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DETAILED REVIEW PAPER ON FISH SCREENING ASSAYS FOR THE DETECTION OF ENDOCRINE ACTIVE SUBSTANCES

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DETAILED REVIEW PAPER ON
FISH SCREENING ASSAYS FOR THE DETECTION
OF ENDOCRINE ACTIVE SUBSTANCES

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

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This publication was produced within the framework of the Inter-Organisation Programme for the Sound Management of Chemicals (IOMC).

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The participating organisations are FAO, ILO, OECD, UNEP, UNIDO, UNITAR and WHO. The World Bank and UNDP are observers. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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PREAMBLE

In 1998, a Task Force on Endocrine Disrupter Testing and Assessment (EDTA) was established at the request of OECD member countries. The EDTA Task Force is a Special Activity of the Test Guidelines Programme and its main objectives 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 testing results and assessments.

The need for new and updated test methods to detect and characterise endocrine disrupting chemicals has been expressed by the Task Force for the assessment of human health effects and environmental effects. At early meetings of the EDTA Task Force, it appeared that existing OECD Test Guidelines would insufficiently cover for endocrine-related effects, especially for the environment. Member countries decided to list test methods which could potentially cover effects of chemicals on the reproductive system (estrogen agonists/antagonists and androgen agonists/antagonists) and on the development (thyroid system), and proposed enhancements where needed. Fish screening assays have been developed for the detection of (anti-) estrogenic and (anti-)androgenic compounds in oviparous animals living in aquatic ecosystems, as there is good evidence that fish in the field are being impacted by endocrine active substances.

This Detailed Review Paper (DRP) is intended to provide the current state-of-the-knowledge in the area of fish screening assays for chemicals active at the endocrine level on the reproductive system of test animals. The main reasons for developing a screening assay is (a) there is good evidence that fish in the field are being impacted by endocrine disrupters, and (b) there are noticeable differences between mammals and fish as regards exposure pathways and metabolism. Also, fish differ in steroid profiles from mammals. For example, 11-ketotestosterone (11-KT), as opposed to testosterone (T), is the most important androgen in fish, and the estrogen receptor (ER) in fish appears to differ structurally and functionally from the mammalian ER. In addition, steroid receptors in eggs and for hepatic vitellogenin (VTG) have no known analogous receptors in mammals, which would suggest sites of endocrine disruption unique to oviparous animals. Therefore, a screening assay in fish is considered essential to address these known endocrine differences.

Two Expert Consultation Meetings on Endocrine Disrupters Testing in Fish were held in 1998 and 2000 to discuss the screening and testing needs. At these meetings, experts recommended endpoints reflective of estrogenic or androgenic activity, which could be used for the detection of endocrine active substances. They consist of morphological, biochemical and histological measurements and observations in either adult fish (males and females analyzed separately) or in juveniles, depending on the assay considered. The DRP has been written to provide a summary of the literature up to 2003, and an overview

of existing approaches relevant for the standardisation and validation of fish screening assays, with their advantages and limitations.

General principles for the conduct of validation studies have been defined following the OECD Stockholm Conference on Validation and Regulatory Acceptance of New and Updated Test Methods in Hazard Assessment. The OECD draft Guidance Document No. 34 describes these guiding principles and addresses the important steps and aspects that must be considered prior to and during the validation process. They include: (i) the definition of the test method and related issues (*e.g.*, purpose, predictions, endpoints, limitations), (ii) the design and conduct of the initial phase of the validation process leading to the optimisation of the test method (often referred to as the pre-validation), (iii) the design and conduct of the broader, multi-laboratory follow-up validation work, based on the outcomes of the initial phases and aiming at accumulation of data on the relevance and reliability of the test method, and (iv) the overall data evaluation and subsequent validation study conclusion, keeping in mind the requirements of regulatory authorities for submission of information relating to new or modified test procedures. It also discusses the need for and the extent of an independent evaluation, or peer review, of validated test methods.

The planning and conduct of a validation study should be undertaken on a case-by-case basis since there may be several ways of assessing the validity of the method. As described in the draft Guidance Document No. 34, the validation process is sufficiently flexible so that it can be applied equally well to a wide variety of tests and procedures. The flexibility also applies regardless of whether tests are for health or environmental effects. Flexibility is also encouraged on issues such as the amounts of information required at each phase, the number of chemicals tested, when and to what extent to use blind testing, and the number of laboratories participating.

A Validation Management Group for Ecotoxicity Testing (VMG-eco) has been established at the OECD level to supervise the planning and conduct of experimental work in fish, birds, amphibians and invertebrates. This VMG-eco reports back to the Task Force on Endocrine Disrupters Testing and Assessment (EDTA). To discuss the technical details of the screening assay, a Fish Drafting Group was created in 2002.

The U.S. Environmental Protection Agency took the lead in preparing the initial version of this Detailed Review Paper for their national programme on endocrine disrupters. The purpose of a Detailed Review Paper is to provide the state-of-the-art knowledge in a particular area, e.g. fish screening assays for the detection of endocrine active substances, to be broadly inclsive of possible methods and approaches without giving preference to a particular approach. A draft of this document was circulated for comments in May 2003 to OECD member countries. Comments were received from several internationally recognised experts. A revised draft taking into account comments received served as the basis for the present OECD Detailed Review Paper. Dr. Gitte Petersen, member of the Fish Drafting Group representing Denmark, assisted the Secretariat in making the final version of the DRP.

There are a number of different approaches to screening for endocrine active substances in fish, but there is currently no overall consensus between different regulatory systems on which of the various existing approaches should be developed for screening chemicals for regulatory purposes. The Detailed Review Paper aims at describing some of these approaches, along with the relevant proposed protocols, as a synopsis for the current activity in this area. These protocols were suggested as candidates for further development under the Conceptual Framework for Testing and Assessment established by the EDTA Task Force. This Conceptual Framework should be considered as a toolbox that provides useful approaches for the Member countries in their regulation of chemicals, rather than a rigid testing scheme. For example, regulatory authorities in some countries may require a reproduction screen as part of their testing strategy, whereas others may advocate the use of an early life-stage approach/assay. Until sufficient data become

available on testing with endocrine disrupters, the question of the relevant assay at the relevant Tier of the test battery remains difficult to address.

Finally, the Deatiled Review Paper presents the state-of-the-science as of 2002. Commenting rounds have been necessary to reach consensus on the document at the OECD level. Therefore, at the stage of the declassification of the document by the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology, some publications and recent scientific findings may not be found in the Detailed Review Paper. Good recent reviews are also available elsewhere (Ankley and Johnson, 2004).

EXECUTIVE SUMMARY

i) The purpose of this detailed review paper (DRP) is to survey and investigate the status of various screening protocols that have been proposed for use in identifying chemicals that act as potential endocrine disrupters in fish. This DRP is a synthesis of relevant scientific peer-reviewed papers which provide a basis for developing a standard transferable protocol for conducting a fish screening assay. It summarizes, explains and documents decisions regarding the relevant principles, methods and techniques used in fish screening assays, and identifies issues that might require prevalidation studies to adequately address.

ii) The rationale and objectives of fish screening assays are described in **Section 1: Introduction.**

iii) Background knowledge on the neuroendocrine system and on the reproductive and thyroid hormones in fish as well as toxicokinetics of chemicals in fish provide the scientific basis for fish screening assays for the detection of endocrine active substances. This is the subject of **Section 2: Overview and Scientific Basis of a Fish Screening Assay for endocrine active substances**.

iv) Three fish species have been identified for their potential to be used in a screening assay for the detection of endocrine active substances: fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). The culturing and handling of all three species have been well documented for many years. All species tolerate a wide range of water-quality and water-temperature conditions, require small culture space, and produce the number of embryos needed for testing. Fathead minnows have a strong regulatory history in the United States, the zebrafish is used for regulatory work and research in Europe, and the medaka is the dedicated species in Japan for regulatory work. The strengths and weaknesses of the three test species were evaluated for ease of culturing and handling, sex determination and sex characteristics, endpoint measurement, length of life cycle, size of organisms in terms of space required for testing, and also in terms of plasma volume collectable. This is reviewed in **Section 3: Candidate Test Species**.

v) Possible endpoints reflective of disruption of the reproductive axis ranging from morphological alterations to biochemical measurements are described in **Section 4: Description of assay endpoints**. These endpoints are not necessarily all measured in each proposed assay, but a combination of recommended endpoints allow the detection of chemicals with diverse modes of action within a single assay, and allows linkages between endpoints of increasing biological complexity and relevance. Following the overview of possible endpoints, an attempt is made to define and document endpoints sensitivity to (anti-) estrogenic or (anti-)androgenic exposures in **Sections 5 and 6**. A large number of environmental pollutants have been hypothesized to cause toxicity by binding to the estrogen receptors (estrogenic compounds) or to produce responses similar to estrogen without binding to estrogen receptors (estrogen-like compounds). Consequently, experimental studies of the responses of fish exposed to natural or synthetic estrogens have received a higher degree of scrutiny than have studies on other fish hormonal systems. Fewer studies have examined the response of fish to treatment with androgenic compounds and thyroid hormone mimics. Similarly, studies of antagonistic effects of xenobiotics on fish hormone pathways are relatively recent. **Section 7** addresses the present knowledge about the thyroid axis in fish, possible active compounds and their effects observed on the thyroid gland and hormone levels.

vi) Parameters to consider in defining a protocol are described in **Section 8: Experimental Design Considerations for Fish Screening Assays**. They include the life-stage and the exposure duration. In choosing the test design, it is important to remember that a screening assay should be relatively short in its duration, sensitive enough, reliable and cost-effective. An acceptable combination of these objectives will lead the choice of the assay.

vii) From the existing literature, four protocols were selected, based on the potential they present to be used for regulatory purpose for the detection of endocrine active substances in fish. They include: 1) 14-day fish reproductive assay, 2) 21-day reproductive test, 3) 21-day fish non- reproductive screen and 4) a partial life-cycle test (or extended early life-stage test). Not all member countries currently accept some of the protocols above as being suitable as screening assays for regulatory purposes, but there is sufficient information available on these protocols for their inclusion, at this time, in this Detailed Review Paper. Each of the proposed protocols is relatively new and has not been through a validation process at the OECD level. Only validated assays may become Test Guidelines, and therefore the protocols described in the Detailed Review Paper should not been seen as OECD Test Guidelines, especially in their current form. Each protocol is described in further details in **Section 9: Candidate protocols** and **Annexes B, C, D and E**. It should be noted that, as more experience is gained in testing, a candidate protocol may cease to be relevant, and conversely other protocols may emerge as progress is made with biomarkers or other endpoints.

viii) Currently there are insufficient data to make a definitive selection on which exposure protocol has the most merit for use as a screen and should be chosen above all others. **Section 10: Additional Data Needs Implementation Considerations** describes the current data gaps in the area. The Fish Drafting of the Validation Management Group for Ecotoxicity Testing is currently working on the validation of a suitable protocol for the fish screening assay, involving the various species used in regulatory work.

