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Topic 4.12

Development of fish tests for endocrine disruptors*

T. H. Hutchinson[1,‡], H. Yokota[2], S. Hagino[3], and K. Ozato[4]

[1]AstraZeneca Global Safety Health & Environment, Brixham Environmental Laboratory, Brixham, Devon TQ5 8BA, UK; [2]Kurume Laboratory, Chemicals Evaluation and Research Institute (CERI), Japan; [3]Sumika Technoservice Corporation, Takarazuka-city, Japan; [4]Nagoya University Bioscience Center, Nagoya, Japan

Abstract: International concern over endocrine active substances (EASs) has led to intensive research programs to establish fish reproductive and developmental toxicity tests for use in environmental (ecological) risk assessment. This chapter gives an overview of key themes of in vivo ecotoxicology research, including fish screening assays, partial life-cycle tests (the draft Organization for Economic Cooperation and Development (OECD) fish reproduction test and the new fish development test) and fish full life-cycle tests. In the context of the OECD test guidelines program, fish species of primary interest include fathead minnow, medaka, and zebrafish, while guppy, rainbow trout, sheepshead minnow, and three-spined stickleback are also of scientific importance. Critical factors for evaluation include baseline reproductive biology and definition of EAS sensitive life-stages. For regulatory applications, a critical review of existing fish EAS data suggests that apical adverse effect endpoints, namely development, growth and reproduction (e.g., fecundity, fertilization rates and hatching success) should be used to derive predicted no effect concentrations (PNECs) for the environmental risk assessment of EASs. In support of these apical adverse effect endpoints, biomarker responses (e.g., vitellogenin, gonadal-somatic index, and gonad histopathology) should be used to provide mechanistic data, compare species (e.g., cyprinids vs. salmonids) and allow extrapolation between laboratory and field studies.

INTRODUCTION

Over the past decade, a growing body of scientific information has highlighted the potential threats of chemicals to the reproductive health of fish [1]. Internationally, the Organization for Economic Cooperation and Development (OECD) is actively coordinating research efforts for EAS testing in fish and other animal species [2]. In this context, the OECD test guideline program seeks to ensure that proposed fish test guidelines measure biologically relevant endpoints and that these endpoints are reproducible between laboratories internationally. This OECD effort is supported by significant regional initiatives in Europe, Japan, and North America that collectively seek to identify a cost-effective battery of fish screening and testing assays for the regional ecological risk assessment of EASs. It is widely recognized that a range of fish tests for EASs are needed, including partial and life-cycle protocols with a range of freshwater and marine species.

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‡Corresponding author

Importantly, different types of fish tests may be required according to different circumstances (e.g., persistent vs. unstable substances; constant release of the substance into surface waters or only limited episodic releases) [3]. Taking three illustrative scenarios: a fish full life-cycle test would be appropriate where an estrogenic chemical was being constantly discharged [4]; a fish development test or a fish reproduction test (both partial life-cycle tests) would be useful in addressing a nonbioaccumulative pesticide such as methoxychlor applied only seasonally [5,6]; while a short-term and inexpensive assay would be invaluable in rapidly screening potential EASs, complex mixtures, or effluents, and thereby help guide the experimental design of long-term studies [7–9].

In terms of test organisms, no single fish species monopolizes the useful attributes for use in environmental risk assessment protocols, consequently it is important to consider a suite of fish species when developing and validating test guidelines for EASs. Freshwater species of primary international interest include fathead minnow (*Pimephales promelas*) [6,9,10], medaka (*Oryzias latipes*) [5,11,12], and zebrafish (*Danio rerio*) [13,14]. Additionally, several other OECD fish species are also being used in EAS research, for example, guppy (*Poecelia reticulata*) [15], rainbow trout (*Oncorhynchus mykiss*) [8], sheepshead minnow (*Cyprinodon variegatus*) [16], and three-spined stickleback (*Gasterosteus aculeatus*) [17]. Against this background, this chapter summarizes key scientific developments in the establishment of new OECD test guidelines using fish, together with the use of such data in environmental risk assessments. Many of the principles are illustrated using the medaka, however, these examples apply equally to other OECD fish species.

