The 3rd International Medaka Symposium, 2003

Development of Test Methods with Medaka to Detect Endocrine Disrupting Chemicals

February 27 and 28, 2003
Okazaki Conference Center, Okazaki, Japan

Organized by: Ministry of the Environment, Government of Japan
Cooperated by: Chemical Evaluation and Research Institute, Japan
Okazaki National Research Institutes, Japan

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Program

PROGRAM

[] February 27, Thursday

Development of Test Methods with Medaka to Detect Endocrine Disrupting Chemicals

9:30-9:45 Opening Address

I. Kazuhiko Adachi	Director of Environmental Health and Safety Division, Ministry of the Environment Japan
II. Taisen Iguchi	Center for Integrative Bioscience Okazaki National Research Institutes, Japan
III. Anne Gourmelon	OECD, Environmental Health and Safety Division

Session I Each Fish's Activity of Evaluation of Endocrine Disrupting Chemicals

(Chairperson: Anne Gourmelon)

9:45-10:00 Medaka (Taisen Iguchi: Okazaki National Research Institutes)
10:00-10:45 Zebra fish (Thomas Braunbeck: Heidelberg University)
10:45-11:30 Fathead minnow (Karen Thorpe; University of Exeter/AstraZeneca)

Lunch 11:30-13:00

Special Lecture Sex Determination and Gonadal Sex Differentiation in Fish

13:00-14:00 Yoshitaka Nagahama: National Institute for Basic Biology

(Chairperson: Taisen Iguchi)

Session II Endocrine Disrupting Effects and Test Methods with Medaka

Part I (Chairperson: Taisen Iguchi)

14:00-14:25 Availability of Receptor Binding Assay for Screening of Endocrine Disrupting Chemicals
(Makoto Nakai: CERI)

14:25-14:55 Endocrine Disrupting Effects and Testing Methods with Medaka
(Masanori Seki: CERI)

14:55-15:25 Partial Life-cycle Test and Full Life-cycle Test in Medaka
(Hirofumi Yokota: CERI)

Tea Break 15:25-15:40

Part II (Chairperson: Koji Arizono)

15:40-16:05 Relationship Between Vitellogenin and Genetic Sex in Medaka Partial Life-cycle Test with the Novel Sex Determining Gene, DMY
(Tatsuo Abe: CERI)

16:05-16:30 Transgenic See-Through Medaka for Monitoring Chemical Substances in Aquatic Environments
(Yuko Wakamatsu: Nagoya University & Masato Kinoshita: Kyoto University)

16:30-16:55 Feeding and Preparing Endocrine-Disrupting Chemicals Free Food
(Koji Arizono: Prefectural University of Kumamoto)

16:55-17:00 Other information, medaka report
(Chisumi Eto : CERI)

17:00 **Closing** Kenjiro Ozato : Emeritus Professor of Nagoya University

[] February 28, Friday

Technical Meeting and Strategy on Phase 1 Test

Session I **OECD's Framework and Recent Studies with Three Fish Species**
(Chairpersons: Taisen Iguchi, Rodney D. Johnson and Anne Gourmelon)

9:30-10:00 **OECD's Framework and Validation Test on the Endocrine Disrupting Chemicals**
(Anne Gourmelon: OECD)

10:00-11:40 **Report on the Results of Positive Controls, such as EE2, E2 and MT etc. with three Fish Species.**

10:00-10:30 Medaka (Masanobu Maeda:CERI)
10:30-11:00 Zebrafish (Gitte Petersen:DHI Water and Environment)
11:00-11:30 Fathead minnow (Rodney Johnson; US EPA)
11:30-11:40 Discussion

Lunch 11:40-12:40

Session II **Sensitivity and/or Variance of Parameters such as Vitellogenin and Gonad Histology for Three Fish Species**
(Chairpersons: Taisen Iguchi, Rodney D. Johnson and Anne Gourmelon)

Part I

12:40-14:10 **Vitellogenin(for methodology, antibody and standard)**
12:40-13:00 Medaka (Norihisa Tatarazako :National Institute for Environmental Studies, Japan)
13:00-13:20 Zebrafish (Thomas Braunbeck: Heidelberg University)
13:20-13:40 Fathead minnow (Karen Thorpe; University of Exeter/AstraZeneca)
13:40-14:10 Discussion

Tea Break 14:10-14:25

Part II

14:25-16:05 **Gonadal Historogy (for methodology and endpoints available for each fish including secondary sex characteristics)**
14:25-14:45 Medaka(Masanori Seki: CERI)
14:45-15:05 Zebrafish (Leif Norrgren:Swedish University of Agricultural Sciences)
15:05-15:25 Fathead minnow (Rodney Johnson; US EPA)
15:25-16:05 Discussion

Part III

16:05-16:30 **Draft Protocol of Phase 1 Test and Other Issues**
General Discussion

16:30 **Closing**

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Abstracts

Opening Address I

Greetings

Endocrine disrupters (EDs) have become the focus of great concern among the general public over their adverse effects on human beings and wildlife, however there is still much to be scientifically explored, which has made EDs a key environmental safety issue.

Therefore, the Ministry of the Environment, Japan (MOE) has been conducting environmental monitoring and hazard assessment, and also has been engaged in the exchange of information with other countries and international organizations under a framework of international coordination.

As a part of these activities, the MOE has started developing testing methods using Medaka, one of the ideal models for the research and testing for toxicology so as to evaluate the effects of endocrine disrupters.

This symposium is planned to discuss the development of testing methods of endocrine disrupters using Medaka, building networks among researchers.

I earnestly hope that it will be a valuable conference that could contribute to new advances in this field.

Kazuhiko ADACHI
Director,
Environmental Health and Safety Division
Ministry of the Environment
Government of Japan

Opening Address II

Activities of Fish Drafting Group under the MOE Program with Endocrine Disruptors

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It is an honor and my pleasure to open the Third International Medaka Symposium at the Okazaki Conference Center. The previous two symposia occurred in Nagoya.

Research and policy activities concerning endocrine disrupter in the Ministry of the Environment (MOE) were initiated in 1998 with the release of the document entitled “Strategic Programs on Environmental Endocrine Disrupters (SPEED’98)”. Following this initial program, a major research project was launched in 1998 .The purpose of the project was to develop screening tests and evaluate the endocrine disruptive effects of 65 substances. Since its initiation in 1998, the MOE project has progressed with research activities focused on mammals and other species important to ecosystem health, including fish, birds and amphibians.

One of these activities initiated by the MOE involves the development of testing methods using medaka. As part of this MOE sponsored research project, the Fish Working Group for Development of Screening Tests and Testing was established in 1999. The aim of the Fish Working Group is to develop testing methods using medaka that can eventually be submitted as draft protocols to the OECD.

At the time this group was established, it was very important to determine what biological effects various endocrine disruptors induced and whether those responses changed based on the sensitivity at any given stage during the fish life cycle. Further, it was important to determine what kinds of chemicals had biological effects and whether the changes induced were similar to those observed in other species. Fish full life cycle tests have been started to confirm the effects of a number of reference chemicals, such as estradiol-17â, ethynylestradiol and methyltestosterone, using **medaka**. For the second stage of these studies, partial fish life stage toxicity testing and reproduction testing has been developed. Further, new **medaka** strains, such as FLF and d-rR have been used for the development of these tests. *In vitro* studies also have been developed in order to screen many of the

chemicals of concern.

