0.429 | 0.83 ± 0.2 | 5.5 ± 3.1 | 20 | 0/13 | 2.4 ± 0.4 | 3.6 ± 0.9 | 3.8 ± 3.4 | 2,100± 1,100 |
| 1.41 | 0.69 ± 0.2 | 2.9 ± 2.6 | 20 | 0/13 | 2.4 ± 0.6 | 3.0 ± 0.5 | 4.7 ± 4.7 | 1,600± 1,400 |
| 4.39 | 0.85 ± 0.3 | 5.8 ± 3.7 | 20 | 0/14 | 2.2 ± 0.6 | 3.6 ± 0.5 | 12 ± 16** | 1,800± 660 |
| 13.3 | 0.76 ± 0.2 | 3.9 ± 2.8 | 20 | 0/11 | 2.1 ± 0.5 | 3.2 ± 0.7 | 1.3 ± 2.0 | 2,400± 1,900 |
| 35.8 | 1.1 ± 0.3** | 5.9 ± 3.1 | 20 | 1/10 | 2.2 ± 0.9 | 3.7 ± 1.0 | 2.7 ± 2.1 | 2,900± 3,300 |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)
Statistically significant differences from solvent control group(※※indicates $p<0.01$, ※ indicates $p<0.05$)

# Di-ethyl phthalate

## 1. Vitellogenin Assay

Table 1. Results

| Treatment (μg/L) | Vitellogenin (ng/mg liver) | | Hepatosomatic index (%) | |
|---|---|---|---|---|
| | 14-d | 21-d | 14-d | 21-d |
| Control | 4.4±1.1 | 1.3±0.1 | 2.05±0.11 | 1.65±0.13 |
| 8.1 | 2.1±0.2 | 2.3±0.6 | 1.95±0.19* | 1.75±0.34 |
| 26.8 | 4.8± 1.5 | 1.8±0.5 | 1.87±0.13* | 1.64±0.11 |
| 119.8 | 2.7± 0.8 | 2.2± 0.8 | 2.00±0.15* | 2.41±0.76 |
| 355.8 | 2.4±0.4 | 1.0± 0.1 | 1.91±0.14* | 1.61±0.10 |
| 1,053.3 | 2.5±0.7* | 1.2± 0.2 | 1.98±0.11* | 1.76±0.06 |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

## 2. Partial Life-Cycle Test

Table 2-A. Results

| Treatment (μg/L) | Hatchability (%) | Time to hatching (day) | Mortality (%) | Total length (mm) | Body weight (mg) |
|---|---|---|---|---|---|
| Control | 81 | 11.2± 0.3 | 8.6 | 20.8± 0.3 | 164.5± 6.6 |
| 0.6 | 80 | 12.3± 0.4 | 3.8 | 20.6± 0.2 | 158.1± 5.4 |
| 2.5 | 83 | 12.4 ± 0.4* | 13.3 | 21.1± 0.2 | 167.7± 4.5 |
| 8.4 | 91 | 12.3± 0.5* | 17.6 | 21.5± 0.2 | 167.8± 4.1 |
| 36.0 | 92 | 11.8± 0.3* | 5.4 | 20.1± 0.2* | 142.0± 3.8* |
| 121.6 | 88 | 11.3± 0.3 | 2.3 | 20.3± 0.2 | 140.5± 3.9* |

Table 2－B. Results (continued)

| Treatment (μg/L) | Gonadosomatic index (%) | | No. of fish | No. of males with testis-ova / No. of males | Hepatosomatic index (%) | | Vitellogenin (ng/mg liver) | |
|---|---|---|---|---|---|---|---|---|
| | ♂ | ♀ | | | ♂ | ♀ | ♂ | ♀ |
| Control | 1.09±0.07 | 7.54±0.19 | 20 | 0/10 | 4.49±0.50 | 4.24±0.60 | 0.16 ± 0.05 | 255.7± 95.0 |
| 0.6 | 0.87±0.10 | 7.40±0.21 | 20 | 0/10 | 4.19±0.36 | 3.85±0.52 | 0.21 ± 0.08 | 160.0± 102.6 |
| 2.5 | 1.02±0.06 | 7.34±0.19 | 20 | 0/10 | 3.89±0.42 | 4.44±0.42 | 1.18 ± 0.76 | 196.7± 80.0 |
| 8.4 | 0.84±0.08 | 7.46±0.15 | 20 | 0/10 | 3.99±0.44 | 3.83±0.49 | 0.52 ± 0.18 | 150.7± 136.4 |
| 36.0 | 0.92±0.11 | 7.09±0.21 | 20 | 0/10 | 4.46±0.45 | 4.83±0.32 | 2.12 ± 1.03 | 75.2± 46.5* |
| 121.6 | 0.90±0.11 | 6.91±0.21 | 20 | 0/10 | 4.96±0.29 | 4.30±0.38 | 0.10 ± 0.02* | 70.9± 50.6* |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

# Butylbenzyl phthalate

## 1. Vitellogenin Assay

Table 1. Results

| Treatment (μg/L) | Vitellogenin (ng/mg liver) | | Hepatpspmatic index (%) | |
|---|---|---|---|---|
| | 14-d | 21-d | 14-d | 21-d |
| Control | 0.6±0.1 | 1.5±0.2 | 2.08±0.56 | 1.87±0.16 |
| 14.0 | 0.6±0.1 | 1.2±0.2 | 2.35±0.13 | 1.67±0.18 |
| 26.7 | 0.7± 0.1 | 1.3±0.1 | 1.93±0.08 | 2.00±0.11 |
| 69.7 | 1.1± 0.2 | 1.5± 0.1 | 1.93±0.11 | 1.72±0.12 |
| 337.1 | 0.8±0.2 | 1.3± 0.1 | 2.37±0.16 | 2.12±0.26 |
| 1,045.4 | 2.6±0.5** | 1.5± 0.1 | 2.46±0.23 | 2.24±0.22 |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

## 2. Partial Life-Cycle Test

Table 2-A. Results

| Treatment (μg/L) | Hatchability (%) | Time to hatching (day) | Mortality (%) | Total length (mm) | Body weight (mg) |
|---|---|---|---|---|---|
| Control | 98 | 12.7 ± 1.0 | 16.3 | 20.1 ± 0.2 | 129.9 ± 3.6 |
| 0.7 | 94 | 11.1 ± 0.7 | 17.0 | 20.3 ± 0.2 | 137.8 ± 3.9 |
| 2.7 | 89 | 14.9 ± 1.1** | 25.8 | 21.4 ± 0.2** | 162.9 ± 4.3** |
| 11.5 | 99 | 15.4 ± 1.1** | 31.3 | 21.4 ± 0.2** | 154.7 ± 3.8** |
| 28.6 | 96 | 12.1 ± 0.7** | 11.5 | 20.1 ± 0.2 | 131.9 ± 3.1 |
| 99.5 | 86 | 14.2 ± 1.1 | 30.2 | 22.0 ± 0.2** | 179.4 ± 4.6** |

Table 2-B. Results (continued)

| Treatment (μg/L) | Gonadosomatic index (%) | | No. of fish | No. of males with testis-ova / No. of males | Hepatosomatic index (%) | | Vitellogenin (ng/mg liver) | |
|---|---|---|---|---|---|---|---|---|
| | ♂ | ♀ | | | ♂ | ♀ | ♂ | ♀ |
| Control | 0.83±0.07 | 7.40±0.26 | 20 | 0/10 | 2.14±0.15 | 2.52±0.19 | 1.12 ± 0.10 | 375.1± 200.6 |
| 0.7 | 0.96±0.11 | 7.60±0.21 | 20 | 0/10 | 2.07±0.22 | 2.55±0.18 | 1.47 ± 0.36 | 457.7± 164.6 |
| 2.7 | 1.09±0.08 | 7.63±0.19 | 20 | 0/10 | 2.68±0.29 | 2.99±0.24 | 1.43 ± 0.24 | 142.3± 96.7 |
| 11.5 | 1.12±0.08 | 7.43±0.28 | 20 | 0/10 | 2.45±0.31 | 3.62±0.38 | 1.58 ± 0.23 | 90.9± 28.4 |
| 28.6 | 1.16±0.09 | 7.52±0.23 | 20 | 0/10 | 2.81±0.37** | 3.21±0.26 | 1.86 ± 0.40 | 330.4± 136.6 |
| 99.5 | 1.17±0.07 | 7.55±0.31 | 20 | 0/10 | 3.30±0.57 | 4.25±0.46 | 1.47 ± 0.35 | 129.3± 69.9 |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

# Di-(2-ethylhexyl) adipate

## 1. Vitellogenin Assay

Table 1. Results

| Treatment (μg/L) | Vitellogenin (ng/mg liver) | | Hepatosomatic index (%) | |
|---|---|---|---|---|
| | 14-d | 21-d | 14-d | 21-d |
| Control | 0.20±0.02 | 0.36±0.02 | 2.31±0.16 | 2.60±0.10 |
| 2.4 | 0.18±0.01 | 0.42±0.04 | 2.49±0.17 | 2.21±0.14* |
| 7.9 | 0.16± 0.05 | 0.38±0.03 | 2.77±0.21 | 2.30±0.12 |
| 21.5 | 0.18±0.01 | 0.37± 0.02 | 2.61±0.16 | 2.47±0.14 |
| 181.7 | 0.15± 0.01 | 0.33± 0.02 | 2.53±0.12 | 2.64±0.10 |
| 453.6 | 0.21±0.04 | 0.46±0.05 | 2.21±0.15 | 2.42±0.20 |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

## 2. Partial Life-Cycle Test

Table 2-A. Results

| Treatment (μg/L) | Hatchability (%) | Time to hatching (day) | Mortality (%) | Total length (mm) | Body weight (mg) |
|---|---|---|---|---|---|
| Control | 97 ± 3.9 | 8.3 ± 0.1 | 3.5 ± 4.0 | 30.1 ± 2.1 | 260 ± 56 |
| Solvent control | 92 ± 8.4 | 8.4 ± 0.2 | 7.5 ± 8.8 | 31.0 ± 1.5 | 277 ± 51 |
| 0.711 | 98 ± 3.3 | 8.1 ± 0.2 | 1.8 ± 3.6 | 30.0 ± 2.4 | 261 ± 64 |
| 2.33 | 95 ± 3.3 | 8.2 ± 0.2 | 6.8 ± 9.4 | 31.0 ± 1.7 | 286 ± 55 |
| 7.88 | 92 ± 3.3 | 8.1 ± 0.3 | 13 ± 13 | 31.2 ± 1.9 | 301 ± 71** |
| 26.3 | 95 ± 6.4 | 8.2 ± 0.1 | 5.1 ± 6.4 | 31.1 ± 1.3 | 280 ± 44 |
| 87.1 | 95 ± 6.4 | 8.3 ± 0.2 | 4.0 ± 4.6 | 31.1 ± 1.6 | 280 ± 54 |

Table 2-B. Results (continued)

| Treatment (μg/L) | Gonadosomatic index (%) | | No. of fish | No. of males with testis-ova / No. of males | Hepatosomatic index (%) | | Vitellogenin (ng/mg liver) | |
|---|---|---|---|---|---|---|---|---|
| | ♂ | ♀ | | | ♂ | ♀ | ♂ | ♀ |
| Control | 1.1 ± 0.2 | 6.9 ± 2.9 | 20 | 0/8 | 1.8 ± 0.5 | 3.6 ± 0.604 | 6.3±13 | 2,100± 680 |
| Solvent control | 1.2 ± 0.4 | 9.1 ± 1.9 | 20 | 1/14 | 1.9 ± 0.5 | 3.3 ± 0.4 | 1.3 ± 1.6 | 2,500± 1,900 |
| 0.711 | 1.1 ± 0.4 | 6.7 ± 2.7 | 20 | 0/10 | 1.9 ± 0.4 | 3.1 ± 0.7 | 1.6 ± 2.0 | 2,000± 1,300 |
| 2.33 | 1.3 ± 0.4 | 8.6 ± 2.7 | 20 | 0/6 | 1.8 ± 0.2 | 3.4 ± 0.8 | 3.0 ± 4.7 | 2,600± 1,300 |
| 7.88 | 1.2 ± 0.2 | 7.5 ± 2.7 | 20 | 1/9 | 1.8 ± 0.3 | 3.7 ± 0.8 | 1.8 ± 1.6 | 1,600± 610 |
| 26.3 | 1.0 ± 0.3 | 6.7 ± 3.0 | 20 | 0/13 | 1.8 ± 0.5 | 3.6 ± 0.8 | 5.4 ± 9.1 | 2,100± 390 |
| 87.1 | 1.0 ± 0.2 | 5.7 ± 3.6 | 20 | 0/12 | 1.8 ± 0.3 | 3.1 ± 0.3 | 1.4 ± 1.2 | 1,500± 980 |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

# Triphenyl tin chloride

## 1. Vitellogenin Assay

Table 1. Results

| Treatment (μg/L) | Vitellogenin (ng/mg liver) | | Hepatosomatic index (%) | |
|---|---|---|---|---|
| | 14-d | 21-d | 14-d | 21-d |
| Control | 1.6±0.3 | 1.7±0.2 | 2.61±0.23 | 2.71±0.49 |
| 0.118 | 1.1±0.1 | 1.2±0.2 | 2.72±0.17 | 3.55±0.38 |
| 0.280 | 1.4± 0.2 | 1.4±0.1 | 2.76±0.19 | 3.19±0.40 |
| 0.928 | 0.8± 0.1** | 0.9± 0.1** | 3.21±0.19 | 4.33±0.70 |
| 2.890 | 0.9± 0.1* | 0.9± 0.1* | 3.55±0.35 | 5.04±1.04 |
| 8.871 | - | - | - | - |

－indicates 100% mortality

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

## 2. Partial Life-Cycle Test

Table 2-A. Results

| Treatment (ng/L) | Hatchability (%) | Time to hatching (day) | Mortality (%) | Total length (mm) | Body weight (mg) |
|---|---|---|---|---|---|
| Control | 95 | 10.4 ± 0.2 | 9.5 | 21.4 ± 0.2 | 170.2 ± 3.9 |
| 27.6 | 93 | 10.2 ± 0.3 | 5.4 | 21.3 ± 0.2 | 162.6 ± 3.9 |
| 80.1 | 87 | 12.0 ± 0.4** | 11.5 | 21.8 ± 0.1 | 179.6 ± 3.8 |
| 178.0 | 80 | 12.0 ± 0.5** | 12.5 | 21.8 ± 0.2 | 181.5 ± 5.1 |
| 619.1 | 83 | 16.7 ± 0.9** | 25.3 | 22.2 ± 0.2* | 194.8 ± 5.9* |
| 1,859.5 | 88 | 12.8 ± 0.6** | 17.1 | 20.3 ± 0.2* | 153.5 ± 4.7* |

Table 2-B. Results (continued)

| Treatment (ng/L) | Gonadosomatic index (%) | | No. of fish | No. of males with testis-ova / No. of males | Hepatosomatic index (%) | | Vitellogenin (ng/mg liver) | |
|---|---|---|---|---|---|---|---|---|
| | ♂ | ♀ | | | ♂ | ♀ | ♂ | ♀ |
| Control | 1.14±0.08 | 7.57±0.17 | 20 | 0/10 | 3.55±0.42 | 4.06±0.25 | 1.3 ± 0.2 | 295.5± 117.9 |
| 27.6 | 1.12±0.11 | 7.66±0.19 | 20 | 0/10 | 3.50±0.40 | 3.96±0.30 | 1.6 ± 0.6 | 251.9± 102.1 |
| 80.1 | 0.98±0.09 | 7.54±0.22 | 20 | 0/10 | 3.25±0.45 | 3.72±0.36 | 1.1 ± 0.1 | 276.0± 99.5 |
| 178.0 | 1.08±0.07 | 7.53±0.22 | 20 | 0/10 | 2.65±0.18 | 3.93±0.29 | 1.2 ± 0.1 | 335.0± 150.7 |
| 619.1 | 1.05±0.09 | 7.22±0.20 | 20 | 0/10 | 3.46±0.39 | 5.12±0.23 | 1.4 ± 0.5 | 183.6± 77.7 |
| 1,859.5 | 1.07±0.11 | 7.29±0.18 | 20 | 0/10 | 4.38±0.30 | 4.91±0.41 | 1.2 ± 0.4 | 43.2± 20.9 |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

# Benzophenone

## 1. Vitellogenin Assay

Table 1. Results

| Treatment (μg/L) | Vitellogenin (ng/mg liver) | | Hepatosomatic index (%) | |
|---|---|---|---|---|
| | 14-d | 21-d | 14-d | 21-d |
| Control | ND | ND | 1.63±0.38 | 1.71±0.34 |
| Solvent control | ND | ND | 1.54±0.30 | 1.82±0.41 |
| 48 | ND | ND | 1.67±0.38 | 1.80±0.41 |
| 160 | ND | ND | 1.62±0.25 | 2.03±0.34 |
| 500 | 4.7±5.9** | 2.3±3.0** | 1.66±0.36 | 2.02±0.50 |
| 1,380 | 700±480** | 1,600±950** | 2.04±0.43** | 2.21±0.56 |
| 4,650 | 4,600±2,900** | 5,400±2,600** | 2.13±0.57** | 2.27±0.92* |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

## 2. Partial Life-Cycle Test

Table 2-A. Results

| Treatment (μg/L) | Hatchability (%) | Time to hatching (day) | Mortality (%) | Total length (mm) | Body weight (mg) |
|---|---|---|---|---|---|
| Control | 100 ± 0 | 9.4 ± 0.4 | 10 ± 3.9 | 29.5 ± 1.9 | 254 ± 49 |
| 5.06 | 100 ± 0 | 9.2 ± 0.1 | 8.3 ± 6.4 | 29.5 ± 1.6 | 253 ± 45 |
| 15.1 | 97 ± 3.9 | 9.3 ± 0.2 | 3.5 ± 4.0 | 29.5 ± 1.6 | 252 ± 39 |
| 47.0 | 93 ± 7.7 | 9.3 ± 0.1 | 8.9 ± 7.0 | 30.0 ± 1.4 | 270 ± 40 |
| 144 | 98 ± 3.3 | 9.3 ± 0.3 | 3.3 ± 3.9 | 29.6 ± 1.3 | 264 ± 33 |
| 435 | 98 ± 3.3 | 9.5 ± 0.3 | 1.7 ± 3.3 | 30.1 ± 1.6 | 265 ± 42 |

Table 2-B. Results (continued)

| Treatment (μg/L) | Gonadosomatic index (%) | | No. of fish | No. of males with testis-ova / No. of males | Hepatosomatic index (%) | | Vitellogenin (ng/mg liver) | |
|---|---|---|---|---|---|---|---|---|
| | ♂ | ♀ | | | ♂ | ♀ | ♂ | ♀ |
| Control | 0.97 ± 0.3 | 4.0 ± 3.1 | 20 | 0/7 | 2.3 ± 0.6 | 4.1 ± 0.9 | 10 ± 9.3 | 1,800± 1,600 |
| 5.06 | 0.55 ± 0.2 | 4.5 ± 2.9 | 20 | 1/10 | 2.3 ± 0.5 | 4.5 ± 0.7 | 17 ± 17 | 2,300± 1,600 |
| 15.1 | 0.77 ± 0.2 | 3.0 ± 2.6 | 20 | 0/8 | 2.6 ± 0.6 | 4.1 ± 0.7 | 5.5 ± 9.3 | 2,100± 1,400 |
| 47.0 | 0.64 ± 0.4 | 6.0 ± 2.5 | 20 | 2/11 | 3.0 ± 0.7* | 3.7 ± 0.6 | 6.4 ± 4.9 | 3,100± 1,600 |
| 144 | 0.58 ± 0.2* | 3.6 ± 3.4 | 20 | 0/11 | 2.2 ± 0.4 | 3.3 ± 0.9 | 3.3 ± 3.2 | 2,200± 1,400 |
| 435 | 0.88 ± 0.4 | 6.2 ± 2.8 | 20 | 1/11 | 2.3 ± 0.4 | 3.8 ± 0.4 | 56 ± 69 | 3,700± 2,000 |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

# Octachlorostyrene

## 1. Vitellogenin Assay

Table 1. Results

| Treatment (μg/L) | Vitellogenin (ng/mg liver) | | Hepatosomatic index (%) | |
|---|---|---|---|---|
| | 14-d | 21-d | 14-d | 21-d |
| Control | ND | ND | 1.33±0.29 | 1.61±0.27 |
| Solvent control | ND | ND | 1.38±0.30 | 1.51±0.20 |
| 0.24 | ND | ND | 1.40±0.38 | 1.42±0.28 |
| 0.49 | ND | ND | 1.56±0.21 | 1.67±0.41 |
| 1.1 | ND | ND | 1.39±0.30 | 1.56±0.21 |
| 2.8 | ND | ND | 1.36±0.32 | 1.53±0.27 |
| 6.6 | ND | ND | 1.53±0.27 | 1.46±0.15 |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

## 2. Partial Life Cycle Test

Table 2-A. Results

| Treatment (μg/L) | Hatchability (%) | Time to hatching (day) | Mortality (%) | Total length (mm) | Body weight (mg) |
|---|---|---|---|---|---|
| Control | 97 ± 3.9 | 9.1 ± 0.2 | 1.8 ± 3.6 | 30.5 ± 1.8 | 267 ± 61 |
| Solvent control | 97 ± 3.9 | 9.2 ± 0.1 | 6.8 ± 5.5 | 30.8 ± 1.9 | 279 ± 55 |
| 0.0519 | 95 ± 6.4 | 9.1 ± 0.1 | 1.8 ± 3.6 | 29.9 ± 1.7 | 280 ± 44 |
| 0.148 | 98 ± 3.3 | 9.0 ± 0.1 | 7.1 ± 10 | 30.4 ± 1.6 | 274 ± 48 |
| 0.388 | 95 ± 3.3 | 9.1 ± 0.2 | 0 ± 0 | 30.5 ± 2.6 | 282 ± 60 |
| 1.30 | 95 ± 3.3 | 9.1 ± 0.1 | 0 ± 0 | 30.5 ± 1.8 | 269 ± 53 |
| 5.31 | 98 ± 3.6 | 9.0 ± 0.0 | 12 ± 9.2 | 30.2 ± 1.4 | 259 ± 45 |

Table 2-B. Results (continued)

| Treatment (μg/L) | Gonadosomatic index (%) | | No. of fish | No. of males with testis-ova / No. of males | Hepatosomatic index (%) | | Vitellogenin (ng/mg liver) | |
|---|---|---|---|---|---|---|---|---|
| | ♂ | ♀ | | | ♂ | ♀ | ♂ | ♀ |
| Control | 0.82 ± 0.3 | 4.7 ± 3.5 | 20 | 0/11 | 2.8 ± 0.3 | 3.5 ± 0.9 | 6.6 ±11.1 | 1,100± 980 |
| Solvent control | 0.78 ± 0.2 | 3.7 ± 3.8 | 20 | 0/9 | 2.8 ± 0.5 | 3.9 ± 1.0 | 7.7 ± 8.8 | 980± 1,100 |
| 0.0519 | 0.82 ± 0.3 | 3.5 ± 3.0 | 20 | 0/7 | 2.2 ± 0.8 | 3.7± 0.9 | 2.0 ± 2.0 | 1,600± 1,400 |
| 0.148 | 0.84 ± 0.6 | 4.9 ± 4.0 | 20 | 0/13 | 2.0 ± 0.8** | 3.6 ± 0.8 | 1.5 ± 3.3** | 1,600± 1,300 |
| 0.388 | 0.84 ± 0.4 | 5.2 ± 4.0 | 20 | 0/12 | 2.5 ± 0.6 | 3.9 ± 0.8 | 1.2 ± 1.8* | 1,800± 1,200 |
| 1.30 | 0.82 ± 0.2 | 3.9 ± 3.6 | 20 | 0/9 | 2.5 ± 0.7 | 3.9 ± 1.1 | 5.0 ± 6.1 | 1,500± 1,100 |
| 5.31 | 0.70 ± 0.3 | 7.7 ± 3.5 | 20 | 0/13 | 2.6 ± 0.7 | 4.3 ± 1.0 | 0.3 ± 0.3** | 1,500± 660 |

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

# *Chapter 4*
# Genetic Approaches to Understanding the Basic Mechanisms of Endocrine Disruptor Actions on Sex Determination/Differentiation in the Medaka, *Oryzias latipes*

*Yoshitaka NAGAHAMA*
*Laboratory of Rreproductive Biology, Okazaki National Research Institutes*
*38 Nishigonaka, Myodaiji-cho, Okazaki-shi, Aichi, Japan*

**Summary**

Various genetic and molecular approaches have been used to establish the madaka as a test animal model for studying the effects of endocrine disrupting chemicals. We identified, for the first time in any non-mammalian vertebrates, *DMY* as the sex-determining gene of medaka. The cloning and sequencing of a number of genes that are thought to be associated with gonadal sex differentiation have been performed and some are still in progress (Fig. 1). These molecular probes provide useful tools to understand not only the molecular mechanisms of sex determination and gonadal sex differentiation but also to determine the mode of action of various endocrine disruptors during early stages of development of medaka. Finally, approximately 25,000 EST clones were obtained from whole embryos and fry during sex determination and differentiation and adult ovary. These EST clones were 5'-end sequenced and those associated with early stages of sex differentiation were re-arrayed in a DNA chip to evaluate their functional changes during the processes of endocrine disruption.

