

Availability of Receptor Binding Assay for Screening of Endocrine Disrupting Chemicals

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Recently, it has suspected that a number of chemicals in environment have adverse effects on endocrine system of wildlife. 65 substances were listed as “chemicals suspected of causing endocrinological disruption,” in a document titled “Strategic Programs on Environmental Endocrine Disrupters '98 (SPEED '98)” by Japan Environment Agency (present appellation; Ministry of the Environment). We examined hormonal potencies of 12 chemicals from the list (tributyltin, triphenyltin, nonylphenol, 4-*t*-octylphenol, di-*n*-butyl phthalate, dicyclohexyl phthalate, di-2-ethylhexyl phthalate, butylbenzyl phthalate, diethyl phthalate, di-2-ethylhexyl adipate, benzophenone, and octachlorostyrene) mediated by binding to medaka hormone receptors using *in vitro* assay methods, hormone receptor binding assay and reporter gene transcriptional assay. As a result, it was found nonylphenol and 4-*t*-octylphenol showed considerably high binding affinities for medaka estrogen receptor (ER) alpha when compared to human ER. These results suggested that there were differences in receptor-binding properties of chemicals between species. Then, we demonstrated the differences in binding properties of 4-*t*-octylphenol and nonylphenol to the estrogen receptors between species; medaka, mammichog, common carp, zebra fish, rainbow trout, red sea bream. The binding potency was evaluated by relative binding affinity (RBA), which was calculated by using following equation.

$$\text{RBA (\%)} = \frac{\text{IC}_{50} \text{ for } 17\beta\text{-estradiol}}{\text{IC}_{50} \text{ for test substance}} \times 100$$

4-*t*-Octylphenol bound to ERs approximately two-fold stronger than nonylphenol with all species examined in this study. They bound to medaka ER at most strong affinities among six fish species. On the other hand, carp ER showed the lowest sensitivity to both 4-*t*-octylphenol and nonylphenol, and their RBA values for carp ER were ca. 1/90 of those for medaka ER. Binding potencies of these two alkylphenols showed wide spectrum to fish ERs, and it was obvious that

there were species differences in receptor binding characteristics. These results show the importance of the differences in sensitivities to chemicals among diverse species to assess the endocrine disrupting effects of chemicals to eco-system. From this point of view, receptor binding assay is very convenient and useful methods to clarify these issues.

Endocrine Disrupting Effects and Testing Methods with Medaka

Vitellogenin Assay / Reproduction Test

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The Ministry of the Environment, Japan (MOE), issued “Strategic Programs on Environmental Endocrine Disrupters '98 (SPEED '98)” in May 1998, and 67 substances were listed as “chemicals suspected of causing endocrinological disruption” in the document (65 are listed at present). Following this program, the research project to develop screening and testing methods for evaluating these 65 chemicals was launched in 1998, and the Fish Working Group for Development of Screening and Testing was established in 1999.

The Fish Working Group has developed vitellogenin assay, reproduction test and partial life-cycle test for screening purposes, and full life-cycle test for definitive test with medaka (*Oryzias latipes*), and also conducted hazard assessment of chemicals listed in SPEED '98 using these test methods. The MOE submitted assessment reports on nonylphenol and tributyltin in Fish to the OECD in 2001, and the activities of the group were reported at the special session of the Endocrine Disrupter Testing and Assessment (EDTA) in 2002. In this part, we would like to present our activities of “vitellogenin (VTG) assay and reproduction test”.

In the VTG assay, adult male medaka are exposed to a chemical for 21d under flow-through conditions. At the end of the exposure, livers removed from the fish are homogenized and centrifuged, and then hepatic VTG concentrations are measured with a medaka vitellogenin ELISA assay kit. We have conducted the VTG assay with 3 reference chemicals and 20 chemicals listed in SPEED '98, and the results indicate that the VTG assay with medaka is applicable to estrogenic chemicals. In the reproduction test, breeding medaka pairs are exposed to a chemical for 21 d under flow-through conditions. Effects on reproductive success of the fish as well as their gonadal condition and VTG induction are assessed. We have conducted the reproduction test with 4 reference chemicals and 2 chemicals listed in SPEED '98. These results indicate that the reproduction test with medaka is applicable to estrogenic chemicals from the observation of the estrogenic activity such as testis-ova and VTG induction, and its effects on reproductive potential. In addition, this test method is also applicable to androgenic chemicals

from the observation of masculinization of secondary sex characteristics in females. Further study is required to develop in vivo screening for detecting anti-estrogen and anti-androgen.