Today, we have the pleasure of listening to a special lecture in the area of fish sexual differentiation. In this presentation, the sex-determination gene of medaka, DMY (Y-specific DM-domain gene) will be discussed. This gene is the first to be found in non-mammalian vertebrates.

In conclusion, I am very pleased to open this meeting where I believe there will be a great opportunity to share useful information and I am confident that this meeting will be useful and successful for all.
Thank you for your attention.

Opening Address III

Status of the OECD Special Activity on Endocrine Disrupters Testing and Assessment

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The OECD endocrine disrupter activity was initiated in November 1996 at the request of OECD Member countries and industry. A Task Force on Endocrine Disrupter Testing and Assessment (EDTA) was then established to manage the OECD activity on endocrine disrupters in late 1997. The objectives of the EDTA Task Force are to:

- Identify the needs and prioritize the development of new and enhanced guidelines for the detection and characterization of endocrine disrupting chemicals;
- Develop a harmonized testing strategy for the screening and testing of endocrine disrupters;
- Manage validation work for newly developed and enhanced Test Guidelines as appropriate; and
- Provide practical tools for sharing of testing results and assessments.

A major achievement of the EDTA Task Force has been the definition of an initial framework capturing the potential screening and testing needs in both human health and ecotoxicological areas. The framework attempts to identify tests at different of levels of biological and regulatory complexity: from their interactions with hormone receptors to tests in whole animals at different sensitive life stages and tests where effects might be passed to the next generation.

The EDTA Task Force validation work on new and enhanced methods aims to ensure the reliability and relevance of testing methods in order to allow regulatory acceptance. This validation work is in accordance with the OECD Solna principles, recognized by many centers involved in the validation of test methods (ECVAM, ICCVAM).

The Validation Management Groups (VMG) have been established to co-ordinate and oversee the conduct of method development and validation in three

basic areas: i) mammalian toxicological tests, ii) ecotoxicological tests, and, in the near future, iii) *in vitro* and other non-animal tests.

The VMG for mammalian tests selected three *in vivo* tests as priorities for further development and international validation: the rodent uterotrophic assay (estrogen and antiestrogen activities), the rodent Hershberger assay (androgen and antiandrogen activities), an enhanced Guideline 407 (Repeated dose toxicity) to evaluate several new endocrine related parameters. Validation work is completed for the rodent uterotrophic assay and an independent peer-review will be organized. Validation work is well underway for the Hershberger assay and the enhanced TG 407.

The VMG for ecotoxicity tests oversees the development and validation of test methods for fish, birds and amphibians and it will soon start discussion on invertebrates testing. The work is at an early stage of development. Pre-validation studies for a fish screening assay will start in 2003, endpoints covering (anti) estrogen, aromatase inhibitors and (anti) androgen activities are included. Methods involving fish sensitive life-stages and full life cycle will be considered in higher tiers. Activities on an amphibian metamorphosis assay were proposed by Members countries to address chemicals disrupting the thyroid function. Two avian reproduction tests are being considered: a 1-generation study and 2-generation study.

The Current Testing Strategy by the Ministry of the Environment, Japan, Requires Evaluating the Estrogenic Activity of Chemicals with Medaka

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In screening, a vitellogenin assay is conducted to detect estrogenic activity of suspected EDs. Then, fish partial life-cycle test (PLC) is performed to elucidate their effects on the sexual differentiation of **medaka**, because this test had higher sensitivity for several estrogens than the reproduction test (REP) with matured fish. If adverse effects were not observed in the PLC, the fish full life-cycle test (FLC) is not necessary. If adverse effects were observed in the PLC, FLC would then be conducted (see attached scheme).

1.Screening

(i) Vitellogenin assay

The test has been completed for 20 substances[1),2),3)].

(ii) Partial life-cycle test (PLC)

The test has been completed for 12 substances[1),2)]. The results and the guidelines are described in the attached papers "Results of Assay and Tests in Evaluation of the Endocrine Disrupting Activities in Fish (*Medaka*)"and "The *Medaka* partial life-cycle test guideline".

(iii) Development of FLF/d-rR *Medaka*

Early life stage study has been developed using *Medaka* (FLF/d-rR) in which the

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[1] 12 chemicals selected in 2000:

Tributyltin, 4-octylphenol, nonylphenol, di-n-butylphthalate, octachlorostyrene, benzophenone, di-cyclohexylphthalate, di-(2-ethylhexyl)phthalate, butylbenzylphthalate, di-ethylphthalate, di-(2-ethylhexyl)adipate and triphenyltin

[2] : The results were announced on 14th June, 2002.

[3] Each 8 chemicals selected in 2001 and 2002:

Pentachlorophenol, amitrole, bisphenol A, 2,4-dichlorophenol, 4-nitrotoluene, di-pentylphthalate, di-hexylphthalate and di-propylphthalate in 2001.
Hexachlorobenzen, Hexachlorocyclohexane, Chlordane, Oxychlordane, trans-Nonachlor, DDT, DDE, DDD in 2002.

genetic sex can be distinguished simply by observing its leucophore type which influences the color of the body.

2. Testing- Full life-cycle test – (FLC)

The test has been completed for 3 substances (nonylphenol, 4-octylphenol and di-n-butyl phthalate). The results for nonylphenol were published as a risk assessment report in August 2001, and the results of the other two substances have been announced[3]. The results and the guideline are described in the attached papers "Results of Assay and Tests in Evaluation of the Endocrine Disrupting Activities in Fish (*Medaka*)"and "The ***medaka*** full life-cycle test guideline".

3. *In vitro* studies

(i) *Medaka* estrogen receptor (*me*ERá and *me*ERâ) binding assay and *Medaka* estrogen receptor (*me*ERá and *me*ERâ) reporter gene assay has been developed and validated, and 12 substances[1),2)] have been tested to date.

(ii) *Medaka* androgen receptor (*me*AR) reporter gene assay has also been developed and validated, and 12 substances[1),2)] have been tested to date.