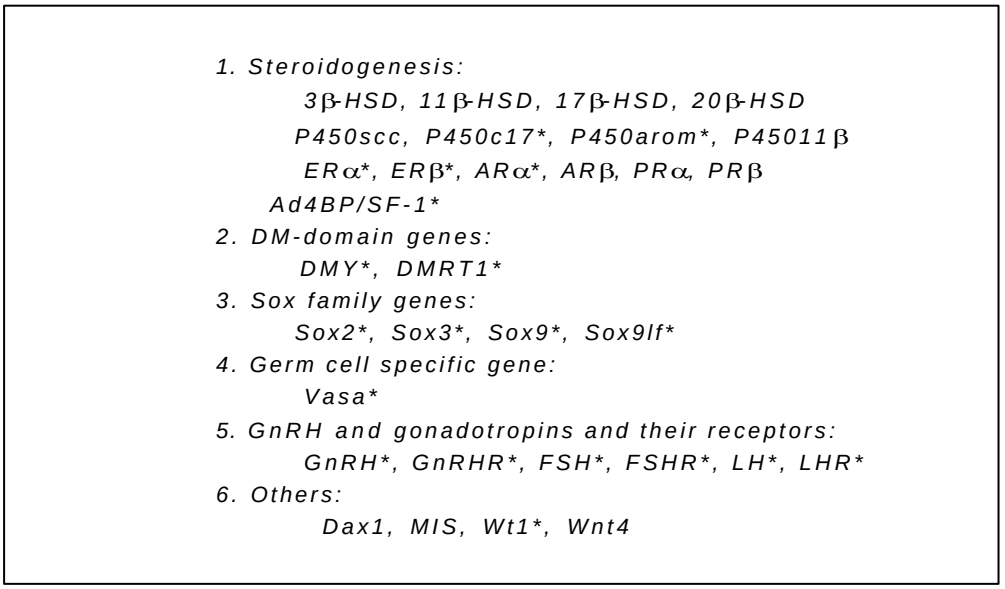


**Fig. 1.** Genes that are thought to be associated with sex determination and gonadal sex differentiation in vertebrates.

*, already cloned or cloning is in progress in medaka.

## 1. Introduction

A number of environmental pollutants have been shown to disrupt reproductive function in both wild populations and laboratory animals through interactions with different endocrine pathways. Primary targets for these endocrine active contaminants are steroid hormone receptors and steroidogenic enzymes. Recent evidence from studies on fish suggests that the periods of sex determination and gonadal sex differentiation are potential targets for endocrine disrupting chemicals present in the environment (Fig. 2). Treatments with exogenous sex hormones around the time of sex differentiation have profound effects on gonadal development including sex reversal, hermaphroditism and sterilization [1]. The gonads are, therefore, extremely sensitive to environmental stimuli around the time of sex differentiation, often with irreversible effects. As a result, environmental endocrine disruptors likely affect sex differentiation in wild fish. To determine causation and the mechanisms of disruption, it is critical to have a working knowledge of normal gonadal sex differentiation and its regulation. The medaka, *Oryzias latipes*, is an ideal model for studies related to endocrine disruption due to several reasons. This fish can spawn daily with regulated photo-thermal conditions, possess short time of maturation allowing the possibility to utilize both parental populations and progeny, and can be manipulated for transgenic studies. Here, I will review some of our recent attempts to establish medaka as a test organism for studying the endocrine disruptors' action together with our findings on this line using the Nile tilapia, *Oreochromis niloticus*, : 1) the identification of sex-determining gene, 2) the cloning of genes involved in gonadal sex differentiation, and 3) an attempt to develop DNA microarrays for the identification of differentially regulated genes during gonadal sex differentiation and for routine use in environmental monitoring.

## 2. The identification and cloning of sex-determining gene

Since sex determination precedes gonadal sex differentiation, it is important to understand the cascade of genetic sex determination. This information will be useful to generate molecular probes for basic understanding of sex determination process and also for studying endocrine disruption. In mammals, the Y chromosome is male determining, and encodes a gene referred to as *TDF* (testis determining factor) which induces the indifferent embryonic gonad to develop as a testis [2]. In 1990, the *SRY* (sex determining region of Y) gene was isolated from the human Y chromosome by positional cloning [3].

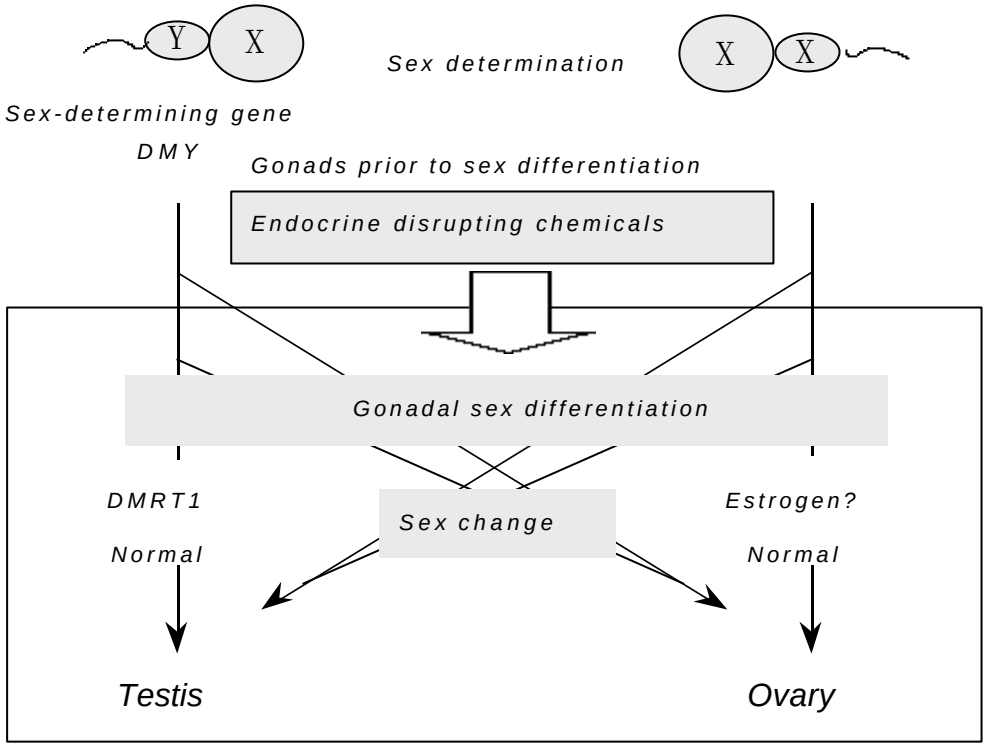


**Fig. 2.** Schematic diagram of the normal cascade of sex determination and possible mechanisms of endocrine disrupting chemical action. Endocrine disrupting chemicals can interchange the normal cascade of gonadal sex differentiation at different levels.

Not only were mutations detected in the *SRY* gene of XY females, indicating that *SRY* is required for normal testis development, but mice transgenic for *Sry* developed into sex reversed males, despite an XX karyotype [4]. Thus, among Y derived sequences *SRY* is both required and sufficient for male sex determination. In contrast, no genes comparable to *SRY/Sry* have been found in non-mammalian vertebrates.

Genetic determination of sex in non-mammalian vertebrates including fish can involve monogenic or polygenic systems, with factors located on the autosomes or on sex chromosomes. In the latter case, both male (XY) and female (ZW) heterogametic systems have been described, as well as many subtle variations on these themes [1]. To identify such a sex-determining gene, a positional cloning approach is suitable. The medaka fish is known to have a stable genetic XX/XY sex determining system, the male being heterogametic and dependent on the presence of the Y chromosome, although the chromosome is not cytogenetically distinct. Another major advantage to working with medaka is a large genetic diversity within the species [5] and the existence of several inbred strains [6]. Alteration of phenotypic sex with no reproductive

consequences, and recombination over the entire sex chromosome pair, suggest that there are no major differences, other than a sex-determining gene, between the X and Y chromosomes.

To clone positionally the sex-determining region, we generated a Y congenic strain to highlight the genetic differences between the X and Y chromosomes from inbred strains of medaka [7]. The Y congenic strain has a sex-determining region derived from the HNI-strain Y chromosome on the genetic background of an Hd-rR strain. Using this strain, we had previously constructed a genetic map of the medaka sex chromosome and constructed a BAC (bacterial artificial chromosome) genomic library [8]. Fluorescence *in situ* hybridization using one of the BAC clones as a probe was used to determine the location of the sex-determining region on the Y chromosome. As shown in Fig. 3, the sex-determining region is located on the centromere side of the long arms of the sex chromosomes.

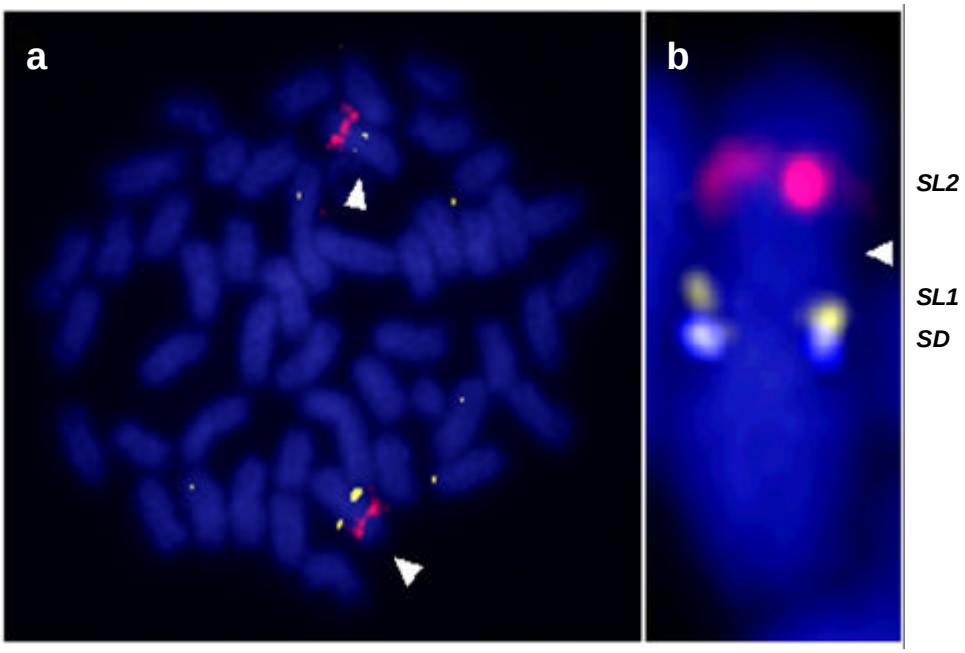


**Fig. 3.** Fluorescence *in situ* hybridization of medaka metaphase chromosome.
**a**, Sex-linked markers *SL1* (yellow) and *SL2* (red) hybridize to both X and Y chromosome.
**b**, A medaka sex chromosome with three different probes(*SL1*, *SL2*, sex-determining region, *SD*).

Shotgun sequencing was used to determine the sequence of the sex-determining region covered by the four BAC clones. The entire sequence of the two, centromere-side BAC clones was determined; however, the remaining

two, telomere-side BAC clones could not be completely sequenced due mainly to numerous repetitive sequences. Consequently, we sequenced a total of 422,202 nucleotides and estimated the four BAC clones covered about 530 kb. Genscan (a gene predicting program, Stanford Univ. ver. 1.0) predicted 52 genes in this region. Deletion analysis of the Y chromosome of a congenic XY female further shortened the region to 250 kb (Fig. 4). Shotgun sequencing of this region predicted 27 genes. Three of these genes were expressed during sexual differentiation. However, only the DM-related *PG17* was Y specific. We thus named it *DMY* [9]. The full-length cDNA sequence (1,320 bp) of *DMY* was obtained by 5' and 3' RACE. The longest open reading frame spans 6 exons and encodes a putative protein of 267 amino acids, including the highly conserved DM domain (Fig. 5). The DM domain was named after a related DNA binding motif found in two proteins, *doublesex* (*dsx*) and *mab-3*, involved in sexual development in the fruitfly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, respectively [10]. Since the initial characterizations of *dsx* and *mab-3*, DM-related genes have been identified from virtually all species examined to date including medaka. In vertebrate species, *DMRT1* (DM-related transcription factor 1), the DM-related gene most homologous to *DMY* (about 80%), correlates with male development [11-13]. Combined with its Y chromosome specificity, this finding suggests that *DMY* plays a pivotal role in testicular differentiation.

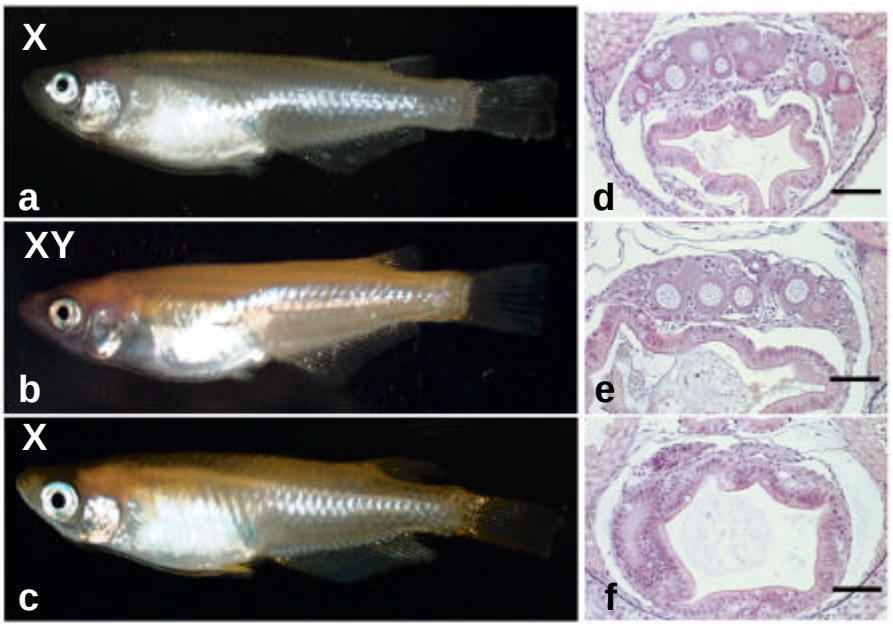


**Fig. 4**. Characteristics of medaka lacking a part of the Y chromosome. Phenotypes of the congenic strain (XX) (**a**), medaka lacking a part of the Y chromosome (XY-) (**b**) and the congenic strain (XY) (**c**). Histological cross-sections of medaka fry sampled at 30 dah. XX (**d**) and XY- (**e**) individuals have ovaries, whereas XY specimen has testes with spermatogonia (**f**). Scale bars, 50 μm.

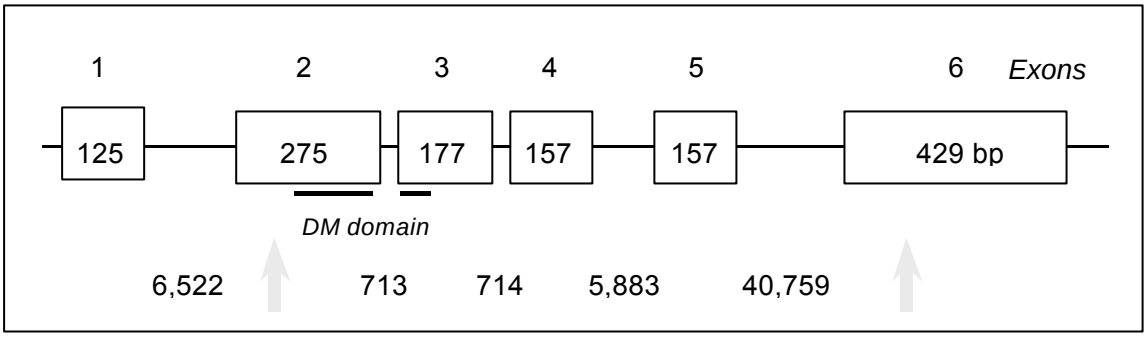


**Fig. 5.** Exon and intron structures of *DMY*.

Two lines of evidence established the critical role of *DMY* in testicular differentiation. Firstly, the analysis of two naturally occurring XY females from two separate populations (Awara and Shirone). One of these mutants contained a single nucleotide insertion in exon 3 of *DMY* that causes premature termination of the DMY protein. When mated, all XY offspring with the mutant Y were female. The absence of about two-thirds of the protein presumably renders *DMY* nonfunctional, thus resulting in XY sex reversal (female phenotype). The other mutant had a severe depression in *DMY* expression in the embryo and 60% of its XY offspring with the mutant Y developed as females. Secondly, specific RT-PCR and *in situ* hybridization were used to determine the temporal expression of *DMY* during sexual differentiation. At hatching and 5 days after hatching, *DMY* mRNA was present in XY embryos. *DMY* signals were detected only in the somatic cells (probably pre-Sertoli cells) surrounding germ cells in XY gonads at the time when sex determination occurs (Fig. 6).

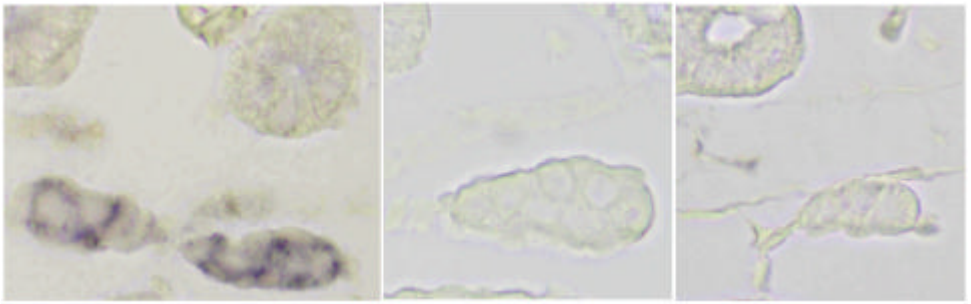

**Fig. 6.** *DMY* mRNA expression in fry gonads shown by *in situ* hybridization of larval sections. **a**, XY gonads on hatching day; antisense probe. Strong signals for *DMY* are seen in cells surrounding the germ cells. **b**, XX gonad on hatching day; antisense probe. *DMY* signal is undetectable in XX in individuals. **c**, XY gonad on hatching day; sense probe. Control hybridization shows no signal. Germ cells (arrows).

*Oryzias curvinotus* has the same sex-determining mechanism (XX-XY) as *O. latipes* and has *DMY* on the Y chromosome, which suggests that *DMY* also has a role in sex determination of *O. curvinotus*. A phylogenetic tree based on the amino acid sequence including the DM-domain shows that *DMY* was derived from *DMRT1* immediately before speciation of *O. latipes* and *O. curvinotus* (Fig. 7). The branch length of *DMY* is longer than that of *DMRT1* in the phylogenetic tree. This means that *DMY* has more mutations than *DMRT1* and suggest that *DMY* accumulated these mutations after it acquired its sex-determining function. It is of interest that the rate of evolution of *Sry*, which is the sex-determining gene on the Y chromosome of mammals, is also higher than that of *Sox* family genes. These results suggest that a new sex-determining gene generated by a gene duplication event in the sex chromosome tends to evolve fast.

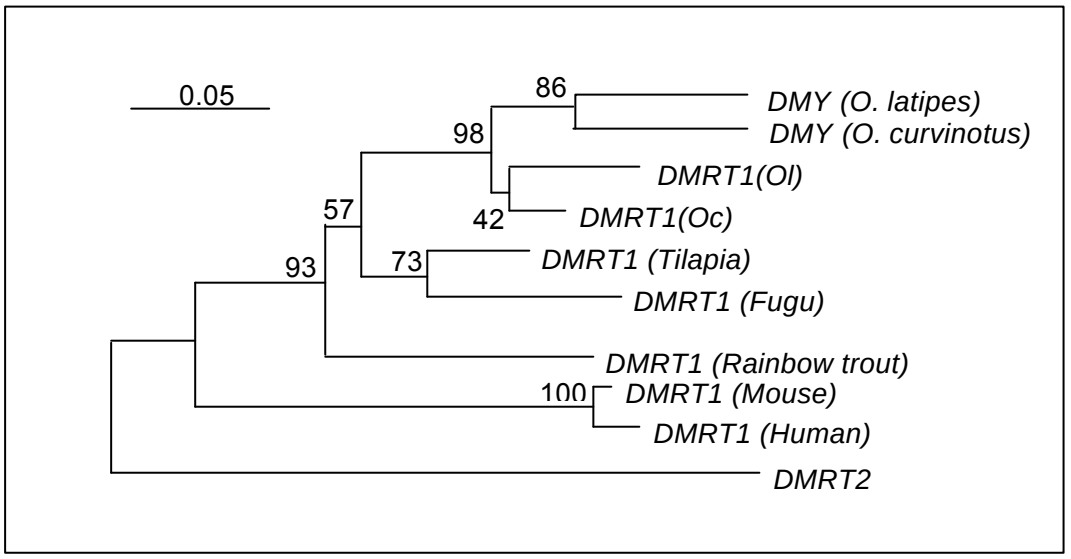


**Fig. 7.** Phylogenetic tree of DMY and its associated DM-domain genes from various

The precise role of *DMY* remains to be elucidated. It would seem likely that the protein product of *DMY* is a regulatory factor, probably DNA binding element that regulates expression of a secondary or downstream testis determinant. The downstream locus could be either Y or X-linked or autosomal. *DMY* is the first identified sex-determining gene in any non-mammalian vertebrates (Fig. 8) and it seems to be a useful molecular probe to understand the effects and molecular mechanisms of endocrine disruptors in wild life.

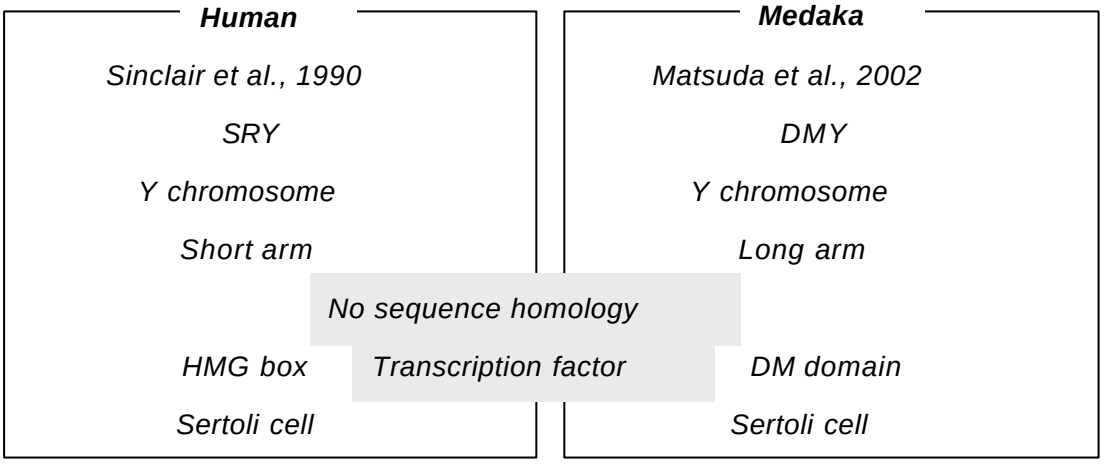


**Fig. 8.** Comparison of two identified sex-determining genes, *SRY* and *DMY*, in vertebrates.

## 3. Gonadal sex differentiation

### 3-1. Germ cells and *vasa* gene

The morphological criteria for judging the initial differentiation of ovaries and testes have been studied in detail in medaka by Yamamoto [14]. Germ cells in putative ovaries outnumber those in putative testes just after hatching, and mitosis and meiosis of germ cells take place earlier in ovaries than in testes [15] (Fig. 9). No sex-specific differences have been identified in the gonadal architecture, including the supporting lineage cells. The sex-specific structure among supporting cells first appears in the acinous structure of males, 10 days after hatching [16]. Thus, unlike mammals, the sex differentiation in medaka is first observed in germ cells and then in supporting cells.

The gene *vasa* encodes a DEAD family of putative RNA helicase and is present in both the polar granules at the posterior end of the oocyte and the nuage structure in the germ cells in *Drosophila*. In several vertebrate species, the *vasa* homologs are found to be expressed specifically in the germ line [17]. We cloned a medaka *vasa* homolog (*olvas*). *In situ* hybridization studies revealed that the transcripts for *olvas* are exclusively detected in the cytoplasm of germ cells in the testis and ovary [18]. We then generated transgenic medaka lines that express green fluorescent protein (GFP) exclusively in germ cells that can be visualized in a living vertebrate. Two medaka strains, himedaka (orange-red variety) and inbred QurtE (this strain lacks most pigments except for leucophores, a Y-linked trait). Around the day

of hatching, the QurtE strain clearly exhibited sexual dimorphisms in the number of GFP fluorescent germ cells [19]. The apparent sexual dimorphism of the gonads indicated by GFP fluorescence can also provide a useful indicator of the effects of environmental substances on gonadal development (Fig. 10).