Partial Life-cycle Test and Full Life-cycle Test in Medaka

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The Organization for Economic Co-operation and Development (OECD) has been key player in the development of many testing methods that can detect and identify endocrine disrupting substances. In this context, the OECD Expert Consultation on Endocrine Disruptors (EDs) testing in fish agreed on a tiered testing scheme and proposed several *in vivo* tests for each tier. One such test, a full life cycle test that evaluates the effects of EDs on the complete life cycle of a species, has been proposed as the definitive testing method. There is also a recognized need for shorter and practical tests, such as fish developmental test and reproduction test, that can detect those of a large number of candidate chemicals promptly. In Japan, Ministry of the Environment has begun assessing the hazard of suspected EDs in fish according to priorities based on the results of environmental monitoring and scientific surveys of endocrine-disrupting effects. The testing system we use for assessing them consists of the several screening and testing methods using medaka (*Oryzias latipes*), which is basically borrowed from the OECD's testing scheme. In this symposium, I present an overview of the higher-tier testing methods in medaka (i.e., partial life-cycle test and full life-cycle test).

Partial life-cycle test. An extensive literature indicates that fish early life-stages, especially the period of sexual differentiation, are very vulnerable to endocrine-disrupting effects. Therefore, a reasonable approach for efficient hazard identification and characterization of suspected chemicals is to establish a new test protocol designed to detect abnormal sexual differentiation and maturation of exposed fish. Medaka is an ideal test species for developing such a test protocol, because it typically develops an inter-sex condition (i.e., testis-ova) in the gonadal tissues when exposed to estrogens or androgens. In light of the above, we have developed a partial life-cycle test using medaka based upon the existing OECD fish early life-stage toxicity test guideline 210. In our test protocol, medaka are exposed to test substances from the fertilized egg stage to early mature stage (about 60-day post-hatch) under flow-through conditions. During exposure period, traditional ecotoxicological endpoints (i.e., embryological development, hatching success, post-hatch survival, and growth) are observed. In addition, mechanistic biomarkers (i.e., secondary sexual characters, gonad histology,

and vitellogenin concentration) are examined at the end of exposure. To date, the partial life-cycle test using medaka has been applied to a variety of estrogenic chemicals, including ethynylestradiol, 17 β -estradiol, alkylphenols, and bisphenol A. Induction of an inter-sex gonad and feminization of secondary sex characters of exposed males were observed in all studies, indicating that the medaka partial life-cycle test has been successfully adapted for the detection of abnormal sexual differentiation caused by estrogenic exposure.

Full life-cycle test. Fish full life-cycle test (FFLC) has already been adopted as an ecological effects test guideline of United States Environmental Protection Agency (U.S. EPA) using Fathead minnow (*Pimephales promelas*) or Sheepshead minnow (*Cyprinodon variegatus*) as recommended test species. Therefore, this EPA protocol represents a good basis for detecting the effects of EDs on fish. However, one needs to consider suitable test species, test condition and endpoints for EDs, because the objective of this protocol is not to evaluate endocrine-disrupting effects of test substances. We have successfully developed an FFLC test using medaka and have begun verifying its applicability as a test for estrogenic chemicals. Our test protocol is designed to elucidate the chronic effects of test substances on the life cycle of medaka over two generations in continuous exposure. The endpoints analyzed in our FFLC study include embryogenesis, hatching success, post-hatch survival, growth and gonadal development (F_0 and F_1), and fecundity and fertility (F_0). As described above, the FFLC test is proposed as a definitive test; therefore, this test must be able to quantitatively assess the concentrations of EDs at which there are developmental and reproductive effects lead to serious damage at a population level. In our FFLC studies with estrogenic chemicals, commonly observed effects related to their estrogenic properties were skewing of the sex ratio toward female and/or testis-ova development in the gonads, as well as decreased fecundity and/or fertility in the reproductive phase. All of these effects appear to impair the reproduction ability of fish communities, indicating the capability of the medaka FFLC test to definitively evaluate the hazard of EDs.

Relationship between vitellogenin and genetic sex in medaka partial life-cycle test with the novel sex determining gene, DMY

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Medaka (*Oryzias latipes*) is one of the most important fish specific for the ecotoxicological tests and basic biological research, and has been recommended as a main OECD model fish. Also medaka has a XX/XY sex determination system like a mammal. To distinguish genetic sex, medaka specific strain has already been used because of the presence of a particular pigment gene. In the d-rR strain, in which the female results in a white body color and the male medaka result in an orange-red color. A sex-linked marker was isolated using an inbred strain, which was coloration locus lf (leucophore free) like a FLF strain. Recently, a sex-determining gene (DMY) was isolated. Using this DMY, we have developed the assay to evaluate the effects of the treatments with endocrine disrupting chemicals (EDCs) on sex differentiation in the medaka partial life-cycle test (PLC).