4. Others (The study on the mechanism of sexual differentiation, DNA chip and DMY)

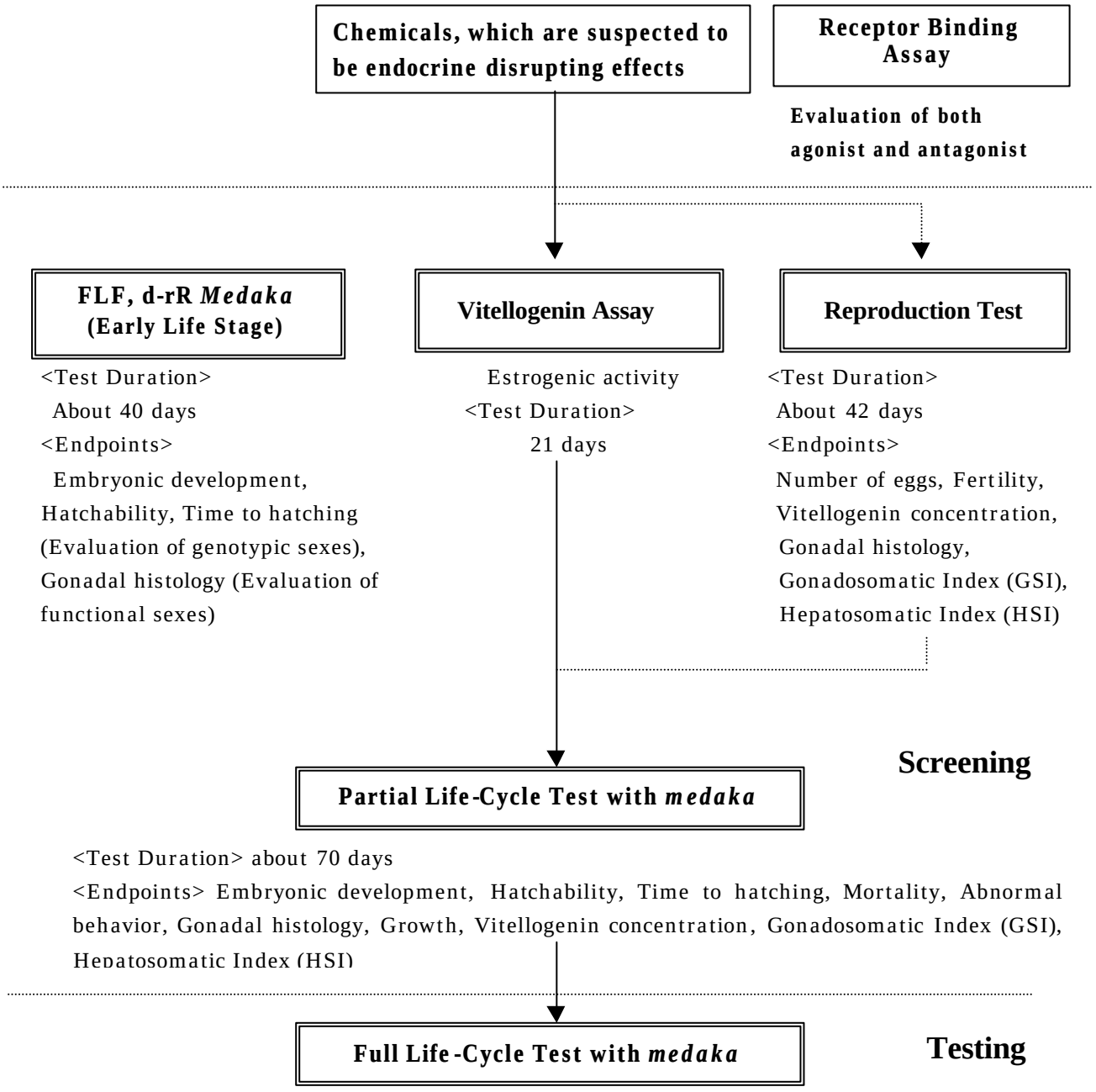
In order to clarify the effects and mechanisms of the EDs on the differentiation of sexing *Medaka*, genetic technology has been used to perform the following studies:

(i) Cloning a series of genes associated with the regulation of the differentiation of sex in *Medaka.*

(ii) The sex-determining gene of *Medaka*, DMY (Y-specific DM-domain gene) has been reported and is the first such gene discovered in a non-mammalian vertebrate. The report of this work was published in the influential international science journal "Nature". (Matsuda *et al.*: DMY is a Y-specific DM-domain gene required for male development in the *medaka* fish. Nature, 417: 559-563, 2002)

(iii)Preparation of a gene, which appears in the period of sex differentiation in order to develop a DNA chip containing a series of genes associated with the differentiation of sex concerning *Medaka.*

Testing Scheme in Evaluation of the Endocrine Disrupting Activities in Fish



Zebrafish as a Test Organism in Endocrine Disrupter Research

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The model character of zebrafish (*Danio rerio*) for a lower vertebrate is mainly due to its extensive use in developmental and neurobiology as well as in, where it has become by far the best-studied organism at all. In ecotoxicology, zebrafish has primarily been used as a test species in life-cycle analysis and early life-stage tests. Moreover, it has been intensively applied in the detection of acute and lethal toxicity of environmental chemicals. As a consequence, together with fathead minnow and Japanese medaka, the zebrafish has been recommended as test species in many existing standard guidelines and has been proposed as a test species for routine endocrine disrupter research.

The zebrafish is an oviparous cyprinid with a relatively short life cycle (4 months from egg to sexual maturation). Spawning is stimulated by the onset of light and occurs every 2nd to 5th day depending on nutrition, temperature, stocking density, age and sex ratio. The number of eggs spawned can be up to 400 eggs/female every day with a fertilization rate of 70 – 80 %. Pheromones play a key role in the reproduction, in that initiation of spawning behavior coincides with the release of female pheromones, whereas the presence of male pheromones triggers ovulation. Hatching is normally accomplished not later than 96h after fertilization at 24 °C to 32 °C.

During development, male zebrafish pass through a stage of juvenile hermaphroditism (undifferentiated gonochorist; juvenile hermaphroditism): After 10 – 12 days, the gonads of all juveniles start to differentiate into ovaries irrespectively of their genetically determined sex; at this stage, gonads consist of clusters of proliferating oogonia and previtellogenic oocytes, each surrounded by granulosa cells. The development of ovaries continues until 23 - 25 days, after which definite sex differentiation starts. The sex-labile period for zebrafish could thus be localized within 20 to 60 days post-hatch. Normally, 50 % of a population

will continue to develop ovaries; in the remaining 50 %, ovaries will degenerate and be transformed into testis. This transformation is characterized by the appearance of degenerating previtellogenic oocytes, granulation and vacuolization of the cytoplasm, as well as irregular oocyte shape. The normal sex ratio may vary and sex ratios of 40:60 (m:f) are not uncommon. After 40 days, sex differentiation is completed, and gonadal maturation will be finished after 60 days. First spawning occurs after about 75 days, when, under optimal conditions, all stages including previtellogenic, vitellogenic and completely mature oocytes are present. In adult post-spawning female zebrafish, oocytes atretic may be present. As an important factor controlling gonadal differentiation in zebrafish, modulation by differential expression of aromatase (cytochrome P45019A and B) has to be taken into consideration. Growth rate may vary with supply of food. and stocking density.

Since zebrafish is a juvenile hermaphrodite, and due to the fact that zebrafish lacks sex chromosomes, development of functional sex in zebrafish is very dynamic and that zebrafish is especially responsive to environmental conditions including exposure to endocrine disrupting chemicals. Exposure to endocrine disrupting chemicals may have great impact on gonad morphology and sex ratios in zebrafish. Exposure to high concentrations of estradiol and certain chemicals, e.g. PCBs, may cause delayed differentiation of oocytes. Exposure to 17á-methyltestosteron may cause estrogen-like effects in testis and reverse the functional sex from male to female. Intersex in zebrafish may be observed in association with exposure to hormonally active agents such as 17a-methyltestosteron, 17a-ethinylestradiol and flutamide. The degree of intersex varies from a sporadic presence of previtellogenic oocytes in the testicular tissue to mature eggs in a large number in close association to spermatids.