1. INTRODUCTION

1.1 Objective of the Fish Screening Assay for the Detection of Endocrine Active Substances

1. The primary objective of fish screening assays is to provide an indication of the potential of a chemical to behave as an endocrine active substance on the organism studied and thus to guide on whether additional testing will be needed to better characterize the potential endocrine disrupter. This objective should be achieved via the conduct of an assay that is relatively short-term, sensitive, reliable and cost-effective (or at least through an acceptable combination of these parameters). The assay is not designed to identify specific mechanisms of hormonal disruption (such as hormone receptor agonistic or antagonistic effects, or disturbance of hormone synthesis or metabolism).

2. Endpoints measured should be reflective of specific disturbances at the (sub-)organism level and will be a combination of e.g. apical, histological and biochemical measurements. This is to establish a link between a response at the sub-organism level and a change observed at the organism level. Such a combination of measurements will also aid in the interpretation of test results and inform on the additional testing needs.

3. A variety of screening assays can be envisaged. Depending on the existing information on the chemical of interest and depending on the priorities that have been established in the testing strategy, each approach presents intrinsic advantages and limitations that will give preferences for a screening assay to be conducted over another, under given circumstances. Importantly, an assay that raises interest for the screening of endocrine active substances should be validated to establish its relevance and reliability. This will allow its regulatory acceptance.

1.2 List of Abbreviations and Nomenclature

11-KT	11-ketotestosterone
17,20-DHP	17α, 20β-dihydroxyprogesterone
17,20,21-THP	17α, 20β, 21-trihydroxyprogesterone
ANOVA	analysis of variance
CPA	cyproterone acetate
CV	coefficient of variation
DDT	dichlorodiphenyl trichloroethane
DES	diethylstilbestrol
DRP	detailed review paper
E2	17β-estradiol
EAC	endocrine-active chemicals
EC50	median effective concentration
ECD	electron capture detector
EDC	endocrine-disrupting chemical
EE2	17α-ethynylestradiol
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
FSH, GTH I	follicle stimulating hormone
GC	gas chromatography
GSI	gonadosomatic index
GTH	gonadotropic hormones
HPLC	high performance liquid chromatography
LC	liquid chromatography
LH, GTH II	luteinizing hormone
LOEC	lowest observed effect concentration
M1	2-[(3,5-dichlorophenyl)]-carbamoyloxy-2-methyl-3-butenoic acid
M2	3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
MATC	maximum acceptable toxicant concentrations
MS	mass spectrometry
MSTFA	N-methyl-N-trimethylsilyl-trifluoroacetamide
MT	17α-methyltestosterone
NOEL	no observed effects level
OECD	Organization for Economic Cooperation and Development
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
QRT-PCR	quantitative reverse transcription-polymerase chain reaction
RIA	radioimmunoassay
T	testosterone
T3	triiodothyronine
T4	thyroxine
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TSH	thyroid stimulating hormone
VTG	vitellogenin

2. OVERVIEW AND SCIENTIFIC BASIS OF A FISH SCREENING ASSAY FOR ENDOCRINE DISRUPTING CHEMICALS

4. This detailed review paper critically evaluates the effect of disturbances in the endocrine system on fish reproduction and overall fitness. A large portion of this document is focused on interpreting published data as they relate to development of a screening assay(s) in three species of fish (fathead minnow, zebrafish, and medaka), which, for a variety of reasons discussed in Section 3.0, are the most likely candidate species for use in regulatory testing. The scientific basis for a fish screening assay, as opposed to the use of a single vertebrate species rat, assumes that endocrine control of reproduction and its response to chemical exposure differs across vertebrate taxa. Although all vertebrates and many invertebrates have an endocrine system, the specific function and action of the various hormones can vary significantly among animal taxa. When considering the effects of chemicals on the endocrine system across a broad vertebrate/invertebrate perspective, differences in pharmacokinetics (absorption, distribution, and elimination) and xenobiotic metabolism will also affect the responses of different species. Therefore, before discussion of the candidate species and various aspects of screening assays, it is pertinent to begin with an overview of the endocrine and reproductive systems in fish.

5. In fish, as with other vertebrates, reproduction requires the coordination of a variety of physiological processes culminating in release of viable gametes and successful fertilization. In many fish species a seasonal reproductive cycle exists whereby the gonads undergo a period of recrudescence or rapid growth prior to spawning. Fish are also similar to other vertebrate groups in that most species are gonochoristic, with separate male and female phenotypes. However, there is great diversity in reproductive strategies among fishes, such as internal or external fertilization, oviparity (fertilized eggs mature outside of the fish) or ovoviviparity/viviparity (internal development) and synchronous (annual) or asynchronous spawning (repeated spawns during a spawning season). Regardless of the reproductive strategy employed, communication between tissues involved in reproduction and the external environment is vital to ensure proper development of the gonads and ultimately, the timing of gamete production and release (Kime 1998). This latter area can be especially important for many fish species as larval/juvenile survival may depend on spawning occurring at a specific time during the year or during optimal environmental conditions (e.g., during the rainy season).

6. In a broad sense, communication between cells and tissues can occur via the central nervous system and/or through release of chemical messengers or signals. Chemical signaling can be further divided into autocrine and paracrine actions to differentiate between effects on similar or different cell types. The endocrine system can be defined as any tissue or cells that release a chemical messenger (hormone) directly into the blood that signals or induces a physiological response in some target tissue (Thomas et al., 2001). In this detailed review paper, the focus is on reproduction and paracrine signaling. However, the function of the endocrine system is much broader and contributes to the regulation many physiological processes such as digestion, metabolism, growth and development. In essence, the endocrine system is involved with all phases of maintenance of homeostasis. Although the focus of this document is on hormones or chemical messengers, it is recognized that the endocrine system is in reality a neuroendocrine system that is integrated with the central nervous system (CNS). Therefore, we initially describe the interaction between the CNS and the reproductive and thyroid systems followed by discussion of control processes involved in regulation of these systems.

2.1 Morphology and Anatomy of the Neuroendocrine System

7. Neuroendocrine control of reproduction is exerted through actions of the brain, pituitary gland, and gonads and is often referred to as the brain-pituitary-gonadal axis. Both external and internal sensory information processed by the brain regulates secretion of gonadotrophic hormones (gonadotropins) from the pituitary gland. Examples of external stimuli are temperature, photoperiod, and olfactory stimulation. Internal stimuli may be basal metabolism or growth and chemical secretions from peripheral tissues (e.g. gonads and sex steroids). Most stimuli that influence reproduction lead to changes in secretion of neurohormones from the hypothalamus (Redding and Patino 1993). In vertebrates, at least ten different peptides and neurotransmitters can be formed by neurons within the hypothalamus (Bently 1998). Under proper stimulation these hormones are secreted and influence the release of pituitary hormones. The pituitary gland in fish as in other vertebrates consists of separate tissues called the neurohypophysis and adenohypophysis (Van Oordt and Peute 1983). The functional relationship between the hypothalamus and regions of the pituitary gland varies significantly among the different fish taxa but in general, the evolutionary trend is towards increased control of pituitary function by neurological connections with the hypothalamus (Scott 1987). This in turn corresponds with greater control by the pituitary of gonad development (Scott 1987). For example, teleost fish differ from other vertebrates in that a well developed portal blood supply between the hypothalamus and the adenohypophysis does not exist (Batten and Ingleton 1987; Peter et al. 1990). Rather, the adenohypophysis is directly innervated with neurosecretory fibers originating in the hypothalamus (Peter et al. 1990). Also in bony fishes, the blood flow to the adenohypophysis passes through the neurohypophysis (Scott 1987). The pituitary gland exerts control through secretion of several hormones the most important of which with respect to reproduction are the gonadotropins (GtHI and GtHII) and thyrotrophin (TSH; Kime 1998).