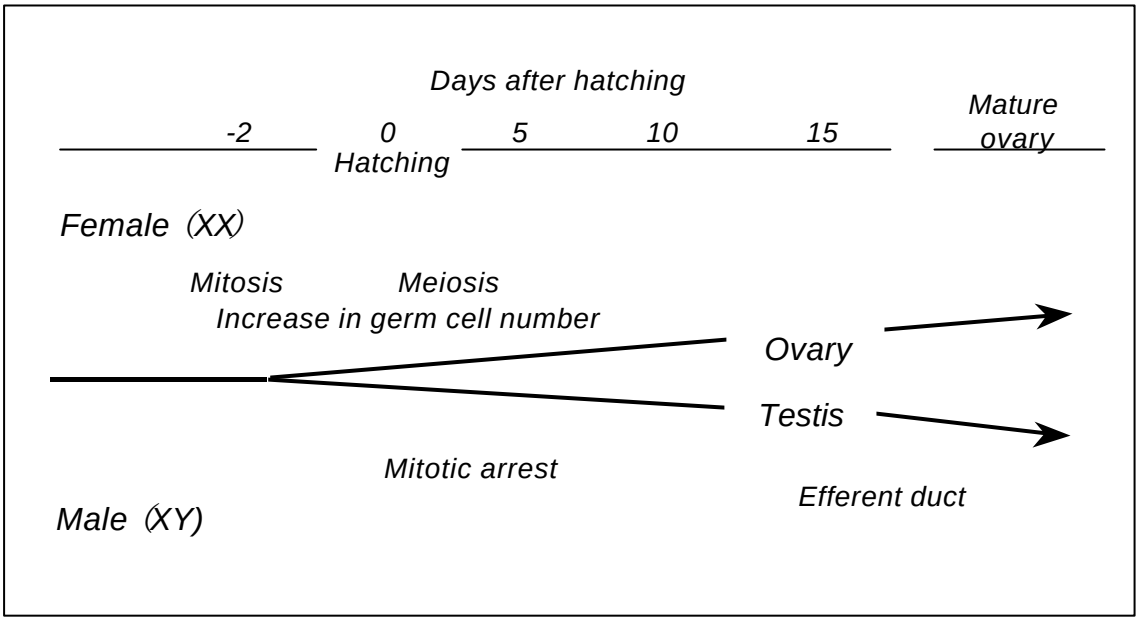


**Fig. 9.** Gonadal sex differentiation in medaka.

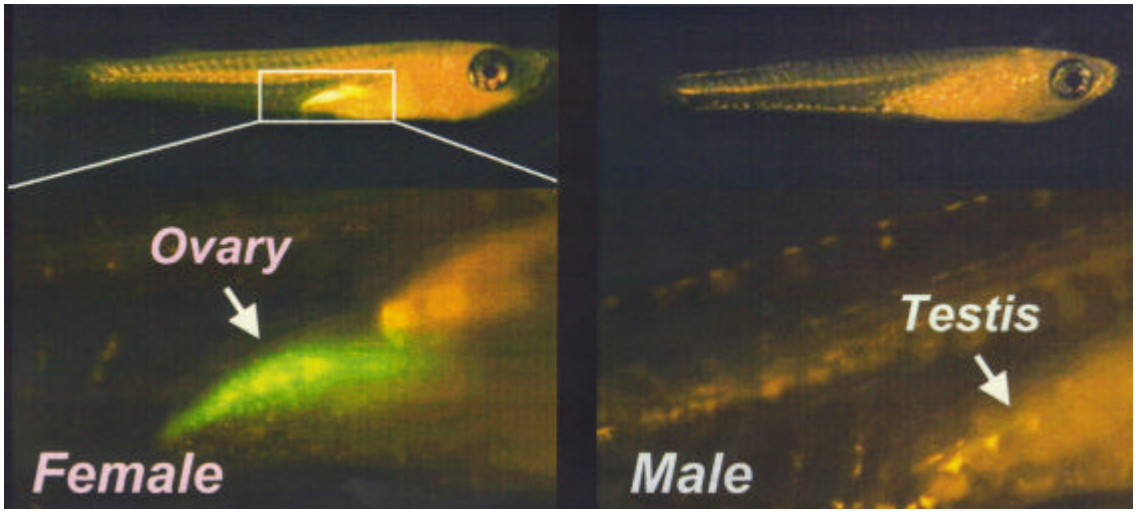


**Fig. 10.** GFP fluorescent germ cells in the ovary and testis of medaka. The ovary contains numerous oocytes which can be seen as a green fluorescence.

### 3-2. Steroidogenic enzymes

As in other vertebrates, a complex series of enzymes are responsible for the biosynthesis of sex steroids in fish [20] (Fig. 11). Several biologically important steroidal mediators have been identified in fish gonads. In females, estradiol-17β and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) are two major steroids which are responsible for oocyte growth and maturation, respectively [20], while in males 11-ketotestosterone has been identified as a spermatogenesis-inducing hormone in the Japanese eel, *Anguilla japonica* [21].

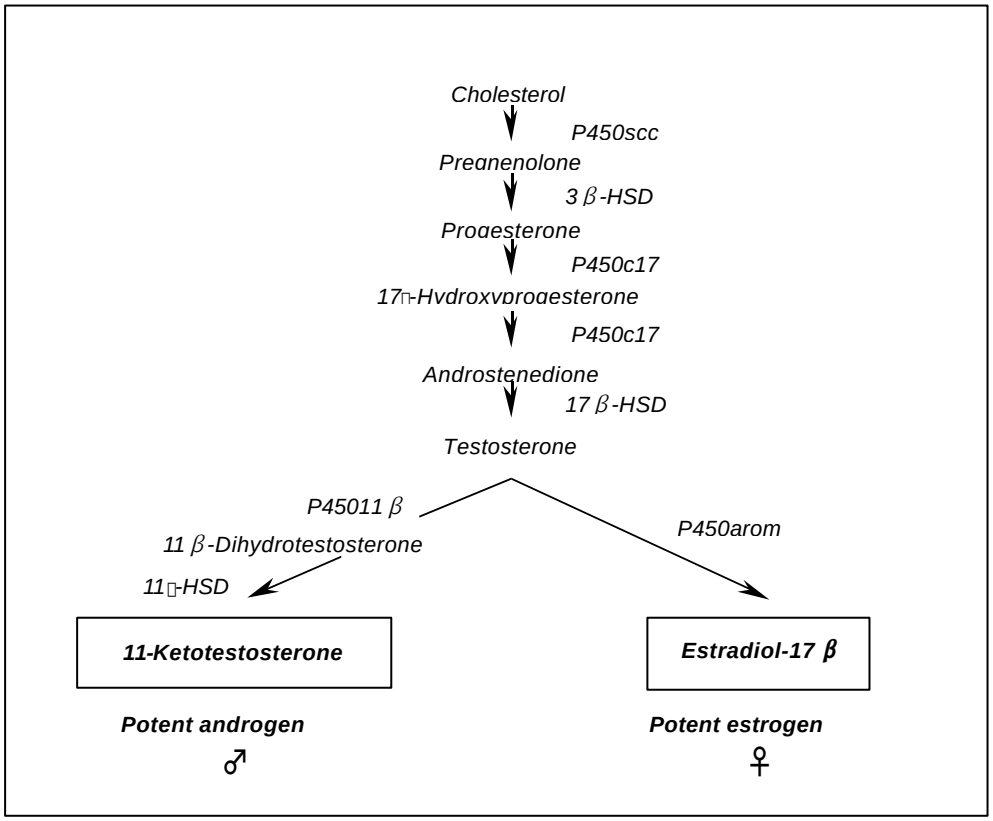


**Fig. 11.** Schematic diagram of steroidogenic pathway in fish

Exogenous sex steroids administered at the time of sex determination can strongly influence the course of sex differentiation in fish, suggesting that they play a critical role in assignment of gonad determination as well as subsequent differentiation [1]. Similarly, if steroid-synthesizing capability is interfered with, sex determination can be disrupted; for example, inhibition of estrogen synthesis in early development using inhibitors of the enzyme aromatase can cause masculinization of several teleost fishes [1]. If steroids are critical for

directing initial sex differentiation rather than being consequence of it, then the appearance of steroid-producing cells and differences in steroid production between the sexes should be apparent prior to morphological differentiation of the gonad. Indeed, immunohistochemistry has shown that P450scc, P450c17, 3β-HSD and P450arom are found at high levels in female gonadal anlagen of tilapia at the undifferentiated and differentiating stages, but is only seen weakly in genetic males and not until 30 days after hatching. These results strongly suggest that endogenous estrogens act as the natural inducers of ovarian differentiation in tilapia [22]. This hypothesis is further supported by evidence of masculinization of genetic female tilapia by inhibition of estrogen synthesis using an inhibitor of P450arom.

On the basis of extensive experiments using various steroid hormones, Yamamoto [23] hypothesized that sex hormones act as endogenous sex inducers during sex differentiation of medaka, androgens acting as testicular inducers and estrogens acting as ovarian inducers. However, some of the morphological studies do not seem to support the critical role of endogenous steroid hormones in gonadal sex differentiation in medaka. For example, it was shown that steroid-producing cells appeared initially long after the completion of sex differentiation [24, 16]. Similarly, 3β-HSD, an important enzyme for steroid hormone biosynthesis, also appeared after sex differentiation. The use of aromatase inhibitors and antiestrogens has also shown that ovarian development may occur independent of the action of estrogens [25]. More recently, using transgenic medaka with over-expressed medaka estrogen receptors, Kawahara *et al*. [26] examined the effects of exogenous estrogen and androgen on sexual differentiation. The results showed that over-expression of estrogen receptors did not affect the sexual development in either male or female after estrogen or androgen treatment, suggesting an estrogen/estrogen receptor-independent female sex determination pathway. Certainly, further studies are required to determine whether endogenous steroids are critical for directing initial sex differentiation in fish including medaka.

Specific genes involved in steroid biosynthesis are differentially expressed in the somatic cells of testis and ovary, which results in the production of an array of sex steroids. To provide a basis for the possible involvement of endogenous sex steroids in gonadal sex differentiation in medaka, it is necessary to examine the expression pattern of various steroidogenic enzymes in gonads before, during and after sex differentiation. We previously isolated two enzymes, P450c17 (D. Kobayashi *et al*., unpublished) and P450arom [27, 28] from medaka ovaries. These cDNA inserts were confirmed to encode each

steroidogenic enzyme by introducing it into non-steroidogenic COS-1 monkey kidney tumor cells. The coding region of the medaka *P450arom* gene is composed of nine exons; human P450arom gene also contains nice exons. Although the intron – exon boundary sequences of medaka P450arom gene are exactly the same as those of mammals, introns are much shorter in medaka than mammals. The promoter region of medaka *P450arom* gene also contains potential Ad4BP/SF-1 sites and estrogen responsive element (ERE) half-sites. These results suggest that the basic structural organization of *P450arom* genes and the regulatory mechanisms of expression are well conserved throughout the vertebrates [27]. Multiple *P450arom* genes have been identified in several fish species, and sequence comparisons have revealed that the forms of *P450arom* expressed in brain and ovary are distinct *P450arom*. Recently, the brain form of *P450arom* has been shown to be expressed at two distinct levels among individual zebrafish embryos around the time of sex differentiation, suggesting a possible role of this gene in controlling sex differentiation [29]. However, no multiple forms of *P450arom* genes have been reported in medaka.

More recently, we have cloned a cDNA encoding the steroidogenic acute regulatory (StAR) protein from medaka ovaries. StAR protein mediates the rate-limiting and acutely-regulated step in steroidogenesis, the transfer of cholesterol from the outer to the inner mitochondrial membrane where P450scc initiates the synthesis of all steroid hormones (M. Tokumoto *et al.*, unpublished).

Using our acquired knowledge of nucleotide sequences, we synthesized peptides and used them to raise polyclonal antibodies against four steroidogenic enzymes essential for the biosynthesis of all major steroid sex steroid hormones including androgens and estrogens [30]. Some of these antibodies can also be used to stain immunohistochemically gonads during the process of sex differentiation in medaka. Our current efforts, in collaboration with Dr. N. Shibata, Shinshu University, center on the cloning and sequencing of the genes encoding several other steroidogenic enzymes such as P450scc, 3β-HSD, and 11β-hydroxylase.

## 3-3. Sex steroid receptors

Steroid hormone action is mediated by binding to specific receptors. Like higher vertebrates, fish also possess two estrogen receptors typical of the α and β forms. In tilapia, while both genes are expressed in early male and female gonads at 10-15 days after hatching, they display different expression patterns suggesting important roles in regulating early sex differentiation. Genomic

and complementary DNA fragments encoding an estrogen receptor homolog have been cloned from medaka and used to examine its expression patterns during gonadal sex differentiation. Estrogen receptor mRNA was expressed in both sexes, but at very low levels, during the period of sex determination [25, 26]. The α and β forms of estrogen receptors are present in medaka ovaries (M. Nakai, see Chapter 2 in this issue; T. Todo, personal communication). Unlike other vertebrates, some of fish have two forms of androgen receptors. Two functional androgen receptors were cloned for the first time from an eel testis cDNA library [31]. A cDNA encoding medaka androgen receptors has also been cloned (T. Todo, unpublished).

## 3-4. Other genes involved in gonadal sex differentiation

Numerous genes have been shown to be involved in sex determination and differentiation [2]. These genes include *Sox9*, *Ad4BP/SF-1*, *DMRT1*, *Dax1*, *WT1*, *MIS* and others. Several of these genes have also been cloned from fish. The *SRY*-related gene *Sox9* is highly conserved in vertebrate evolution. At the onset of gonadal sex differentiation, *Sox9* is up-regulated in testes but down-regulated in ovaries. *Sox9* is an autosomal gene involved in the differentiation of Sertoli cells in mouse and chicken, two species with different genetic mechanisms of sex differentiation. We isolated several kinds of *Sox* sequences and then investigated the expressions of *Sox* transcripts in medaka oocytes and embryos [32]. Medaka *Sox* sequences are present in both males and females and can be classified into five subgroups based on the analysis of the amino acid homologue in the HMG box and further that one of the medaka *Sox* genes is expressed in unfertilized eggs. More recently, two more *Sox* transcripts (*Sox9* and *Sox9lf*) were also isolated from a testis cDNA library [33]. The *Sox9* encodes 487 amino acids and shows approximately 70% amino acid identity with known vertebrate SOX9 proteins. The *Sox9lf* is a longer from of the *Sox9*, which is transcribed from an additional exon in the 5' upstream region. Interestingly, the expression of medaka *Sox9* is predominantly observed in the adult ovary by northern blot and *in situ* hybridization, whereas in the testis, its expression is detectable only by RT-PCR. During medaka embryogenesis, its expression is observed in the cranial cartilage and pectoral fin endoskeleton. These observations suggest that the function of *Sox9* in the cartilage is conserved among vertebrates, while that in the gonad is quite different in medaka.

A cDNA encoding Ad4/BP/SF-1 (FTZ-F1) has also been cloned from ovarian follicles of medaka [34]. Distribution of *Ad4BP/SF-1* transcripts in various

tissues of medaka was very similar to that of mammalian *Ad4BP/SF-1*. The expression pattern of *Ad4BP/SF-1* transcripts during oogenesis coincides with that of *P450arom* transcripts. Transfection assays further suggest a potential transcriptional regulatory activity of Ad4BP/SF-1 in the transcriptional regulation of *P450arom* in the ovarian follicle of medaka. Sequence analyses revealed three putative Ad4BP/SF-1 binding motifs in the medaka *P450arom* promoter region. Since the role of Ad4BP/SF-1 in P450arom regulation in fish granulosa cells during vitellogenesis proved essential, the usage of this probe might be further helpful to test whether estrogens are necessary for ovarian differentiation.

Another gene family (the family of genes that contain DM-domain) involved in insects and nematodes has also been shown to be involved in vertebrate sex determination and gonadal sex differentiation. One member of this group, *DMRT1*, has been shown to be essential for postnatal testis differentiation [35]. In tilapia, *DMRT1* possesses the male-specific motif present in *doublesex*, and expression appears in testis (Sertoli cells) specific, whereas another DM homologue (*DMO*) lacked this motif and was found to be expressed only in ovary [13]. Masculinization of tilapia genetically female (XX) gonads with androgen induces *DMRT1* expression, whereas treatment of genetically male (XY) with estrogen resulted in reduced expression [13, T. Kobayashi, unpublished]. These results suggest that *DMRT1* genes are expressed in response to testis differentiation induced by other factors (i.e. steroids), and in these species, are located somewhat downstream in the determining pathway.

There are several forms of DM-domain genes in medaka. Brunner *et al*. [36] cloned the DM gene family from medaka such as *DMRT1*, *DMRT2*, and *DMRT3*, and determined their structural and functional characteristics. Gene structure and order are highly conserved within a larger region of synteny with human chromosome 9. While the chromosomal localization of the *DMRT* cluster argues against its role as a primary sex-determining locus, the comparative analysis uncovered putative regulatory elements that might be important during sex differentiation and sex reversal [36]. *DMRT1* mRNA has been found expressed in embryos of male medaka around 20 days after hatching [37, Kobayashi *et al*., unpublished].

## 4. EST and DNA microarray

Up to now, I discussed the process of sex determination and identification of the sex-determining gene, together with several other genes involved in

gonadal sex differentiation in medaka. Since this did not account for the entire gene cascade of sex determination/differentiation, we cloned a series of genes and generated EST clones during the early stages of development of medaka. Later we re-arrayed these EST clones in a DNA chip for DNA microarray evaluation of genes. By this we will be able to distinguish the molecular mechanisms of endocrine disruptors to know how it bring about its biological effects. Thus DNA microarray-based gene expression profiling might be highly useful gene technology to understand mRNA-level control of sex differentiation and also to examine endocrine disruptors in medaka embryos.

## 4-1. EST clones

The first step towards microarraying is to isolate gene fragments. Gene targets for arraying may be randomly picked from a cDNA library, characterized by sequencing to produce ESTs (Exposed Sequence Tag) and used for arraying. Embryos are from the Hd-rR strain. For library construction, embryos two days before hatching (7 days after fertilization), 0 day after hatching (dah), 5 dah, 10 dah, 15 dah, and ovary (adult) were used (Table 1). Total RNA was extracted from whole body of embryos and an ovary. Bacterial clones were plated, colonies picked robotically and glycerol stocks constructed in 384-well format. Random clones (3,072 - 6,528) were 5'-end sequenced. Within 24,960 sequence reads, 19,290 sequences were evaluated as high-quality sequences (Table 1). Batch sequence alignments were performed on a computer workstation using a program (programmed by Computer Laboratory of National Institute for Basic Biology). As a result, 7,513 sequences were nonoverlapping sequences. Glycerol stocks correspond to these 7,513 sequences were re-arrayed to 20 plats of 384-well microtiter plates using a GENESIS workstation (Tecan).

**Table 1.** EST clones obtained from medaka embryos during sex determination and gonadal sex differentiation adult ovary

| | 2dbh* | 0dah* | 5dah | 10dah | 15dah | Ovary | All |
|---|---|---|---|---|---|---|---|
| Reads | 6,528 | 3,840 | 3,840 | 3,840 | 3,840 | 3,072 | 24,960 |
| Available | 4,935 | 3,419 | 3,148 | 2,577 | 2,462 | 2,749 | 19,290 |
| Clusters | 3,548 | 1,282 | 2,259 | 1,847 | 1,784 | 1,479 | 7,51 |

* days before hatching; ** days after hatching

## 4-2. DNA arrays

Amplification of target cDNA clones was carried out. PCR amplification was performed using 384-well formatted glycerol stocks. Amplified PCR products were purified using the 384-well glass filter membrane (Corning). The quality and quantity of purified PCR products were analyzed by 1% agarose gel electrophoresis, and the PCR was repeated if required. PCR products were dried under a 37 ℃ oven and dissolved in Microarray Crosslinking Reagent D (Amersham). DNA solutions were spotted on metal coated Microarray slides Type 7 Star (Amersham) using a Molecular Dynamics GenIII Arrayer (Amersham). Each slide had two identical sets of up to 4,608 DNA spots (Fig 12). Spotted slides were UV cross-linked at 100 mJ. Target DNA spotted on slides was stored dry until used.

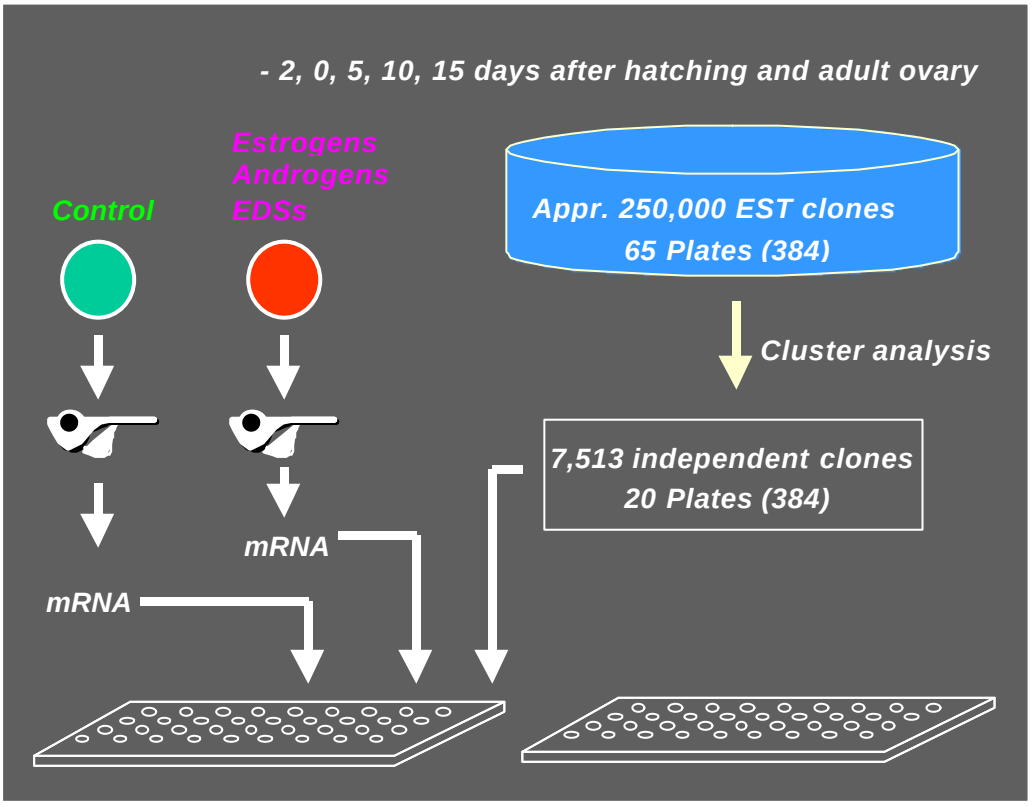


**Fig. 12.** Procedures and methodology to assess normal and endocrine disrupting chemicals' mechanisms of sex determination and differentiation.

## 5. Future directions

As described above, 7,513 individual EST clones that are thought to be essential for gonadal sex differentiation in medaka were selected and re-arrayed. In the next step, we are planning to use this microarray DNA chip to detect mRNA changes after the treatment for several days either with

estrogenic or androgenic compounds or endocrine disruptor chemicals. In brief, we prepare RNA from both from the control and treated groups and then label them with two different dyes (fluorophores Cy3 and Cy5) followed by hybridization on the DNA chip. The difference in the dye reveals those genes that had either undergone up or down regulation due to the treatments. The localization of these genes will be further analyzed by whole mount *in situ* hybridization. Later we plan to prepare the DNA chip containing only these important genes. This will later serve as more comprehensive DNA chip for our future studies related to sex differentiation as well as screening of endocrine disrupting chemical in wild life.

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***Further works***

We have developed several screening and testing methods with medaka for evaluating the hormonal activity of natural steroid hormones and endocrine disrupting chemicals (EDCs). Although the developed screening and testing methods are applicable to estrogen and androgen agonists, the applicability of the test methods for their antagonist are unknown. Therefore, we need to verify the applicability of the methods to the antagonists. Furthermore, we should investigate the usablity of the test methods for weak EDCs, although it has been demonstrated in some test methods. Hereafter, we need to study in order to choose the test method that is the best in terms of sensitivity and cost effectiveness. So far we understand that partial life-cycle test is sensitive compared to reproduction test in both points of general toxicity and endocrine disruption. However, some chemicals may display more toxicity to the next generation than the parent. We also have to consider the differences in susceptibility to chemicals between medaka and other fish and/or organisms. In this point of view, the mechanism causing the different susceptibility to chemicals between two generations and among organisms should be studied out.

A number of works may be necessary for the demonstration of those. However, recent biotechnological tools may help and accelerate to resolve the matter in question. Furthermore, it is not clarified that how relevant the change observed in such endpoints as secondary sex characteristics, gonadal histology, VTG and so on is involved in the ecological effects based on endpoints like survival, growth and reproduction. Therefore, we need to discuss and consider how to evaluate the change of these endpoints and how to use the results obtained by the developed test methods.

*In vitro* studies also have been developed in order to screen many of the chemicals of concern. As part of studies, we found that species difference in the receptor binding affinity shows importance of the differences in sensitivities to chemicals among diverse species to assess the endocrine disrupting effects of chemicals to ecosystem. In conclusion, we should clarify this issue by receptor binding assay using various fish estrogen receptors.

In this report, the sex-determinating gene of medaka, DMY (Y-specific DM-

domain gene) and fish sexual differentiation are discussed. This gene is the first to be found in non-mammalian vertebrates. We have much effort on the area of the DNA chip containing a series of genes associated with the sexual differentiation in **medaka**. A series of genes associated with regulating the sexual differentiation have been cloned and prepared to develop a DNA macro array. The results of these studies with utilizing the gene technology will be useful to make clear the effects of EDCs on the mechanism of sexual differentiation in **medaka** and also the species difference in sensitivities.

# *Appendix*

**Appendix Ⅰ：**

**Ⅰ.：The medaka(*Oryzias Latipes*) Partial Life-Cycle Test Guideline**

**Ⅱ.：The medaka(*Oryzias Latipes*) Full Life-Cycle Test Guideline**

**Appendix Ⅱ：**

**Ⅰ.：Effect of ethinylestradiol on the reproduction and induction of vtellogenin and tests-ova in Medaka(*Oryzias Latipes*)**

**Ⅱ.：Life-cycle toxicity of 4-nonylphenol to Medaka (*Oryzias Latipes*)**

**Appendix Ⅲ：Other information regarding Medaka studies**

# *Appendix I*

**Ⅰ. : The medaka(*Oryzias Latipes*) Partial Life-Cycle Test Guideline**

**Ⅱ. : The medaka(*Oryzias Latipes*) Full Life-Cycle Test Guideline**

# The medaka (*Oryzias latipes*) Partial Life-Cycle Test Guideline

**November 2002**
**Ministry of the Environment, Japan**

# Fish Partial Life-Cycle Test Guideline

## OBJECTIVE

This test guideline describes a fish full life-cycle test that can be used to evaluate the potential chronic effects of chemicals on fish populations. The method gives primary emphasis to potential population relevant effects (namely, adverse impacts on survival, development, growth and reproduction) for the calculation of the No-Observed Effect Concentration ($NOEC_{adverse}$). These effect observations should be augmented by secondary mechanistic biomarker responses (namely, vitellogenin, gonad somatic index [GSI], and gonad histology). The method is applicable to a variety of chemicals, including endocrine disrupters and general toxicants. The medaka (*Oryzias latipes*) is a suitable species for use in this test guideline, however, other species such as fathead minnow (*Pimephales promales*), sheepshead minnow (*Cyprinodon variegatus*), three spined stickleback (*Gasterosteus aculatus*) and zebrafish (*Danio rerio*) are also suitable.