Beside gonad morphology and sex ratio, vitellogenin has been proposed as key endpoints in ecotoxicological test protocols submitted to the OECD. For zebrafish, two vitellogenin genes have been identified, both of which are expressed sex- and organ-specifically. For Z-Vg1, there is an 87 % homology with the vitellogenin-mRNA of fathead minnow. At least on the basis of the amino acid sequence, Z-Vg2 can also be unequivocally assigned to the group of fish vitellogenins. In adult zebrafish exposed to, e.g., 17á-ethynylestradiol, both vitellogenins as well as estradiol receptor and zona pellucida proteins in the liver and testes strongly increase within 1 - 2 days to reach a plateau after 5 days. In unexposed larval zebrafish, estradiol receptors á and â mRNAs can be detected as early as 1 and 2 day(s) after fertilization, respectively, but vitellogenin mRNA could only be visualized from day 4 in larvae stimulated by exposure to 17á-ethynylestradiol. There is evidence that the zebrafish estradiol receptor â has to be subdivided into two subtypes, zfERâ1 and zfERâ2.

Vitellogenin measurement by means of RT-PCR is especially useful for small

fish species such as zebrafish or medaka, since the liver of even subadult specimens is large enough to gain sufficient material for amplification. Exposure of adult zebrafish to reference endocrine disrupters such as nonylphenol, methoxychlor, and 17á-ethynylestradiol resulted in an induction of both vitellogenins as well as the estradiol receptor á. The LOECs for the induction of Z-Vg1-mRNA by nonylphenol and 17á-ethynylestradiol were 25 µg/L and 2,5 ng/L, respectively. For zfERá, corresponding LOECs were 25 µg/L and 2,5 ng/L.

To achieve sufficiently low detection limits for vitellogenin as a protein, immunochemical methods such as RIA, ELISA or immunohistochemistry are required. Protocols for the quantification of fish vitellogenins have been published for a range of different species, and, at the moment, three homologous ELISA systems are available for zebrafish, part of which are based on lipovitellin as antigen. Due to the small size of zebrafish, most studies have used whole-body homogenates rather than blood. Practical detection limits for zebrafish-based vitellogenin ELISAs are in the range 40 ng vitellogenin/g fish; LOECs for 17â-estradiol and 17á-ethynylestradiol are 21 and 1.67 - 3 ng/L, respectively. EC_{10}, EC_{50} and EC_{90} values are 15.4, 41.2 and 67.1 ng/L for 17â-estradiol and 0.1 - 0.92, 2.51 and 4.09 ng/L for 17á-ethynylestradiol.

Vitellogenin induction in zebrafish is thus an important an early warning signal, since it coincides with or precedes effects at higher levels of organization, namely effects on growth delay in spawning, alterations in mating behavior, reduction in egg number and fertilization success (all 1,67 ng/L) as well as gonadal histology in whole life-cycle experiments (3 ng/L). Most importantly, the antibody (DR264) shows cross-reactivity with a range of other commonly used cyprinids including carp, fathead minnow and roach. In addition, there are heterologous systems based on the cross reactivity with antibodies raised against antigens from closely related species such as carp. Whole-body immunohistochemical detection of vitellogenin in combination with image analysis has been developed for zebrafish, but requires substantial additional effort for calibration and validation before use.

In the zebrafish, potent endocrine disrupters such as 17â-estradiol and 17á-ethynylestradiol skewed the sex ratio of siblings of exposed females, caused necrosis of the testes and induced production of the estrogen-regulated vitellogenin. In addition, arrest of embryo development in eggs from exposed mature females has been observed. Exposure of zebrafish during the sex-labile period of 20 to 60 days post-hatch has been shown to be sufficient to detect the influence of relatively low concentrations of estrogens (< 0.6 ng/L 17α-ethynylestradiol) and androgens (22 ng/L 17á-methyltestosterone). Thus, by abbreviating exposure periods to early life stages, running arising from prolonged exposures might be reduced considerably. Assays in which zebrafish has been used cover full life-cycle assays, non-spawning assays with variable exposure periods and partial life-cycle assays. Major endpoints

were reproduction, vitellogenin response, histopathology, GSI and effects on sex ratio (Table 1).

Most results have been obtained with a partial life-cycle assay protocol, which has also been proposed to the OECD as a candidate for a screening assay. The test proposal is based on a homogenous group of individuals originating from a group of broad fish. The number of fish in each group is 60, and exposure starts at day 20 post-hatch, a stage when initial normal mortality has passed. The exposure is conducted between days 20 to 60 post-hatch, i.e. the sensitive window for endocrine disrupting effects in zebrafish. Sampling for vitellogenin measurements (20 fish per concentration) is performed on day 34 post-hatch (i.e., 14 days after the onset of exposure). Exposure of the remaining fish is continued until 60 days post-hatch (i.e., 40 days of exposure in total), after which the fish are sampled and fixed for histopathology, immunochemistry and sex ratio determination. Core endpoints include vitellogenin induction, gonad histopathology and sex ratio, with focus on germ cell maturation, intersex development, etc. The protocol proposed minimizes the use of animals and maximizes the utilization of scarified animals, since a large number of endpoints reflect various aspects of endocrine disruption. In addition, general toxicological impact can be detected. Positive control substances such as 17á-ethynylestradiol, 17â-estradiol, estriol, estrone and 17á-methyltestosterone have all been evaluated in the partial life-cycle assay.

Table 1. Results obtained in the different zebrafish assays

Chemical	Life-stage	End-point	Duration (days)	NOEC (µg/L)	LOEC (µg/L)	EC_{50} (µg/L)	Reference
17á-Ethynyl-estradiol	AD	Vtg	10	0.0008	0.0011	-	Duis et al. 2003
	AD	Vtg	8	0.0002	0.004	0,00251	Rose et al. 2002
	AD	Vtg	21		0.0017	-	Fenske et al. 2001
	LA – JU	Vtg	18	<0.0006	0.0015	-	Örn et al. 2003
	LA – JU	Sex ratio	40	<0.0006	0.0006	-	Örn et al. 2003
	AD	Vtg	4		0.0025	-	Islinger et al. 2003
	AD	GSI	6		0.0010	-	Van den Belt et al. 2002
Estradiol	AD	Vtg	8	0.0129	0.0214	0,0412	Rose et al. 2002
	LA – JU	Vtg	18	0.024	0.054	-	Petersen et al. 2003
	LA – JU	Sex ratio	40	Coming	Coming	-	Petersen et al. 2003
Estriol	LA – JU	Vtg	18	2.9	6.7	-	Petersen et al. 2003
Estrone	LA – JU	Vtg	18	0.036	0.049	-	Petersen et al. 2003
	LA – JU	Sex ratio	40	0.036	0.049	0.044	Petersen et al. 2003
17á-Methyl-testosterone	LA – JU	Vtg	18	0.022	0.042	-	Petersen et al. 2003
	LA – JU	Sex ratio	40	<0.022	0.022	-	Petersen et al. 2003
Bisphenol A	LA – JU	Vtg	18	600	> 600	-	Holbech et al. 2003
	LA – JU	Sex ratio	40	400	600	-	Holbech et al. 2003
Octylphenol	AD	GSI	21	12.5	25	-	Van den Belt et al. 2001
	AD	Vtg	12	3.5	5.1	-	Rose 2001
Butylparaben	AD	Vtg	8	25	50	-	Olesen 2003
Propylparaben	AD	Vtg	8	25	50	-	Olesen 2003

LA = Larvae; JU = Juvenile; AD = Adult

Duis, K. et al. (2003) Manuscript in prep.