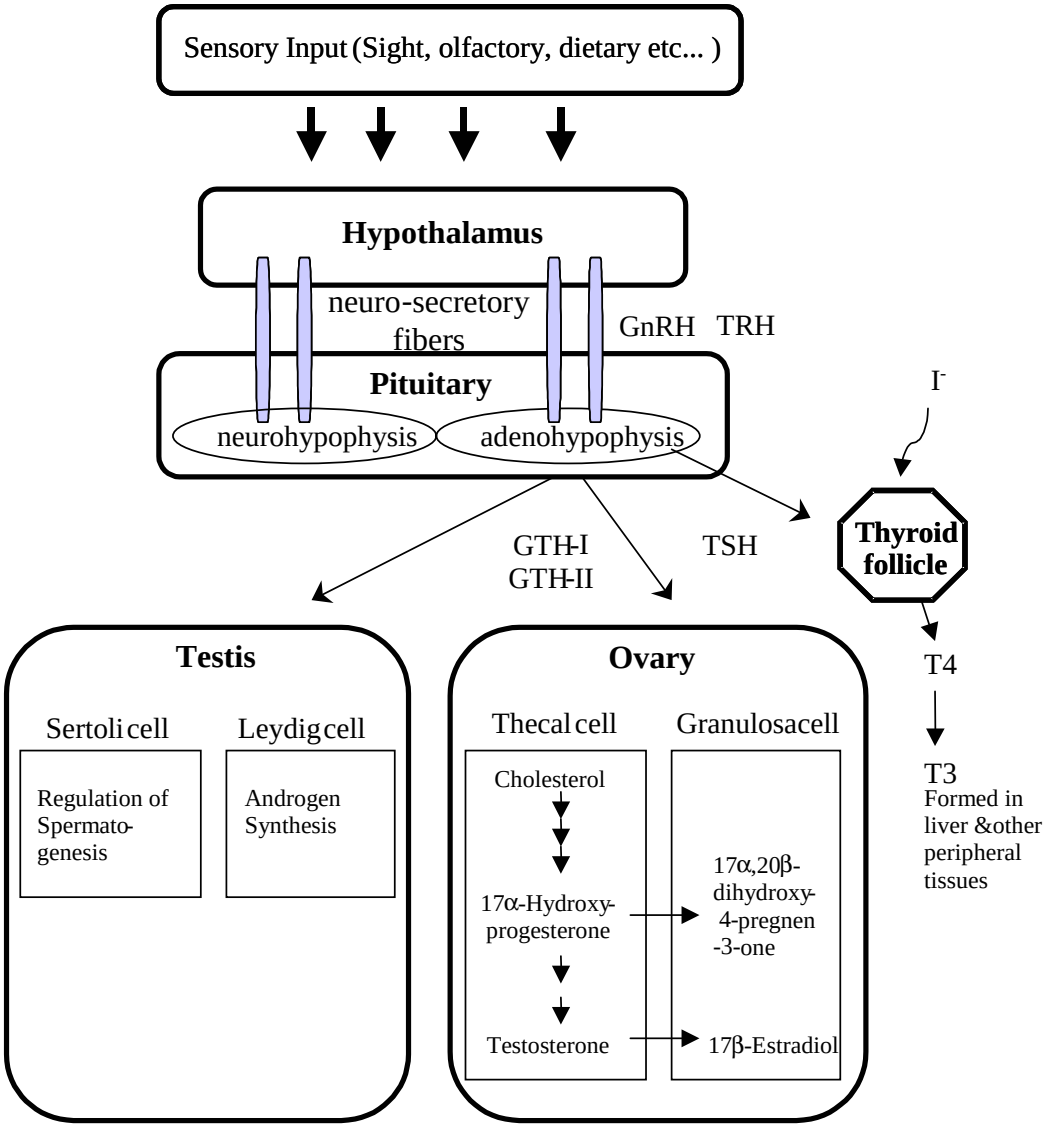
8. The most important peripheral tissue involved in neuroendocrine control of reproduction is the gonads which consist of the ovaries or testes. The thyroid system is also considered to aid in regulation of reproduction, although its specific role is less defined than that of the brain- pituitary-gonadal axis. The gonads in fishes are normally paired structures frequently lying adjacent to the air bladder or the kidney. However, numerous exceptions to this arrangement exist among fishes as in the case of the medaka, where only a single ovary develops (Redding and Patino 2000). In many fishes, the ovaries differentiate sooner in juveniles possessing a female genotype compared to the testes in genotypic males. The fish ovary has been described as essentially a hollow organ containing many lamellae that resemble the pages of a book (Scott 1987). Oogenesis, which is the process of egg development, occurs within the lamellae and can be divided into several discrete phases. For example, in the zebrafish (*Danio rerio*), five stages of egg development have been described beginning with initial growth, a pre-vitellogenesis phase, vitellogenesis, maturation, and then ovulation (Selman et al. 1993). Further delineation of oogenesis is possible, as oocyte maturation itself is now thought of as a two-stage process (Patino et al. 2001). An important change that occurs during pre-vitellogenesis is the development of granulosa and theca cell layers around the oocyte, which synthesize estradiol and the maturation- inducing hormone, 17_,20_-dihydroxy-4-pregnen-3-one (17_,20_-DP; Janz 2000). The diameters of mature eggs from most freshwater fishes range between 0.4 and 3 mm (Redding and Patino 2000) and during ovulation eggs are released into the visceral cavity or lumen of the ovary where they remain until time of spawning (Scott 1987).

9. The morphology of the testes can vary considerably among the different taxa of fish. In many fishes, the testes has a characteristic whitish appearance and elongated lobular shape within which exist tubules that are surrounded by a basement membrane that divides the space into intra- and inter-tubular compartments (Nagahama 1983; Scott 1987). In teleosts, two distinctive forms of testes structure have been described and are termed tubular or lobular (Grier 1981), although technically speaking, tubules are present in both types. Most teleosts such as salmonids and cyprinids possess a lobular type testes characterized by spermatogonia being distributed along the full length of the tubules (Grier 1981). Spermatogenesis is the process of spermatozoa development and occurs through proliferation of primary

spermatogonia within the tubule. Primary spermatogonia arise from germ cells typically embedded in cysts formed by Sertoli cells (Scott 1987). Another important cell type in the testes are the Leydig cells, which are found in connective tissue near Sertoli cells and are involved with synthesis of testosterone, 11-keto- testosterone, and other androgens (Scott 1987; Redding and Patino 1993). Mature spermatozoa are released into the lumen of the tubule and eventually into the sperm duct, which merges with a system of ducts from those of other tubules to form a primary duct for each testis (Redding and Patino 2000). The process of spermatozoa release into the sperm duct is called spermiation. The primary sperm ducts of teleost fish are different from those of other vertebrates in that they are anatomically distinct from the kidney (Redding and Patino 2000).

10. The functional unit of the thyroid system in all vertebrates is the follicle, which consists of epithelial cells (called thyrocytes) that enclose an extracellular space forming a lumen that is filled with a glycoprotein called thyroglobulin (Bently 1998). Thyroid follicles actively scavenge inorganic iodide from the blood, which is then incorporated into tyrosine residues within thyroglobulin. Thyroglobulin is produced by the thyrocytes and secreted into the lumen of the follicle by exocytosis. Successive rounds of oxidation of thyroglobulin by the enzyme thyroid peroxidase leads to formation of the thyroid hormone, thyroxine (T4), which remains in the follicle lumen until secretion into the bloodstream (Raine et al. 2001). Fish are distinct from other vertebrates in that little T3 is synthesized in the follicles. It is interesting to note that the thyroid system is the only endocrine tissue that stores its hormones in an extracellular space (Bently 1998).

Figure 2.1: Outline of the Brain-Pituitary-Gonadal Axis and Thyroid System in Fish.



11. Most teleost fish species differ from other vertebrate groups in that thyroid follicles rarely form a concentrated mass or gland (Bonga 1993). Rather, follicles are dispersed in connective tissue near the pharyngeal region as is the case for fathead minnows (*Pimephales promelas*) or in the head kidney, a hematopoietic and immune organ (Wabuke-Bunoti and Firling 1983; Bonga 1993). In the medaka (*Oryzias latipes*), thyroid follicles appear next to the ventral aorta (Raine et al. 2001).

2.2 Reproductive and Thyroid Hormones

2.2.1 Hypothalamic, Pituitary, and Thyroid Hormones

12. A schematic representation of brain-pituitary-gonadal axis and thyroid system is depicted in Figure 3-1. Important hormones secreted by the hypothalamus are gonadotropin releasing hormone (GnRH), thyrotropin releasing hormone (TRH), and neurotransmitters such as dopamine. These hypothalamic hormones regulate release of gonadotropins and thyrotropins by the pituitary gland. Additional types of hypothalamic secretagogues (i.e., substances released by a tissue or neurosecretory fiber) that are known to influence gonadotropin release are neurotransmitters, of which the best characterized are the monoamines dopamine and serotonin (Vitale and Chiocchiol 1993; Vacher et al. 2000). GnRH is a decapeptide with at least 6 different forms described from cartilaginous and bony fish species plus an additional 2 forms isolated from lampreys (Goos et al. 1998; Dubois et al. 2002). Most GnRHs in fish are structurally conserved (i.e., common across many animal taxa; not changed over evolutionary time) differing only in one or two amino acids (Goos et al. 1998). Teleost fish are similar to other vertebrate groups with the exception of placental animals in that multiple GnRHs are present in the brain (Bently 1998). Apart from the lamprey, all fish appear to possess a common GnRH identical to that found other vertebrates, plus additional GnRH's that are specific to fish taxa (Dubois et al. 2002). Each GnRH is apparently transcribed from different genes (Dubois et al. 2002). There is recent evidence from experiments using the African catfish (*Clarias gariepinus*) that multiple GnRHs work in concert to regulate gonadotropin secretion by the pituitary (Bosma et al. 2000). One and possibly two distinct GnRH receptors are found in fish (Goos et al. 1998). All GnRH receptors are cell surface proteins although the primary sequence of fish GnRH receptors indicate significant differences from their mammalian counterparts (Goos et al. 1998). Consistent with previous studies in the African catfish, the GnRH receptor cloned from striped bass (*Morone saxatilis*) and transfected into a Chinook salmon (*Oncorhynchus tshawytscha*) cell line exhibited reduced activation when incubated with mixtures of native GnRHs as opposed to incubation with a single GnRH (Alok et al. 2001). Interestingly, GnRH receptors are expressed in other tissues besides the pituitary including the testis (Goos et al. 1998; Alok et al. 2000). These findings may in part be related to GnRH additional roles for as a neurotransmitter and autocrine functions within certain tissues (reviewed in Habibi and Huggard 1998).

13. In contrast to GnRH, TRH is a tripeptide that is highly conserved across all vertebrate groups (Bently 1998). In higher vertebrates, TRH functions to regulate pituitary release of TSH in addition to other pituitary hormones (Bently 1998). In fish, the functional role of TRH in regulating TSH release is less established (Janz and Weber 2000). However, recent experiments using pituitary cells isolated from the bighead carp (*Aristichthys nobilis*) indicated TRH exposure could upregulate TSH mRNA levels (Chatterjee et al. 2001). Two TRH pituitary receptors have been identified from fish and are analogous to GnRH receptors in that they are cell surface proteins (Harder et al. 2001). Given the conserved nature of TRH across vertebrate groups, it is not surprising that the two fish TRH receptors characterized are structurally similar to their mammalian counterparts (Harder et al. 2001).