## GLOSSARY OF TERMS

dph = days post-hatch; GSI = gonad somatic index; VTG = vitellogenin

## INTRODUCTION

This test guideline describes a fish partial life-cycle test from fertilized eggs to sexual differentiation and early gametogenesis for identifying and detecting endocrine disrupting effects of substances. This test is also expected to give data directly relevant to ecological risk assessment of substances. This protocol is derived from work on medaka (*Oryzias latipes*) in Chemicals Evaluation and Research Institute, Japan (CERI) based on OECD TG 210 (OECD, 1992) supplemented with gonadal histology and vitellogenin (VTG) analyses. This test has been evaluated through testing with several endocrine disrupting chemicals (EDCs) including bisphenol A (Yokota et al, 2000), 4-nonylphenol and 4-*tert*-octylphenol (preparing the manuscripts for publication in a journal). In addition, this test has been partially evaluated in fish full-life cycle study by testing with OECD reference compounds (4-*tert*-pentylphenol; Seki et al, 2002 [submitted], ethinylestradiol and methyltestosterone; preparing the manuscripts for publication in a journal). In principal, the approach is also applicable to other OECD fish species, notably fathead minnow (*Pimephales promelas*) (Tyler et al, 1999), sheepshead minnow (*Cyprinodon variegatus*) (Zillioux et al, 2001), three-spined stickleback (*Gasterosteus aculatus*) Katsiadaki et al., 2002) and zebrafish (*Danio rerio*).

2. This test guideline measures several biological endpoints. Primary emphasis is given to potential adverse population effects including survival, gross development, growth and reproduction. Secondarily, in order to provide secondary mechanistic (biomarker) information and provide linkage between results from other kinds of field and laboratory studies, where there is *a priori* evidence for a chemical having potential endocrine disrupter activity (eg androgenic or oestrogenic activity) then other useful information can be obtained by measuring vitellogenin (VTG) levels, gonad somatic index [GSI] and evaluating gonad histology.

## PRINCIPAL OF THE TEST

3. Overviews of the relevant test conditions in medaka are provided in Annex 1. For medaka,

exposure is normally initiated with healthy embryos within several hours post-fertilization. This test is conducted at a minimum of five chemical concentrations, as well as appropriate controls with a minimum of 60 embryos for each treatment separated into four groups. Chemical delivery can be via an aqueous route (with or without carrier solvents). Monitoring continues for up to 60-d posthatch and includes embryological development, hatching (hatchability and time to hatch), posthatch survival, growth (total lengths and body weight), sexual differentiation (secondary sex differentiation and gonadal histology), hepatic VTG levels, and gonadal development (GSI).

## DESCRIPTION OF THE METHOD

### Test animals and exposure system

#### Test animals

4. This test should be started with embryos within several hours post-fertilization from a ready supply of known-quality animals. Candidate species for this assay include medaka (*Oryzias latipes*), fathead minnow (*Pimephales promelas*), sheepshead minnow (*Cyprinodon variegatus* Lacépède) and zebrafish (*Danio rerio*). Relevant modification is necessary for test fish species other than medaka. Where possible, field-collected fish generally should not be used to initiate cultures or for the testing.

5. Parental fish for the spawning eggs subjected to exposure should be selected from a population of a single stock. The fish should be paired and acclimatized for at least two weeks prior to the test under conditions of water quality and illumination similar to those used in the test (Annex 1). Fish should be fed exclusively with *Artemia* nauplii (<24 h after hatching) twice a day.

#### Water

- Any water in which the test species shows suitable long-term survival and growth may be used as test water. It should be of constant quality during the period of the test. In order to ensure that the dilution water will not unduly influence the test result (for example by complexation of test substance) or adversely affect the performance of the brood stock, samples should be taken at intervals for analysis. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl, $SO_4$), pesticides, total organic carbon and suspended solids should be made, for example every six months where a dilution water is known to be relatively constant in quality. Some chemical characteristics of an acceptable dilution water are listed in Annex 2.

#### Exposure system

7. The design and materials used for the exposure system are optional. Glass, stainless steel, or other chemically inert material should be used for construction of the test system. For medaka, an exposure system is well suited as follows (Yokota et al, 2000; Yokota et al, 2001a; Kang et al, 2002; Seki et al, 2002): The exposure system consists of a continuous-flow mini-diluter system (Benoit et al, 1982). Stock solution of the test chemical should be injected in this exposure system by an appropriate pump. The flow rate of the stock solution should be calibrated in accordance with analytical confirmation of the test solutions before the initiation of exposure, and checked volumetrically periodically during the test. The test solution in each chamber is renewed adequately depending on the test chemical stability and water quality.

## Experimental Design

8. It is recommended to use a minimum of five chemical concentrations. Prior knowledge of the test substance (e.g. results of the test at tier 1 in OECD testing scheme, in vitro receptor binding assay, subchronic toxicity, and range-finding test) should help in selection of appropriate test concentration.

9. For the fish partial life-cycle test in weak estrogen, the highest concentration used in the definitive test should be selected to cause significant subchronic toxicity, because weak estrogens may exert subchronic toxicity and abnormal sex differentiation at similar concentration ranges at 60 dph (Seki et al, 2002 [submitted]). If possible, the range-finding test may be conducted under conditions (water quality, test system, animal loading) similar to those used for the definitive test (Annex 1). It should be conducted with newly hatched larvae, and focus on lethality over the course of at least a 7-d assay. For the test in strong estrogen or androgen, histological measurements are much more responsive than subchronic effects, therefore, the highest concentration used in the definitive test should be selected to cause considerable sex reversal. The lowest concentration should be a factor of 10- to 100-times lower than the highest concentration. The use of five concentrations in this test enables not only some dose-response relationships, but also providing the lowest-observed-effect concentration and no-observed-effect concentration which are necessary for risk assessment. At present, a minimum of four replicate test chambers (each containing 15 fish) is recommended per treatment in medaka (Yokota et al, 2000). It has been demonstrated that this sample size enables detection of statistically-significant differences for the majority of the endpoints in control fish versus animals treated with "model" EDCs, including bisphenol A (Yokota et al, 2000), 4-nonylphenol and 4-*tert*-octylphenol (preparing the manuscripts for publication in a journal). Based on this test, a minimum of 360 fish (15 fish in each of four replicates for five treatments, plus one control) is required per assay. This would, of course, increase when additional control treatments are required.

## Administration of test substance via water and analytical determination

10. It is recognized that the environmentally relevant routes of exposure are via the water or the diet. For water soluble chemicals, theoretically there are several options for delivery of test chemical to the fish via the water.

11. Test solutions of the chosen concentrations are prepared by dilution of a stock solution. The stock solution should preferably be prepared by simply mixing or agitating the test substance in the dilution water by using mechanical means (e.g. stirring or ultrasonication). Saturation columns can be used for achieving a suitable concentrated stock solution. It is recommended that, whenever possible, solvents not be used to generate stock solutions for conducting chemical exposures via the water.

12. In some cases it may be necessary to use solvents to generate stock solutions for aqueous testing; this could occur when a chemical is very insoluble, unstable in a saturator system, or so expensive/limited in availability that the use of saturators is not practical. Generally, examples of suitable solvents in fish chronic toxicity test are acetone, dimethylformamide, dimethylsulfoxide, ethanol, methanol and triethylene glycol. However, there are relatively few studies on the possible effects of these solvents on the fish endocrine system. Hence, it is essential that any test utilizing a carrier solvent include both solvent-exposed and non-exposed control.

- A new section on dietary exposure would be useful

### Analytical determination and measurements

13. During the test, the concentrations of the test substance are determined at appropriate intervals (e.g. every week in each of the test treatments).

14. During the test, the flow rates of diluent and stock solution should be checked at intervals accordingly (e.g. five times a week). It is recommended that the test substance concentrations fall within ± 20 % of nominal values (i.e. within the range 80-120 %). If the concentrations of the test substance are not maintained within ± 20 % of nominal values, it is preferable within ± 20 % of mean measured values. However, in case of some chemicals which can markedly accumulate in fish body, the concentrations of the test substance may be decreased depending on fish growth. In that case, it is recommended that renewal rate of the test solution in each chamber be raised.

15. It is recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance has been satisfactory maintained within ± 20 % of the nominal, the results can be based on nominal or measured values.

16. During the test, dissolved oxygen, pH, and temperature should be measured in one test vessel of all treatment groups and the controls. As a minimum, these measurements should be measured once a week through the exposure periods.

## Performance of the test

### Embryological phase

17. Exposure should be initiated shortly after fertilization (at the latest, < several hours post-fertilization). The 60 embryos employed for each treatment are randomly separated into four groups of 15 for testing in quadruplicate. Each group of embryos may be placed directly in the test chamber or in a cylindrical glass egg cup (e.g. diameter, 5 cm; depth, 10 cm), which is covered with stainless steel mesh (e.g. No. 32) on the bottom. If the egg cups are used, these may be swung vertically at a slow pace (e.g. 20-25 times/min.) in the test chamber. The developing embryos should be observed daily under a stereoscopic microscope. Any dead embryo should be discarded; live ones should be returned to the chamber. This procedure should be repeated until all the living embryos have hatched. Generally, hatchability and time to hatch of medaka in the controls at 24 °C are ≥ 90 % and 9-10 d, respectively (Yokota et al, 2000; Yokota et al, 2001a).

### Larval–juvenile phase

18. After hatching, the larvae should be fed an adequate amount of *Artemia* nauplii (<24 h after hatching) twice a day; some nauplii remain for at least 1 h after feeding. Daily observation should be made to examine mortality, abnormal behavior and appearance until 60-d posthatch, and dead fish should be removed as soon as possible.

19. At 60 dph, all fish should be taken out and be killed with an adequate anesthetic (e.g. FA-100, Tanabe Seiyaku, Osaka, Japan). Then external secondary sex characteristics should be observed under a stereoscopic microscope, based on the shape of the dorsal and anal fins. Notably, some chemicals cause skewed occurrence of secondary sex characteristics in medaka; for example, when exposed to androgen receptor agonist, methyltestosterone, the sex ratio estimated from secondary sex characteristics at 60-d posthatch can skew toward male (preparing the manuscripts for publication in a journal). Then the fish are drained on filter paper; body weight and total length should be measured.

20. After the measurement of the fish growth, five individuals from each of the four test chambers (i.e. 20 individuals from each treatment group) should be randomly selected and their gonads and livers should be removed and weighted for gonadal histology, GSI calculation (100 x gonad wt/body wt) and measurement of hepatic VTG concentration.

**Gonadal histology**

21. After removal of the gonads, those should be placed in an appropriate fixative (e.g. Bouin's solution, 4% formaldehyde or 1% glutaraldehyde) and embedded in paraffin or plastic after dehydrated. Fish should be sectioned longitudinally at 3-5 μm in thickness. Stepwise sectioning of the gonad ensures respective sections of the ensure gonad but does not guarantee detection of testis-ova (Gray et al, 1999a). It is not clear that how many sections should be prepared to satisfy both detecting abnormal tissues and be cost-effective. The section can be stained with hematoxylin and eosin, mounted with adequate agent (e.g. malinol, Muto pure chemicals, Tokyo, Japan) and then examined under a light microscope.

22. When exposed to estrogens in medaka, induction of testis-ova composed of both testicular germ cells and oocytes in the gonad has been well described in many in vivo studies (Egami, 1955; Yamamoto, 1965; Wester and Canton, 1986; Gary et al., 1997; Gronen et al, 1999; Gray et al, 1999a; Gray et al, 1999b; Metcalfe et al, 2001; Kang et al, 2002; Seki et al, 2002). This condition can be observed when exposed to estrogens from embryo to 60-d posthatch in medaka (Yokota et al, 2000; Yokota et al, 2001a; Seki et al, 2002 [submitted]). The rate of testis-ova induction can be increased dose-dependently, and the extent of oocyte-containing parenchyma in each testis-ova specimen can increase with increasing estrogen concentrations (Yokota et al, 2001a; Seki et al, 2002 [submitted]). Since small testis-ova appear similar in structure to enlarged spermatogonia, it is recommended that oocyte surrounded by an obvious band of follicular epithelium in the testis be testis-ova (Gray et al, 1999a). On occasion, abnormal connective tissues can develop in medaka when exposed to estrogens (Egami, 1955; Gray et al, 1999a; Kang et al, 2002; Seki et al, 2002). This condition can be observed when exposed to estrogens from fertilized eggs to 60-d posthatch in medaka (Yokota et al, 2001a; Seki et al, 2002 [submitted]). The ovary is evaluated based on relative number of previtellogenic, vitellogenic, and postvitellogenic oocytes. Strong estrogen, ethinylestradiol can inhibit ovarian development or cause many previtellogenic oocytes in female medaka (Scholz et al, 2000; Seki et al, 2002). We observed testis-ova condition when exposed to androgen, methyltestosterone from fertilized eggs to 60-d posthatch in medaka (preparing the manuscripts for publication in a journal). However, it was not clear whether this condition had been developed in genetically female fish by androgenic activity of methyltestosterone or in genetically male fish by estrogenic effect of aromatized chemical. We recommend nonaromatizable androgen may be suitable for androgenic reference compound. When exposed to anti-androgen, vinclozolin in medaka, induction of testis-ova has been reported (Kogar et al, 1999). Further research to characterize the effect of sex steroid antagonists (anti-androgen and anti-estrogen) on sexual differentiation and to verify the applicability of the present protocol to these chemicals is needed.

**Vitellogenin (VTG)**

23. The livers removed should be stored at −70°C until the VTG measurements. For the assay, they should be individually homogenized with enzyme-linked immunosorbent assay (ELISA) buffer and centrifuged, and the collected supernatants are used for measuring VTG in ELISA (Yokota et al, 2001b).

24. VTG is an estrogen-dependent glycoprotein which is usually only synthesized in liver of

mature females (Denslow et al, 1999). Therefore, when detected in the serum of male fish, VTG can be used as a biomaker of exposure to estrogenic chemicals in a variety of fish species, including medaka (Gronen et al, 1999; Metcalfe et al, 2000; Yokota et al, 2001b; Kang et al, 2002; Seki et al, 2002), fathead minnow (Ankley et al, 2001; Harries et al, 2000; Panter et al, 1998; Kramer et al, 1998; Parks et al, 1999; Tyler et al, 1999), and rainbow trout (Jobling. et al, 1996; Thorpe et al, 2000). Different methods are available to assess VTG production in fish; a measurement technique that is both relatively sensitive and specific is determination of protein concentrations in plasma or liver via ELISA or radioimmunoassay (RIA). For ELISA, monoclonal medaka VTG antibody and purified VTG protein from medaka are utilized (Yokota et al, 2001b). In fathead minnow, polyclonal fathead minnow VTG antibody and VTG protein have also been purified (Korte et al, 2000; Parks et al, 1999). Until now, the VTG induction assay using plasma have been developed and validated in fathead minnow (Tyler et al, 1999; Ankley et al, 2001) and rainbow trout (Thorpe et al, 2000). In small fish species such as medaka, collecting the plasma is technically difficult. Therefore hepatic VTG induction assay have been developed and validated in medaka (Yokota et al, 2001b).

## VALIDITY OF THE TEST

25. This test has not been performed extensively, but based on our study with medaka to date, we suggest the following criteria for the test acceptability:

- the dissolved oxygen concentration must be between 60 and 100 % of the air saturation value throughout the test;
- the water temperature must not differ by more than ± 2.0 °C between test chamber or between successive days at any time during the test;
- there should be more than 80 % survival of control animals after hatching through 60-d posthatch.

26. As experience is gained with this test, additional biological performance criteria can be incorporated.

## DATA REPORTING

### Statistical analysis

27. The primary goal of the data analyses are to calculate the No-Observed Effect Concentration and the Lowest Observed Effect Concentration based on potentially adverse population effects ($NOEC_{adverse}$ and $LOEC_{adverse}$, respectively). These calculations should address the effects on survival, gross development, growth and reproduction. Secondarily, the data for VTG, GSI and gonad histology may be used to calculate the No-Observed Effect Concentration and the Lowest Observed Effect Concentration based on mechanistic biomarker reponses ($NOEC_{biomarker}$ and $LOEC_{biomarker}$, respectively).

New paragraph. Different options are available for data analysis, we suggest the following flow acceptability; If carrier solvent is used in the test, appropriate analytical method may be used prior to data analysis to determine whether there are differences between the solvent control and control groups. If no differences are found, these groups are pooled for subsequent analysis. If differences are found, the control group without solvent is excluded from the subsequent analyses because of few studies on the possible effects of the solvents on the fish endocrine system. To identify potential endocrine activity by a chemical, biological responses except for sex ratios may be compared between in treatments versus controls groups using analysis of variance (ANOVA) followed by multiple comparison test (e.g. Dunnett's multiple comparison test). If the required

assumptions for parametric methods are not met, non-parametric test may be conducted. The data on sex ratios may be assessed by chi-squared analysis.

28. Any endpoints that are significantly impacted by the test chemical should be reported.

**Test report**

29. The test report must include the following:

Test substance:

- physical nature and, where relevant, physicochemical properties;
- chemical identification data.

Test species:

- scientific name, strain, source and method of collection of the fertilized eggs and subsequent handling.

Test conditions:

- photoperiod(s);
- test design (e.g. chamber size, number of test chambers and replicates, number of embryos per replicates);
- method of preparation of stock solutions and frequency of renewal (the solubilising agent and its concentration must be given, when used);
- the nominal test concentrations, the means of the measured values and their standard deviations;
- dilution water characteristics: pH, hardness, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon, suspended solids and other measurements made;
- water quality within test vessels, pH, temperature and dissolved oxygen concentration;
- detailed information on feeding (e.g. type of foods, source, amount given and frequency).

Results:

- data for the control (plus solvent control when used) and the treatment groups as follows, embryological development, hatching (hatchability and time to hatch), posthatch survival, growth (total length and body weight), sexual differentiation (secondary sex characteristics and gonadal histology), reproduction (fecundity and fertility), gonadosomatic index (GSI) and hepatic vitellogenin (VTG);
- statistical analysis used and results of this.

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## ANNEX 1

### Test Conditions For The Fish Partial Life-cycle Test Guideline

| | |
|---|---|
| 1. Recommended species | Medaka (*Oryzias latipes*)* |
| 2. Test type | Flow-through |
| 3. Water temperature | 24 ± 2°C |
| 4. Illumination quality | Fluorescent bulbs (wide spectrum) |
| 5. Light intensity | 10-20 μE/$M^2$/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels) |
| 6. Photoperiod | 16 h light, 8h dark |
| 7. Loading rate | <5g per L |
| 8. Test chamber size | 2.5 L |
| 9. Test solution volume | 1.8 L |
| 10. egg cup | (If possible,) glass cylinder (e.g. diameter, 5 cm; depth, 10 cm), with a base of stainless steel mesh (e.g. No. 32) |
| 11. Volume exchanges of test solutions | Minimum of 6 daily |
| 12. Age of test organisms | Fertilized eggs (< several hours post-fertilization) |
| 13. No. of fish per test vessel | 15 |
| 14. No. of treatments | ≥5 (plus appropriate controls) |

## ANNEX 1 continued

| | |
|---|---|
| 15. No. of vessels per treatment | 4 |
| 16. No. of fish per test concentration | 60 |
| 17. Feeding regime | Live *Artemia* nauplii (<24h after hatching) 2x daily |
| 18. Aeration | None unless DO reaches <5.0 mg/L |

| | |
|---|---|
| 19. Dilution water | Clean surface, well, reconstituted water, or dechlorinated tap water |
| 20. Dilution factor | ≤ 3.2 |
| 21. Chemical exposure duration | 180-days |
| 22. Primary endpoints | embryological development, hatching (hatchability and time to hatch), posthatch survival, growth (total length and body weight), sexual differentiation (secondary sex characteristics and gonadal histology), gonadosomatic index (GSI) and hepatic vitellogenin (VTG) |
| 23. Test acceptability | Dissolved oxygen ≥ 60 % of saturation; mean temperature of 24 ± 2°C; ≥ 80 % post-hatch survival of fish at 60-d posthatch in the controls |

* Other recommended species such as fathead minnow, sheepshead minnow and zebrafish is not mentioned here.

## ANNEX 2

## SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

| SUBSTANCE | CONCENTRATION |
|---|---|
| Particular matter | < 20 mg/L |
| Total organic carbon | <2 mg/L |
| Unionized ammonia | <1 µg/L |
| Residual chlorine | <10 µg/L |
| Total organophosphorus pesticides | <50 ng/L |
| Total organochlorine pesticides plus polychlorinated biphenyls | <50 ng/L |
| Total organic chlorine | <25 ng/L |

# The medaka (*Oryzias latipes*) Full Life-Cycle Test Guideline

**November 2002**
**Ministry of the Environment, Japan**

# Fish Full Life-Cycle Test Guideline

## OBJECTIVE

This test guideline describes a fish full life-cycle test that can be used to evaluate the potential chronic effects of chemicals on fish populations. The method gives primary emphasis to potential population relevant effects (namely, adverse impacts on survival, development, growth and reproduction) for the calculation of the No-Observed Effect Concentration ($NOEC_{adverse}$). These effect observations should be augmented by secondary mechanistic biomarker responses (namely, vitellogenin, gonad somatic index [GSI], and gonad histology). The method is applicable to a variety of chemicals, including endocrine disrupters and general toxicants. The medaka (*Oryzias latipes*) is a suitable species for use in this test guideline, however, other species such as fathead minnow (*Pimephales promales*), sheepshead minnow (*Cyprinodon variegatus*), three spined stickleback (*Gasterosteus aculatus*) and zebrafish (*Danio rerio*) are also suitable.

## GLOSSARY OF TERMS

dph = days post-hatch; GSI = gonad somatic index; VTG = vitellogenin

## INTRODUCTION

This test guideline describes a confirmatory test based on full life-cycle of fish over two generations to give data relevant to ecological risk assessment of endocrine disrupting chemicals (EDCs). Fish full life-cycle test for ecotoxicological studies has already been established by the U.S. Environmental Protection Agency (U.S. EPA) in fathead minnows (*Pimephales promelas*) and sheepshead minnows (*Cyprinodon variegatus*) (U.S. EPA, 1986). Present protocol in this guideline is derived from work on medaka (*Oryzias latipes*) in Chemicals Evaluation and Research Institute, Japan (CERI) based on the original protocol by the U.S. EPA. This test described in this guideline has been evaluated by testing with OECD reference compounds (4-*tert*-pentylphenol; Seki et al., 2002 [submitted], ethinylestradiol and methyltestosterone; preparing the manuscripts for publication in a journal) and conducted with the known EDCs (4-nonylphenol; Yokota et al., 2001a, 4-*tert*-octylphenol and 17β-estradiol; preparing the manuscripts for publication in a journal) for their hazard assessments. In principal, the approach is also applicable to other OECD fish species, notably fathead minnow (Länge et al., 2001), sheepshead minnow (*Cyprinodon variegatus*) (Zillioux et al, 2001), three-spined stickleback (*Gasterosteus aculatus*) Katsiadaki et al., (2002) and zebrafish (*Danio rerio*).

2. This test guideline measures several biological endpoints. Primary emphasis is given to potential adverse effects on population relevant parameters including survival, gross development, growth and reproduction. Secondarily, in order to provide secondary mechanistic information and provide linkage between results from other kinds of field and laboratory studies, where there is *a priori* evidence for a chemical having potential endocrine disrupter activity (eg androgenic or oestrogenic activity) then other useful information can be obtained by measuring vitellogenin (VTG) levels, gonad somatic index [GSI] and evaluating gonad histology.

## PRINCIPAL OF THE TEST

3. Overviews of the relevant test conditions in medaka are provided in Annex 1. For medaka,

exposure of parental fish ($F_0$) is normally initiated with healthy embryos within several hours post-fertilization. This test is conducted at a minimum of five chemical concentrations, as well as appropriate controls with a minimum of 60 embryos for each treatment separated into four groups. Chemical delivery can be via an aqueous route (with or without carrier solvents). Monitoring continues for up to 100 dph and includes embryological development, hatching (hatchability and time to hatch), posthatch survival, growth (total length and body weight), sexual differentiation (secondary sex differentiation and gonadal histology), reproduction (fecundity and fertility), hepatic VTG levels, and gonadal development (GSI). Eggs spawned from the $F_0$ fish at least 3 d of the $F_0$ exposure are also exposed to the test substance and examined for hatching, and the progeny generation ($F_1$) is examined for survival after hatching, as well as growth, sexual differentiation, and hepatic VTG levels, until 60 dph.

## DESCRIPTION OF THE METHOD

### Test animals and exposure system

#### Test animals

4. This test should be started with embryos within several hours post-fertilization from a ready supply of known-quality animals. Candidate species for this assay include medaka (*Oryzias latipes*), fathead minnow (*Pimephales promelas*), sheepshead minnow (*Cyprinodon variegatus*) and zebrafish (*Danio rerio*). Relevant modification is necessary for test fish species other than medaka. Field-collected fish generally should not be used to initiate cultures for the testing.

5. Parental fish for the spawning eggs subjected to exposure should be selected from a population of a single stock. The fish should be paired and acclimatized for at least two weeks prior to the test under conditions of water quality and illumination similar to those used in the test (Annex 1). Fish should be fed exclusively with *Artemia nauplii* (<24 h after hatching) twice a day.