Fenske, M., van Aerle, R., Brack, S., Tyler, C.R., Segner, H. (2001) Development and validation of a homologous zebrafish (*Danio rerio* Hamilton - Buchanan) vitellogenin enzyme-linked immunosorbent assay (ELISA) and its application for studies on estrogenic chemicals. Comp. Biochem. Physiol 129C: 217-232.

Islinger, M., Willimski, D., Völkl, A., Braunbeck, T. (2003) Effects of 17a-ethinylestradiol on the expression of three estrogen-responsive genes and cellular ultrastructure of liver and testes in male zebrafish. Aquat. Toxicol., in press.

Örn, S., Holbech, H., Madsen, T.H., Norrgren, L., Petersen, G.I. (2003). Exposure of juvenile zebrafish (*Danio rerio*) to ethynylestradiol and methyltestosterone; effects on gonad development and vitellogenin induction. Aquat. Toxicol., submitted.

Olesen, A. (2003). Masters thesis. In preparation.

Petersen, G.I., Kinnberg, K., Holbeck, H., Norrgren, L. (2003) Validation of the partial life cycle assay with zebrafish (*Danio rerio*) by exposure to ethynylestradiol, 17â-estradiol, estriol, estrone. In preparation.

Rose, J., Holbech, H., Lindholst, C., Norum, U., Povlsen, A., Korsgaard, B., Bjerregaard, P. (2002) Vitellogenin induction by 17ß-estradiol and 17a-ethinylestradiol in male zebrafish (*Danio rerio*). Comp. Biochem. Physiol. 131: 531-539.

van den Belt, K., Verheyen,R., Witters, H. (2002) Reproductive effects of ethinylestradiol and 4*t*-octylphenol on the zebrafish (*Danio rerio*). Arch. Environ. Contam. Toxicol. 41: 458-467.

Fathead minnow

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Overview

This short chapter describes a test method that is undergoing parallel development in the USA and UK with the fathead minnow (*Pimephales promelas*) suitable for assessing potential reproductive effects of chemicals, with an emphasis on endocrine pathways controlled by estrogens and androgens. The test is conducted with reproductively-mature animals for 21 d. Endpoints assessed include: adult survival, reproductive behaviour, secondary sex characteristics, gonadosomatic index, gonadal histology, plasma concentrations of vitellogenin and sex steroids (â -estradiol, testosterone, 11-ketotestosterone), fecundity, fertility, and, if desired, F_1 viability. In addition to describing the test method, guidance is presented as to interpretation of test results with respect to identification of specific classes of endocrine-disrupting chemicals.

Background and Scope

There has been recent concern for the potential effects of endocrine-disrupting chemicals (EDCs) on reproduction and development of humans and wildlife species (Colborn *et al.* 1996). The Office of Research and Development of the U.S. Environmental Protection Agency (U.S. EPA) has identified EDC issues as one of six high priority research areas (Kavlock *et al.* 1996; Ankley *et al.* 1997). Further, in response to legislation passed by the U.S. Congress (Food Quality Protection Act, PL 104-170; Safe Drinking Water Act, PL 104-182), the U.S. EPA is implementing a screening program for EDCs with specific mechanisms/modes of action (MOA). To aid in the development of this screening program, the U.S. EPA cosponsored a series of expert workshops on screening methods (Gray *et al.* 1997;

Ankley *et al.* 1998; DeVito *et al.* 1999), and convened a multi-stakeholder advisory committee (Endocrine Disruptor Screening and Testing Advisory Committee; EDSTAC) to recommend specific test methods and screening paradigms for EDCs (U.S. EPA 1998). The focus of these methods is on chemicals that may affect reproduction and/or development through disruption of physiological processes controlled by estrogen, androgen, and thyroid hormones. Parallel activities for the development of effective methods for detection of endocrine disrupting chemicals are also of high priority in Europe. Working under the guidance of the OECD (Organization for Economic Co-operation and Development), in Europe there is a major scientific effort to strengthen the current testing guidelines available for fish chronic toxicity testing to more adequately include EDCs. One screening assay recommended in both the U.S. EPA (1998) and Europe is a short-term (21 d) reproduction test with the fathead minnow (*Pimephales promelas*, Rafinesque). This test is designed to identify chemicals that affect processes controlled by estrogens and androgens. A screening test with fish is considered particularly important for two reasons: (1) estrogenic/androgenic controls on reproduction/development in fish may differ significantly enough from that of higher vertebrates such that mammalian (rat) screening methods may not identify potential EDCs in this important class of animals, and (2) as opposed to human health effects, there is currently significant evidence of adverse EDC effects in a variety of wildlife species, including fish (Crisp *et al.* 1997; Ankley and Giesy 1998; Tyler *et al*., 1998).

From an ecological perspective, determination of effects of toxicants on reproductive fitness and, hence, possible population-level impacts clearly is critical (Suter *et al.* 1987). However, in terms of screening for chemicals that cause toxicity via MOA of known concern, endpoints specific to these pathways also are important. In recognition of this, endpoints suggested as desirable for EDC screening in fish models include effects on reproductive behavior, secondary sex characteristics, gonadosomatic index, gonadal histology, and plasma concentrations of vitellogenin and sex steroids (â-estradiol, testosterone, 11-ketotestosterone) (Ankley *et al.* 2001; Jensen *et al.* 2001; Harris *et al.*, 2000; Ankley et al. 1998; U.S. EPA 1998). Recent studies have assessed the use of these endpoints in EDC studies with the fathead minnow, confirming their utility in this species, and providing important baseline data in terms of interpretation of results obtained from these standard test protocols. Specifically, induction of vitellogenin in response to estrogen receptor agonists (Kramer *et al.* 1998; Panter *et al.* 1998; Parks *et al.* 1999; Tyler *et al.* 1999; Harries *et al.* 2000; Korte *et al.* 2000; Ankley *et al.* 2001; Länge *et al.* 2001), and alterations in gonadal histology or secondary sex characteristics associated with exposure to estrogen or androgen receptor agonists (Smith 1974; Miles-Richardson *et al.* 1999a,b; Harries *et al.* 2000; Ankley *et al.*

2001; Länge *et al.* 2001), have been characterized in EDC screening studies with the fathead minnow. Finally, there also is an emerging database concerning the effects of EDCs with known MOA on patterns of circulating sex steroids in this species (Giesy *et al.* 2000; Makynen *et al.* 2000; Ankley *et al.* 2001; 2002; 2003; Jensen *et al.* 2003).

Summary of Test Protocol

This test is designed as a short-term reproduction assay suitable for identifying chemicals that affect reproduction or, potentially, development through disruption of any of a number of pathways, including those controlled by estrogens and/or androgens. Several potentially sensitive endpoints are assessed. An overview of specific test conditions is given in Table 1. The test is initiated with mature male and female fish that have a documented history of reproductive success as measured both by fecundity (number of eggs) and by embryo viability (e.g., hatch). This is established during a pre-exposure phase of 14 to 21 d in the same system/test chambers as will be utilized for the chemical exposure. During the subsequent 21-d chemical exposure, survival, reproductive behavior, and secondary sex characteristics are observed, and fecundity (number of spawns and number of eggs/spawn) monitored daily. Viability of resultant embryos (e.g., hatching success, developmental rate, occurrence of malformations) can be assessed in animals held either in clean water or in the same treatment regime to which the adults were exposed. At conclusion of the 21-d test, blood samples are collected from the adults for determination of plasma vitellogenin and sex steroids, the gonads sampled for measurement of the gonadosomatic index (GSI) and histological analyses, and secondary sex characteristics quantified. Effects in the treatment groups are assessed by comparison to control groups to determine if any of the endpoints in the exposed fish are significantly different from those in controls. Those chemicals identified as positive in this test may be advanced for more comprehensive testing.