SPECIES AND STRAIN SELECTION

Fathead minnow (*Pimephales promelas*)

This species is representative of the ecologically widespread Cyprinidae and has long been used to support water quality criteria through OECD and other test guidelines. This gonochoristic species (with no reports of natural hermaphrodites under natural conditions) is highly amenable to laboratory culture, and the life cycle is relatively rapid (4–5 months from embryo hatching to adult breeding at 25 °C). Fathead minnows are fractional spawners (typically, females produce batches of 50–100 transparent eggs every 3–5 days), the embryo-larvae are sensitive to EASs, and there is a growing body of information on the reproductive physiology and molecular endocrinology of this species [10,18–20]. To our knowledge there are no specific strains of fathead minnow available for EAS research, and wild-type populations are commonly used.

Medaka (*Oryzias latipes*)

The Japanese medaka is representative of the subtropical medaka (family Oryziatidae) and is widely used as small fish model in laboratory research and in ecotoxicology. The genetic sex determination, oogenesis, fertilization, and embryonic development of this species have been extensively studied [21,22]. In this gonochoristic species (showing no hermaphroditism under natural conditions), the generation time is short (typically 2–3 months), year-round spawning is daily under artificial conditions. Medaka sex is determined by XY chromosomes (i.e., female and male sex is determined by XX and XY chromosomes, respectively). It is known that some genes for pigmentation and DNA sequences identified by PCR are linked to sex chromosomes and very recently, a male determining gene on the Y chromosome (Dmy) was identified and cloned [23]. Using such markers, the genotypic sex of medaka can be accurately determined. Several medaka strains have been established in Japan for research into EASs (see <http://biol1.bio.nagoya-u.ac.jp:8000 for photographs>).

Orange-red variety or "Himedaka"

This strain with orange body color is commercially available and widely used for research and testing because it is the easiest to be maintained in laboratory conditions. This out-bred (wild-type) strain is well suited to testing EASs [24].

d-rR or S-rR (Yamamoto) strain

The genotypic sex of this strain can be identified using a color marker and the strain is therefore well suited to EAS testing [11]. The sex-specific body color is due to the location of the recessive gene (*r*) for the orange-red color in xanthophores on the X chromosome and the wild-type allele (*R*) on the Y chromosome (crossing-over rate between X and Y chromosomes is ca. 0.3 %). Expression of the body color occurs rather late, that is, some two weeks after hatching (depending on the laboratory conditions and feeding regime).

Qurt, FLF, and FLFII

In both the Qurt strain [26] and FLF strain [27] , the genotypic sex can be identified at early embryonic stages since leucophore differentiation occurs at the 2-day-old embryo stage in the male, but not in the female. This is due to the location of the recessive mutant gene (*lf*) for the white color in leucophores on the X chromosome and the wild-type allele (*LF*) in the Y chromosome (crossing-over rate of the color marker is rather high, ca. 3 to 4 %).

The Hd-rR.Y^{HNI} strain has the orange-red body and SL1 as markers for identification of the genotypic sex [28]. The genotypic sex can be identified accurately because the crossing-over rate of SL1 is almost zero. The FLFII strain was generated by crossing FLF with Hd-rR.Y^{HNI} . In the FLFII, the genotypic sex can be screened at early embryonic stages by the presence of leucophores, confirmed at larval stages by the presence of xanthophores, and finally reconfirmed by the PCR marker to eliminate errors in identification using pigment cells [29]. However, the *Dmy* sex marker may be used for all medaka strains, whereas the SL1 exists only in some specific strains. The FLF strain has been also used for studies of EASs [30].

See-through medaka

The see-through medaka is a fish model with a transparent body in the adult stage, as well as during embryonic stages [27]. 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. Importantly, the see-through medaka will provide an opportunity for noninvasive studies of morphological and molecular events caused by exposure to EASs.