#### Water

Any water in which the test species shows suitable long-term survival and growth may be used as test water. It should be of constant quality during the period of the test. In order to ensure that the dilution water will not unduly influence the test result (for example by complexation of test substance) or adversely affect the performance of the brood stock, samples should be taken at intervals for analysis. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl, $SO_4$), pesticides, total organic carbon and suspended solids should be made, for example, every six months where a dilution water is known to be relatively constant in quality. Some chemical characteristics of an acceptable dilution water are listed in Annex 2.

#### Exposure system

7. The design and materials used for the exposure system are optional. Glass, stainless steel, or other chemically inert material should be used for construction of the test system. For medaka, an exposure system is well suited as follows (Yokota et al., 2000, 2001a; Kang et al., 2002; Seki et al., 2002): The exposure system consists of a continuous-flow mini-diluter system (Benoit et al., 1982). Stock solution of the test chemical should be injected in this exposure system by an appropriate pump. The flow rate of the stock solution should be calibrated in accordance with analytical confirmation of the test solutions before the initiation of exposure, and checked volumetrically

periodically during the test. The test solution in each chamber is renewed adequately depending on the test chemical stability and water quality.

## Experimental Design

8. It is recommended to use a minimum of five chemical concentrations. Prior knowledge of the test substance (e.g. results of the tests at tier 1 and 2 in OECD testing scheme, in vitro receptor binding assay, subchronic toxicity, and range-finding test) should help in selection of appropriate test concentrations.

9. For the fish full life-cycle test in weak estrogens, the highest concentration used in the definitive test should be selected to cause significant subchronic toxicity, because weak estrogens may exert subchronic toxicity and abnormal sex differentiation at similar concentration ranges (Seki et al., 2002 [submitted]). If possible, the range-finding test may be conducted under conditions (water quality, test system, animal loading) similar to those used for the definitive test (Annex 1). For the test in strong estrogen or androgen, histological measurements are much more responsive than subchronic effects, therefore, the highest concentration used in the definitive test should be selected to cause considerable sex reversal. The lowest concentration should be a factor of 10- to 100-times lower than the highest concentration. The use of five concentrations in this test enables not only dose-response relationships, but also providing the lowest-observed-effect concentration and no-observed-effect concentration which are necessary for risk assessment. At present, a minimum of four replicate test chambers (each containing 15 fish) except the reproductive trial (a minimum of six replicate test chambers) is recommended per treatment in medaka (Yokota et al., 2001a). It has been demonstrated that this sample size enables detection of statistically-significant differences for the majority of the endpoints in control fish versus animals treated with "model" EDCs, including 4-nonylphenol (Yokota et al., 2001a), 4-*tert*-pentylphenol (Seki et al., 2002 [submitted]), ethinylestradiol, methyltestosterone, 4-*tert*-octylphenol and 17β-estradiol (preparing the manuscripts for publication in a journal). Based on this test, a minimum of 360 fish (15 fish in each of four replicates for five treatments, plus one control) is required per assay. This would, of course, increase when additional control treatments are required.

## Administration of test substance via water and analytical determination

10. It is recognized that the environmentally relevant routes of exposure are via the water or the diet. For water soluble chemicals, theoretically there are several options for delivery of test chemical to the fish via the water.

11. Test solutions of the chosen concentrations are prepared by dilution of a stock solution. The stock solution should preferably be prepared by simply mixing or agitating the test substance in the dilution water by using mechanical means (e.g. stirring or ultrasonication). Saturation columns can be used for achieving a suitable concentrated stock solution. It is recommended that, whenever possible, solvents not be used to generate stock solutions for conducting chemical exposures via the water.

12. In some cases it may be necessary to use solvents to generate stock solutions for aqueous testing; this could occur when a chemical is very insoluble, unstable in a saturator system, or so expensive/limited in availability that the use of saturators is not practical. Generally, examples of suitable solvents in fish chronic toxicity test are acetone, dimethylformamide, dimethylsulfoxide, ethanol, methanol and triethylene glycol. However, there are relatively few studies on the possible effects of these solvents on the fish endocrine system. Hence, it is essential that any test utilizing a carrier solvent include both solvent-exposed and non-exposed controls.

- A new section on dietary exposure would be useful

### Analytical determination and measurements

13. During the test, the concentrations of the test substance are determined at appropriate intervals (e.g. every other week in each of the test treatments).

14. During the test, the flow rates of diluent and stock solution should be checked at intervals accordingly (e.g. five times a week). It is recommended that the test substance concentrations fall within ± 20 % of nominal values (i.e. within the range 80-120 %). If the concentrations of the test substance are not maintained within ± 20 % of nominal values, it is preferable within ± 20 % of mean measured values. However, in case of some chemicals which can markedly accumulate in fish body, the concentrations of the test substance may be decreased depending on fish growth. In that case, it is recommended that renewal rate of the test solution in each chamber be raised.

15. It is recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance has been satisfactory maintained within ± 20 % of the nominal, the results can be based on nominal or measured values.

16. During the test, dissolved oxygen, pH, and temperature should be measured in one test vessel of all treatment groups and the controls. As a minimum, these measurements should be measured once a week through the exposure periods.

## PERFORMANCE OF THE TEST

### Embryological phase

17. Exposure should be initiated shortly after fertilization (at the latest, < several hours post-fertilization). The 60 embryos employed for each treatment are randomly separated into four groups of 15 for testing in quadruplicate. Each group of embryos may be placed directly in the test chamber or in a cylindrical glass egg cup (e.g. diameter, 5 cm; depth, 10 cm), which is covered with stainless steel mesh (e.g. No. 32) on the bottom. If the egg cups are used, these may be swung vertically at a slow pace (e.g. 20-25 times/min.) in the test chamber. The developing embryos should be observed daily under a stereoscopic microscope. Any dead embryo should be discarded; live ones should be returned to the chamber. This procedure should be repeated until all the living embryos have hatched. Generally, hatchability and time to hatch of medaka in the controls at 24 °C are ≥ 90 % and 9-10 d, respectively (Yokota et al, 2000; Yokota et al, 2001a).

### Larval-juvenile phase

18. After hatching, the larvae should be fed an adequate amount of *Artemia* nauplii (<24 h after hatching) twice a day; some nauplii remain for at least 1 h after feeding. Daily observation should be made to examine mortality, abnormal behavior and appearance until 60-d posthatch, and dead fish should be removed as soon as possible.

19. At 60-d posthatch, 20 individuals from each treatment group (e.g. five individuals from each of the four test chambers) should be randomly removed and euthanized with an adequate anesthetic (e.g. FA-100, Tanabe Seiyaku, Osaka, Japan). The fish should be observed for external secondary sex characteristics based on the shape of the dorsal and anal fins under a stereoscopic microscope.

Notably, some chemicals cause skewed occurrence of secondary sex characteristics in medaka; for example, when exposed to androgen receptor agonist, methyltestosterone, the sex ratio estimated from secondary sex characteristics at 60-d posthatch can skew toward male (preparing the manuscripts for publication in a journal). Then the fish are drained on filter paper; body weight and total length should be measured.

**Gonadal histology**

20. After observation of secondary sex characteristics and measuring growth, the fish should be devoted for gonadal histology. The fish should be placed in an appropriate fixative (e.g. Bouin's solution, 4% formaldehyde or 1% glutaraldehyde) and embedded in paraffin or plastic after dehydrated. Fish should be sectioned longitudinally at 3-5 μm in thickness. Stepwise sectioning of the gonad ensures respective sections of the ensure gonad but does not guarantee detection of testis-ova (Gray et al, 1999a). It is not clear that how many sections should be prepared to satisfy both detecting abnormal tissues and be cost-effective. The section can be stained with hematoxylin and eosin, mounted with adequate agent (e.g. malinol, Muto pure chemicals, Tokyo, Japan) and then examined under a light microscope.

21. When exposed to estrogens in medaka, induction of testis-ova composed of both testicular germ cells and oocytes in the gonad has been well described in many in vivo studies (Egami, 1955; Yamamoto, 1965; Wester and Canton, 1986; Gary et al, 1997; Gronen et al, 1999; Gray et al, 1999a; Gray et al, 1999b; Metcalfe et al, 2001; Kang et al, 2002; Seki et al, 2002). This condition can be observed when exposed to estrogens from embryo to 60-d posthatch in medaka (Yokota et al, 2000; Yokota et al, 2001a; Seki et al, 2002 [submitted]). The rate of testis-ova induction can be increased dose-dependently, and the extent of oocyte-containing parenchyma in each testis-ova specimen can increase with increasing estrogen concentrations (Yokota et al, 2001a; Seki et al, 2002 [submitted]). Since small testis-ova appear similar in structure to enlarged spermatogonia, it is recommended that oocyte surrounded by an obvious band of follicular epithelium in the testis be testis-ova (Gray et al, 1999a). On occasion, abnormal connective tissues can develop in medaka when exposed to estrogens (Egami, 1955; Gray et al, 1999a; Kang et al, 2002; Seki et al, 2002). This condition can be observed when exposed to estrogens from fertilized eggs to 60-d posthatch in medaka (Yokota et al, 2001a; Seki et al, 2002 [submitted]). The ovary is evaluated based on relative number of previtellogenic, vitellogenic, and postvitellogenic oocytes. Strong estrogen, ethinylestradiol can inhibit ovarian development or cause many previtellogenic oocytes in female medaka (Scholz et al, 2000; Seki et al, 2002). We observed testis-ova condition when exposed to androgen, methyltestosterone from fertilized eggs to 60-d posthatch in medaka (preparing the manuscripts for publication in a journal). However, it was not clear whether this condition had been developed in genetically female fish by androgenic activity of methyltestosterone or in genetically male fish by estrogenic effect of aromatized chemical. We recommend nonaromatizable androgen may be suitable for androgenic reference compound. When exposed to anti-androgen, vinclozolin in medaka, induction of testis-ova has been reported (Kogar et al, 1999). Further research to characterize the effect of sex steroid antagonists (anti-androgen and anti-estrogen) on sexual differentiation and to verify the applicability of the present protocol to these chemicals is needed.

**Reproductive trial**

22. At 70-d posthatch, the sex of the surviving fish should be distinguished by their external secondary sex characteristics, and six mating pairs from each treatment and the controls should be selected to examine fecundity and fertility. If sex ratio is skewed toward male or female, no pairs can be selected. Each pair should be assigned to a test chamber and exposed until 100-d posthatch. The water temperature may be increased from 24 °C to 28 °C to stimulate spawning at 70-d

posthatch. The eggs spawned from each female should be counted, and assessed for viability microscopically for 30 consecutive days (71-100 d posthatch). Using weak estrogen, this test protocol has been verified to detect significant reproductive impairment (Seki et al, 2002 [submitted]).

23. On the day after the end of the reproductive examination (101-d posthatch), all pairs examined should be removed from their chambers and euthanized. If possible, the number of androgen-dependent papillary processes on the anal fin in males may be counted under a stereoscopic microscope. After body weights are measured, the gonads and livers should be removed and weighed for GSI calculation (100 x gonad wt/body wt) and measurement of hepatic VTG concentrations. For example, typical GSI values for reproductively active medaka at about 100-d posthatch range from 7 to 10 % for females, and 0.9 to 1.3 % for males (Yokota et al, 2001a; Seki et al, 2002 [submitted]). Nonylphenol that reduce fertility also can inhibit the maturation of the testis (Yokota et al, 2001a).

**Vitellogenin (VTG)**

24. The livers removed should be stored at –70 °C until the VTG measurements. For the assay, they should be individually homogenized with enzyme-linked immunosorbent assay (ELISA) buffer and centrifuged, and the collected supernatants are used for measuring VTG in ELISA (Yokota et al, 2001b).

25. VTG is an estrogen-dependent glycoprotein which is usually only synthesized in the liver of mature females (Denslow et al, 1999). Therefore, when detected in the serum of male fish, VTG can be used as a biomaker of exposure to estrogenic chemicals in a variety of fish species, including medaka (Gronen et al, 1999; Metcalfe et al, 2000; Yokota et al, 2001b; Kang et al, 2002; Seki et al, 2002), fathead minnow (Ankley et al, 2001; Harries et al, 2000; Panter et al, 1998; Kramer et al, 1998; Parks et al, 1999; Tyler et al, 1999), and rainbow trout (Jobling et al, 1996; Thorpe et al, 2000). Different methods are available to assess VTG production in fish; a measurement technique that is both relatively sensitive and specific is determination of protein concentrations in plasma or liver via ELISA or radioimmunoassay (RIA). For ELISA, monoclonal medaka VTG antibody and purified VTG protein from medaka are utilized (Yokota et al, 2001b). In fathead minnow, polyclonal fathead minnow VTG antibody and VTG protein have also been purified (Korte et al, 2000; Parks et al, 1999). Until now, the VTG induction assay using plasma have been developed and validated in fathead minnow (Tyler et al, 1999; Ankley et al, 2001) and rainbow trout (Thorpe et al, 2000). In small fish species such as medaka, collecting the plasma is technically difficult. Therefore hepatic VTG induction assay have been developed and validated in medaka (Yokota et al, 2001b).

**$F_1$ embryo phase**

26. Eggs spawned by females in the last 3 d of the reproductive trial (98 to 100-d posthatch) should be subjected to the exposure study of the progeny generation ($F_1$). To evaluate their hatchability and time to hatch, the fertilized eggs from each spawning in treatment groups and in the controls should be collected and exposed until hatching, under the same flow-through conditions as those used for the $F_0$ generation.

**$F_1$ larval–juvenile phase**

27. The newly hatched larvae in the treatments and the controls should be randomly transferred to four test chambers in each treatment and kept in the chambers until the last hatching. Then 15

larvae in each chamber should randomly be selected (60 larvae in each treatment group). Then they may be treated until 60-d posthatch in the same flow-through system for the $F_0$ generation. The fish should be checked daily until 60-d posthatch for mortality, abnormal behavior, and appearance. Dead fish should be removed as soon as possible. At 60-d posthatch, the external secondary sex characteristics of all the surviving $F_1$ fish should be observed, and their weights and lengths should be measured after overdosing with anesthetic. They should be fixed and prepared for the gonadal histology. Some estrogenic chemicals can have adverse effects at lower concentrations in the progeny than in their parent generations (Yokota et al, 2001a; Seki et al, 2002 [submitted]). Further work to elucidate the enhanced response in the progeny by transgenerational exposure need to be investigated.

## VALIDITY OF THE TEST

28. This test has not been performed extensively, but based on our study with medaka to date, we suggest the following criteria for the test acceptability:

- the dissolved oxygen concentration must be between 60 and 100 % of the air saturation value throughout the test;
- the water temperature must not differ by more than ± 2.0 °C between test chamber or between successive days at any time during the test;
- there should be more than 80 % survival of control animals after hatching through 60-d posthatch in each $F_0$ and $F_1$ generation.

29. As experience is gained with this test, additional biological performance criteria can be incorporated.

## DATA REPORTING

### Statistical analysis

30. The primary goal of the data analyses are to calculate the No-Observed Effect Concentration and the Lowest Observed Effect Concentration based on potentially adverse population relevant effects ($NOEC_{adverse}$ and $LOEC_{adverse}$, respectively). These calculations should address the effects on survival, gross development, growth and reproduction. Secondarily, the data for VTG, GSI and gonad histology may be used to calculate the No-Observed Effect Concentration and the Lowest Observed Effect Concentration based on mechanistic biomarker reponses ($NOEC_{biomarker}$ and $LOEC_{biomarker}$, respectively).

New paragraph. Different options are available for data analysis, we suggest the following flow acceptability; If carrier solvent is used in the test, appropriate analytical method may be used prior to data analysis to determine whether there are differences between the solvent control and control groups. If no differences are found, these groups are pooled for subsequent analysis. If differences are found, the control group without solvent is excluded from the subsequent analyses because of few studies on the possible effects of the solvents on the fish endocrine system. To identify potential endocrine activity by a chemical, biological responses except for sex ratios may be compared between in treatments versus controls groups using analysis of variance (ANOVA) followed by multiple comparison test (e.g. Dunnett's multiple comparison test). If the required assumptions for parametric methods are not met, non-parametric test may be conducted. The data on sex ratios may be assessed by chi-squared analysis.

31. Any endpoints that are significantly impacted by the test chemical should be reported.

## Test report

32. The test report must include the following:

Test substance:

- physical nature and, where relevant, physicochemical properties;
- chemical identification data.

Test species:

- scientific name, strain, source and method of collection of the fertilized eggs and subsequent handling.

Test conditions:

- photoperiod(s);
- test design (e.g. chamber size, number of test chambers and replicates, number of embryos per replicates);
- method of preparation of stock solutions and frequency of renewal (the solubilising agent and its concentration must be given, when used);
- the nominal test concentrations, the means of the measured values and their standard deviations;
- dilution water characteristics: pH, hardness, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon, suspended solids and other measurements made;
- water quality within test vessels, pH, temperature and dissolved oxygen concentration;
- detailed information on feeding (e.g. type of foods, source, amount given and frequency).

Results:

- Data for the control (plus solvent control when used) and the treatment groups as follows, embryological development, hatching (hatchability and time to hatch), posthatch survival, growth (total length and body weight), sexual differentiation (secondary sex characteristics and gonadal histology), reproduction (fecundity and fertility), gonadosomatic index (GSI) and hepatic vitellogenin (VTG);
- Statistical analysis used and results of this.

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## ANNEX 1

### Test Conditions For The Fish Full Life-cycle Test Guideline

| | |
|---|---|
| 1. Recommended species | Medaka (*Oryzias latipes*)* |
| 2. Test type | Flow-through |
| 3. Water temperature | 24 ± 2 °C (28 ± 2 °C at reproductive phase) |
| 4. Illumination quality | Fluorescent bulbs (wide spectrum) |
| 5. Light intensity | 10-20 μE/$M^2$/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels) |
| 6. Photoperiod | 16 h light, 8h dark |
| 7. Loading rate | <5g per L |
| 8. Test chamber size | 2.5 L |
| 9. Test solution volume | 1.8 L |
| 10. egg cup | (If possible,) glass cylinder (e.g. diameter, 5 cm; depth, 10 cm), with a base of stainless steel mesh (e.g. No. 32) |
| 11. Volume exchanges of test solutions | Minimum of 6 daily |
| 12. Age of test organisms | Fertilized eggs (< several hours post-fertilization) |
| 13. No. of fish per test vessel | 15 (from embryo to 69-d posthatch)<br>1F + 1M (reproductive trial from 70 to 100-d posthatch) |
| 14. No. of treatments | ≥5 (plus appropriate controls) |

**ANNEX 1 continued**

| | |
|---|---|
| 15. No. of vessels per treatment | 4 (6 at reproductive trial) |
| 16. No. of fish per test concentration | 60 (6F + 6M at reproductive trial) |
| 17. Feeding regime | Live *Artemia* nauplii (< 24 h after hatching) 2x daily |
| 18. Aeration | None unless DO reaches <5.0 mg/L (<4.7 mg/L at reproductive trial) |

| | |
|---|---|
| 19. Dilution water | Clean surface, well, reconstituted water, or dechlorinated tap water |
| 20. Dilution factor | ≤ 3.2 |
| 21. Chemical exposure duration | 180-days |
| 22. Primary endpoints | $F_0$ and $F_1$: embryological development, hatching (hatchability and time to hatch), posthatch survival, growth (total length and body weight), sexual differentiation (secondary sex characteristics and gonadal histology) and hepatic vitellogenin (VTG)<br>$F_0$: reproduction (fecundity and fertility) and gonadosomatic index (GSI) |
| 23. Test acceptability | Dissolved oxygen ≥ 60 % of saturation; mean temperature of 24 ± 2 °C (28 ± 2 °C at reproductive phase); ≥ 80 % post-hatch survival of fish at 60-d posthatch in the controls |

* Other recommended species such as fathead minnow, sheepshead minnow and zebrafish is not mentioned here.

## ANNEX 2

## SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

| SUBSTANCE | CONCENTRATION |
|---|---|
| Particular matter | < 20 mg/L |
| Total organic carbon | <2 mg/L |
| Unionized ammonia | <1 µg/L |
| Residual chlorine | <10 µg/L |
| Total organophosphorus pesticides | <50 ng/L |
| Total organochlorine pesticides plus polychlorinated biphenyls | <50 ng/L |
| Total organic chlorine | <25 ng/L |

# *Appendix II*

Ⅰ.：**Effect of ethinylestradiol on the reproduction and induction of vtellogenin and tests-ova in Medaka(*Oryzias Latipes*)**

Ⅱ.：**Life-cycle toxicity of 4-nonylphenol to Medaka (*Oryzias Latipes*)**

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# EFFECT OF ETHINYLESTRADIOL ON THE REPRODUCTION AND INDUCTION OF VITELLOGENIN AND TESTIS-OVA IN MEDAKA (*ORYZIAS LATIPES*)

MASANORI SEKI,*† HIROFUMI YOKOTA,† HARUKI MATSUBARA,† YUKINARI TSURUDA,‡ MASANOBU MAEDA,† HIROSHI TADOKORO,† and KUNIO KOBAYASHI†

†Chemicals Evaluation and Research Institute, Japan, 19-14, Chuo-machi, Kurume-shi, Fukuoka, 830-0023, Japan
‡Faculty of Agriculture, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-8581, Japan

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**Abstract**—Mature medaka (*Oryzias latipes*) were exposed to ethinylestradiol ($EE_2$) at measured concentrations of 32.6, 63.9, 116, 261, and 488 ng/L for 21 d under flow-through conditions. Effects on reproductive success of the fish as well as on gonadal condition and vitellogenin (VTG) induction were assessed. A significant decrease in fecundity was observed only at the highest $EE_2$ concentration, whereas hepatic VTG concentration in males was increased at concentrations of 63.9 ng/L and greater. In addition, an intersex condition (testis-ova) of the gonad was observed in male medaka exposed to $EE_2$ concentrations of ≥63.9 ng/L. Overall, these results indicate that the lowest-observed-effect concentrations of $EE_2$ based on reproduction versus induction of both VTG and testis-ova in the medaka were 488 and 63.9 ng/L, respectively. Thus, the physiological and histological measurements were approximately eightfold more responsive to the $EE_2$ exposure than were overt reproductive effects. This suggests that the elevated VTG levels and testis-ova may not be definitely responsible for reproductive impairment of the fish.

**Keywords**—Ethinylestradiol Medaka Reproduction Testis-ova Vitellogenin

## INTRODUCTION

During the past two decades, global concern about environmental pollution of some chemicals mimicking the effects of steroid hormones (particularly estrogens) has increased because of the possibility for adverse effects on sexual development and reproduction in wildlife [1,2]. Many reports have appeared regarding estrogen-related developmental and physiological effects, such as a high prevalence of intersex gonads and/or induction of the female-specific protein (vitellogenin [VTG]) in male fish inhabiting aquatic environments receiving effluents from sewage treatment works [3–8]. In addition, several studies have determined that the estrogenic activity of sewage treatment works effluents may be due to the presence of natural and synthetic estrogens in the effluents [9–11].

A synthetic estrogen, 17α-ethinylestradiol ($EE_2$), induces VTG and intersex gonads (i.e., testis-ova) in male fish at extremely low concentrations. Purdom et al. [3] found that a 10-d immersion exposure of male rainbow trout (*Oncorhynchus mykiss*) to $EE_2$ induced the VTG response at doses ranging from 0.1 to 10 ng/L, and those authors suggested that $EE_2$ could be one of the major contributors to the estrogenic response observed during exposure to sewage treatment works effluents. Metcalfe et al. [12] reported that the lowest-observed-effect concentration (LOEC) for testis-ova induction in male medaka exposed to $EE_2$ from hatching to approximately 100-d posthatch was 0.1 ng/L, which was well within the concentrations detected in many sewage effluents. However, it is not clear to what extent the induction of VTG and testis-ova gonads in wild male fish exposed to $EE_2$ causes reproductive impairment and subsequent decrease in their populations.

A short-term fish reproduction assay has been developed in fathead minnow (*Pimephales promelas*) by Harries et al. [13] and by Ankley et al. [14]. This assay is useful for detecting the effects of endocrine-disrupting chemicals on fish reproduction and for relating these effects to physiological and histological measurements. This protocol may be applicable to other fish species as well.

Medaka is an ideal model organism for studying reproductive toxicity because of its short life cycle (maturation within six to eight weeks) [15]. This species can spawn 10 to 40 eggs daily under an optimal photoperiod, temperature, and food regime [15]. Medaka is also a suitable test organism for investigations of developmental abnormalities of gonads, because this species develops an intersex condition (testis-ova) in the gonads even if exposed to estrogenic substances after the period of sex differentiation [16,17]. Moreover, VTG induction has already been observed in male medaka after treatment with environmental estrogens [18,19]. Therefore, toxicity testing with mature medaka offers an integrative approach for determining the relationship between reproductive impairment and gonadal intersexuality as well as for determining physiological alterations, such as VTG induction in males exposed to environmental estrogens.

The present study was conducted to elucidate the effects of $EE_2$ at various concentrations on reproduction, gonadal development, and VTG induction in adult medaka and to assess whether physiological and histological measures (i.e., induction of VTG and testis-ova) correlate with reproductive impairment. We exposed mating pairs of medaka to $EE_2$ for 21 d under flow-through conditions and examined the fecundity, fertility, mortality, abnormal responses, hepatic VTG induction, and gonadal histology of these animals.