14. As previously stated, the most important pituitary hormones that regulate reproduction are the gonadotropins and thyrotrophin. These hormones are synthesized in specific cell types within the adenohypophysis of the pituitary. Both gonadotropins and thyrotrophin are heterodimer glycoproteins

similar in structure, possessing a common, species-specfic α-subunit and a hormone-specific β-subunit (Janz 2000). There are two gonadotropins produced in fish, termed GTH-I and GTH-II. The consensus among endocrinologists appears to be that GTH-I is functionally similar to the mammalian follicle stimulating hormone (FSH) and GTH-II is similar to leutinizing hormone (LH; Schulz et al. 2001). The primary cellular targets of the gonadotropins are the granulosa and theca cells surrounding the oocyte within the ovarian follicle and sertoli and Leydig cells in the testes (Janz and Weber 2000). These cells are stimulated by the gonadotropins to synthesize the sex steroids (estrogen and androgens) and the maturational steroid 17α,20β-DP. In the two cell-type model for estrogen synthesis proposed by Nagahama (1983), thecal cells convert cholesterol to testosterone through a series of reactions that also produce 17α-hydroxyprogesterone, a precursor to 17α,20β-DP. The granulosa cells then convert testosterone into estradiol (Nagahama 1983). It is now also established that granulosa cells synthesize 17α,20β-DP (Scott 1987). Of the two gonadotropins, GTH-II is more clearly established with stimulating granulosa cells to produce estradiol and 17α,20β-DP (Janz and Weber 2000; Patino et al. 2001). In isolated thecal cells collected from ovarian follicles removed from coho salmon, GTH-I stimulated testosterone formation but has no apparent effect on aromatase activity (Planas et al. 2000). In the testes, cell-specific actions of the different gonadotropins is less defined as compared to mammals, but in general, sertoli cells are more responsive toward GTH-I and Leydig cell respond to GTH-II (Schulz et al. 2001). In the latter case, GTH-II primarily regulates Leydig cells production of androgens (Schulz et al. 2001). Although the function of GTH-I is less defined, experimental studies in salmonids indicate that circulating levels of GTH-I are much higher than those of GTH-II during the vitellogenesis and spermatogenesis portions of the reproductive cycle (Prat et al. 1996). In contrast, circulating levels of GTH-II appear to increase only during final oocyte maturation and spermiation (Swanson et al. 1989; Schulz et al. 2001). Thus, GTH-I appears to be the primary gonadotropin responsible for directing growth of the gonads during the majority of the reproductive cycle. Consistent with the presence of two types of gonadotropins, two separate membrane bound gonadotropin receptors are present in the gonads. One gonadotropin receptor termed GTH-RI binds both GTH-I and II while GTH-RII preferentially binds to GTH-II (Schulz et al. 2001). In the testes, GTH-RI is expressed by sertoli cells and GTH-RII is expressed by Leydig cells during spermiation (Schulz et al. 2001). The tendency for cell-specific expression of GTH receptors also occurs in the ovary, with GTH-RI found in both thecal and granulosa cells while the GTH- RII is expressed only by granulosa cells (Nagahama et al. 1994).

15. In contrast to central nervous system control (e.g. GTH-I and II) of sex steroid synthesis by the gonads, thyroid hormone levels in fish are regulated to a much larger extent by peripheral tissues (Eales and Brown 1993). The functional activity of TSH is limited to regulating the release of T4 and iodide uptake by the thyroid follicles (Eales et al. 1999). Secreted T4 is converted to the active thyroid hormone T3 by an outer ring deiodination (ORD) process that is catalyzed by at least two different selenocysteine type, microsomal enzymes (Leatherland et al. 1990; Eales et al. 1999). In fish, ORD activity is typically highest in the liver, but is also present in other peripheral tissues as well (Darras et al. 1998; Eales et al. 1999). As mentioned previously, fish are different from mammals in that in fish, thyroid follicles primarily secrete T4 and circulating levels of T3 are derived solely from ORD activity in peripheral tissues (Eales and Brown 1993). Only a single TSH receptor has been described in fish, and in some species is expressed only in thyroid tissue (Oba et al. 2001), while in others, gonadal expression of a TSH receptor has been reported (Kumar et al. 2000). The biological significance of gonadal expression of the TSH receptor is unknown.

2.2.2 Gonadal Hormones

16. The biological consequences of stimulation and inhibition of sex steroids forms the basis for most endocrine disruptor screening assays and is discussed in detail in Sections 5 and 6. In the present section, the main sex steroids and their synthesis are described.

17. The sex steroids are derivatives of cholesterol and possess a four-ring structure. Many variations of substituent groups on the rings are possible, although the specific addition of methyl or ethyl substituents provides the basic structural skeleton for the estrogens, androgens, and progestogens (Kime 1987). Estrogens are C_{18} steroids, the most important of which are 17β- estradiol and estrone. Estrogen is primarily synthesized in the ovary, although enzymes involved in estrogen synthesis are also present in the brain, which suggests that small quantities are formed there (Halm et al. 2001). Androgens are C_{19} steroids with the 11-oxygenated derivatives, such as 11-ketotestosterone being the most important in male sexual development in fish (Borg 1994; Fostier et al., 1983). This is in contrast to other vertebrates, where testosterone is the more biologically active androgen. The testis is the primary site of androgen synthesis, although testosterone and androstenedione are precursors for estrogen synthesis and as such, are also formed in the ovaries. 11-keto-testosterone is generally present at higher levels than testosterone in males, and found at barely detectable levels in females. Unlike testosterone, 11-keto- testosterone cannot be converted to an estrogen. The testes of teleost fishes is distinctive with respect to the high capacity for glucuronide conjugation with androgens (Scott 1987). In other vertebrate groups such as mammals, steroid conjugation occurs in the liver and is considered a deactivation and elimination pathway (Parkinson 1996). Thus, the biological significance of testicular glucronidation in fish is uncertain. However, an interesting function for steroid conjugates may be as male sex pheromones. For example, experiments using the zebrafish suggest excreted steroid-glucronide conjugates from male fish are capable of inducing ovulation in females (Vandenhurk and Resnik 1992). Progesterones are C_{21} steroids that have received less study in fish compared to estrogen and androgens, but are likely formed in the gonads of most fish species. The most important progesterone in teleost fishes appears to be 17α,20β-DP. This hormone is involved with oocyte maturation and spermiation in males and at time of spawning is present in the gonads at much higher concentrations than estrogen or androgens (Scott 1987).

18. The synthesis and in some cases interconversion of steroids is quite complicated. Many enzymes are involved, and their subcellular location varies between the mitochondria, endoplasmic reticulum (e.g., microsomal) and the cytoplasm. In general, sex steroid biosynthesis can be divided into seven types of enzyme-catalyzed reactions. These are lyase, hydroxylase, hydroxysteroid dehydrogenase, isomerase, aromatase, reductase, and conjugation. Of these types of reactions, aromatase, hydroxysteroid dehydrogenase, and conjugation appear to be the most important as possible targets of endocrine disruption as they are either the final step in 17β- estradiol or 11-keto-testosterone synthesis or aid in excretion of steroids. For example, inhibition of aromatase activity can greatly diminish estrogen synthesis and produce anti-estrogenic effects in fish. This latter topic is discussed in detail in Section 5.2.2. Steroid conjugation may be important in the production of pheromones as was mentioned previously for androgens, but there is also evidence that progesterone conjugates may function as pheromones as well (Vermeirssen and Scott 2001).

19. A significant physicochemical difference among the sex steroids, thyroid hormones, and the hypothalamus-pituitary hormones is that steroid and thyroid hormones are poorly soluble in water. In theory, this property would limit their concentration in extracellular fluids. This potential limitation is overcome by binding of steroids and thyroid hormones to both specific carrier proteins (termed steroid binding proteins) and nonspecific proteins such as albumin and vitellogenin (Hobby et al. 2000; Monteverdi and Di Giulio 2000; Zeginiadou et al. 1997). High affinity binding proteins are likely present in all fishes as in other vertebrates such that greater than 99% of the circulating hormone is bound (e.g. < 1% is free in solution) (Fostier and Breton 1975; Petra 1991). This may contribute to regulation of hormone activity as it is generally considered that only the unbound or free fraction of the steroid/thyroid hormone is biologically active. Thus, modulation of hormone binding and transport in the blood are potential targets for endocrine disruptors.

2.2.3 Feedback Control Mechanisms

20. As discussed in Section 2.1, release of pituitary hormones is controlled by the hypothalamus and sensory input to higher brain centers. Additional regulation of hormone synthesis and secretion within the brain-pituitary-gonadal axis and thyroid system occurs from feedback control exerted by the secreted hormone(s) or hormones that are induced by hypothalamic-pituitary hormones. Feedback control mechanisms are well documented in vertebrates and can be manifested as either long or short feedback loops (Batten and Ingleton 1987). In fish, long feedback loops with the neuroendocrine system are well described with a good example being the effect of sex steroids on gonadotropin release. Gonadotropins (the tropic hormones) can stimulate the synthesis and release of sex steroids (the target hormones) by the gonads, which in turn alter the release of tropic hormones by the pituitary. A short feedback loop occurs when the secreted hormone auto-regulates its own release. Both positive and negative feedback can occur to either stimulate or inhibit release of the tropic hormone. Complicating factors that limit attempts to generalize about the type and extent of feedback loops within the brain-pituitary-gonadal axis amongst fishes are significant interspecies differences and the stage of reproductive development. In particular, there is a relative lack of studies using asynchronous spawning fishes such as those typically used in regulatory testing. The group of fish that are the best characterized with respect to feedback control mechanisms are the salmonids. For example, castration of mature Atlantic salmon (*Salmo salar*) reduced both pituitary and circulating levels of GTH-I and II suggesting a positive feedback effect of androgens (Borg et al. 1998). However, when castrated salmon were given testosterone replacement therapy during the normal time period for spawning, an initial suppression of GTH-I release occurred, which was subsequently stimulated several months later near the end of the spawning season (Borg et al. 1998). In the Atlantic croaker (*Micropogonias undulatus*), gonadectomy and subsequent replacement therapy with testosterone or estrogen increased the GnRH induced GTH-II secretion from the pituitary during the early phases of gonad recrudescence (Khan et al. 1999). After maturation of the gonads, steroid treatment inhibited the responsiveness of the pituitary towards GnRH and release of GTH-II (Khan et al. 1999). Other studies involving gonadectomy have generally indicated that sex steroids have a negative feedback on gonadotropin release (Kobayashi and Stacey 1990; Larsen and Swanson 1997). In additional studies with salmonids, it would appear that the most pronounced feedback is exerted by testosterone and estrogen. In sexually maturing coho salmon (*Onchorynchus kisutch*), exposure to testosterone or estrogen but not 17α,20β-DP, has a negative feedback effect on GTH-I secretion (Dickey and Swanson 1998). In contrast, testosterone and estrogen treatments increased pituitary content of GTH-II (Dickey and Swanson 1998). Additional evidence suggests that feedback effects of testosterone are meditated in part by conversion to estrogen as aromatase inhibitors or administration of nonaromatizable androgens can block or exert a reduced effect (Trudeau et al. 1991; Khan et al. 1999).