Zebrafish (*Danio rerio*)

This tropical species (family Cyprinidae) is native to the Indian subcontinent, is well suited to laboratory culture, and the life cycle is relatively rapid (3–4 months from embryo hatching to adult breeding at 25 ± 2 °C). Zebrafish are considered to be a gonochoristic-undifferentiated species (namely, both juvenile males and females pass through an ovary like stage before differentiating into the phenotypic sex). In males, this includes a period of juvenile hermaphroditism, followed by a histological intersex phase with both immature ovaries and testes prior to development of the mature male gonads [31]. This process of oocyte apoptosis in juvenile zebrafish has recently been reported by Uchida et al. [32]. Zebrafish are sensitive to EASs, and there is a growing body of information on the reproductive toxicology of this species [13,14].

SCREENING FOR POTENTIAL ENDOCRINE ACTIVITY

Based on current experience, we propose that the most promising approach to date is the establishment of a new screening protocol adapted from OECD test guidelines 204 and 215. The concept protocol is

referred to as a "Non-spawning Fish Screening Assay" and is based upon the measurement of three core endpoints, namely vitellogenin, gonado-somatic index (GSI) and gonad histopathology [2]. For example, Panter et al. [33] reported a 21d adult fathead minnow assay measuring VTG and GSI response to estrogens. More recent work in Europe has extended this approach to juvenile fathead minnows using a variety of weak and potent antiestrogens [9]. Similarly, the Japanese Ministry of Environment [34] has successfully used a 14 to 21-day medaka screening assay to comprehensively detect benzophenone, di-cyclohexyl phthalate, di-ethyl phthalate, octylphenol, and triphenyltin chloride. Moreover, this approach has been successfully adopted for the rapid detection of xenoestrogens in sheepshead minnows [16] and zebrafish [13,14]. In addition to inducing VTG titres, xenoestrogen exposure has also been shown to reduce gonadosomatic index and cause histological changes in gonads of zebrafish after between 6 to 24 days [14]. In summary, available data indicate that the nonspawning fish screening test can be successfully in OECD fish species for the rapid detection (within 14–21 days) of a range of EASs. Other protocols that may have a future role in chemical testing programs are described below.

FISH DEVELOPMENT TEST (EXTENDED ELSs TEST)

It is now well established from aquaculture that fish early life-stages (ELSs) are sensitive to EASs. This fact, together with a reliance in regulatory ecotoxicology on the fish ELSs test for chronic hazard assessments [35] has led to the concept of a new "Fish Development Test" which can be seen as an extended ELSs test [36]. Depending on the suspected mode of action of an EAS (based on information gained in mammalian and fish screening assays), the assessment of developmental affects caused by EASs should include survival, growth, development, gonad histology, and VTG up to 90–100 days post-hatch. Demonstrations of aspects of this test concept have recently been reviewed [36]. Further details of the approach are illustrated for medaka.

Medaka development test

As medaka is a gonochoristic species (showing no hermaphroditism under natural conditions) a medaka development test could effectively address EAS-induced sex-reversal, one of the main endpoints of endocrine-disrupting effects. For example, the medaka sex-reversal assay is based on the d-rR and S-rR strains (see above). In the sex-reversal test, the sex-linked colors are unchanged by exposure to EASs but sex-reversal is identified by the gonad histology as well as the secondary sexual characteristics (dorsal and anal fins).

Fish are exposed to a chemical during their susceptible periods from the prelarva just after hatching to 28 days post-hatch and then reared for an additional 14 days in clean dilution water until their functional sexes become detectable. At the age of 42 days (at 24 °C), fish with a total length more than 20 mm are fixed and examined for secondary sexual characteristics on the dorsal fin (maximum length, and cleft depth between the last ray and preceding one) and anal fin [maximum length, length of second ray from the last, and appearance of small anal papillary processes (app) on the posterior region]. Thereafter, serial cross sections of gonads of the fish are made and observed microscopically.