## MATERIALS AND METHODS

### *Test fish*

Medaka (*Oryzias latipes*) used in this study were originally purchased from a local fish farm in Kumamoto, Japan, and a

* To whom correspondence may be addressed (seki-masanori@ceri.jp).

breeding stock of these medaka has been maintained in our laboratory for at least three years. Medaka selected for this study were approximately six months posthatch and were fully mature (mean body wt, 356 ± 46 mg SD; mean total length, 33 ± 2 mm SD). Each of 60 breeding medaka pairs was acclimated for three weeks in a 1-L chamber with flow-through dechlorinated tap water (pH 7.2–7.6; hardness, 44.0–61.0 mg $CaCO_3$/L) at 24 ± 1°C. During the acclimation, the fish were placed under a summer photoperiod (16:8-h light:dark) and fed exclusively with *Artemia* nauplii (<24 h after hatching) twice a day. During the last week of acclimation, eggs spawned from each pair were collected, counted, and checked for fertility daily. At the start of the exposure, we selected 36 pairs to use in this study based on their superior fecundity and fertility during the last acclimation week. These 36 pairs were allocated to each of six treatment groups (i.e., six pairs/treatment) according to total egg numbers as determined by a stratified sampling method conducted manually on Microsoft Excel 97 (Microsoft, Tokyo, Japan).

*Test chemicals*

Ethinylestradiol (100% pure) was obtained from Wako Pure Chemical (Tokyo, Japan). An $EE_2$ aqueous stock solution was prepared as follows: $EE_2$ was dissolved in acetone to prepare a 100 mg/L solution. Next, 5 ml of this solution was added to a 500-ml Erlenmeyer flask (Iwaki Glass, Chiba, Japan), and the solvent was then evaporated to dryness under a stream of nitrogen. After evaporation, 500 ml of dechlorinated tap water was added to the flask and stirred for approximately 24 h by using a magnetic stirrer to dissolve the $EE_2$ in the water. An appropriate volume of this aqueous solution was diluted with dechlorinated tap water to prepare a $EE_2$ stock solution of 20 μg/L.

*Exposure conditions*

The exposure system consisted of a continuous-flow mini-diluter system as described by Benoit et al. [20]. In this exposure system, the $EE_2$ stock solution was delivered to a mixing vessel by a glass-plunger pump (EYELA GMW-A; Tokyo Rikakikai, Tokyo, Japan) and diluted with dechlorinated tap water before entering cylindrical glass test chambers (diameter, 15.0 cm; depth, 17.5 cm). The flow rate of the stock solution was calibrated based on analytical confirmation of the test solutions before the initiation of exposure. The test chamber was designed to contain approximately 1.8 L of the test solution by maintaining an overflow level of 10 cm, renewing 12 times a day. The flow rate of the stock solution and dechlorinated tap water was checked volumetrically once a day.

To determine the exposure concentration of $EE_2$, a 14-d preliminary study was performed using paired medaka exposed to $EE_2$ concentrations ranging from 10.0 to 500 ng/L. In the experiment, the effective concentrations on fecundity and fertility were 500 and >500 ng/L, respectively. From this result, we selected 31.3, 62.5, 125, 250, and 500 ng/L as the nominal concentrations of $EE_2$ for this study. Six pairs allocated to each treatment were divided among three test chambers separated into two compartments with stainless-steel mesh (no. 20). The eggs spawned from each pair were collected, counted, and microscopically evaluated daily for fertility for 21 d during the exposure. Fertilized eggs were judged by the presence of a perivitelline space located between the chorion and plasma membrane under a light microscope. The paired medaka also were observed daily for survival and abnormal responses. Any dead fish were removed as soon as possible. The photoperiod was 16:8-h light:dark. Water temperature was maintained at 24 ± 1°C. The test equipment and chamber were cleaned at least once a week to prevent any dense bacterial or algal growth. Residual food and feces in the test chamber were removed daily.

*Histological examination of the gonads*

At the end of the exposure, all surviving fish were anesthetized in a 2,000-fold–diluted FA-100 solution (Tanabe Seiyaku, Osaka, Japan). Their livers were removed for VTG measurement (see next paragraph), and their gonads also were removed and subjected to gonadal histology. The gonads were fixed in Bouin's solution and then embedded in Technovit 7100 (Heraeus Kuizer, Wehrheim, Germany) and cut into serial sagittal sections (thickness, 5 μm) with a microtome. The sections were stained with hematoxylin and eosin, mounted with Eukitt (O. Kinder, Freiburg, Germany), and then examined under a light microscope.

*Hepatic VTG concentrations*

The livers were weighed and stored at −70°C until VTG assay. For VTG measurement, livers were individually homogenized in 200 μl of ice-cold enzyme-linked immunosorbent assay buffer (10 mM phosphate-buffered saline, pH 7.1, containing 0.05% v/v Tween-20 [Cayman Chemical, Ann Arbor, MI, USA] and 1 mg/ml of albumin from bovine serum) by using a glass, handheld homogenizer on ice. The homogenized samples were centrifuged at 13,000 *g* for 10 min at 5°C, and the supernatants were collected and frozen at −70°C until enzyme-linked immunosorbent assay. Hepatic VTG concentrations were measured by the method of Yokota et al. [21], which had been validated for measuring VTG in medaka, using a sandwich enzyme-linked immunosorbent assay involving anti-medaka VTG antibodies.

*Determination of $EE_2$ concentrations in test solutions*

The concentrations of $EE_2$ in the test solutions were measured once a week during the exposure. Equal volumes of test solutions taken from all test chambers of each treatment group were pooled, and 500 ml of the solutions were applied individually to preconditioned MEGAbont ElutC18 solid-phase extraction cartridges (Varian, Harbor City, CA, USA). After rinsing each cartridge with 10 ml of water:methanol (5:2, v/v), the $EE_2$ was eluted with 5 ml of ethylacetate:methanol (5:1, v/v), and the eluate was dried under a stream of nitrogen at 40°C. The residue was dissolved in 1 ml of methanol, and then the mixture was shaken for 30 s and centrifuged for 2 min at 3,000 rpm. The solvent was evaporated under a stream of nitrogen at 40°C. The residue was redissolved in 1 ml of a methanol:acetonitrile:water (3:1:3, v/v/v) solution containing 100 ng/ml of estradiol-d4 as an internal standard and then shaken for 30 s and centrifuged (3,000 rpm, 2 min). Next, the solution was filtered through a 0.1-μm Ultrafree-MC Filter Unit (Millipore, Tokyo, Japan), and the filtrate was analyzed by high-performance liquid chromatography with a Hewlett-Packard HP-1100 (Avondale, PA, USA) equipped with L-column octadecylsilylated silica gels (length, 150 mm; inner diameter, 2.1 mm; particle size, 5 μm; Chemicals Evaluation and Research Institute, Tokyo, Japan) at 40°C. Each 20-μl sample was injected into the chromatograph and eluted in an isocratic mode at a flow rate of 0.2 ml/min in a mobile phase of methanol:acetonitrile:water (3:1:3, v/v/v) and at 0.02 ml/

min in a postcolumn solution of triethylamine:methanol (1:9, v/v). After being eluted from the column, the sample was analyzed with a Quattro-LC mass spectrometer (Micromass, Beverly, MA, USA) equipped with a turbo ion spray source operated in the negative-ion ionization mode. The cone voltage was 60 V for $EE_2$ and 50 V for the internal standard; the collision energy was 40 eV. The temperature of the ion source was 120°C, and that for desolvation was 350°C. Ions of $EE_2$ were monitored from 295 to 145 m/z, and the internal standard was monitored from 275 to 146 m/z.

### *Statistical analysis*

All statistical analyses were carried out with SPSS Base 8.0J (SPSS, Tokyo, Japan). The experimental data, except for the hepatic VTG concentrations, were checked for homogeneity of variances across treatments by using Levene's test. When the assumptions were met, the data were subjected to one-way analysis of variance followed by Dunnett's multiple-comparison test. When no homogeneity was observed, the nonparametric Kruskal-Wallis test was used, followed by the Mann–Whitney *U* test with Bonferroni's adjustment. The data on fertility was stabilized for variance by applying arcsine transformation before the statistical analysis. The data on hepatic VTG concentrations were assessed by the Mann–Whitney *U* test with Bonferroni's adjustment. Data for VTG concentrations lower than the determination limit were transformed to half the value of the determination limit for the analysis [22]. Differences were considered to be significant at $p \leq 0.05$; however, Bonferroni's $p$ was used in nonparametric tests.

## RESULTS

### *Concentration of $EE_2$ in test solution*

The means (coefficients of variation) of measured $EE_2$ concentrations in the test solutions during the exposure period were 32.6 (5.9), 63.9 (4.2), 116 (4.8), 261 (5.1), and 488 (9.3) ng/L, indicating that the nominal concentrations of $EE_2$ in the respective treatments remained consistent throughout the exposure period. The $EE_2$ concentration in the control treatment was less than the determination limit (2.0 ng/L) in all analyses. The following results are expressed as average values of each measured concentration.

### *Reproduction*

During the three weeks of exposure, the fecundity of paired medaka decreased with increasing $EE_2$ concentration higher than 116 ng/L, resulting in a significant difference ($p = 0.006$) at 488 ng/L compared to controls (Fig. 1A). In the 488 ng/L treatment group, the fecundity of one of the six pairs dramatically decreased after 6 d of exposure, and thereafter, this mating pair produced no eggs. However, no significant difference was observed in the fecundity during the first week of the exposure period (Fig. 1B). The reduction in fecundity of the other pairs in this treatment group occurred after the second week of exposure, resulting in a significant decrease in the fecundity during the second ($p = 0.003$) and third ($p = 0.002$) weeks of the exposure period (Fig. 1C and D). The mean fecundity of the six pairs in the 488 ng/L treatment during the three-week exposure was reduced to approximately 50% of that of the control pairs (Fig. 1A). Many parent medaka, especially males, in this treatment exhibited symptoms such as light body color, swollen abdomen, and/or epidermal hemorrhage. Five of the 12 parents died within a few days after cessation of spawning. Notably, four of the five dead

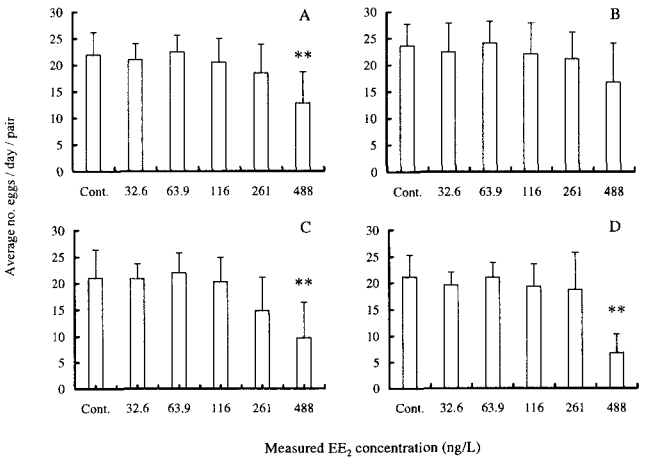


Fig. 1. Overall fecundity (**A**) and its weekly changes (first [**B**], second [**C**], and third [**D**] weeks) in the average number of spawned eggs per day per pair ($n = 6$; except $n = 5$ for the 261 ng/L group and $n = 2$ for the 488 ng/L group in **D**). Error bars represent the standard deviation of the mean. Asterisks (**) denote significant differences from that of the controls at $p = 0.006$ (**A**), $p = 0.003$ (**C**), and $p = 0.002$ (**D**). Cont. = control; $EE_2$ = ethinylestradiol.

parents were male, and all of them died after the abdomen began to swell (Table 1). In addition, the fertility of eggs spawned from the pairs in the 488 ng/L treatment decreased after the second week of exposure, but no statistically significant difference was found in the mean fertility throughout the exposure period (Fig. 2). An apparent reduction in fertility was associated with cessation of spawning in the pairs that displayed the above-described symptoms; therefore, few data were available regarding fertility in the 488 ng/L treatment group.

In the 261 ng/L treatment group, one of the six pairs completely stopped breeding after 9 d of exposure, and another pair, in which the male displayed a light body color and epidermal hemorrhage (Table 1), had a reduced spawning frequency. However, compared with controls, no difference was observed in either the fecundity or the fertility of this group. In pairs exposed to $EE_2$ at ≤116 ng/L, both the fecundity and fertility remained at the respective values recorded in the same breeding pairs before the exposure; these data did not differ significantly from those of the controls.

### *Hepatic VTG concentration*

Hepatic VTG increased in medaka, especially in males, in all treatment groups in a concentration-dependent manner (Fig.

Table 1. Mortality and symptoms observed in paired medaka during the 21-d ethinylestradiol ($EE_2$) exposure period

| $EE_2$ concn.[a] (ng/L) | $n$[b] | No. of males and females | | | |
|---|---|---|---|---|---|
| | | Mortality | Light body color | Swollen abdomen | Epidermal hemorrhage |
| Control | 6 | 0, 0 | 0, 0 | 0, 0 | 0, 0 |
| 32.6 | 6 | 0, 0 | 0, 0 | 0, 0 | 0, 0 |
| 63.9 | 6 | 0, 0 | 0, 0 | 0, 0 | 0, 0 |
| 116 | 6 | 0, 0 | 0, 0 | 0, 0 | 0, 0 |
| 261 | 6 | 0, 1 | 3, 0 | 1, 0 | 1, 0 |
| 488 | 6 | 4, 1 | 6, 4 | 4, 0 | 2, 0 |

[a] Mean measured concentrations through the exposure period.
[b] $n$ = Number of pairs.

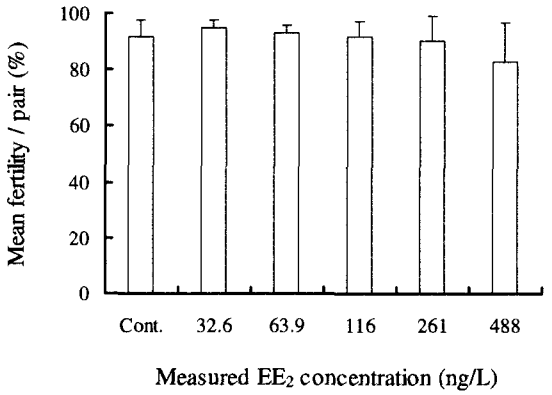


Fig. 2. Mean fertility of eggs produced by females in the control and treatment groups during the exposure period ($n$ = 6). Error bars represent the standard deviation of the mean. Cont. = control; $EE_2$ = ethinylestradiol.

3). The VTG concentrations in the control males were not detectable (<18.9 ng/mg liver), whereas noticeable VTG production was observed in the males exposed to $EE_2$ at ≥63.9 ng/L. Compared with the control data, these differences were significant ($p$ = 0.002) for each of the treatments, except for the highest treatment (488 ng/L) because of the small sample number ($n$ = 2). Hepatic VTG levels in the males exposed to ≥116 ng/L were higher than those for females in the respective treatment groups. Although VTG induction in female medaka was observed in all treatment groups, it was less pronounced than in males, and no significant difference was observed between any treatment group and the controls (Fig. 3).

*Histological examination*

Histological analysis showed the production of testis-ova in the testes of males exposed to $EE_2$ at ≥63.9 ng/L (Table 2). A high incidence (83%) of testis-ova was found in the 116 and 261 ng/L treatment groups, whereas that in the 63.9 ng/L treatment group was low (33%). Figure 4A and D show typical sections of the gonads of control male and female fish, respectively. In the testis-ova gonads in the males of the 63.9, 116, and 261 ng/L treatment groups, spermatocytes and spermatids could still be differentiated, indicating active spermatogenesis (Fig. 4B). However, in the highest treatment group (488 ng/L), testis-ova developed in only one of the two testes, and almost the whole area in each specimen was composed of abnormally developed connective tissues, with only a few spermatozoa and spermatocytes (Fig. 4C) compared to control (Fig. 4A). In the ovaries of females in the 488 ng/L group, many previtellogenic oocytes were observed in all specimens (Fig. 4E) compared to control (Fig. 4D), indicating a regressed condition of the ovaries (Table 2). However, no histological abnormalities were observed in the ovaries of female medaka exposed to $EE_2$ at ≤261 ng/L.

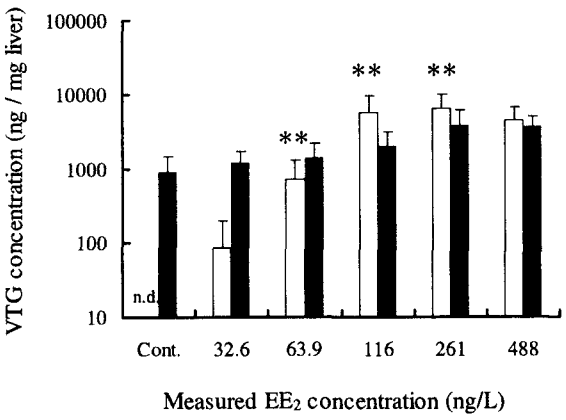


Fig. 3. Hepatic vitellogenin (VTG) concentration of medaka at the end of the 21-d ethinylestradiol ($EE_2$) exposure. Values are shown as the mean VTG concentration of male (open bars) and female (solid bars) fish ($n$ = 6; except $n$ = 5 for the 261 and 488 ng/L female groups and $n$ = 2 for the 488 ng/L male group). Error bars represent the standard deviation of the mean. Asterisks (**) denote significant differences from controls at $p$ = 0.002. Values of all control males and of one male from the 32.6 ng/L group were less than the determination limit (<18.9 ng/mg liver).

Table 2. Histological examination of the gonads of medaka at the end of 21-d ethinylestradiol ($EE_2$) exposure

| $EE_2$ concn.[a] (ng/L) | Males $n$[b] | Males Testis | Males Testis-ova | Females $n$[b] | Females Developed ovary | Females Regressed ovary[c] |
|---|---|---|---|---|---|---|
| Control | 6 | 6 | 0 | 6 | 6 | 0 |
| 32.6 | 5[d] | 5 | 0 | 6 | 6 | 0 |
| 63.9 | 6 | 4 | 2 | 6 | 6 | 0 |
| 116 | 6 | 1 | 5 | 6 | 6 | 0 |
| 261 | 6 | 1 | 5 | 5 | 5 | 0 |
| 488 | 2 | 1 | 1 | 5 | 0 | 5 |

[a] Mean measured concentrations through the 21-d exposure period.
[b] $n$ = Number of individuals.
[c] Ovary with many previtellogenic oocytes.
[d] One testis was not successfully removed at dissection.

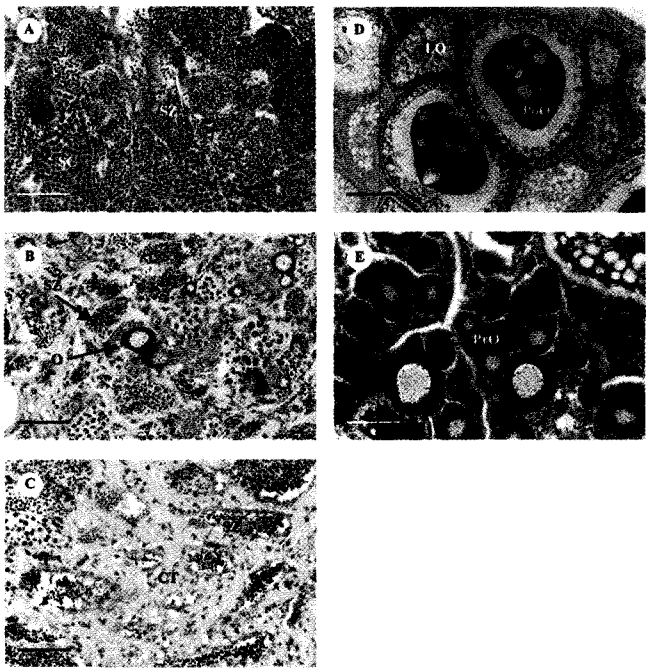


Fig. 4. Sagittal sections (thickness, 5 μm) through gonads at the end of the 21-d ethinylestradiol ($EE_2$) treatment and stained with hematoxylin and eosin. **A.** Testis of a control male. **B.** Testis-ova of a male exposed to 261 ng/L of $EE_2$. **C.** Abnormal testis of a male exposed to 488 ng/L of $EE_2$. **D.** Ovary of a control female. **E.** Regressed ovary of a female exposed to 488 ng/L of $EE_2$. CT = abnormal connective tissues; LO = late-vitellogenic oocytes; O = oocytes; PoO = postvitellogenic oocytes; PrO = previtellogenic oocytes; SC = spermatocytes; SZ = spermatozoa. Bar = 100 μm (**A–C**), 500 μm (**D**), and 200 μm (**E**). Magnification ×100 (**A–C**), ×20 (**D**), and ×50 (**E**).

## DISCUSSION

The present study clearly demonstrates that $EE_2$ at a wide range of concentrations affects reproduction, gonadal development, and physiological status in paired medaka. In this study, the fecundity of paired medaka decreased with increasing $EE_2$ concentrations (≥116 ng/L), resulting in a significant difference in the fecundity of medaka at 488 ng/L compared with that of the controls, although the fertility of the spawned eggs decreased only at 488 ng/L. Furthermore, the hepatic VTG level in exposed males was induced in all treatments, indicating significant induction at concentrations as low as 63.9 ng/L. Additionally, male medaka exposed to $EE_2$ concentrations of ≥63.9 ng/L developed testis-ova. Overall, these results indicate that in medaka, the LOEC of $EE_2$ on reproduction was 488 ng/L and that on induction of both VTG and testis-ova gonads was 63.9 ng/L. Therefore, the physiological and histological measurements (i.e., VTG and testis-ova induction) were approximately eightfold more responsive to $EE_2$ exposure than was reproductive failure (i.e., reduction of fecundity and fertility).

The VTG induction in fish exposed to estrogens has not yet been clearly correlated with reproductive impairment. Gronen et al. [18] found that male serum VTG levels were significantly correlated with fertilization after adult male medaka were exposed to octylphenol (OP) for 21 d. However, Kramer et al. [23] showed that VTG production in male fathead minnow treated with estradiol ($E_2$) for 19 d was not correlated with egg production. In the present study, although the amount of VTG induced in males exposed to ≥116 ng/L of $EE_2$ was higher than that induced in females in each treatment group, no reproductive impairment was observed, except in the 488 ng/L treatment group. Thus, the relatively increased VTG levels in male fish exposed to estrogens may not always correlate with decreased reproduction. However, some reports have appeared concerning toxic effects of excessive VTG induction in male fish. Dietary exposure of juvenile rainbow trout to $E_2$ induced excessive VTG as well as increased liver and kidney damage and mortality [24]. Schwaiger et al. [25] reported that intramuscular administration of $EE_2$ to common carp (*Cyprinus carpio*) predominantly caused hypertrophy of the hepatocytes as a consequence of $EE_2$ stimulating VTG synthesis. Gray et al. [17] reported that exposure of male medaka to OP during early life induced swollen abdomens and, subsequently, complete mortality before maturation. These authors suggested that general peritoneal edema related to ascites caused the mortality in males, because the male medaka has no natural process to excrete VTG accumulated in excess, causing pathological effects. In the present study, four of six males in the 488 ng/L treatment group died after the abdomen began to swell. In this treatment group, VTG level also increased remarkably. Thus, excessive VTG production in males exposed to estrogens may cause lethal and sublethal toxicity.

Testis-ova induction in medaka exposed to estrogens has been well described in many in vivo studies; however, the toxicological significance of this altered condition with regard to reproductive ability is still obscure. Gray et al. [26] reported that one male medaka having testis-ova induced by exposure to OP (100 μg/L) for six months after hatching was able to fertilize eggs from an unexposed female. In the present study, testis-ova were observed in male medaka exposed to $EE_2$ concentrations of ≥63.9 ng/L, with an especially high incidence (83%) of induction in the 116 and 261 ng/L treatment groups. In the testes of fish, however, spermatocytes and spermatids were still present with testis-ova, and no significant difference was found in reproductive parameters. On the other hand, in the 488 ng/L treatment group, the fecundity and fertility of paired medaka were reduced, and in each of the two testes, almost the whole area was composed of abnormally developed connective tissues, with few spermatozoa or spermatocytes. This observation suggests that the reproductive activity depends on the state of spermatogenesis rather than on the induction of testis-ova, but of course, the formation of testis-ova disturbs spermatogenesis.

Some studies have reported the development of abnormal connective tissues and/or inhibition of spermatogenesis in medaka exposed to estrogens. Egami [27] exposed medaka to various concentrations of $E_2$ and estrone and found that, when testis-ova were induced in the testes of fish with increasing estrogen concentration, the amounts of spermatids and spermatozoa decreased whereas that of connective tissue increased. Egami [27] also found that the testes that were strongly suppressed by the estrogen treatment did not contain testis-ova and that, in some of the gonads, the testicular tissue had been mostly converted into masses of connective tissue. Gray et al. [17] reported that the testes of male medaka treated with OP were mostly fibrotic, with little spermatogonial tissue remaining, and that a delicate balance may exist between exposures that induce testis-ova and those that damage testicular tissue to an extent that inhibits testis-ova development. Kang et al. [28] reported that exposure of medaka to 463 ng/L of $E_2$ induced decreasing fecundity and fertility, which were accompanied by histological lesions of the testis, indicating a replacement by connective tissues and a lack of spermatogenesis. Therefore, exposure to estrogens may induce development of abnormal connective tissues in the testis, which inhibits spermatogenesis depending on the exposure concentration and results in reproductive failure in fish.

Although to our knowledge no report has appeared regarding a relationship between histological lesions in the ovaries of fish exposed to estrogens and their reproduction, several studies regarding abnormalities of oocytes or ovary development have been published. Papoulias et al. [29] found that a single injection of $EE_2$ into eggs of d-rR medaka resulted in a high incidence of atretic follicles in XX females. Gray et al. [17] reported that exposure of medaka to 100 μg/L of OP during the early developmental stage caused an atretic condition in some oocytes surrounded with previtellogenic ones. Miles-Richardson et al. [30] found that oocyte differentiation is inhibited in sexually mature fathead minnow females treated with $E_2$ for two weeks. Scholz and Gutzeit [31] reported that treatment with $EE_2$ induced decreased fecundity accompanied by gonadal somatic index reduction in d-rR female medaka. These authors suggested that $EE_2$ may exert ovary-specific toxicity and/or interfere with the release of gonadotropins, which have been shown to stimulate ovarian development [32]. In the present study, the exposure of paired medaka to 488 ng/L of $EE_2$ caused many previtellogenic oocytes in female medaka, concurrent with decreasing fecundity. Therefore, exposure to estrogens also may lead to developmental abnormalities of oocytes, and especially to inhibition of oocyte maturation in the ovary, resulting in decreased fecundity.