Medaka life-cycle test and reproduction test developed by us were designed for detection assay of EDCs on fish. In the present study, PLC was developed as a short-term test of full life-cycle test (FLC). To verify the applicability of PLC to weak estrogens, 4-*tert*-pentylphenol (4-PP) was employed as weak estrogen and was one of the recommended reference chemicals by OECD Expert Consultation on endocrine-disrupter testing in fish. Medaka were exposed to 4-PP at concentrations of 62.2, 121, 238, 413 and 783 µg/L (measured by HPLC) from fertilized eggs to 60-d posthatch under a flow-through condition to evaluate the endocrine disrupting effects based on the genetic sex in medaka partial life-cycle test. At 60-d posthatch, gonadal histology and hepatic vitellogenin (VTG) induction were assessed in 20 fish randomly selected from each treatment group. Medaka also individually investigated by DNA marker, DMY. For determining genetic sex, a polymerase chain reaction (PCR) method was employed. In genetically male (XY) medaka, induction

of testis-ova was observed in the fish exposed to 4-PP concentrations of $\geq 121 \mu\text{g/L}$, in which statistically significant induction of hepatic VTG was also observed. In genetically female (XX) medaka, although no effects were observed on gonadal histology, the hepatic VTG levels in fish exposed to $\geq 121 \mu\text{g/L}$ PP were significantly higher than those of controls.

Consequently, detection of the endocrine disrupting effects based on the genetic sex with DMY determination in medaka partial life-cycle test allows us to assess the hazard of the endocrine disruptors with higher accuracy than ordinary tests such as FLC, PLC and so on.

Transgenic See-Through Medaka for Monitoring Chemical Substances in Aquatic Environments

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Transgenic fish with reporter genes to visualize effects of chemical substances are a potentially powerful biomonitor for detecting them in aquatic environments. Here, we show two examples of such transgenic fish constructed in the gonad and liver in medaka.

The see-through medaka is a vertebrate model with a transparent body in the adult stages, as well as during embryonic stages, that was generated from a small laboratory fish, medaka (*Oryzias latipes*). In this fish model, most of the pigments are genetically removed from the entire body by a combination of recessive alleles at four loci. The main internal organs, namely, heart, spleen, blood vessels, liver, gut, gonads, kidney, brain, spinal cord, lens, air bladder, and gills, in living adult fish are visible to the naked eye or with a simple stereoscopic microscope. This fish is healthy and fertile.

A transgenic see-through medaka was produced by using the green fluorescent protein gene (*GFP*) fused to the regulatory region of the medaka *vasa* gene (*vasa/GFP*), in which germ cell specific expression of GFP was visualized. In the transgenic see-through medaka, development, growth, maturation and aging of the gonad were observed from the outside of the body throughout life. When the *vasa/GFP* see-through medaka was exposed to EE2, the effects could be evaluated as changes in the fluorescent imaging of the gonad.

Next, we developed a method to detect estrogen-like substances by the fluorescent imaging of the choriogenin H (ChgH) synthesis in the liver. Choriogenin H (ChgH) is a female-specific protein and composes the egg envelope (chorion). In mature female medaka, estrogens from the ovary induce ChgH synthesis specifically in the liver. A transgenic medaka was established that carried a plasmid containing the *GFP* gene regulated by the 5' and 3' flanking region of the ChgH gene (*ChgH/GFP*). In the male of F1 generation of this line, GFP and ChgH-mRNA were induced specifically in the liver by the exposure of E2 at the concentration of 10-100 ppm for 24-72 hours. Thus male of this transgenic medaka

tells us the existence of estrogen-like substances with the fluorescent imaging in the liver. The *ChgH/GFP* is to be introduced to the see-through medaka.

In the next step, we can produce a transgenic medaka exhibiting the fluorescent imaging in the gonad and liver as a response to environmental chemicals. Our goal is to construct a transgenic see-through medaka exhibiting the fluorescent image in multiple organs, which is used as a “transgenic fish chips” for environmental research and testing.

