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)

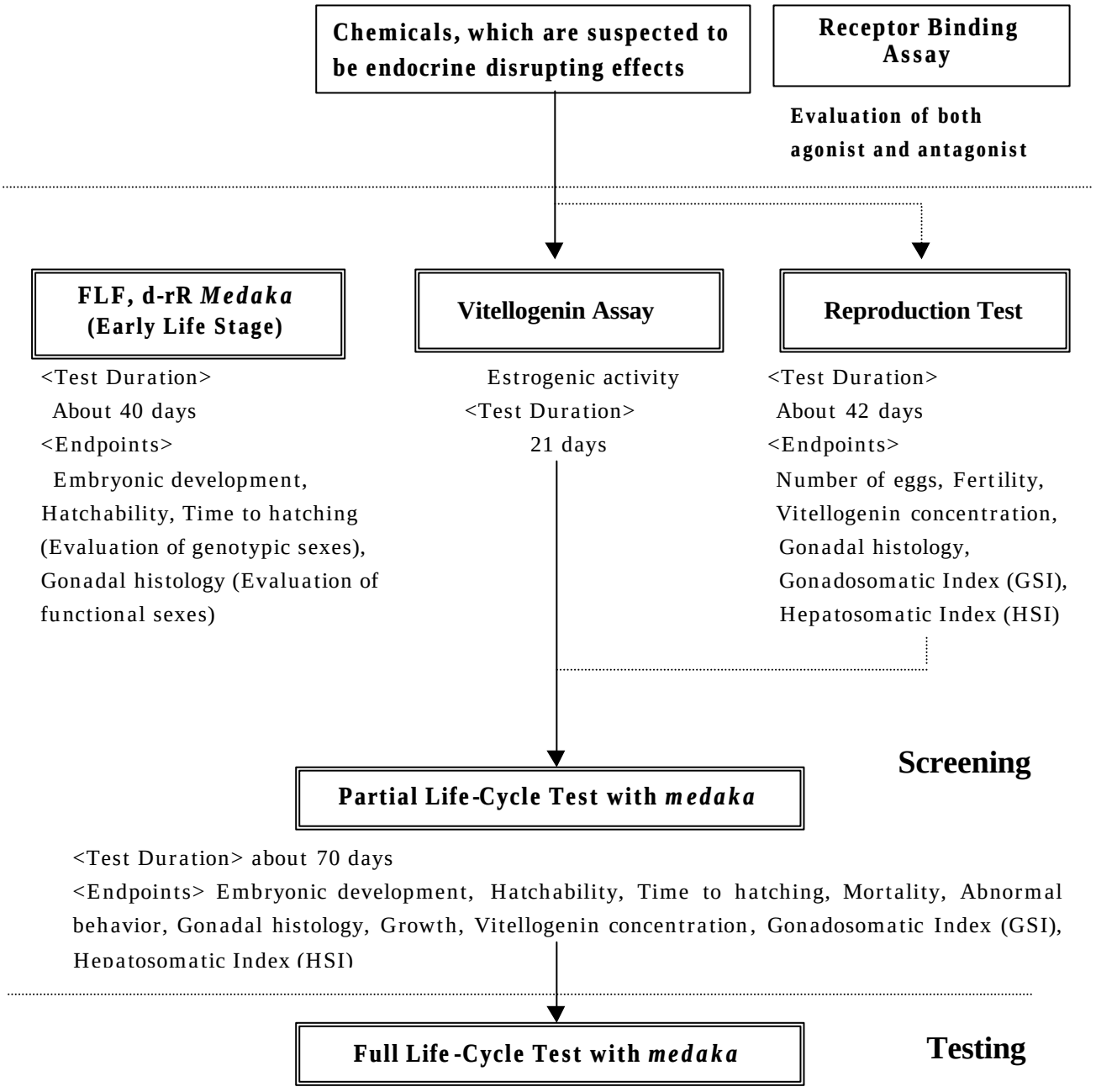
In order to clarify the effects and mechanisms of the EDs on the differentiation of sexing *Medaka*, genetic technology has been used to perform the following studies:

(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.

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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.

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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.