A comparison of the relative sensitivity of responses to $EE_2$ among various fish species is important in risk assessment. Although to our knowledge no reports have appeared concerning the reproductive effects of $EE_2$ exposure in fish other

than medaka, a few studies on reproduction in fish exposed to $E_2$ have appeared. Kang et al. [28] reported that exposure of medaka to 463 ng/L of $E_2$ for three weeks decreased fecundity, as mentioned, but also that exposure to 227 ng/L had no effect. When fathead minnows were exposed to $E_2$ for 19 d, the $E_2$ concentrations expected to cause 50% and 10% inhibition of egg production were 120 and 6.6 ng/L, respectively [23]. Therefore, medaka may be less sensitive than fathead minnow with regard to fecundity.

Several studies on VTG induction in fish exposed to $EE_2$ have also been conducted. Although in the present study the LOEC of $EE_2$ for VTG induction in medaka was 63.9 ng/L, Purdom et al. [3] found that a 10-d immersion exposure of male rainbow trout to $EE_2$ caused VTG induction at concentrations ranging from 0.1 to 10 ng/L. Furthermore, Jobling et al. [33] reported that exposure of adult rainbow trout to 2 ng/L of $EE_2$ for three weeks caused significant induction of VTG. Länge et al. [34] reported that the LOEC of $EE_2$ for VTG induction over the full life cycle of fathead minnow was 16 ng/L. These differing sensitivities in terms of VTG induction may be caused partly by dissimilarities in the period and stage of exposure or by different methods of VTG assessment. Although medaka seems to be less susceptible to $EE_2$ than rainbow trout and fathead minnow in terms of VTG induction, further work is needed to determine the relative sensitivity of responses to $EE_2$ among various fish species.

## CONCLUSION

The present study demonstrated that the exposure of paired medaka to $EE_2$ decreased their fecundity and fertility, although fecundity at the highest concentration (488 ng/L) was only significantly lower than that of the controls for the 21-d exposure. On the other hand, the induction of hepatic VTG in exposed males was significant at $EE_2$ concentrations as low as 63.9 ng/L. In addition, testis-ova were observed in the gonad of male medaka exposed to $EE_2$ concentrations of $\geq$63.9 ng/L. These results indicate that, in medaka, the LOEC of $EE_2$ on reproduction was 488 ng/L and on induction of both VTG and testis-ova was 63.9 ng/L. Therefore, the physiological and histological measures were approximately eightfold more responsive to $EE_2$ exposure than was reproductive impairment. These results suggest that the high VTG level and testis-ova induced in the testes of medaka may not be a main factor for the impairment of reproductive ability in fish.

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# LIFE-CYCLE TOXICITY OF 4-NONYLPHENOL TO MEDAKA (*ORYZIAS LATIPES*)

HIROFUMI YOKOTA,*† MASANORI SEKI,† MASANOBU MAEDA,† YUJI OSHIMA,‡ HIROSHI TADOKORO,† TSUNEO HONJO,‡ and KUNIO KOBAYASHI†

†Kurume Research Laboratory, Chemicals Evaluation and Research Institute, Japan 19-14, Chuo-machi, Kurume-shi, Fukuoka 830-0023, Japan

‡Faculty of Agriculture, Kyushu University, Hakozaki, Higashi-ku, Fukuoka 812-0053, Japan

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**Abstract**—We studied the chronic effects of 4-nonylphenol (4-NP) on reproductive status of medaka (*Oryzias latipes*) over two generations of continuous exposure. The exposure study of the parental ($F_0$) medaka was begun on embryos within 24 h postfertilization and continued with monitoring through embryological development, hatching, posthatch survival, growth, sexual differentiation, and reproduction under flow-through exposures to mean measured 4-NP concentrations of 4.2, 8.2, 17.7, 51.5, and 183 μg/L for up to 104 d. Eggs spawned from the $F_0$ fish at 102 and 103 d posthatch were also examined for hatchability, survival after hatching, growth, and sexual differentiation until 60 d posthatch. The 183-μg/L treatment significantly reduced the embryo survival and swim-up success of the $F_0$ fish. The cumulative mortality after swim-up of the $F_0$ fish exposed to 17.7 and 51.5 μg/L were significantly higher than the control mortality. No concentration-related effect of 4-NP was observed on the growth of surviving $F_0$ fish at 60 d posthatch. However, the sex ratio estimated from the appearance of their secondary sex characteristics was skewed toward female in the 51.5-μg/L treatment. Additionally, gonadal histology showed that 20% of the fish in the 17.7-μg/L treatment and 40% in the 51.5-μg/L treatment had testis–ova, indicating that 4-NP affects the gonadal development and survival of medaka at similar concentrations in juveniles. The sex ratio of the $F_0$ fish in the 51.5-μg/L treatment was completely skewed toward female; subsequently, the effects on fecundity and fertility in this generation were monitored at mean measured concentrations of 4.2, 8.2, and 17.7μg/L from 71 to 103 d posthatch. Fecundity was unaffected by any of the treatments examined. The mean fertility in the 17.7-μg/L treatment was reduced to 76% of that in the controls, although no statistically significant differences were determined. Overall, these results indicate that the lowest-observed-effect concentration (LOEC) and no-observed-effect concentration (NOEC) of 4-NP through the life cycle of the $F_0$ medaka were 17.7 and 8.2 μg/L, respectively. In the $F_1$ medaka, no significant effects were observed on hatching success, posthatch mortality, or growth, but sexual differentiation at 60 d posthatch was affected. Induction of testis–ova in the gonads of the $F_1$ fish was observed in both the 8.2- and the 17.7-μg/L concentrations. The results indicate that 4-NP can have significant effects on reproductive potential of medaka at concentrations as low as 17.7 μg/L.

**Keywords**—4-Nonylphenol Environmental estrogen Medaka Testis–ova Life-cycle toxicity

## INTRODUCTION

Global concern about environmental pollution of exogenous estrogenic substances has increased over the past two decades. These substances have the potential to interfere with the endocrine system of wildlife, resulting in altered sexual development and reproduction [1]. Endocrine-disrupting effects have been well reported in aquatic fish species in association with the discharge of treated sewage to rivers in several countries [2–6]. Throughout the United Kingdom, large populations of roach (*Rutilus rutilus*) inhabiting the settlement lagoons of sewage treatment works (STWs) have hermaphroditic gonads composed of both testicular germ cells and oocytes [7]. Domestic semi-field studies have confirmed that most effluents from STWs can exert estrogenic activity by the induction of vitellogenin, a female-specific protein, in caged male rainbow trout (*Oncorhynchus mykiss*) [8]. Although a study using bioassay fractionation techniques has identified that natural and synthetic hormones (17β-estradiol, estrone, and ethynylestradiol) are responsible for the estrogenicity of effluents from seven STWs in the United Kingdom [9], the participation of alkylphenolic chemicals has been also implicated [2,8].

* To whom correspondence may be addressed (yokota-hirofumi@kurume.cerij.or.jp).

Alkylphenol polyethoxylates are widely used as nonionic surfactants in the manufacturing of cleaning agents, plastics, paper, cosmetics, and food products [10]. Nonylphenol ethoxylates (NPEOs) have been used predominantly, amounting to about 80% of the production of alkylphenol surfactants. Although the NPEOs in drainage flowing into STWs are nontoxic and hydrophilic, bacterial degradation produces toxic hydrophobic compounds such as nonylphenols (NPs) during sewage treatment [11]. The detected concentrations of NPs are generally very low (<1 μg/L), but in some sites they exceed 10 μg/L [12–17].

In both in vitro and in vivo assays, alkylphenolic chemicals, including NPs, have been shown to possess estrogenic activity [18–21]. Several studies using intact fish have confirmed that NPs have physiological and developmental effects related to their estrogenic properties. The exposure of sexually mature male rainbow trout to alkylphenol polyethoxylates, including 4-nonylphenol (4-NP), caused a dose-dependent elevation of plasma vitellogenin levels accompanied by a reduction in testicular weight [21]. Christiansen et al. [22] found that treatment with 4-*tert*-NP caused a significant increase in vitellogenin synthesis and a marked reduction in the gonadosomatic index (GSI) of male eelpout (*Zoarces viviparus*). Gray and Metcalfe [23] reported that treatment with *p*-NP induced testis–ova in Japanese medaka (*Oryzias latipes*). Many studies have been

conducted on the ecotoxicity of NPs, and these data have been reviewed by Staples et al. [24]. These authors pointed out that the threshold NP levels required to produce adverse effects in fish exceed the concentrations observed in rivers and lakes and even in most sewage effluents. However, no reports have been made of how continuous exposure to NPs may affect the entire life cycle, including the sexual differentiation and reproductive phases. Further work needs to be done to elucidate dose–response relationships and to determine NOECs for the chronic effects of NPs on the full life cycle of fish, including the developmental and reproductive phases.

Medaka is an ideal test species for evaluating life-cycle toxicity in the parental and progeny generations because of its short life cycle [25]. This fish can spawn 10 to 40 eggs daily under optimal conditions, which facilitates studies of reproductive effects. Medaka is also sensitive to estrogenic substances because it typically develops an intersex condition (testis–ova) in the gonadal tissue when exposed to estrogenic chemicals [23,26,27]. Multigeneration toxicity testing in medaka can be valuable and definitive for determining the estrogenic effects of synthetic chemicals and substances present in the aquatic environment on the complete life cycles of fish.

This study was conducted to elucidate the chronic effects of 4-NP on the life cycle of medaka over two generations in continuous exposures. We examined embryological development, hatching success, posthatch survival, growth, sexual differentiation, and reproduction of the $F_0$ generation as well as those of the progeny generation ($F_1$).

## MATERIALS AND METHODS

### *4-NP*

The test substance (analytical grade 4-NP; 97.4% purity as a mixture of isomers) was obtained from Kanto Chemical, Tokyo, Japan. A 4-NP stock solution of 1,500 mg/L was prepared by dissolving in ethanol.

### *Test fish*

Medaka were originally purchased from a local fish farm in Kumamoto, Japan, and a breeding stock of these medaka has been maintained for several years in our laboratory. About 100 breeding medaka pairs (mean ± standard deviation of body wt and total length were 233 ± 50 mg and 29.9 ± 1.8 mm, respectively) were kept for at least two weeks in 1-L chambers with flow-through dechlorinated tap water (pH, 7.4–7.5; hardness, 44.0–73.5 mg $CaCO_3$/L) at 24 ± 1°C. During mating, the fish were placed under a summer photoperiod (16: 8-h light:dark) and fed exclusively with *Artemia* nauplii (<24 h after hatching) twice a day. Eggs spawned from each female were carefully collected within a few hours after fertilization, pooled in a petri dish containing about 5 ml of dechlorinated tap water, checked for fertilization and development (to the early morula stage) under a light microscope, and then subjected to the 4-NP exposure.

### *Exposure design*

The exposure system consisted of a continuous-flow mini-diluter system modified from Benoit et al. [28]. The 4-NP stock solution was injected in this exposure system by a mini-chemical pump (SP-D-2000; Nihon Seimitsu Kagaku, Tokyo, Japan). The flow rate of the stock solution was calibrated in accordance with our analytical confirmation of the test solutions before the initiation of exposure. The stock solution and dechlorinated tap water were delivered to a mixing vessel before entering a cylindrical glass test chamber (diameter, 15.0 cm; depth, 17.5 cm). The test chamber was designed to contain about 1.8 L of the test solution by maintaining an overflow level of 10 cm. The flow rate of dechlorinated tap water was checked volumetrically once a day. The renewal rate of the test solution in each chamber was 14 times a day, and the stock solution was newly prepared daily. Nominal treatment concentrations of 4-NP at 1.85, 5.56, 16.7, 50, and 150 μg/L were selected for this study based on a 7-d preliminary exposure with newly hatched larvae. The controls received tap water alone, and the solvent controls received dechlorinated tap water containing ethanol (100 μl/L) at a concentration equivalent to that of the test solution in the highest 4-NP treatment. The photoperiod was 16 h light: 8 h dark. Water temperature was controlled with a thermostat and kept at 24 ± 1°C for the reproductive phase, when temperatures were increased to 28 ± 1°C. The test equipment and chambers were cleaned at least twice a week to prevent dense bacterial growth. Residual food and feces in the test chamber were removed daily.

### *Biological protocols*

*Embryological phase.* Exposure was initiated at less than 24 h postfertilization. The 60 embryos employed for each treatment were randomly separated into four groups of 15 in each test chamber for testing in quadruplicate. The developing embryos were observed daily under a stereoscopic microscope. Any dead embryos were discarded; live ones were returned to the chamber. This procedure was repeated until all the living embryos had hatched.

*Larval–juvenile phase.* After hatching, the larvae were fed an adequate amount of *Artemia* nauplii (<24 h after hatching) twice a day; some nauplii remained for at least 1 h after feeding. Daily observation was made to examine mortality and abnormal behavior and appearance until 60 d posthatch, and dead fish were removed as soon as possible.

At 60 d posthatch, five individuals from each of the four test chambers (i.e., 20 individuals from each treatment group, except for the highest treatment group in which complete mortality occurred), were randomly removed and observed for external secondary sex characteristics based on the shape of the dorsal and anal fins. The fish were euthanized with an overdose solution (a 1,000-fold aqueous solution) of anesthetic FA-100 (Tanabe Seiyaku, Osaka, Japan) and drained on filter paper; body weight and total length were measured. They were then fixed in Bouin's solution and prepared for gonadal histology according to standard procedures [29]. The fish embedded in paraffin wax were sectioned longitudinally at 3 μm in thickness providing at least five sections per gonad. The sections were stained with hematoxylin and eosin, mounted with malinol (Muto pure chemicals, Tokyo, Japan), and then examined under a light microscope.

*Reproductive phase.* At 70 d posthatch, the sex of the surviving fish was distinguished by their external appearances, and six mating pairs from each of the two low treatments (4.2 and 8.2 μg/L) and the controls and solvent controls were selected to examine fecundity and fertility. No pairs from the 51.5-μg/L treatment and only three pairs from the 17.7-μg/L treatment could be selected because of a skewed sex ratio and/or the limited number of surviving fish. Each pair was assigned to a test chamber and exposed until 104 d posthatch. The water temperature was increased from 24°C to 28°C to stimulate spawning. The eggs spawned from each female were collected, counted, and assessed for viability microscopically for 33 con-

secutive days (71–103 d posthatch). On the day after the end of the reproductive examination (104 d posthatch), all pairs examined were removed from their chambers, and their body weights and total lengths were measured after overdosing with FA-100. Their gonads were removed and weighed, and the GSI was calculated (i.e., 100 × gonad weight/body weight).

*$F_1$ embryo phase.* The eggs spawned on the last 2 d of the reproductive phase (102 and 103 d posthatch) were subjected to the following semistatic 4-NP exposure until hatching. The fertilized eggs from each spawning in the 4.2-, 8.2-, and 17.7-μg/L treatments and the controls and solvent controls were collected and placed in a 100-ml beaker containing 80 ml of test solution and incubated in a water bath at 24 ± 1°C. The test solutions were prepared by dilution of 4-NP stock solution with dechlorinated tap water and renewed every day until the last hatching. In the control and the solvent control treatments, tap water alone and tap water containing ethanol (100 μl/L) were used. The developing embryos were observed daily and dead ones removed.

*$F_1$ larvae–juvenile phase.* The newly hatched larvae in the two low treatments (4.2 and 8.2 μg/L) and the controls and solvent controls were randomly transferred to four test chambers in each treatment and kept in the chambers until the last hatching. Then 15 larvae in each chamber were selected (60 larvae in each treatment group). In the 17.7-μg/L treatment, the larvae were transferred to two test chambers because of fewer embryos from three pairs of $F_0$ fish, and 15 larvae in each chamber were selected (30 larvae in this treatment group). Then they were treated until 60 d posthatch in the same flow-through system described previously for the $F_0$ generation. The fish were checked daily until 60 d posthatch for mortality, abnormal behavior, and appearance. Dead fish were removed as soon as possible.

At 60 d posthatch, the external secondary sex characteristics of all the surviving $F_1$ fish were observed, and their weights and lengths were measured after overdosing with FA-100. They were then fixed in Bouin's solution and prepared for gonadal histology as previously described for the $F_0$ generation.

### *Determination of 4-NP concentration in test solution*

*Sample preparation.* The concentration of 4-NP in each test solution was determined approximately every two weeks through the exposure period, except for the semistatic period of $F_1$ embryo exposure. Equal volumes of test solution collected from all test chambers of each treatment were combined and applied individually to preconditioned Sep-Pak PS-2 Plus solid-phase extraction cartridges (Waters, Tokyo, Japan). The 4-NP was eluted with 6 ml of methyl acetate. The eluate was centrifuged (3,000 rpm, 10 min) and the methyl acetate phase collected. Next, 5 ml of hexane were added to the water phase, and the mixture was shaken for 1 min. The hexane phase was combined with the collected methyl acetate phase, and then the solvents were removed by drying in a stream of nitrogen. The residue was dissolved in 1 ml of the hexane solution containing 50 μg/ml of 4-*tert*-octylphenol as an internal standard and then analyzed by gas chromatography/mass spectrometry (GC-MS). The calibration curves were plotted as the ratios of the peak areas of 4-NP to that of the internal standard.

*GC-MS conditions.* The samples were analyzed by GC-MS with a Shimadzu QP-5,000 equipped with a 30-m DB-1 column (250-μm i.d., film thickness 0.25 μm; J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas at a flow rate of 50 ml/min. One-microliter injections were made in a splitless mode with a 1-min purge-off. The injection-port temperature was maintained at 280°C to ensure complete volatilization of the sample. The temperature program for the column began with a 1-min hold at 80°C, followed by a 30°C/min ramp-up to 180°C, a 5°C/min ramp-up to 200°C, and a 35°C/min ramp-up to 280°C, which was held for 5 min. After being eluted from the GC column, the samples were carried through a 250°C transfer line into the ion source of the mass spectrometer held at 250°C. The electron energy was 70 eV and electron multiplier voltage 1,000 V. Ions of 4-NP were monitored at *m/z* 107 and 135 and the internal standard at *m/z* 135.

Table 1. Geometric mean concentrations of 4-nonylphenol measured in the test solutions through the exposure period

| Nominal concn. (μg/L) | *N* | Mean measured concn. (μg/L) | SD[a] | CV[b] (%) |
|---|---|---|---|---|
| Control | 12 | ND[c] | | |
| Solvent control | 12 | ND | | |
| 1.85 | 12 | 4.2 | 1.6 | 38.5 |
| 5.56 | 12 | 8.2 | 3.7 | 44.6 |
| 16.7 | 12 | 17.7 | 3.4 | 19.1 |
| 50 | 6 | 51.5 | 7.1 | 13.8 |
| 150 | 2 | 183 | | |

[a] SD = standard deviation.
[b] CV = coefficients of variation.
[c] ND = not determined at 0.5 μg/L.

### *Statistical analysis*

Either a chi-square test, Student's *t* test (parametric data), or Bonferroni's *U* test (nonparametric data) were used prior to data analysis to determine whether differences existed between the solvent control and control groups. If no differences were found, these groups were pooled for subsequent analysis. If differences were found, the control group without solvent was excluded from the subsequent analyses. The experimental data, except for the sex ratios in each treatment for the $F_0$ and $F_1$ generations, were checked for homogeneity of variances and assumptions of normality across treatments by Levene's test and Shapiro–Wilk's test, respectively [30,31]. When the assumptions were met, the data were subjected to analysis of variance followed by Dunnett's multiple comparison test [32]. When the data failed to meet either one or both of the assumptions, a nonparametric Wilcoxon's rank sum test was used with Bonferroni's adjustment [33]. The data on hatchability, swim-up failure, and cumulative mortality were transformed to arcsine for variance stabilization before analysis [34]. The data on sex ratios distinguished from the gonads were assessed by chi-square analysis. All statistical analyses were conducted with SPSS® Base 8.0 J [30]. Differences were considered to be significant at the $p \leq 0.05$ level.

## RESULTS

### *Concentration of 4-NP in the test solution*

The 4-NP nominal concentrations >16.7 μg/L remained consistent throughout the exposure period (Table 1). However, the two low nominal concentrations (1.85 and 5.56 μg/L) were not stable, and the geometric mean concentrations in these treatments were 4.2 μg/L (*CV* = 38.5%) and 8.2 μg/L (*CV* = 44.6%) (Table 1). The concentrations in the control and solvent control treatments were all below the determination limit (<0.5 μg/L).

Table 2. Mean ± standard deviation ($n = 4$) of hatchability, time to hatch, and swim-up failure in the $F_0$ generation

| 4-Nonylphenol concn. (μg/L)[a] | Hatchability (%) | Time to hatch (d) | Swim-up failure (%) |
|---|---|---|---|
| Control | 90.0 ± 8.6 | 9.4 ± 0.2 | 5.5 ± 6.9 |
| Solvent control | 93.3 ± 5.4 | 9.9 ± 0.3 | 7.0 ± 5.8 |
| 4.2 | 95.0 ± 6.4 | 9.5 ± 0.02 | 3.6 ± 4.2 |
| 8.2 | 90.0 ± 8.6 | 9.6 ± 0.2 | 16.2 ± 7.7 |
| 17.7 | 88.3 ± 6.4 | 10.2 ± 0.4 | 18.7 ± 9.7 |
| 51.5 | 93.3 ± 7.7 | 10.2 ± 0.6 | 14.1 ± 5.1 |
| 183 | 46.7 ± 18.1[b] | 9.6 ± 0.3 | 100[c] |

[a] Mean measured concentrations.
[b] Significantly different from the pooled controls ($p < 0.001$).
[c] Significantly different from the pooled controls ($p = 0.004$).

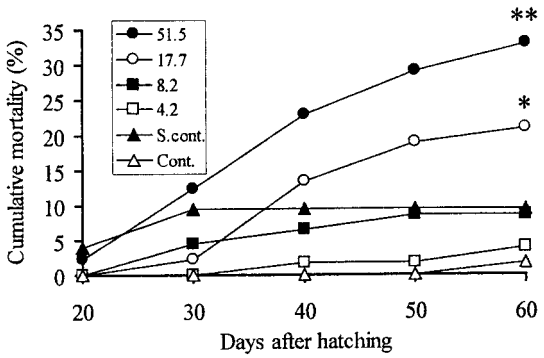


Fig. 1. Post-swim-up cumulative mortality from 20 to 60 d posthatch in each 4-nonylphenol (4-NP) treatment (4.2–51.5 μg/L) of the $F_0$ generation. Concentrations are expressed as mean measured concentrations (μg/L). * and ** denote significant differences from the pooled controls at $p = 0.031$ and 0.002, respectively.

### *$F_0$ generation*

*Mortality, abnormal behavior, and appearance.* Embryo development and hatching of medaka eggs were affected by 4-NP exposure. Hatchability was significantly decreased in the highest treatment (183 μg/L) relative to the pooled controls ($p < 0.001$), but most hatchabilities in the lower treatments were >90%, with no significant difference from the controls (Table 2). In the 183-μg/L treatment, about 50% of embryos died after 8 d postfertilization, exhibiting a reduction in their heart rate and blood circulation before dying. The time to hatching was about 10 d in all treatments (Table 2). However, no hatched larvae swam up successfully in the 183-μg/L treatment, resulting in a significant difference from the swim-up data for the pooled controls ($p = 0.004$) (Table 2).

Post-swim-up mortality was positively correlated with 4-NP concentration (mortality = 12.1 log $e$ [4-NP μg/L] − 14.7, $r^2 = 0.99$). Mortalities in the 17.7- and 51.5-μg/L treatments increased after 20 d posthatch, and the cumulative mortalities at 60 d posthatch were significantly different from those of the pooled controls ($p = 0.031$ and 0.002, respectively) (Fig. 1). Dying fish displayed a lightened body color. Although a few dead fish were also observed in the two low treatments and the controls during exposure, their cumulative mortalities were <10% at 60 d posthatch, and no differences were observed between these treatments and the pooled controls.

*Growth, external secondary sex characteristics, and gonadal histology.* No significant differences were observed in either mean total length or body weight of the $F_0$ fish at 60 d posthatch in any treatments (Table 3).

From our observation of external secondary sex characteristics, the sex ratios of males to females in the controls were about 1:1.3, and those in the three groups treated with 4-NP at ≤17.7 μg/L were slightly different from each other, whereas no males were distinguished in the 51.5-μg/L treatment (Table 3). This result was not statistically analyzed because external secondary sex characteristics may not be reliable for intersex medaka.