The medaka sex-reversal test has been applied to a variety of chemicals, including 17α-ethinylestradiol (EE2), 17β-estradiol (E2), diethylstilbestrol (DES) 4-*t*-pentylphenol (4*t*PP), methyltestosterone (MT), and flutamide (Flu). The existence of a secondary sexual characteristic of males, small papillary processes on the anal fin, was confirmed in almost all genotypic males but not in control females (Fig. 1). In contrast, this character increased in genotypic females exposed to MT at 0.1 μg/l. No change was observed in either males or females exposed to Flu even at the highest concentration (1000 μg/l). The gonads of male and female control fish naturally differentiated to testis or ovary, respectively (Fig. 2). Gonads of genotypic males exposed to EE2, DES, 4tPP, and E2 differentiated into ovaries with LOECs of 0.032 μg/l, 0.032 μg/l, 10 μg/l, and 0.1 μg/l, respectively. On the con-

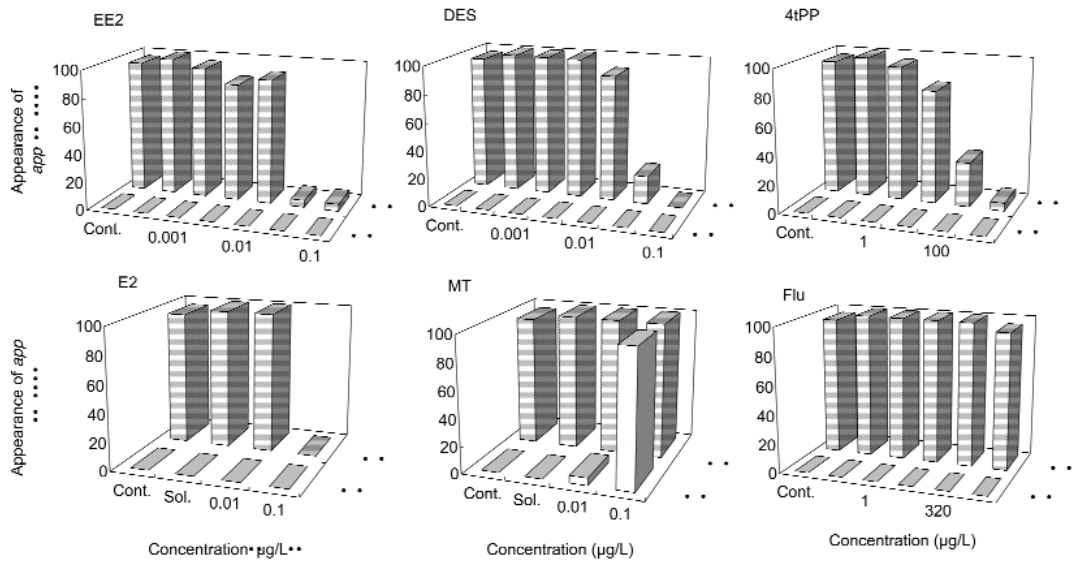

Fig. 1 Percentage of appearance of small papillary processes on anal fin of S-rR strain medaka exposed to ethynylestradiol (EE2), diethylstilbestrol (DES), 4-*t*-pentylphenol (4*t*PP), 17β-estradiol (E2), methyltestosterone (MT) and flutamide (Flu) (from Hagino et al., 2001).