Test Results and Interpretation

The basic fathead minnow reproduction test described in this document has been conducted with a number of EDCs representative of several MOA. In this section we provide an overview of responses in the test to chemicals with known endocrine MOA, as a basis for interpretation of study results with unknown chemicals. Some individual responses are very diagnostic in terms of identification of a specific endocrine MOA (e.g., induction of vitellogenin in males caused by estrogen receptor agonists), but in many cases it is/will prove necessary to consider patterns of responses in the whole suite of endpoints to assess which (if any) endocrine pathway has been affected. It must be noted that the database from

which this interpretive guidance was developed is limited. For example, tests with chemicals with mixed (endocrine) MOA have been rare, and likely would result in unanticipated patterns of responses (e.g., see methyltestosterone example below). Another important shortcoming in the current knowledge base is a lack of data for chemicals which affect reproduction, but not through alterations in the endocrine systems of concern. The assumption in these cases is that some generic measure of reproductive potential would be affected (e.g., fecundity, GSI) in the absence of changes in other, more diagnostic, endpoints such as secondary sex characteristics, plasma vitellogenin and sex steroid concentrations, and gonadal histopathology.

Table 2 summarizes responses of fathead minnows to different EDCs in the context of the suite of endpoints described in this document. The most work, by far, has been with estrogen receptor agonists. Strong agonists, such as â-estradiol, reduce fecundity of actively-spawning animals, and consistently induce vitellogenin in males (Table 2; Kramer *et al.* 1998; Panter *et al.* 1998; Tyler *et al.* 1999; Korte *et al.* 2000). Other endpoints that have been reported to be affected by strong estrogen receptor agonists in sexually-mature fathead minnows include gonadal (testicular and ovarian) histopathology and alterations in secondary sex characteristics (Panter *et al.* 1998; Miles-Richardson *et al.* 1999a). Exposure of fathead minnows to chemicals that are weaker estrogen receptor agonists (e.g., alkylphenols, methoxychlor) elicit a qualitatively similar pattern of effects similar to those observed after exposure to stronger agonists, although the magnitude of the effects (not surprisingly) differs between weak and strong estrogens (Miles-Richardson *et al.* 1999b; Giesy *et al.* 2000; Harries *et al.* 2000; Ankley *et al.* 2001). For example, methoxychlor significantly decreased (but did not completely inhibit) spawning of fathead minnows at a concentration of about 5 ì g/L (Ankley *et al.* 2001). At this concentration, a significant induction of vitellogenin in male fathead minnows was observed; however, the response was much less pronounced than when adult male fathead minnows were exposed to strongly estrogenic substances (Panter *et al.* 1998; Korte *et al.* 2000; Ankley *et al.* 2001). There also have been descriptions of alterations in secondary sex characteristics and ovarian histopathology in adult fathead minnows exposed to weak estrogens (Miles-Richardson *et al.* 1999b; Harries *et al.* 2000; Ankley *et al.* 2001). Plasma concentrations of sex steroids also can be affected (in a sex-specific manner) by weak estrogen receptor agonists (Table 2; Ankley *et al.* 2001); presumably, if comparable data were available, this also would be observed in exposures with strong estrogens.

Two androgen receptor agonists have been evaluated using the short-term fathead minnow reproduction test (Table 2; Ankley *et al.* 2001; 2003). The synthetic androgen methyltestosterone elicited a suite of responses indicative of a chemical with a mixed estrogenic and androgenic MOA rather than a “pure”

androgen. Exposure to methyltestosterone at concentrations ≥ 0.2 mg/L caused an immediate cessation of spawning. Consistent with previous demonstrations (from aquaculture studies) that methyltestosterone is androgenic in fish, the adult females were clearly masculinized, exhibiting pronounced nuptial tubercle development within about 6 d of exposure. However, methyltestosterone also caused a large induction of vitellogenin in both males and females, which is a response consistent with (and relatively specific to) an estrogen receptor agonist. This likely occurred because methyltestosterone can be converted via aromatase to a methyl-estradiol analogue, which resulted in the fish actually being exposed to an estrogen/androgen mixture (Hornung *et al.* 2003). Given this, it is difficult to say whether other responses observed in the test (e.g., reduced steroid concentrations, reduced GSI, abnormal gonadal histology; Table 2) were due to the androgenic or estrogenic (or combined) nature of methyltestosterone. To address this uncertainty, additional testing was conducted with a non-aromatizable androgen, trenbolone actetate, a synthetic growth promoter used for livestock production (Ankley et al. 2003). An in vitro competitive binding study with the fathead minnow androgen receptor demonstrated that 17-â-trenbolone had a higher affinity for the receptor than that of the endogenous ligand, testosterone. The fish were exposed to target concentrations of 17-â-trenbolone ranging from 0.005 to 50 ìg/L. Fecundity of the fish was significantly reduced by exposure to measured test concentrations ≥ 0.027 ìg/L (Table 2). The 17-â-trenbolone was clearly androgenic in vivo at these concentrations, as evidenced by the de novo production in females of dorsal (nuptial) tubercles, structures normally present only on the heads of mature males. Plasma steroid (testosterone, â-estradiol) and vitellogenin concentrations in the females all were significantly reduced by exposure to 17-â-trenbolone (Table 2). The 17-â-trenbolone also altered reproductive physiology of male fathead minnows, albeit at concentrations much higher than those producing effects in females. Males exposed to 41 ìg 17-â-trenbolone/L (measured) exhibited decreased plasma concentrations of 11-ketotestosterone and elevated concentrations of â-estradiol and vitellogenin (Table 2). Overall, the studies with methyltestosterone and trenbolone clearly illustrate the utility of the 21-d fathead minnow assay for identifying androgenic chemicals.

Two putative anti-androgens have been evaluated in 21-d reproduction studies with the fathead minnow (Table 2). Makynen *et al.* (2000) assessed the effects of vinclozolin on fathead minnows held in a paired-breeding situation. Due to minimal reproduction in controls from that experiment, it was difficult to determine whether exposure to the mammalian androgen receptor antagonist affected fecundity of the fish. Vinclozolin did not markedly affect plasma steroid concentrations in males or females, and vitellogenin was not measured in that

experiment. At a concentration of about 700 ìg/L vinclozolin did, however, cause a significant reduction in GSI of the females, which was accompanied by retarded oocyte maturation and atresia. Because neither vinclozolin or its primary metabolites bound to the fathead minnow androgen receptor *in vitro* (Makynen *et al.* 2000), it was uncertain whether the responses observed in the gonads of the females were truly indicative of an anti-androgen. Therefore, the results of a reproduction test with the androgen receptor antagonist flutamide (which does bind to the fathead minnow androgen receptor; Makynen *et al.* 2000) may be more descriptive of the expected pattern of responses associated with exposure of reproductively-active fathead minnows to an anti-androgen. At concentrations ranging from 60 to 600 ìg/L, flutamide caused a concentration-dependent decrease in fecundity which, as was observed with vinclozolin, was accompanied by retarded oocyte maturation in the female fathead minnow (Table 2; Jensen *et al.* 2003). Flutamide also affected steroid concentrations and appeared to cause a slight increase in vitellogenin concentrations in both sexes. In addition, flutamide exposure resulted in subtle indications of gonadal histopathology in the males, which was comprised of cellular degeneration and necrosis. Based on these results, the most consistent effects of the anti-androgens in this test appear to be expressed in the gonads of the females (histopathology) and alterations in steroid concentrations.