21. An explanation for the differential feedback of steroids, that is supported from research on synchronous spawning fishes such as salmonids, is based on differing effects of steroids on the pituitary gland and hypothalamus. Several studies have demonstrated that steroid treatment can increase the expression of the beta subunits for gonadotropins and pituitary content of the hormones (Querat et al. 1991; Antonopoulou et al. 1999; Mateos et al. 2002). These results imply that steroid feedback upon the pituitary is primarily mediated at the gene transcription level. In the hypothalamus of rainbow trout, steroid receptors are not expressed in GnRH neurons but instead appear to occur on neighboring fibers (Navas et al. 1995). Given this pattern of hypothalamic expression of steroid receptors, their role in feedback of GnRH release would appear to be limited to actions on neurons capable of regulating the activity of GnRH secretory neurons. In this regard, additional data from rainbow trout suggest that a negative feedback of estrogen on gonadotropin secretion is linked to effects on the hypothalamus and dopaminergic activity (Linard et al. 1995; Saligaut et al. 1998). Alternatively in other fishes such as the Atlantic croaker, neuronal fibers under gamma-aminobutyric acid (GABA) neurotransmitter control may be involved (Khan and Thomas 1992). Despite the limited data in fishes, some researchers have proposed as a general mechanism that positive feedback of steroids is mediated through the pituitary and induced synthesis of

gonadotropins while negative feedback is achieved by decreasing the secretion of GnRH by the hypothalamus (Mateo et al. 2002). Although this generalization is attractive for simplifying the feedback effects of steroids on gonadotropin synthesis and release, other studies examining the synthesis of the various forms of GnRH in immature male tilapia (*Oreochromis nilotica*) suggest a variety of actions including changes in biosynthesis of GnRHs occur within different regions of the hypothalamus (Parhar et al. 2000).

22. Feedback control for thyroid hormone secretion is less complicated compared to steroid feedback actions and appears to be regulated primarily by a long feedback loop. In the few fish species studied (all teleosts), both T4 and T3 have a negative feedback effect on TSH secretion by the pituitary (Yoshiura et al. 1999). Consistent with findings for steroids, both T4 and T3 appear to decrease transcription of the beta subunit for TSH in the pituitary gland (PradetBalade et al. 1997; 1999). It is unknown whether T4 or T3 influence hypothalamic release of TRH however T3 is known to decrease the synthesis of GnRH in tilapia (Parhar et al. 2000).

2.3 Toxicokinetics of Chemicals in Fish

23. The toxicity of any chemical is based on its mode of action (toxicodynamics) in conjunction with its delivery and concentration at target tissues (toxicokinetics). Although the focus of this detailed review paper is largely on toxicodynamic concerns, some understanding of toxicokinetics is important as significant interspecies differences in toxicity can occur due to toxicokinetic differences (Watanabe et al. 1988). The fundamental processes that determine the toxicokinetics of a chemical are common to all vertebrates and involve absorption, tissue distribution, metabolization and elimination. In fish, absorption of xenobiotics occurs primarily by branchial (across the gills), oral (via ingestion of contaminated food or water), and dermal routes. In reality, chemicals are almost always absorbed by fish through a combination of these processes, although for some chemicals, a specific exposure pathway may predominate.

24. The underlying physiological processes that influence the gill absorption of chemicals in fish is well characterized. Physicochemical properties such as lipophilicity, molecular size or molecular volume and binding to plasma proteins are the primary determinants of the rate of passive absorption of chemicals across the gills (Mckim et al. 1985; Opperhuizen et al. 1985 Hayton and Barron 1990; Sijm et al. 1994). In general, chemicals that are relatively small (e.g < 200 to 300 M.U.), lipophilic (e.g., a log octanol-water partition coefficient [log P] between 3 to 6 and highly protein bound typically exhibit the highest absorption rates in fish (Mckim et al. 1985). Many of the aforementioned physicochemical and biological properties are linked. For example, super lipophilic substances tend to be larger in molecular size. Plasma protein binding of xenobiotics increases with increasing lipophilicity in both fish and mammalian blood plasma (Schmieder and Henry 1988). Allometric influences on the surface area to weight ratio favor a shift to dermal absorption over gill absorption in smaller sized fish (Mckim et al. 1996). The latter effect would be expected to be enhanced with chemicals exhibiting blood flow limiting uptake (e.g., log P between 1 and 3 and protein binding < 60 to 90%; Schmieder and Weber 1992; Hayton and Barron 1990). Physicochemical factors influencing oral absorption of chemicals are more complex, although basic physicochemical properties that influence oral uptake are similar to those that affect gill absorption (Kleinow and James 2001). The influence of molecular size on oral absorption is better characterized than for gill absorption, with molecular weights exceeding 600 or molecular volumes exceeding 0.3 nm^3 greatly reducing absorption (Niimi and Oliver 1988). In general, oral absorption can be the predominant exposure route with chemicals that exhibit significant biomagnification in the food chain or accumulate in sediments (reviewed in Kleinow and James 2001).

25. The tissue distribution of chemicals is influenced by various physiological processes such as tissue blood flow rates and chemical-specific properties such as lipophilicity and binding to macromolecules. For many lipophilic pollutants, the highest tissue burden is in adipose and muscle tissues

(Shultz et al. 1975; Birkholz et al. 1989; Bloom 1992). These tissues are not metabolically active towards xenobiotics and are not thought of as target organs for toxicity. Furthermore, it has been demonstrated that for lipophilic xenobiotics, adipose tissue can have a protective effect; e.g., individuals with high fat content frequently exhibit decreased toxicity Lassiter and Hallam 1990; Geyer et al. 1993; 1994). However, this latter statement applies primarily to acute effects of toxicants which may not be relevant to endocrine active chemicals. Furthermore, mature gonads are often high in lipid content (Jorgensen et al. 1997) thus enhancing concentrations of lipophilic chemicals at potential endocrine target organs. Additional generalizations regarding tissue distribution and physicochemical properties of chemicals are difficult to make. A number of chemicals have been documented to display unusual patterns of tissue distribution that can usually be attributed to tissue-specific binding proteins and/or sequestering by certain tissues. For example, the concentration of the bipyridylium herbicide diquat is 70 times higher in the kidney compared to blood and most other tissues in channel catfish (*Ictalurus punctatus*) (Schultz et al. 1995). Certain metals such as cadmium and inorganic mercury are also preferentially accumulated by the liver and kidney, presumably through interactions with metal binding proteins such as metallothienin (Roesijadi 1992; Schultz et al. 1996). In fish such as brown trout, concentrations of highly chlorinated PCB congeners in well perfused tissues tend to be higher in the brain, in contrast to mammals where the liver typically has the highest levels (Bachour et al. 1998). For other potential toxicants which are neither lipophilic nor exhibit tissue-specific binding properties, the pattern of tissue distribution is expected to be more uniform and favor tissues that are well perfused, e.g. liver, kidney, and spleen, or are at the site of absorption (gills and gastrointestinal tract). It is also important to recognize that for many lipophilic chemicals that exhibit rapid absorption, distribution from blood to peripheral tissues can be a comparatively slow process. Thus, the time to reach a steady-state body burden will be controlled by blood flow to storage tissues (e.g., by distribution and not by the uptake rate; Hayton and Barron 1990).

26. Elimination of contaminants can occur either as the intact chemical or through biotransformation. Direct elimination can occur by the gills, kidney, through the skin (dermal) and/or through excretion in the bile. For lipophilic chemicals, many of the factors influencing gill uptake will also influence branchial elimination (Fitzsimmons et al. 2001). For lipophilic, highly protein-bound chemicals that are poorly metabolized, branchial elimination can be the most significant elimination pathway (Mckim et al. 1986; Cravedi et al 1993). For some chemicals, urinary or biliary excretion can be the most important elimination pathway (Guarino and Anderson 1976; Pritchard and Bend 1991). Conjugation with glucoronide or sulfate and subsequent excretion in bile and urine is known to be an important elimination route for natural and synthetic steroids (Truscott 1983; Schultz et al. 2001; Vermeirssen and Scott 2001). For many chemicals, biotransformation is not only important as an elimination pathway but also in bioactivation to more toxicologically potent forms. A good example of the latter is with the fungicide vinclozolin, which is metabolized into two potent anti-androgenic compounds (Gray et al. 1999; see also Section 6.2.1). Furthermore, the greatest interspecies differences in elimination rates are often attributable to differences in biotransformation. Across vertebrate groups, biotransformation rates can vary up to several thousand fold for specific reactions even when measured under identical experimental conditions (Gregus et al. 1983). Within fishes, somewhat lower inter-species variations are more typically observed, although large differences can still occur when an important metabolizing enzyme is absent or expressed at a minimal level. A good example is with flavin-containing monooxygenases, which catalyze the oxidation of certain hetero-atom containing xenobiotics and are poorly expressed in many freshwater fishes (Schlenk 1998). With other types of reactions, inter-species differences are typically lower but may still be important in controlling accumulation. For example, the difference in metabolism of trifluralin (a pre-emergent herbicide) among seven different fish species was 35 fold, which contributed to a 30 fold range in bioconcentration values (Schultz and Hayton 1999). In some species such as channel catfish, branchial elimination appeared to be the most important elimination pathway, while in others such as bluegills (*Lepomis machrochirus*), biotransformation was more important (Schultz and Hayton 1999). These results and those of other studies indicate that a wide variation among fishes can exist in rates of metabolism of specific chemical substrates (for other examples see Buhler and Rasmussen 1968 or Funari et al. 1987).

Thus, differences in biotransformation can be the most important determinant of elimination rates in fish, not to mention the potential for differential activation of chemicals to endocrine-active metabolites. From a toxicokinetic perspective, this latter point is probably the most important contributing factor for interspecies differences in sensitivity to endocrine active compounds.

2.4 Endocrine Disruption as a Target for Chemical Toxicity

27. Recently, an extensive survey of U.S. surface waters identified the presence of many pharmaceutical agents including natural and synthetic hormones (Kolpin et al. 2002). This report follows decades of increasing reports of reproductive disturbances in fish and other wildlife that was attributed to exposure-specific chemical agents or waste water effluents. Perhaps the most well known example is that of the feminizing effects of the pesticide DDT in wildlife (Bitman et al. 1969). Later observations of masculinization of female mosquitofish residing in or near disposal sites for paper mill effluents indicated that hormonal disturbances in fish can occur beyond disturbances of female hormones (Howell et al. 1980). Additional studies on white suckers (*Catostomus commersoni*) exposed to bleached kraft mill effluents identified changes in sex hormone levels and abnormal reproductive development that were suggestive of a more generalized endocrine disturbance (Munkittrick et al. 1991). These studies in fish in addition to numerous reports of reproductive effects in fish-eating birds, alligators, Great Lakes mink, frogs, invertebrates, and perhaps humans, led to several workshops in the 1990s discussing the effects of chemicals on reproduction (Colborn and Clement 1992; Ankley et al. 1998). The publication of the book *Our Stolen Future* (Colborn et al., 1996) popularized the hypothesis that man-made chemicals may be unintentionally altering the endocrine system and reproduction. These chemicals were specifically proposed to act, even at environmentally relevant doses (i.e., usually lower than laboratory doses), as agonists or antagonists to endogenous endocrine hormones to disrupt the hormonal control of homeostasis, differentiation, growth, and development, including effects on reproductive structures and functions. These agents were called endocrine-active chemicals (EACs), then endocrine-disrupting chemicals (EDCs), and now, most popularly, endocrine disruptors (EDSTAC 1998).