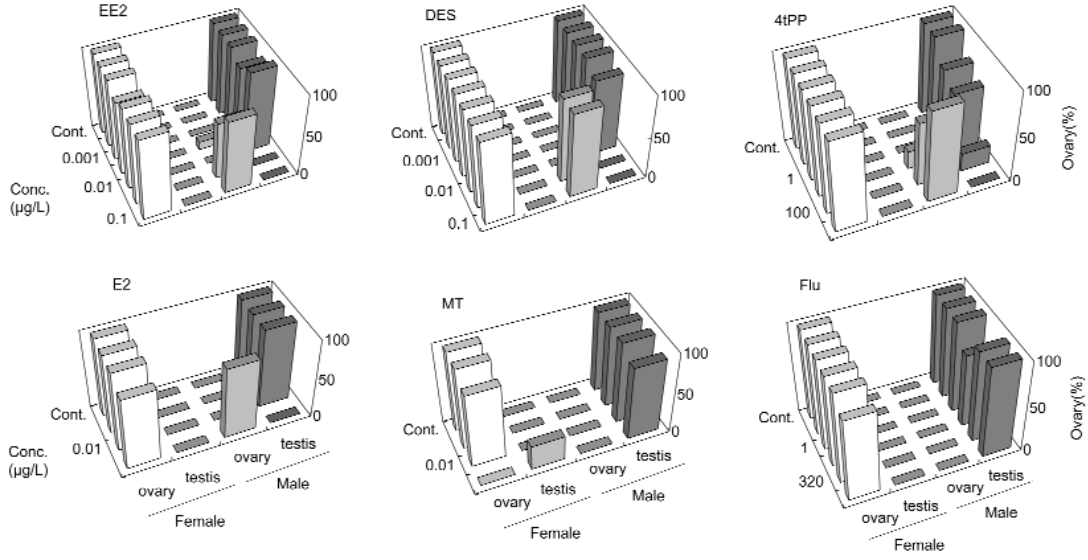

Fig. 2 Sex-reversal of gonads of S-rR strain medaka exposed to ethynylestradiol (EE2), diethylstilbestrol (DES), 4-*t*-pentylphenol (4*t*PP), 17α-estradiol (E2), methyltestosterone (MT), and flutamide (Flu) (from Hagino et al., 2001).

trary, gonads of genotypic females exposed to MT differentiated into testes at 0.1 µg/l. Gonads of both genotypic males and females exposed to Flu differentiated to testis and ovary, respectively.

Comparison between the no observable effect concentration (NOEC) or lowest observable effect concentration (LOEC) values for sex-reversal and the 96 h acute LC_{50} values of a chemical is useful to understand types of effect and mode of action. Potent (mammalian) estrogens and androgens caused sex-reversal on fish at extremely low levels, with 42d NOECs for sex-reversal being >100 000 times

lower than 96 h LC_{50} values. The weak estrogenic chemicals produced slight effects on fish, with NOEC:LC_{50} ratios of up to 2600 (Table 1). Other chemicals suspected to have weak estrogenic effects had NOEC:LC_{50} ratios <100 [11]. Hagino et al. [37] reported that the androgenic action of MT was inhibited by Flu. When S-rR strain medaka were exposed to Flu at 1000 μg/l, methyltestosterone (MT) at 0.1 μg/l, or Flu at 1000 μg/l plus MT at 0.1μg/l, Flu alone induced neither estrogenic nor androgenic effects, MT alone induced sex-reversal from female to male, but no *app* were observed in any females simultaneously exposed to Flu and MT.

Table 1 Overall NOEC and LOEC (42-day values) of ethinylestradiol, diethylstilbestrol, 4-*t*-pentylphenol, 17ß-estradiol, methyltestosterone and flutamide on sex-reversal of S-rR strain medaka with acute toxicity/sex-reversal effect ratio.

Compound	Genotypic sex	96 hr-LC_{50} (μg/l)	NOEC on sex-reversal (μg/l)	LOEC on sex-reversal (μg/l)	LC_{50}/NOEC ratio	LC_{50}/LOEC ratio
Ethinylestradiol	male	1500	0.01	0.032	150 000	46 875
	female		0.1	>0.1	15 000	<15 000
Diethylstilbestrol	male	1400	0.01	0.032	140 000	43 750
	female		0.1	>0.1	14 000	<14 000
4-*t*-Pentylphenol	male	2600	1	10	2600	260
	female		1000	>1000	2.6	<2.6
17ß-Estradiol	male	3900	0.01	0.032	390 000	121 875
	female		0.1	>0.1	39 000	<39 000
Methyltestosterone	male	>10 000	0.1	>0.1	>100 000	100 000
	female		<0.01	0.01	>1 000 000	>1 000 000
Flutamide	male	3600	1000	>1000	3.6	<3.6
	female		1000	>1000	3.6	<3.6

FISH REPRODUCTION TEST

The concept of an adult fish reproduction test is seen by scientists who support the OECD work on EASs as a promising future test guideline [2]. This partial life-cycle protocol has been successfully demonstrated using the fathead minnow [6], medaka [7,38], and zebrafish [13,14]. For illustrative purposes, the approach using medaka is now outlined.