Aromatase (CYP19) is a cytochrome P450-based enzyme that, under normal physiological conditions, converts testosterone to â-estradiol. There is emerging evidence that the MOA via which some EDCs exert their effects is through alterations in steroid synthesis associated with inhibition of aromatase activity (i.e., CYP19). Fadrozole, a classical inhibitor of aromatase activity, was evaluated using the protocol described in this document (Table 2; Ankley *et al.* 2002). The chemical caused a concentration-dependent reduction in fecundity at concentrations ranging from about 1.5 to 50 ìg/L. Consistent with the presumed MOA, there also was a concentration-dependent decrease in both plasma â-estradiol and vitellogenin in the female fathead minnow. In addition, plasma concentrations of testosterone and 11-ketotestosterone were increased in the males, and histological alterations observed in the gonads of both sexes. Given the specificity of aromatase inhibitors, the decreases in â-estradiol and, subsequently, vitellogenin in the female fathead minnow should be an excellent diagnostic response for this class of EDCs. Previous studies with fish have emphasized vitellogenin induction in males as a highly-specific indicator of an endocrine MOA (estrogen receptor agonists); these data indicate an equally useful and diagnostic response associated with vitellogenin reductions in (sexually-mature) females. This endpoint presumably would reflect effects of chemicals, not only on â-estradiol synthesis (as for fadrozole), but the action of chemicals that act as estrogen receptor antagonists.

The patterns of responses summarized in Table 2 clearly represent only a small subset of possible outcomes associated with exposure to EDCs. Adverse effects associated with some MOAs (estrogen receptor agonists, androgen receptor agonists, and aromatase inhibitors) should be easily identified. Identification of chemicals as anti-androgens may be more equivocal (although these types of chemicals would clearly be "flagged" as endocrine-active through alterations in gonadal histology and, perhaps, steroid concentrations). As this test is conducted with additional chemicals reflective of the MOA discussed above, as well as other MOA, guidance in interpreting test results will expand.

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Table 1. Overview of recommended exposure conditions for the fathead minnow 21-d reproduction test. Test conditions for two slightly different versions of the assay (Ankley *et al.* 2001; Harries *et al.* 2000), are differentiated by italics (Harries *et al.* is italicized).

1. Test type	Flow-through
2. Water temperature	25 ± 1°C
3. Illumination quality	Fluorescent bulbs (wide spectrum)
4. Light intensity	10-20 ìE/M^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)
5. Photoperiod	16 h light, 8 h dark
6. Test chamber size	18 L (40 x 20 x 20 cm) (minimum) *16 L (35 x 15 x 30 cm)*
7. Test solution volume	10 L *12.5 L*
8. Volume exchanges of test solutions	Minimum of six daily
9. Flow rate	Approximately 3.5 L/g fish/day
10. Age of test organisms	Reproducing adults (120 d minimum)
11. No. of fish per test chamber	Four females and two males *One female and one male*
12. No. of treatments	Two minimum (plus appropriate controls)
13. No. of replicates per treatment	Four minimum *Six minimum*
14. No. of fish per test concentration	Minimum of 16 females and 8 males *Minimum of 6 females and 6 males*
15. Feeding regime	Frozen adult brine shrimp twice daily *Frozen adult brine shrimp twice daily and pelleted fish food once daily*
16. Aeration	None unless dissolved oxygen concentration falls below 4.9 mg/L
17. Dilution water	Clean surface, well, or reconstituted water
18. Chemical dilution factor	Variable
19. Chemical exposure duration	≤ 21 d
20. Primary endpoints	Adult survival, reproductive behavior, secondary sex characteristics, gonadosomatic index (GSI) and gonadal histology, plasma vitellogenin and sex steroid concentrations, fecundity, and fertility
21. Optional endpoints	Embryo hatch, larval survival, and morphology
21. Test acceptability	Dissolved oxygen ≥60% of saturation; mean temperature of 25 ± 1°C; 90% survival in the controls; successful egg production in controls

Table 2. Performance of fathead minnow screening tests with known endocrine-disrupting chemicals.

MOA/Chemical	Effects Observed						
	Fecundity	**Sex**	**Secondary Sex Characteristics**	**Plasma Steroids**	**Vitellogenin**	**GSI**	**Gonadal Histology**
ER Agonist							
â-Estradiol [a]	–	*Male*	– Tubercle size	ND [b]	-	–	Sertoli cell proliferation, degenerative changes
		Female	None	ND	-	ND	Oocyte atresia, retarded oocyte maturation
Methoxychlor [c]	–	*Male*	None	– T, KT	-	None	None
		Female	None	– E	None	None	Oocyte atresia
AR Agonist							
Methyltestosterone [c]	–	*Male*	- Tubercle size	– T, KT	-	–	Germ cell necrosis, reduced spermatogenesis
		Female	Masculinized	– E, T	-	–	Oocyte atresia, retarded oocyte maturation
Trenbolone [d]	–	*Male*	None	– KT, - E	-	None	Increased spermatogenesis
		Female	Masculinized	– E, T [e]	–[e]	None	Oocyte atresia, retarded oocyte maturation
AR Antagonist							
Vinclozolin [f]	ND	*Male*	None	- E	ND	None	None
		Female	None	None	ND	–	Retarded oocyte maturation
Flutamide [g]	–	*Male*	None	- E	-	None	Cellular degeneration and necrosis
		Female	None	- T	-	None	Oocyte atresia, retarded oocyte maturation
Aromatase Inhibitor							
Fadrozole [h]	–	*Male*	None	- T, KT	None	-	Increased spermatogenesis
		Female	None	– E	–	None	Oocyte atresia, retarded oocyte maturation

[a] Data obtained from Kramer *et al.* 1998; Panter *et al.* 1998; Miles-Richardson *et al.* 1999a; and Korte *et al.* 2000.
[b] Not determined.
[c] Data obtained from Ankley *et al.* 2001.
[d] Data obtained from Ankley *et al.* 2003.
[e] Non-monotonic concentration-response relationships observed.
[f] Data obtained from Makynen *et al.* 2000.
[g] Data obtained from Jensen *et al.* 2003.
[h] Data obtained from Ankley *et al.* 2002.

Special Lecture

Sex Determination and Gonadal Sex Differentiation in Fish

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Sex determination and gonadal sex differentiation in fish are plastic. Since its first discovery in the medaka, *Oryzias latipes*, sex reversal using exogenous steroid hormones around the time of sex determination has been duplicated in other species. Because of these features, the fish is an excellent model for the study of endocrine disrupting chemicals. We have used three major fish species to study the genetic, hormonal and environmental aspects of sex determination and gonadal sex differentiation in fish.