28. Although sufficient observations in wildlife support the endocrine disruptor hypothesis, it is nonetheless controversial due in part to difficulties in establishing links among environmental exposure to chemicals, changes in endocrine function, and altered reproduction (Van der Kraak 1998: WHO, 2002). As discussed in previous sections, the endocrine system is complex with many potential target sites and hormonal systems may be affected. Disturbances could be directed at the brain- pituitary or at the gonadal level or a combination of both. However, there is only limited experimental data available on the vulnerability of the hypothalamus-pituitary system to chemicals. Several studies have demonstrated that gonadotropin secretion can be altered, but it is unclear whether this is due to a specific endocrine disruptor type interaction or some other non- specific toxicological effect (Kime 1998). In contrast, endocrine disruptor effects on thyroid and gonadal hormones are more established. An excellent example of the latter is a set of recent studies on wild fish living in U.K. rivers that indicate unnatural exposure to estrogenic substances (e.g., the natural hormone estradiol, the synthetic one ethinylestradiol, used in contraceptive pills, alkylphenol ethoxylates and nonylphenol originating from the textile industry) caused impaired reproduction (Jobling et al. 1998; van Aerle et. al. 2001; Sheahan et al, 2002).

29. In conclusion, evidence exists that environmental concentrations of EDCs adversely affect fish sexual differentiation, development and reproduction. Greatest focus on potential hormone targets are the sex steroids and, to a lesser extent, the thyroid hormones. The remaining sections of this detailed review paper will provide information on screening assays directed toward three species of fish (i.e., fathead minnow, zebrafish, and medaka). This paper will outline the relevant principles, methods, and techniques needed for one or more initial protocols, and identifies issues that might require additional validation studies.

3. CANDIDATE TEST SPECIES

30. This review paper will focus on three species of fish that are the most likely candidates for use in screening assays: fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*). These fish share several attributes that make them ideal test species for reproductive toxicity testing, including small size at maturity (which reduces maintenance costs) and overall ease of culture. All three species are asynchronous spawners, meaning the ovaries contain oocytes at all stages of maturity, allowing spawning to occur repeatedly over an extended time period. This reproductive attribute is important as it allows groups of fish to be tested for spawning success before and after chemical treatments during a relatively short time period. It should be recognized, however, that the presence of gametes in all developmental stages may make identification of gonadal cellular targets more difficult.

3.1 Fathead Minnow (Pimephales Promelas)

3.1.1 Culture and Handling

31. The fathead minnow is a common freshwater minnow belonging to the largest family of fish, the Cyprinidae. Their original range was limited to central North America, but they have since been introduced into most regions of North America (Pflieger 1975). Fathead minnows can live in a wide range of habitats, including shallow turbid waters, and they are an important bait- fish species. As a result, fathead minnows are easily cultured and are readily available from commercial sources. The fathead minnow has been used extensively in aquatic toxicity testing in the United States, and a number of testing guidelines exist, including detailed information on their laboratory culture (Denny 1987).

32. Fathead minnows are small (35 to 75 mm in total length) and offer ease of culture in laboratory aquaria. Adult males are territorial but are still tolerant of a number of other adult male and female fish in an aquarium. Usually the “normal” mating situation with fatheads does not involve a pair wise pairing but rather a higher female to male ratio, however, recent investigations have shown that the coefficient of variation for fecundity was very similar regardless of whether the fatheads were housed in pairs or in groups (Jaber, personal communication). This means that replicates consisting of one pair of fish can be used instead of replicates consisting of e.g. 6 females and 2 males.They can tolerate low dissolved oxygen and a variety of water temperatures and water quality parameters, but for optimal growth and reproduction, water temperatures should be within a range of 24ºC to 25ºC, and dissolved oxygen should remain above 60% of saturation. Adult fathead minnows are sexually dimorphic and can easily be sexed in the aquarium. The development of aggressive behavior and secondary sex characteristics are signs of sexual maturity in males, and the development of a papilla is a distinct sign of sexual maturity in females. However, the time of sexual differentiation is not well documented for the fathead minnow. The generation time of fathead minnows is about 4 months. They can be kept in breeding condition all year without manipulating light cycles and water temperature. Fathead minnows can be induced to spawn by placing spawning substrate in the aquarium, which is guarded by the male. Better spawning synchronization can be obtained by withholding the spawning substrates for several days. Spawning is usually initiated just before light and can last through the morning; therefore, it is advisable not to disturb the fish during the morning hours except to feed and collect embryos. Each spawning female will produce 50 to 250 embryos per spawn. Embryos are collected from the aquarium by removing the spawning substrate. The embryos can be incubated on the spawning substrate or they can be removed by well established procedures without harm to the developing individuals from the substrate and incubated in a container or in egg cups (glass cylinders with mesh bottoms). Fertilization can be assessed immediately with light magnification or it can be assessed 24 hours after spawning by counting the number of opaque and white (nonfertile) embryos versus the number of translucent eggs (fertile). Fertilization rates of 80%

to nearly 100% are typical. Embryo incubation time is 4.5 to 6 days at 25ºC. The larvae are reared in separate aquaria and should be offered live brine shrimp nauplii (*Artemia*) immediately after hatch. Juvenile fish can be fed a mix of frozen brine shrimp and live nauplii, whereas adults can be fed a mixture of frozen brine shrimp and commercial flake food. All life stages should be fed *ad libitum* two to three times per day.

3.2 Medaka (Oryzias Latipes)

3.2.1 Culture and Handling

33. The medaka (*Oryzias latipes*) is a freshwater killifish belonging to the family of Asian ricefishes, Adrianichthyidae, indigenous to areas of Japan, Taiwan, and southeastern Asia, where ambient temperatures range from 5ºC to 35ºC (Kirchen & West 1976). The medaka has a long history as an experimental animal and a complete presentation of their biology can be found in Yamamoto (1975) and on the website http://biol1.bio.nagoya-u.ac.jp/, which lists over 1000 references dating from the early 1900s. The culture and handling of the medaka has been exhaustively studied, and detailed guidelines are available in EPA/600/3- 91/064 (Denny et al. 1991). Medaka are small (25 mm to 50 mm in total length) and are easy to rear and maintain in laboratory aquaria. Adults can be maintained in an aquarium with little space and can tolerate low dissolved oxygen and a wide range of temperatures and salinity (Yamamoto 1975). Medaka are sexually dimorphic, and although the sex of fully grown fish can be determined by observing the outline of the anal and dorsal fins, the observation of an anesthetized fish under a low-power microscope is recommended for confirmation and for sexing an immature fish (Yamamoto 1975). In the medaka, the sexual differentiation of the female gonad begins before hatching, whereas the male differentiates during a critical window around 13 days post-hatching (Kime 1998).

34. Medaka have a generation interval of 2 to 3 months, and can be induced to spawn throughout the year by controlling the temperature and photoperiod. Breeding tanks are ideally set up with a ratio of 4 males per 6 females. Spawning activity normally occurs through 14 to 15 months of age. Spawning is highly predictable in time, usually within 1 hr of first daylight, and can be controlled in the laboratory by adjusting the timing of the recommended cycle of 16-hour light: 8-hour dark (Hirshfield 1980; Koger et al., 1999). Measures of medaka reproductive output, including gonadal morphology, fecundity, and fertility, demonstrate that through laboratory control of temperature and photoperiod, reproductive capacity of breeding groups can be calibrated before exposure, thereby establishing a baseline from which to assess effects (Koger et al., 1999). Individual females can lay an average of 25 eggs/day for up to 4 months under proper conditions of photoperiod, temperature and food supply (Hirshfield 1980). A cluster typically contains 10 to 30 eggs that are attached to the female's vent by filaments for a number of hours until they are brushed off onto a spawning substrate that simulates aquatic plants (Yamamoto 1975). Even when spawning substrates such as a spawning sponge are provided, some females retain their embryos and require manual stripping (Denny et al., 1991). The eggs should be collected as soon as possible after spawning to prevent their predation by adults. As described in the medaka Web site, the adhesion fibers firmly hold individual eggs as a cluster. To prevent harm by fungi, and to ensure uniform potential for oxygenation, the individual eggs must be separated. Two methods exist; the first involves gently rolling the egg mass between moistened fingertips, thereby breaking attachment fibers and separating individual eggs. A slight variation of the same approach is to place the egg mass on a paper towel, add 1 to 2 drops of water and place a second towel over the egg mass, and then perform gentle circular motion with the fingertips to separate eggs. The other method involves grasping the central band of adhesion fibers with tiny forceps, then with opened iridectomy scissors, separate one egg from the cluster by gently stretching attachment fibers and cutting them near the egg surface. This process is repeated until clean eggs with very short attachment fibers are separated from the original mass. Once separated, the eggs can be incubated either separately or in individual wells, without the addition of anti-bacterial or anti-fungal agents. Because of the transparency of the egg chorion, fertilization can be easily assessed with a dissecting

microscope. The egg-incubation period is approximately 1 week when kept at 28ºC (Yamamoto 1975), and 9 to 10 days when at 25°C to 26°C.

35. There is a concern with the treatment of embryonated eggs by addition of substances to the embryo-rearing medium. These are designed to afford protection from bacteria and invertebrate organisms that may damage eggs. In Japan where emphasis has been more on developmental biology, 0.5 ppm methylene blue addition to the embryo-rearing medium is common and was also recommended by Kirchen and West (1976). Another approach recommended by Kirchen and West (1976) is to use a brief hypertonic saline rinse followed by culture in embryo rearing with methylene blue treatment. Alternatively, eggs can be separated by the moistened fingertip method, placed in a separatory funnel in water and aerated vigorously (Dave Hinton pers. comm.). This approach yields satisfactory quantities of embryonated eggs. When pigmentation of eyes and other features indicative of late embryonic stages has been reached, eggs were placed in petri dishes until hatching was completed (Dave Hinton pers. comm.). The embryos will tolerate a temperature range of 7ºC to 38ºC (Kirchen & West 1976). The larvae are reared in separate aquaria and begin feeding on the day of hatch with a recommended diet of live brine shrimp nauplii (*Artemia*) ad libitum twice daily for all life stages. A feeding approach used by Japanese researchers at the Laboratory of Freshwater Fish Stocks, Bioscience Center, University of Nagoya, is to place 10 hatchlings in small (4 liter) aquaria, where they are fed a finely ground commercial ration plus two feedings of paramecium infusion. Larvae are not fed brine shrimp until day 15 after hatch. The commercial ration is used from day 1 throughout the first 30 days. After one month, larvae are transferred to larger tanks and placed on system water. Feeding is now by brine shrimp, once per day and commercial ration for the afternoon feeding each day.