Recently, Japanese researchers have reported a 21-day medaka reproduction test adopted from the test design for fathead minnows and applied to estrogens. In this test protocol, mating pairs of reproductively mature medaka (3 to 6 month post-hatch) are exposed to EASs, and their reproductive performance (fecundity and fertility), mortality, behavior, and appearance are examined over a 21-day period. At the end of the exposure period, hepatic VTG is measured by ELISA [39] and gonads are examined microscopically.

Optionally, if potential trans-generational effects of EASs are to be assessed in medaka, the eggs spawned from females are collected and transferred to test chambers containing water with or without a test substance and then cultured until the early life stage or early mature stage (30 to 60 days post-hatch). Results from testing of estrogens by CERI suggests that the physiological and histological measures used were more sensitive to change than the fish's overt reproductive system; in other words, the elevated VTG levels and induction of testis-ova (mechanistic biomarkers) may not intrinsically be responsible for reproductive impairment (the adverse effect endpoint) [38]. Overall, these studies indicate that a 21-day medaka reproduction test can be used to detect the effects of estrogenic substances in terms of fecundity, gonadal histology, and VTG levels. More work is needed to extend this approach to a wider range of environmentally relevant EASs (including aromatase inhibitors, androgens, and antiestrogens), taking into account not only medaka but also other OECD fish species.

FISH FULL LIFE-CYCLE TEST

For EASs identified in mammalian and fish screening assays and which are expected to be continuously discharged into rivers or streams, the fish full life-cycle (FFLC) test represents the highest-tier single-species test [3]. Typically, fish are exposed to the test compound from newly fertilized (<24 h) embryo (F0 generation) to juvenile stage of the F1 generation offspring. At maturation of the F1 generation, breeding pairs are randomly selected in order to promote and record spawning activity. The endpoints analyzed in the existing FFLC study include spawning frequency, number of eggs produced, F0 fertility, viability of embryos, hatching success, growth and development (F0 and F1) [4,40,41].

To date, test substances evaluated in the medaka FFLC tests include bisphenol A (BPA), 4-nonylphenol (4-NP), E2 and EE2 (results summarized in Tables 2 and 3). In the BPA test, Yokota and coworkers did not evaluate the reproductive effects in the F0 fish. As described above, the FFLC test is proposed as a definitive test; therefore, this test must be able to quantitatively assess the concentrations of EASs at which there are developmental and reproductive effects that could lead to serious population impact. In CERI FFLC tests with these four estrogens, 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 (Table 2). 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 aquatic hazard of such EASs.

CONCLUSIONS AND RESEARCH RECOMMENDATIONS

It is clear that the numerous global observations of sexual disruption in wild fish populations have led to significant investment in fisheries research into EASs [1]. Given the wide range of testing scenarios envisaged (including single substances and complex effluents), it is proposed that the OECD effort toward validating fish test guidelines should continue to emphasize common scientific principles but remain inclusive of a modest range of important fish species: namely, fathead minnow, medaka, and zebrafish [2,36]. Four concept protocols seem useful: (1) nonspawning fish screening test; (2) fish development (extended ELSs) test; (3) fish reproduction test; and (4) fish full life-cycle test. Further priority research recommendations are outlined below:

Environmentally realistic exposures and integrated chemistry

Environmentally relevant routes of EAS exposure (primarily via the water or diet) should be used, with nominal concentrations verified by chemical analysis wherever feasible. Also, given reports of endocrine activity in commercial fish diets [42–44], these chemical analyses should not be restricted to the test substance per se but should also be applied to the fish diets. The purpose here is not to eliminate the use of such commercial fish diets but rather to gather critical baseline information on background levels of EASs (e.g., PCBs, organochlorine pesticides, phytoestrogens, steroids) that will help in the interlaboratory validation of test guidelines for OECD purposes.