The induction of testis–ova composed of both testicular germ cells and oocytes in the gonad was observed in the 17.7- and 51.5-μg/L treatments but not at lower concentrations. The sex ratios determined by histological examination were similar to those found on external examination (Table 3). In the 17.7-μg/L treatment, 4 of 20 fish (20%) had testis–ova, and all these fish displayed externally male characteristics. Moreover, 8 of 20 fish (40%) in the 51.5-μg/L treatment had progressed testis–ova and exhibited externally female characteristics (Table 3). The extent of oocyte-containing parenchyma in each testis–ova specimen increased with increasing of 4-NP concentrations (Fig. 2). Although the oocytes in testis–ova observed in the 17.7-μg/L treatment occurred frequently and were often in clusters, spermatocytes and spermatids could still be differentiated, and spermatogenesis was observed (Fig. 2A). In testis–ova of the 51.5-μg/L treatment, almost the whole area was composed of oocytes that accompanied small testicular tissues interspersed with few testicular germ cells (Fig. 2B). Furthermore, abnormal connective tissues were observed in the testis–ova gonads. However, no histological abnormalities were observed in the ovaries of medaka in the 17.7- and 51.5-μg/L

Table 3. Total length and body weight of $F_0$ fish at 60 d posthatch and their sex ratios as determined by gross examination of secondary sex characteristics and by gonadal histology

| 4-Nonylphenol concn. (μg/L)[a] | Total length (mm)[b] | Body weight (mg)[b] | Sex ratio (♂:♀) | Gonadal histology: Number of fish with | | |
|---|---|---|---|---|---|---|
| | | | | Testis | Ovary | Testis–ova |
| Control | 26.4 ± 1.7 | 178 ± 36 | 9:11 | 9 | 11 | 0 |
| Solvent control | 25.9 ± 1.8 | 170 ± 45 | 8:12 | 8 | 12 | 0 |
| 4.2 | 26.0 ± 1.4 | 167 ± 33 | 12:8 | 12 | 8 | 0 |
| 8.2 | 26.9 ± 2.0 | 197 ± 44 | 13:7 | 14 | 6 | 0 |
| 17.7 | 26.1 ± 2.3 | 179 ± 48 | 9:11 | 5 | 11 | 4 |
| 51.5 | 25.2 ± 1.9 | 167 ± 42 | 0:20 | 0[c] | 12[c] | 8[c] |

[a] Mean measured concentrations.
[b] Data expressed as mean ± standard deviation ($n = 20$).
[c] The sex ratio obtained from gonadal histology differed significantly from that of the pooled controls ($p < 0.001$).

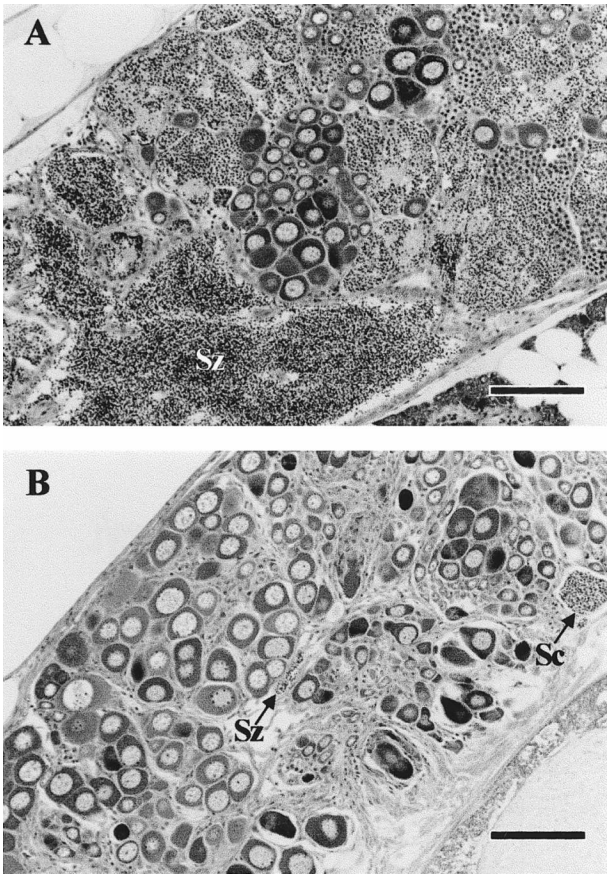


Fig. 2. Longitudinal sections of testis–ova in the gonads of $F_0$ generation medaka in the 17.7-μg/L (**A**) and 51.5-μg/L (**B**) treatment at 60 d posthatch. Each bar shows 100 μm length. (**A**) Oocytes appear in clusters within the testicular tissue. Numerous spermatozoa (Sz) are still present in a compacted mass in this section. (**B**) A more progressed testis–ova. Almost the entire area is composed of oocytes, accompanying small testicular tissues interspersed with a few spermatocytes (Sc) and spermatozoa (Sz).

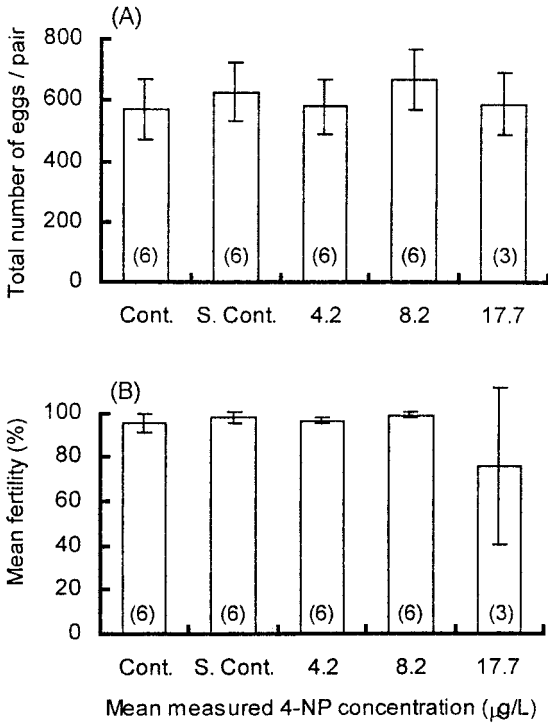


Fig. 3. Fecundity (**A**) and fertility (**B**) of paired $F_0$ generation medaka in the reproductive phase from 71 to 103 d posthatch. Data were expressed as mean ± standard deviation. The number of pairs in each treatment was indicated on each bar.

treatments. The gonads of medaka treated with <17.7 μg/L appeared histologically identical to those of the controls. Chi-square analysis indicated a significant difference between the sex ratios obtained from histological examination of the pooled controls and of the fish in the 51.5-μg/L treatment ($p < 0.001$).

*Reproduction.* The fecundity of paired medaka during the reproductive phase from 71 to 103 d posthatch was not affected by 4-NP treatment; however, mean fertility was decreased in the 17.7-μg/L treatment (Fig. 3). The breeding pairs in the two low treatments and in the controls spawned every day, and their mean fertility maintained >90%. In the 17.7-μg/L treatment, however, the fertility in one of three pairs declined from 14 d after initiation of the reproductive phase (94 d posthatch), and thereafter few fertilized eggs were observed in this mating pair. Consequently, the mean fertility of the breeding pairs in the 17.7-μg/L treatment was reduced to 76% of the control pairs, but no statistically significant difference was determined because of the small sample size ($n = 3$).

The GSI of male medaka at the end of the reproductive phase was reduced in the 17.7-μg/L treatment, although no statistically significant difference was found because the values within this treatment varied considerably (Fig. 4A). The GSI in female fish increased with an increase in 4-NP concentration, resulting in a significant elevation of GSI in the fish

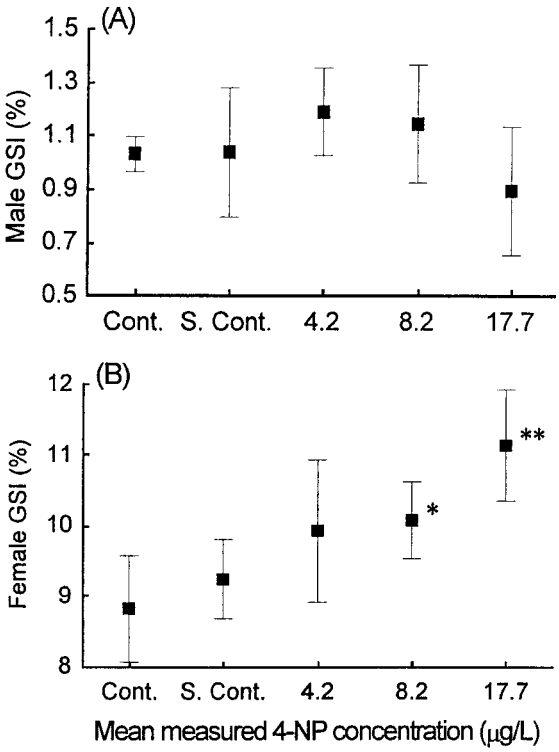


Fig. 4. Gonadosomatic index (GSI) in male (**A**) and female (**B**) of paired medaka ($F_0$) at the end of the reproductive phase. Data were expressed as mean ± standard deviation. The sample size in each treatment was 6, except in the 17.7-μg/L treatment ($n = 3$). * and ** denote significant differences from the pooled controls at $p = 0.050$ and 0.002, respectively.

Table 4. Hatchability and time to hatch in $F_1$ embryos collected during the last 2 d of the reproductive phase of $F_0$[a]

| 4-Nonylphenol concn. (μg/L)[b] | Hatchability (%) of $F_1$ embryo (days to hatch) | | | | |
|---|---|---|---|---|---|
| | *n*[c] | From $F_0$ at 102 d posthatch | *N*[c] | From $F_0$ at 103 d posthatch | Mean |
| Control | 99 | 64.8 ± 33.9 (10.5 ± 0.4) | 114 | 84.9 ± 18.8 (10.0 ± 0.4) | 74.4 (10.2) |
| Solvent control | 139 | 76.3 ± 35.3 (11.0 ± 0.6) | 115 | 76.5 ± 15.7 (10.4 ± 0.3) | 76.4 (10.7) |
| 4.2 | 125 | 80.5 ± 13.0 (10.7 ± 0.5) | 95 | 67.5 ± 22.5 (10.1 ± 0.6) | 74.0 (10.4) |
| 8.2 | 101 | 71.8 ± 21.0 (11.2 ± 0.8) | 130 | 81.9 ± 16.2 (10.0 ± 0.5) | 76.9 (10.6) |
| 17.7 | 35 | 73.4 ± 9.4 (12.2 ± 0.2) | 35 | 97.5 ± 3.5 (11.1 ± 0.4) | 85.4 (11.6) |

[a] Data expressed as mean ± standard deviation ($n = 3$ in the 17.7-μg/L treatment and $n = 6$ in the others).
[b] Mean measured concentrations.
[c] Number of fertilized eggs.

treated with 4-NP at 8.2 and 17.7 μg/L (compared with that in the pooled controls) (Fig. 4B).

### *$F_1$ generation*

*Mortality, abnormal behavior, and appearance.* No embryological abnormalities or hatching failures of fertilized eggs ($F_1$ embryos) were observed in any treatment (Table 4). The hatchabilities in all treatments were >70%, and the time to hatch in most embryos was 10 to 12 d in every treatment, with no significant differences between the treatments and the pooled controls (Table 4).

Although a few dead fish were observed in all treatments, the $F_1$ juvenile mortality rate for 60 d posthatch was not significantly higher than that of the pooled controls (Table 5).

*Growth, external secondary sex characteristics, and gonadal histology.* The growth of 4-NP-exposed $F_1$ juveniles at 60 d posthatch was not affected at the concentrations tested. The mean total lengths (range 25.9–27.0 mm) and body weights (range 169–178 mg) of fish exhibited no significant differences between treatments and controls (Table 5).

Morphological examination of secondary sex characteristics showed that the sex ratios were approximately 1:1 in the control and ≤8.2 μg/L in the 4-NP treatment. In the 17.7-μg/L treatment, however, more females than males were identified, and the sex ratio of males to females was about 1:2 (Table 5).

Induction of testis–ova in the gonads of the $F_1$ generation was observed at lower 4-NP concentrations than at those in the $F_0$ generation. In the 8.2- and 17.7-μg/L treatments, testis–ova were observed in two (10%) and five (25%) among 20 fish examined, respectively (Table 5). However, all these fish with testis–ova clearly displayed external male characteristics. The degree of development of oocytes in each testis–ova specimen was not as severe as that in the $F_0$ medaka in the 17.7-μg/L treatment, and developing spermatids and spermatozoa were observed in the gonads with testis–ova. In the 4.2-μg/L treatment and the controls, no testis–ova were observed in any of the $F_1$ medaka examined. A significant difference was observed between the sex ratios obtained from histological examination of the pooled controls and of the fish in the 17.7-μg/L treatment ($p = 0.002$).

## DISCUSSION

Our study clearly demonstrates that 4-NP exerts numerous estrogenic and nonestrogenic effects on each life stage of medaka, depending on the concentrations tested. In our study, the embryo survival and hatching success of the $F_0$ medaka were significantly reduced only at the highest 4-NP concentration (183 μg/L). However, a 4-NP concentration as low as 17.7 μg/L affected the mortality of the $F_0$ medaka after swim-up. Although our study found no effect of 4-NP on the growth of the $F_0$ medaka at 60 d posthatch (even in the 51.5-μg/L treatment), the sex ratio estimated from secondary sex characteristics was completely skewed toward female at a concentration of 51.5 μg/L. Furthermore, gonadal histology revealed that abnormal sexual differentiation, as shown by the presence of oocytes in the testis (testis–ova), occurred in the 17.7- and 51.5-μg/L treatments. These results show that 4-NP affects the gonadal development and survival of medaka at similar concentrations in juveniles. In the reproductive phase, the mean fertility of the breeding pairs was reduced (although not statistically significant) in the 17.7-μg/L treatment. Overall, these results indicate that the LOEC and NOEC of 4-NP throughout

Table 5. Cumulative mortality, growth, and sex ratios as determined by gross examination of secondary sex characteristics and by gonadal histology at 60 d posthatch of $F_1$ medaka

| 4-Nonylphenol concn. (μg/L)[a] | | | | Growth | | Sex ratio | | Gonadal histology | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Number of fish | | |
| | *n* | Mortality (%)[b] | *N* | TL (mm)[c] | BW (mg)[d] | (♂:♀) | *N* | Testis | Ovary | Testis–ova |
| Control | 60 | 1.7 | 59 | 26.5 ± 1.4 | 169 ± 38 | 28:31 | 20 | 7 | 13 | 0 |
| Solvent control | 60 | 10 | 54 | 26.6 ± 1.8 | 174 ± 40 | 26:28 | 20 | 11 | 9 | 0 |
| 4.2 | 60 | 10 | 54 | 26.9 ± 1.5 | 177 ± 35 | 25:29 | 20 | 9 | 11 | 0 |
| 8.2 | 60 | 18.3 | 49 | 27.0 ± 2.2 | 178 ± 46 | 24:25 | 20 | 10 | 8 | 2 |
| 17.7 | 30 | 6.7 | 28 | 25.9 ± 1.9 | 171 ± 38 | 9:19 | 20 | 4[e] | 11[e] | 5[e] |

[a] Mean measured concentrations.
[b] Cumulative mortality at 60 d posthatch.
[c] Total length.
[d] Body weight.
[e] The sex ratio obtained from gonadal histology differed significantly from that of the pooled controls ($p = 0.002$).

the complete life cycle of the $F_0$ medaka were 17.7 and 8.2 μg/L, respectively.

In the progeny generation, we observed no concentration-related effect of 4-NP on hatching success in embryos, mortality, behavior, appearance, or growth of hatched larvae. However, we could demonstrate an effect on sexual differentiation at 60 d posthatch: Histological examination of the gonads showed that 10% of fish in the 8.2-μg/L treatment and 25% in the 17.7-μg/L treatment had testis–ova. This result suggests that exposure to 4-NP beginning in ovo and continuing throughout the early life stage of the $F_1$ generation induced the formation of hermaphroditic gonads at lower concentrations than in the $F_0$ generation. Two explanations for this finding are possible. First, 4-NP may exert enhanced effects to the progeny generation through maternal transfer of 4-NP into the $F_1$ embryos, although threshold concentrations in the other endpoints, such as mortality, did not increase. Second, transgenerational exposure to 4-NP may potentiate its estrogenic response by increasing the number of estrogen receptors or by enhancing the affinity of the estrogen receptor of the progeny generation. Metcalfe et al. [35] reported a significantly greater induction of hepatic vitellogenin in male medaka exposed to DDT by maternal transfer compared with that in control males when all males were subsequently exposed to 17β-estradiol (12 mg/L) at 10 months old. However, our study neither determined the concentrations of 4-NP in the spawned eggs nor measured the number of estrogen receptors in the gonads of the $F_1$ medaka. Because we observed a low incidence (10%) of testis–ova in $F_1$ medaka exposed to 8.2 μg/L, we cannot substantiate this possible enhanced response without further study. We will investigate in further studies whether the progeny after transgenerational exposure to 4-NP suffer reproductive impairment as adults at lower concentrations than those that caused reproductive impairment in the parental generation.

It has been reported that 4-NP exerts lethal toxicity in fish at levels of tens to hundreds of micrograms per liter. Gray and Metcalfe [23], using a static assay, reported that the embryo-larval median lethal concentration (LC50) of *p*-NP for medaka was 460 μg/L. Our study demonstrated that flow-through exposure of medaka to 183 μg/L 4-NP caused embryological abnormality and swim-up failure in the $F_0$ medaka, resulting in significant mortality. The reason for these different values in embryo-larval toxicity is probably due to differences in stability of 4-NP in the static exposure system used by Gray and Metcalfe, which did not maintain the nominal concentrations [23]. The embryological toxicity of another related alkylphenol substance, octylphenol, has also been examined in medaka and caused embryological abnormalities, including slowed heart rate, blood circulation, and swim bladder inflation, in hatched larvae at the nominal concentrations ≥500 μg/L [36]. In our study, we observed the same symptoms in embryos at 8 d postfertilization in the 183-μg/L treatment. Therefore, it appears that toxicity values of alkylphenols, including 4-NP, range from about 200 to 500 μg/L for the early life stage of fish. Some subacute toxicity tests with fish have shown that 4-NP is lethal to hatched larvae and juveniles at concentrations lower than the previously mentioned embryological toxicity. For example, the LOEC in survival of fathead minnow (*Pimephales promelas*) for 33-d early-life-stage exposure was 14 μg/L [24]. In our study, the mortality of $F_0$ medaka was increased by prolonging exposure beyond 30 d posthatch, resulting in significant mortality at 4-NP concentrations ≥17.7 μg/L, compared with the pooled controls. This time-dependent mortality may result from the bioaccumulation of 4-NP, which can accumulate in the lipid tissues of exposed fish [37]. The bioconcentration factors for NP in fish range widely from <1 to 1,250 (average 300), depending on fish species [21,24]. Therefore, prolonged exposure might be required to accumulate a sufficient threshold concentration of 4-NP in the fish body, culminating in delayed mortality of the juvenile medaka. Schwaiger et al. [38] reported that exposure of juvenile carp (*Cyprinus carpio*) to NP (1–15 μg/L) over a 70-d period caused severe anemia. Therefore, chronic exposure of NP might affect general health status by inducing hematological alterations in the fish, leading to reduced survival.

Alkylphenolic substances have been also reported to affect the sexual differentiation of fish. Gimeno et al. [39] exposed genetically male common carp to 4-*tert*-pentylphenol and then observed the formation of oviducts in the gonads. Hermaphrodite gonads (testis–ova) in medaka are induced by treatment with 4-*tert*-octylphenol [40,41]. In our study with 4-NP, testis–ova were observed at concentrations ≥17.7 μg/L, in agreement with the results of a previous *p*-NP study by Gray and Metcalfe [23]. Although the mechanism of the development of testis–ova with these alkylphenols in medaka is unknown, the abnormal development in the gonads might be induced in a concentration-dependent manner by the estrogenic activity of these chemicals. Jobling et al. [21] observed a dose-dependent elevation of plasma vitellogenin levels, accompanied by a reduction in testicular weight, in male rainbow trout exposed to alkylphenol polyethoxylates, including NP. Our results also showed that the incidence and severity of testis–ova in the gonads of $F_0$ medaka were increased in a concentration-dependent manner. In $F_0$ fish in the 17.7-μg/L treatment, the incidence of testis–ova was not high (20%), and numerous spermatozoa were observed in the gonad of hermaphroditic fish, indicating active spermatogenesis. These fish still exhibited male secondary sex characteristics. In the 51.5-μg/L treatment, however, 40% of the fish had testis–ova, but none had external male characteristics. This completely skewed sex ratio suggests that the occurrence of testis–ova in this study is due to abnormal sexual differentiation in genetically male medaka exposed to 4-NP. The degree of intersexuality in the gonads of some hermaphroditic fish exposed at 51.5 μg/L was more severe than that at 17.7 μg/L. In specimens from fish exposed to 51.5 μg/L, almost the whole area examined was composed of oocytes, accompanying small testicular tissues interspersed with few spermatocytes and spermatozoa, suggesting inhibition of spermatogenesis. The magnitude of the effects of 4-NP on the sexual differentiation of medaka would depend on the concentrations of 4-NP in the water and probably that in the fish body, and the intersex condition of the gonads in medaka might influence spermatogenesis and subsequently reproductive function.

In our study, the fertility of paired medaka was reduced at a 4-NP concentration of 17.7 μg/L, although it was less pronounced. The GSI of male medaka at the end of reproductive phase was also reduced in the 17.7-μg/L treatment, some of which had testis–ova in the gonads. The GSI reductions have already been found in adult male trout exposed to 4-NP, accompanied by a dose-dependent elevation of plasma vitellogenin levels [21]. Therefore, our results suggest that 4-NP may inhibit testicular development in fish through its estrogenic properties, inhibiting maturation of the testis and potentially reducing fertility in male fish. Gonadal histology of male medaka at 60 d posthatch in the 17.7-μg/L treatment revealed

hermaphrodism, although spermatogenesis was still observed. The development of the intersex condition might depend on the duration of exposure. Gray et al. [40] reported that exposure of medaka to octylphenol at 100 μg/L from hatching for a period of one or two months did not induce testis–ova, but exposure for three months resulted in 3 of 50 males developing this condition. Gimeno et al. [42] also reported that exposure of sexually mature male carp to 4-*tert*-pentylphenol for three months resulted in the progressive disappearance of spermatozoa and spermatogenic cysts with increasing exposure time. Shibata and Hamaguchi [43] exposed sexually matured male medaka to 17β-estradiol (160 μg/L) for 30 d and then observed the increasing development of testis–ova and inhibition of spermatogenesis in the testes with increasing duration of exposure. Therefore, in our study, prolonged exposure (for 60 d posthatch) of male medaka with testis–ova may have preceded oogenesis, as well as deficient sperm quality and quantity. The reduction in both fertility and GSI in male medaka exposed to 17.7 μg/L, although not significant, indicates that the reproductive abilities of these fish may have been affected by the estrogenicity of 4-NP and suggests that further examination of this response is needed. On the other hand, exposure to 4-NP (4.2–17.7 μg/L) had no effect on fecundity of female medaka and enlarged their ovarian size (as indicated by the GSI of spawning females in our study). This indicates that 4-NP did not have adverse effects on the reproductive abilities of female medaka at the concentrations tested. Nimrod and Benson [44] reported that the fecundity of medaka exposed to NP at 0.5, 0.8, or 1.9 μg/L from hatch to one month did not change among treatment groups and suggested that this factor would not be compromised by larval exposure to NP.

Several monitoring studies on NP levels in aquatic environments have generally shown concentrations less than 1 μg/L, but in some monitoring sites NP levels exceed 10 μg/L. A survey of rivers in the United Kingdom found $<10$ μg/L in most rivers examined, and only one river had up to 180 μg/L [12]. In the United States, NP concentrations in rivers and STW effluents are generally lower than those found in the United Kingdom; a survey of 30 U.S. rivers found that 70% of the sites had concentrations of 0.1 μg/L or less [13]. Snyder et al. [14] reported that NP was detected in 17 of 23 samples of effluents and surface waters collected from several locations in the Trenton Channel of the Detroit River, with concentrations ranging from $<0.011$ to 37 μg/L. Hale et al. [15] detected NPs at concentrations of $<1.0$ μg/L in 20% of the 59 effluents from sewage treatment plants in Virginia, USA, and concentrations of $>10$ μg/L in 10% of effluents. One treated-effluent water sample from Cape Cod (MA, USA) contained NP at 15.9 μg/L [16]. In Japan, the Environmental Agency surveyed NP levels in various rivers and estuaries and reported that NP was less than 1.1 μg/L in all the 42 samples examined [17]. The LOEC of 4-NP obtained in our study (17.7 μg/L) was at least one order of magnitude greater than the ambient levels of NP present in most rivers and effluents. However, our study suggests that 4-NP may affect wild fish populations in some sites in which it is detected at $>10$ μg/L. Furthermore, Routledge et al. [4] reported that exposure to 10 ng/L of 17β-estradiol elevated plasma vitellogenin levels in the male rainbow trout but not in the male roach, indicating species differences in sensitivity toward estrogen exposure. Therefore, the life-cycle toxicity of NP in other fish species remains to be investigated.

In summary, this study demonstrates that life-cycle exposure of 4-NP to medaka causes lethal toxicity and also affects adversely its sexual differentiation and reproduction as shown by the induction of hermaphroditic gonads and reduced fertilization rates, respectively. Overall, this study establishes a LOEC and NOEC of 4-NP through the life cycle of the $F_0$ medaka of 17.7 and 8.2 μg/L, respectively. In the progeny generation ($F_1$) exposed to those concentrations of 4-NP, no significant effects were observed on hatching success, posthatch mortality, or growth, whereas sexual differentiation at 60 d posthatch was affected. An intersex condition of the gonads in the $F_1$ fish was induced not only in the 17.7- but also in the 8.2-μg/L treatment, suggesting the enhanced effect of 4-NP on sexual differentiation in the progeny generation compared with $F_0$, by continuous exposure from beginning in ovo to the juvenile stage.

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# *Appendix III*

**Other information regarding Medaka studies**

1. Atlas of Medaka Gonadal Histology
http://www.nies.go.jp/edc/edcdb/index_j.html
2. Medakafish Home Page
http://biol1.bio.nagoya-u.ac.jp:8000/ (Nagoya University)
3. Medaka ToxiNet
URL: http://medaka.fish.agr.kyushu-u.ac.jp/ (Kyusyu University)
4. Medaka Genome Database
http://mbase.bioweb.ne.jp/~dclust/medaka_top.html
5. The Ministry of the Environment, Japan
URL: http://www.env.go.jp/en/index.html
6. Chemicals Evaluation and Research Institute, Japan
URL: http://www.cerij.or.jp/ceri_en/index_e4.shtml
7. National Institute for Environmental Studies (NIES)
URL: http://www.nies.go.jp/

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CERI

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