3.2.2 Application of Genetically Engineered/Inbred Strains in Toxicity Testing

36. There are estimated to be over 500 cultivated strains of medaka. Specific information for many of these strains can be obtained at the website (http://biol1.bio.nagoya-u.ac.jp:8000/), which is maintained by researchers at Nagoya University, Nagoya, Japan. Two inbred strains that have characteristics that would make them particularly useful for a reproductive screen are the d-rR and STIII or "see-through medaka." The d-rR medaka was first described in 1953 (Yamamoto 1953) and is a mutant strain possessing sex-linked pigmentation, with males having an orange-red coloration and females having white coloration. Recent experiments have shown that hormone-induced reversal of phenotype does not alter pigmentation, making it much easier to identify individuals that have undergone phenotype reversal (Edmunds et al., 2000; Papoulias et al., 2000). Thus, genetic males that have developed female characteristics such as ovaries and secondary sex characteristics (e.g., dorsal fin notch) still retain the orange-red pigmentation (Papoulias et al., 2000). This feature would be a particularly valuable aid in identifying phenotype reversal as an assay endpoint, as it can sometimes be difficult to determine whether a phenotypic female is in fact a genetic (XX) female or phenotypically reversed male (XY female). However, it would be unlikely, if not impossible, that complete phenotype reversal would occur in short-term exposures with sexually mature individuals, and the d-rR strain might have greater utility in screening assays using juvenile life stages and partial-life-cycle or multi-generational tests in which chemical exposures occur prior to sexual differentiation. Although the d-rR medaka strain may not be useful in traditional approaches to short-term screening assays, this strain would be useful in tests in which single-sex exposure of a test chemical is desired. Because of the ease in identifying the genetic sex of individuals, a reproductive assay in which only male or female individuals are exposed to the test chemical would be relatively easy to perform. This type of test would be useful in assessing the specific effects of chemical exposure on gamete viability. For example, exposed males could be mated with unexposed females and fertilization rate measured as an indicator of sperm viability. This type of experimental design has been attempted with a non-mutant strain of medaka (Tabata et al., 2001), although the use of the d-rR strain would likely reduce error in sex identification.

37. One of the effects of "endocrine disruption" is the appearance of oocytes in testicular tissue of male fish. At present there is an ongoing discussion whether or not a natural background of "testes-ova or ova-testes" exist in medaka. In view of the more than 500 strains of medaka, it is more than likely that the presence of background incidences of'testes-ova or ova-testes" is strain dependent.

38. The see-through medaka is a recently described strain that has an almost complete absence of pigmentation in all life stages (Wakamatsu et al., 2001). The transparency of the fish allows direct visualization of the internal organs, including the reproductive organs. The introduction of a transgenic version of the STIII strain expressing the green fluorescent protein gene in primordial germ cells (Tanaka et al., 2001) permits detailed observations of the ovaries and testes in a non-invasive manner over the life span of the individual without harming the fish and would allow repeated observations of gonad morphology during an exposure. This capability would be particularly valuable in monitoring changes in morphology of the gonads during a short-term exposure in sexually mature fish. In addition, because of the exceptional transparency of this mutant medaka, oocyte maturation can be monitored in the ovary of an individual fish (Wakamatsu et al., 2001). Application of this strain may allow the inclusion of an additional endpoint in screening assays, specifically, in-vivo examination of the effect of a chemical on normal oocyte maturation. However, it shall be taken into account that although "See-through" medaka have been established as a stable strain, it may be expensive or otherwise difficult to use for routine testing. More detailed discussions on the practicality of this strain is needed.

3.3 Zebrafish (Danio Rerio)

3.3.1 Culture and Handling

39. The zebrafish, *Danio rerio*, like the fathead minnow, is a member of the Cyprinidae family and is native to East India and Burma. The zebrafish has been used for aquatic toxicity regulatory purposes in Europe and worldwide as a model for studying vertebrate development and genetics. Zebrafish have been extensively studied since the 1930s, and a detailed review of their biology and laboratory use is presented by Laale (1977). Zebrafish are easy to culture and are available from commercial suppliers; detailed methods for their care in the laboratory are available in Westerfield (2000) and at http://zfin.org/zf_info/zfbook/zfbk.html. They can easily be maintained in aquaria at a temperature of 26±2ºC Hisaoka & Battle (1958).

40. Adult zebrafish are vigorous swimmers, reaching a total length of 4 cm to 5 cm and should be fed a variety of food, dry flake food, and live nauplii of or adult brine shrimp 2 times per day for optimal nutrition. Indistinct secondary sexual characteristics can make it difficult to sex zebrafish; however adult zebrafish can be sexed by observation of the external morphology of individual fish. There is normally a very good agreement between the phenotypic sex and the gonadic sex., According to Eaton and Farley (1974), the first spawning occurs at an age of 75 days, when the females and males have reached a standard length of 24.9 mm and 23.1 mm, respectively.. Optimal spawning occurs every 5 to 10 days, producing a maximum of 1800 eggs, with an average of 150 to 400 eggs per female. However, zebrafish will continuously produce a small number of eggs (20 to 50 eggs per female daily when paired in equal numbers of well-fed males and females under a 12-hour-light:12-hour-dark cycle (Westerfield 2000). Spawning takes place shortly after dawn, and the transparent, non-adhesive eggs will naturally fall to the bottom of the tank, where screens can protect the eggs from consumption by the adult fish. Eggs and sperm can be collected from individual zebrafish for controlled fertilization studies (Westerfield 2000). Viable embryos can be readily observed through the very transparent egg chorion, and the embryos normally hatch after 3 days of development. Takahashi (1977) described the normal development including sex differentiation. During development, male zebrafish pass through a stage of juvenile hermaphroditism. Ten to twelve days post-hatch, the gonads of all juveniles starts to differentiate into ovaries irrespectively of their genetically determined sex. The development of ovaries continues until they

reach an age of 23 - 25 days, after which definite sex differentiation begins. Usually, 50 % of a population will continue to develop ovaries, whereas in the remaining 50 % of the individuals, ovaries will start to degenerate and will be transformed into testis. The sex ratio may vary and Örn et al. (2000) as well as Fenske et al. (1999) have reported a female to male ratio of 32 to 68 and 40 and 60, respectively. Forty days post-hatch, the process of sex differentiation and reversal is completed, and final maturation of the gonads will generally be finished at 60 days of age (Takahashi (1977); Andersen et al., (2003)).

3.3.2 Application of Genetically Engineered/Inbred Strains in Toxicity Testing

41. In recent years, reliable methods for the development of transgenic zebrafish have become widely available (Moav et al., 1993). The use of transgenic fish in aquatic toxicological research has been limited until recently, but offers great potential for the development of sensitive biomarkers and aid in the identification of mode of action. One approach has been to transfect zebrafish with DNA motifs that, once activated, will also activate a reporter gene such as luciferase that can be readily detected. Among the motifs successfully introduced into the zebrafish include dioxin, heavy metal, and estrogen responsive motifs (Carvan et al., 2000a; Legler et al., 2000). This approach would be particularly valuable in the development of an androgen sensitive biomarker. With the exception of the protein spiggin, produced by the male stickleback (discussed in Section 6.0), no suitable biochemical biomarker has been developed for androgenic substances. A less explored area of research is the generation of a knock-out zebrafish. More specifically, generation of an ER null mutant zebrafish would be useful for assessing the role of E2 in sexual development in general and the impact anti-estrogens can have on reproduction.

3.4 Strengths and Weaknesses of Test Species

42. The culture and handling of the three species is well defined and well documented. All species tolerate a wide range of water-quality and water-temperature conditions, require small culture space, and produce the number of embryos needed for testing. The fathead minnow has a strong regulatory history in the United States, whereas the zebrafish has been similarly used in Europe and the medaka in Japan. Some of the major strengths and weakness of the three species are summarized in Table 3-1.

43. Given the diverse physiology and life histories of the approximately 20,000 species of fishes, it is unrealistic to assume that a single fish species can be considered “representative”of all fishes. Nonetheless, the high costs involved in screening chemicals may require the use of just one test species. Therefore, it is important that the chosen species is at least representative with respect to sensitivity toward endocrine disruptors. In many respects, it would be desirable to have the selected species be more sensitive towards toxicants, as this can provide an additional safety factor for the protection of more obscure fish species for which little if any toxicity data will ever be obtained. This latter point is especially important for screening assays measuring a specific mode of action of toxicants, such as endocrine disruption. Analyses of aquatic species’ sensitivity toward toxicants have identified that the largest variation occurs with toxicants that exert their effect through a specific biochemical mode of action (Vaal et. al. 2000).

44. As for the strengths and weaknesses of the candidate species, most of the information presented in Table 3-1 focuses on culturing, morphological, and biochemical related concerns. It is difficult to generalize about relative toxicant sensitivities among the candidate species as no thorough side-by-side evaluations have been made with these species. Some authors have suggested that salmonid species are more sensitive than the cyprinid species considered for testing (Nagel and Isberner, 1998). Attempts to compare species differences in toxicant sensitivity are commonly fraught with contradictory results. For example, the guppy (*Poecilia reticulata*) is 50 times more sensitive toward the acute toxicity of parathion than the medaka (Vaal et al. 1997). However, the medaka is 95 times more sensitive toward

trichloroethylene acute toxicity than the guppy (Vaal et al. 1997). Despite the tendency for paradoxical results, one extensive study suggests that the medaka and fathead minnow are very similar in their toxic responses toward diverse chemicals (Holcombe et al. 1995). There have been compilations of data summarizing acute toxicity data among both fishes (for example see Mayer and Ellersieck 1986). Data summaries can be useful in identifying unusual sensitivities or resistance of particular species toward certain organic or inorganic compounds. Statistical analyses of these databases have indicated that fathead minnows and medaka are very similar in their average sensitivity toward acute toxicity of toxicants (Vaal et. al. 1997). There is less comparative information for the zebrafish on this subject. However, based on findings from other cyprinids, the zebrafish should be similar in sensitivity to the other candidate species. It would appear that selection of a candidate species should not be based on toxicant sensitivity as no obvious differences are apparent.