Biologically relevant endpoints

Research needs to better define which endpoints can be used to directly measure the adverse effects of EASs in fish populations and therefore which endpoints should be used for setting water quality criteria. These adverse effect endpoints (suitable for the calculation of the PNEC) include fecundity, fertilization and hatching success, development, and growth. Further research is needed into the validation and interpretation of biomarkers (including vitellogenin, GSI, and gonadal histology) so as to help link data from both field and laboratory studies and help in pattern recognition across particular classes of chemicals.

Table 2 Summary of the effects observed in the FFLC test for medaka with several estrogens.

Test substance	Developmental stage										
	Parental generation (F0)						Progeny generation (F1)				
	Embryo		Larvae – Juvenile			Adult	Embryo	Larvae – Juvenile			
	Survival	Hatching	Survival	Growth	Sex diff.	Reprod.	Survival	Hatching	Survival	Growth	Sex diff.
Bisphenol A[1]	–	–	–	+ (Reduct.)	+ (Femin. and testis-ova induction)	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
17β-Estradiol[2]	–	–	+ (Reduct.)	+ (Reduct. and stimulation)	+ (Femin.)	+ (Low fecund.)	–	–	–	–	–
Ethinylestradiol[3]	–	–	–	–	+ (Femin. and testis-ova induction)	+ (Low fecund. and fertility)	+ (Reduct.)	–	–	–	–
4-Nonylphenol[4]	+ (Reduct.)	+ (Reduct.)	+ (Reduct.)	–	+ (Femin. and testis-ova induction)	+ (Low fertility)	–	–	–	–	+ (Femin. and testis-ova induction)

[1]Yokota et al. [12].
[2]Yokota [47].
[3]Yokota [48].
[4]Yokota et al. [41].
– : No adverse effects were observed.
+ : Adverse effects were observed.
n.t. : Not tested.

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Table 3 Summary of LOEC determined for various developmental endpoints in the full life-cycle tests with several estrogens.

Endpoint	LOEC (μg/l)			
	Bisphenol A[1]	17β-Estradiol[2]	Ethynylestradiol[3]	4-Nonylphenol[4]
F0 generation				
Embryo survival	>1820	>0.891	>0.0306	183
Hatching	>1820	>0.891	>0.0306	183
Post-hatch survival	>1820	0.891	>0.0306	17.7
Growth	1820	0.0094	>0.0306	>51.5
Sex differentiation	1820	0.0927	0.0306	17.7
Fecundity	n.d.	0.0094	0.0101	>17.7
Fertility	n.d.	>0.0094	0.0101	17.7
Ratio of				
sex differentiation to post-hatch survival	<1	0.10	<1	1
fecundity or fertility to post-hatch survival	n.t.	0.011	<0.33	1
fecundity or fertility to sex differentiation	n.t.	0.10	0.33	1
F1 generation				
Embryo survival	n.t.	>0.0094	0.0101	>17.7
Hatching	n.t.	>0.0094	>0.0101	>17.7
Post-hatch survival	n.t.	>0.0094	>0.0101	>17.7
Growth	n.t.	>0.0094	>0.0101	>17.7
Sex differentiation	n.t.	>0.0094	>0.0101	8.2

[1]Yokota et al. [12]
[2]Yokota [47]
[3]Yokota [48]
[4]Yokota et al. [41]
n.t. : Not tested

Research beyond antiestrogens

While the focus of current debate is on the EASs affecting androgen, estrogen, and thyroid function in animals, it should be recognized that to date much of the published work on fish is limited to antiestrogens. For example, there are relatively few data, supported by chemical analysis, quantifying the effects of antiandrogens or aromatase inhibitors on different life-stages of OECD fish species, although some aromatase data have been recently published [45,46]. The use of other fish species and novel endpoints (e.g., the spiggin biomarker for androgens) [17] suggests a potentially useful way forward.

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