Feeding and Preparing Endocrine-Disrupting Chemicals Free Food

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In this study, we analyzed contents of phytoestrogens (genistein, daidzein, equol, and coumestrol) in two commercial fish diets (a diet for trout (TD) and a diet for ornamental carp (CD)) using Liquid Chromatography-Mass Spectroscopy/Mass Spectroscopy (LC-MS/MS), and these contents were compared with that of a casein-based formulated fish diet (FD) which does not contain soya bean or fish meal. The contents of phytoestrogens were generally high in CD, TD, and low in FD. Among these samples, CD showed the highest phytoestrogen contents: genistein, 390,800 ng/g; daidzein, 416,800 ng/g; coumestrol, 1,325 ng/g; equol, 6.4 ng/g. We also determined the estrogenic activity of the fish diets using male goldfish *Carassius auratus* by measuring plasma vitellogenin (VTG) levels as a biomarker of estrogen exposure. When male goldfish were fed one of these diets for 31 days, plasma VTG was detected in CD-fed fish (78.01 ± 48.18 µg/ml) and TD-fed fish (3.51 ± 3.83 µg/ml), whereas plasma VTG was not detected in FD-fed fish (less than 0.040 µg/ml). These results indicate that the commercial fish diets examined contain a large amount of phytoestrogens and showed estrogenic activity that were strong enough to induce VTG production in male goldfish.

Table 1. Ingredient and composition of the three diets

Fish diets	Ingredients
FD	casein 80 %, wheat flour 18 %, vitamin mix 0.5 %, mineral mix 1.5 %
TD	fish meal 60 %*, wheat flour 29 %, soya bean 4 %, rice bran 2 %, yeast and vegetable oil 5 %. vitamin mix, mineral mix
CD	wheat flour**, soya bean meal, fish meal, alfalfa meal, rice bran, shrimp meal, spirulina. vitamin mix, mineral mix, methionin

* Composition which does not include vitamin mix and mineral mix.

** Composition of the ingredients is not shown.

It is necessary to eliminate estrogenic substances other than test chemicals in the screening test system for estrogenic endocrine-disrupting chemicals (EDCs). Since

the formulated diet developed in the present study contain less phytoestrogens than the commercial fish diets and has low estrogenic activity, it is suggested that VTG production using male goldfish in combination with the low estrogen fish diet is a good *in vivo* system for evaluation of estrogenic effects of EDCs. Table 2. Concentrations of phytoestrogens in fish diets using LS-MS/MS analysis.

Diet	Genistein	Daidzein	Equol	Coumestrol
FD	93.2	129.6	1,027.2	8.8
TD	47,680.0	41,120.0	117.2	226.4
CD	390,800.0	416,800.0	6.4	1324.8

The data of fish diets and commercial infant powdered milks were represent total ng/g diet and total ng/ml milk, respectively. N.D.: Not Detected. Data represents the mean (n=3).

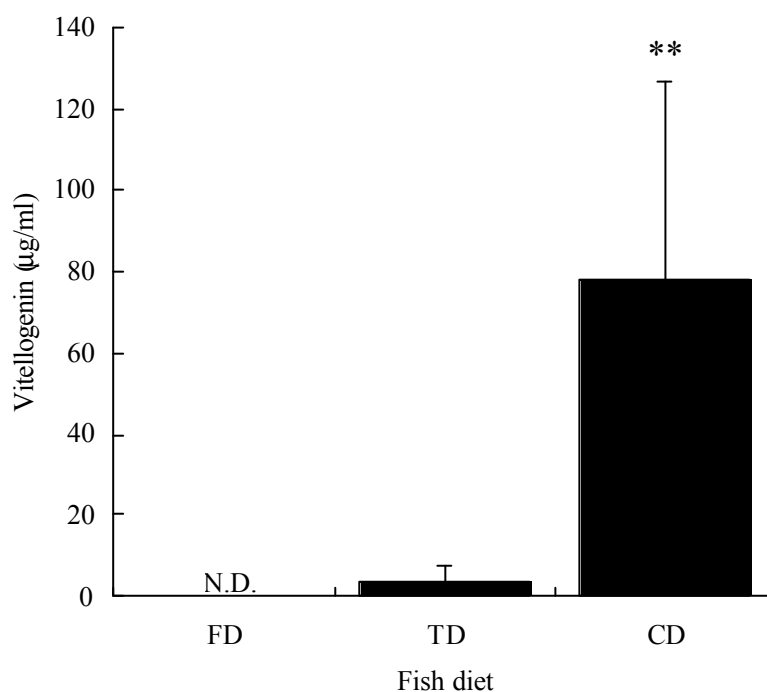


Figure 1. Plasma VTG levels in adult male goldfish.

Fish were fed 1.0 % body weight volume of one of the three diets (TD, CD and FD) every two days for 31 days. N.D. = Not Detected (less than 0.040 µg/ml). Columns and bars represent the mean and standard deviation. **, Significant difference compared to TD-fed fish ($p < 0.01$).