Nile tilapia, *Oreochromis nitolicus*, is an excellent example of the precise nature of steroidogenic actions during gonadal sex differentiation. Using all genetic male and female tilapia, we have shown that steroid-producing cells in ovaries prior to and during sex differentiation express all of the steroidogenic enzymes required for estradiol-17â production. These results, together with evidence of masculinization of genetic females by fadrozole or tamoxifen, strongly suggest that endogenous estrogens act as the natural inducers of ovarian differentiation in tilapia. In contrast, the ability of steroid-producing cells to synthesize steroid hormones in all-male fry appears after testicular differentiation. *DMRT1* is expressed male-specifically in testicular Sertoli cells during sex differentiation, suggesting an important role of *DMRT1* in testicular differentiation in tilapia.

Protogynous hermaphroditism (i.e. female to male sex change) exemplifies the plasticity of teleost sex determination and sexual differentiation. Sex change takes place following alteration of social dominance, such being dependent upon differences in relative body size. In *Thalassoma duperrey*, down-regulation of gonadal aromatase correlates with ovarian degeneration during sex change indicating estrogens maintain female function and development. The subsequent sex change, i.e. testis formation, may be the default pathway in the absence of estrogens, the result of up-regulation of or increased responsiveness to putative testis-determining factors (e.g. androgens, DMRT1), or some combination.

The medaka has two major advantages for genetic research: a large genetic

diversity within the species and the existence of several inbred strains. Using positional cloning and detailed sequence analysis of BAC clones by shotgun sequencing, we have recently identified *DMY* (DM-related gene on the Y chromosome) as a strong candidate for the sex-determining gene of medaka, which possess a stable genetic XX/XY sex determining system. *DMY* contains the highly conserved DM domain found in other genes involved in sexual development in both vertebrates and invertebrates. Expression analysis during early embryogenesis shows that *DMY* is found in the somatic cells (Sertoli cells) of the XY gonads at the time when sex determination occurs. Two naturally occurring XY female mutants established *DMY*'s critical role in male development. One of these mutants contained an insertion that causes premature termination of the DMY protein. When mated, all of XY offspring with the mutant Y were female. The other mutant had a severe depression in *DMY* in the embryo and 60% of its XY offspring with the mutant Y developed as females. *DMY* provides the first example of a sex-determining gene in non-mammalian vertebrates.

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.

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

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, 17â-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 determing 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 ≥ 121 μg/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 ≥ 121 μg/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 lever. 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 phytoestorogens 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).

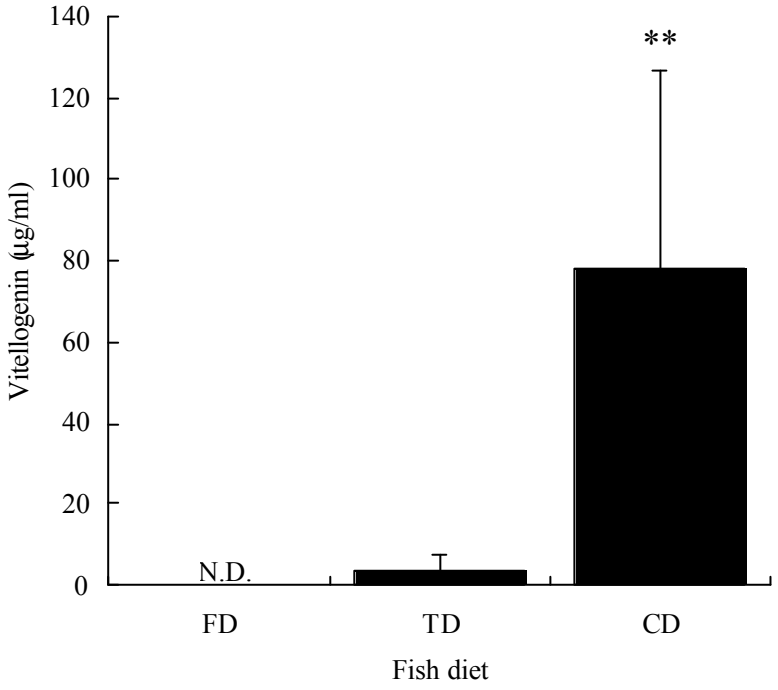

Figure 1. Plasma VTG levels in adult male goldfish.
Fish were fed 1.0 % body weight volume of one of the three diets (TD, CD and FD) every two days for 31 days. N.D. = Not Detected (less than 0.040 μg/ml). Columns and bars represent the mean and standard deviation. **, Significant difference compared to TD-fed fish (p<0.01).

Other information, MEDAKA report

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Since 1998, there has been a large effort to develop and validate procedures to identify EDs. Our research activity in the Strategic Programs on Environmental Endocrine Disrupters (SPEED'98) has been focusing on medaka. So far, fish full life-cycle test, partial life-cycle test and reproduction test have been conducted. Hazard assessment data of tributyltin, 4-nonylphenol and other 10 chemicals were circulated to the OECD in 2001 and 2002. This set of data is available from MOE's web site:

http://www.env.go.jp/en/topic/edcs/approach/2002.html

In September 2002, the Fish Gonad Histology Workshop took place at the RIVM in Bilthoven, the Netherlands. The purpose of the workshop is to exchange information and experience and also reach common understandings among experts of what would be considered as normal and abnormal fish histopathology. At the workshop, we agreed that histopathology of gonads is a relevant endpoint for screening tests.

In conclusion, we summarized our activities and compiled medaka test and results as the MEDAKA report and the database. I believe these would be a very helpful guidance for fish labs especially for the laboratories that are initiating a research study using medaka.

MEDAKA *report*

The MEDAKA report will be to provide scientific background on **medaka** bioassay for screening of EDs. This will contribute to the development of modification of early life-stage toxicity test for endocrine disrupting properties and also other general fish toxic properties.

Database

There are about 60 pictures regarding normal testis and ovary development and secondary sexual characteristics from embryo to almost matured adult stage. This database is still under construction. The name of database is the *Atlas of Medaka Gonadal Histology* and will be available from:

http://www.nies.go.jp/edc/edcdb/index_e.html

Closing

50 Years from Sex Reversal

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In the end of 2002, the Ministry of Environment, Japan, announced that they will launch a plan to generate a global standard of protocols for endocrine disrupters testing using medaka in Japan, fathead minnow in the United States, and zebrafish in Europe. The vitellogenin assay proposed as the key protocol reminds me that 2002 is the 50th anniversary of discovery of sex reversal by Tokio Yamamoto. First, he generated the d-rR strain by crossing of orange-red and white strains for five years in 1946-1950, in which the genetic sex is identified by the body color, that is, genetic males exhibit the red body and genetic females exhibit the white body. Then, he exposed this strain to estrogens or androgens, and obtained functional males with the white body and functional females with the red body. The sex reversal was proved in 1953. I recognize that our discussions of the endocrine disrupters in three medaka symposia from 2000 to 2003 are based on his framework of sex determination and differentiation.

In 1953, he noticed the possibility of mutation of sex-determining gene to interpret the presence of a small number of females with the red body or males with white body in control populations of the d-rR strain. The sex determining gene and its mutant genes were identified and cloned in 2002, which certainly becomes a root of toxicogenomics of endocrine disrupters. I also remind that the d-rR strain have been a key that induces the explosion of the medaka biology very recently, Y chromosme mapping, genome-wide mutagenesis, whole genome sequencing etc.

I hope, reminding Yamamoto's contribution in this field, that the third medaka symposium becomes the starting point for standardization of protocols by collaboration of three groups of medaka, fathead minnow and zebrafish.