Table 3.1: Strengths and Weaknesses of Test Species

Species	Strengths	Weaknesses
Fathead minnow (*Pimephales promelas*)	- Large enough to collect individual blood plasma samples - Distinct secondary sex characteristics in both sexes, clearly responsive to endocrine active substances, - Large historical regulatory database - Frequently used in the U.S.A. for regulatory purposes - Spawn on a substrate - High fertilization rate - Indigenous to North America	- Relatively long life cycle - Relatively high variability in fecundity - Genome poorly characterized
Medaka (*Oryzias latipes*)	- Relatively short life cycle - Distinct secondary sex characteristics in both sexes, clearly responsive to endocrine active substances - Relatively small fish, making culture and testing possible in smaller space - Female sex-determined during embryo stage vs. male sex-determined after hatch - High fertilization rate - Relatively low variability in fecundity - Sex-linked color strain - Anticipated that entire genome will be sequenced soon. - Frequently used in Japan for regulatory purposes - Genome is well-characterised	- Difficulty of collecting sufficient blood sample from adult (liver is sampled instead) for VTG analysis.
Zebrafish (*Danio rerio*)	- Short life cycle - Small fish, making culture and testing possible in smaller spaces - Male fish go through a hermaphroditic phase as juveniles - Widely used in other medical and genetic research - High fertilization rate - High amount of eggs (150-400) per spawn - Relatively low variability in fecundity - Frequently used in Europe for regulatory purposes - Transgenic fish increasingly available - Anticipated that entire genome will be sequenced soon.	- Minimal secondary sex characteristics, making it difficult to separate males and females - Difficulty of collecting sufficient blood sample from adult (although not impossible) for VTG analysis - harder to work with in a spawning assay because a) a group-spawning setting is necessary, and b) the zebrafish are broadcast spawners.

4. DESCRIPTION OF ASSAY ENDPOINTS

45. There is wide consensus among experts that a fish screening assay should cover several core endpoints and supplementary endpoints as appropriate. Apical endpoints like sexual behavior and sexual characteristics should be complemented by specific endpoints indicative of the mode of action (MOA). Three manifest core endpoints are gross morphology (including determination of the gonado-somatic index GSI, and disappearance or appearance of secondary sexual characters), vitellogenin (VTG) measurement and gonad histology. A flexible stepwise approach should be used to measure those endpoints, i.e. no priority order is proposed: all core endpoints should be measured unless one particular core endpoint shows a significant response. In addition to the three core endpoints, supplementary endpoint(s) might be included optionally (e.g. steroid titres, fecundity, fertility, etc.)

Gross morphology

46. Morphological changes are easy to measure and are not time consuming. All relevant morphologic parameters can be easily included in the list of observations made (e.g. body colour, body shape, etc.). It is to note that endpoints related to secondary sex characteristics and gonadal status (GSI, histopathology) would be meaningless and/or impossible to measure in juvenile fish. Gross morphology data should include, as far as possible (depending on the fish species), the following:

- Gonado-somatic index (GSI);
- Macroscopic examination of gonads;
- Secondary sexual characteristics (in adults of sexually dimorphic fish species or in juveniles where the genetic sex is known);

Vitellogenin measurement

47. VTG is a phospholipoglycoprotein precursor to egg yolk protein that normally occurs in sexually-active females of all oviparous species; the production of VTG is controlled by interaction of estrogens with the estrogen receptor. Significantly, males maintain the capacity to produce VTG in response to stimulation with estrogen receptor agonists; as such, induction of VTG in males and immature females has been successfully exploited as a biomarker specific for estrogenic compounds in a variety of fish species. Yet, the endpoint can be equally as valuable in females for anti-estrogens, both direct (i.e., estrogen receptor agonists; Panter et al. 2000a) and indirect (e.g., inhibitor of aromatase; Ankley et al. 2002) as well as some types of androgens (Ankley et al. 2003). However, there is a need for more research to understand and interpret what an increase of VTG means in biological terms. Likewise, the issue of the "noise of biomarker" requires more research to clarify the range of endogenous VTG levels in adult and juvenile fish.

48. The measurement of VTG should be based upon a validated method with sufficient sensitivity and precision. Information should be available upon the intra-assay and inter-assay variability and on the lower detection limit of the method. It is recommended to use homologous VTG as standard protein when applying direct methods of VTG quantification. A list of techniques to measure plasma VTG in several fish species, is provided in section 1.2 of Annex A.

Gonadal histology

49. Baseline histology data on gonads exist for fathead minnow, medaka, and zebrafish. Efforts have been made to compile into a single practical document (OECD, 2004) a) receommended histological techniques for the sampling, fixation, embedding and sectioning of fish gonads, and b) recommendations and examples for the staging of gonads and the interpretation of lesions. This guidance document will assist pathologists in their work, especially in the validation of the endpoint, as part of the screening assay for endocrine active compounds.

4.1 Growth and Morphological Alterations

4.1.1 Gonadosomatic Index

50. Condition and tissue-somatic indices, such as the gonadosomatic index (GSI), are a general measure of the overall condition of the fish or growth status of a specific tissue. Tissue- somatic indices are commonly reported in fisheries studies because of the relative ease of determination and the general belief that certain indices, such as the liver-somatic index, can be an excellent predictor of adverse health in fish (Adams & McLean 1985). The GSI is also frequently reported as a general measure of gonad maturation and spawning readiness and is based on the broad assumption that proportionally larger gonads indicate greater development (West 1990).

51. The GSI can be potentially useful as part of a reproductive screen, because reduction in relative gonad mass can occur as a response to certain types of EACs. Although frequently reported, the appropriateness for comparison of gonadosomatic indices between control and treatment groups is based on several specific assumptions, many of which may be difficult to validate in fish such as those considered in this review. Specifically, one key assumption is that linear relationships between gonad weight and body weight are constant throughout varying stages of gonadal development (Devlaming et al., 1982). This criterion can be challenging to meet with females of fish species that are asynchronous, fractional spawners, as inter-individual variation in ovarian weight can be high during the spawning cycle. Two approaches researchers have used to overcome this limitation are synchronization of spawning cycles through environmental manipulations (Soyano et al., 1993; Van den Belt et al., 2001), and more commonly, simply including sufficient numbers of replicates in an exposure tank to “average” out the variation across individuals.

52. The general procedure for determining the GSI is simple and involves humanely euthanizing the fish, removing excess moisture and determining the total mass, and then removing and weighing the gonads. The index is then calculated as GSI = 100 x gonad weight / body weight. Typical values for reproductively active fathead minnows, medaka, and zebrafish are as follows: fathead minnows: females 8% to 12%; males ~1% (Jensen et al., 2001); medaka: females 8.5%; males 1.5% (Scholz & Gutzeit 2000); zebrafish: females 6.7% ± 1.6%; males 0.98% ± 0.2 % (Van den Belt et al., 2001).

53. In laboratory studies using oestrogen agonists, concentration-dependent reductions in GSI have been reported in fathead minnows exposed to 17β-oestradiol and oestrone (Panter *et al*., 1998), medaka exposed to alkylphenols (Yokota *et al*., 2001; Japanese Ministry of Environment 2002), zebrafish exposed to octylphenol (van den Belt *et al*., 2001) and in rainbow trout exposed to either 17α-ethinylestradiol (Billard *et al*., 1981) or alkylphenols (Jobling *et al*., 1996). No GSI data are available for anti-oestrogens. There are few studies on androgens, and no data could be obtained for medaka or zebrafish. In breeding groups of fathead minnows, Ankley *et al* (2001) demonstrated impacts on GSI from methyltestosterone while Panter *et al* (unpublished data) have observed similar results in non-spawning fish exposed to dihydrotestosterone. In terms of anti-androgens, Makynen *et al*., (2000) showed impacts on GSI in fathead

minnows. Panter *et al* (unpublished data) have also seen GSI impacts after 14 or 21 days exposure to flutamide.

54. More data on reference chemicals are needed to investigate the sensitivity of the GSI endpoint in all three most common laboratory species (namely fathead minnow, medaka and zebrafish).

4.1.2 Sexual Differentiation and the Sex Labile Period

55. The sexual differentiation relates to the events that occur during early development, and that allow the expression of the genetic sex into the phenotypic sex. The earliest signs of ovarian differentiation are the entry of oogonia into meiosis, the position of somatic germ cells and/or the proliferation of somatic sex to form the ovarian cavity (oviduct). The earliest signs of testicular differentiation are the appearance of spermatogonia, the arrangement of germ/somatic cells in lobules, differentiation of vascular system and/or sperm duct (vas deferens, efferent duct). After the gonads have differentiated into either ovaries or testes in gonochoristic fish, there may be a considerable period of time during which these tissues develop before gametes are produced for the first time (the sex labile period). In oviparous female fishes, oogenesis must be completed, which involves vitellogenesis, before oocytes are fully formed and ready for ovulation. The testes of the male must complete the sequential processes of spermatogenesis and spermiation to produce viable sperm. The development of the gonads is regulated by endocrine signals originating in the hypothalamus and pituitary, and sex steroids, particularly 11-KT in the male are known to be important for male germ cell production (Borg 1994). The intraovarian role for estrogens in the female is not known, but reduced E2 titers will affect VTG production by the liver. Therefore, EACs that affect any aspect of the reproductive endocrine system causing reduced synthesis or release of gonadotropins, or that interfere with gonadal steroidogenesis, will delay or prevent reproduction from occurring (Arcand-Hoy & Benson 1998). This can be observed by examining the state of development of gonads of exposed fish relative to a suitable control, via the gonadosomatic index or histological staging (Le Gac et al., 2001). A delay in spawning (i.e., time to first spawning/sexual maturity) and spawning frequency are commonly assessed endpoints in toxicological lifecycle studies of fish that presumably result from this mode of action. Fecundity might also be impacted in both sexes when fewer germ cells develop or mature. This can be detected by quantifying the number of eggs spawned in females or assessing sperm number in males.

56. The sex labile period is species-specific (Piferrer, 2001). It has been found that the maximum sensitivity to estrogens occurs earlier than the observed maximum sensitivity to androgens (Hackman and Reinboth, 1974; Piferrer and Donaldson, 1989). These studies imply that the sex labile period to estrogens is located earlier in the development than that to androgens. This is supported by the fact that sexual differentiation of females occurs before that of males in fish (Bruslé and Bruslé, 1983; Nakamura *et al*., 1998; Nagahama, 2000).

57. The localization of the sex labile period has been determined for several species. The sex labile period determined in different species is presented in figure 4.1.

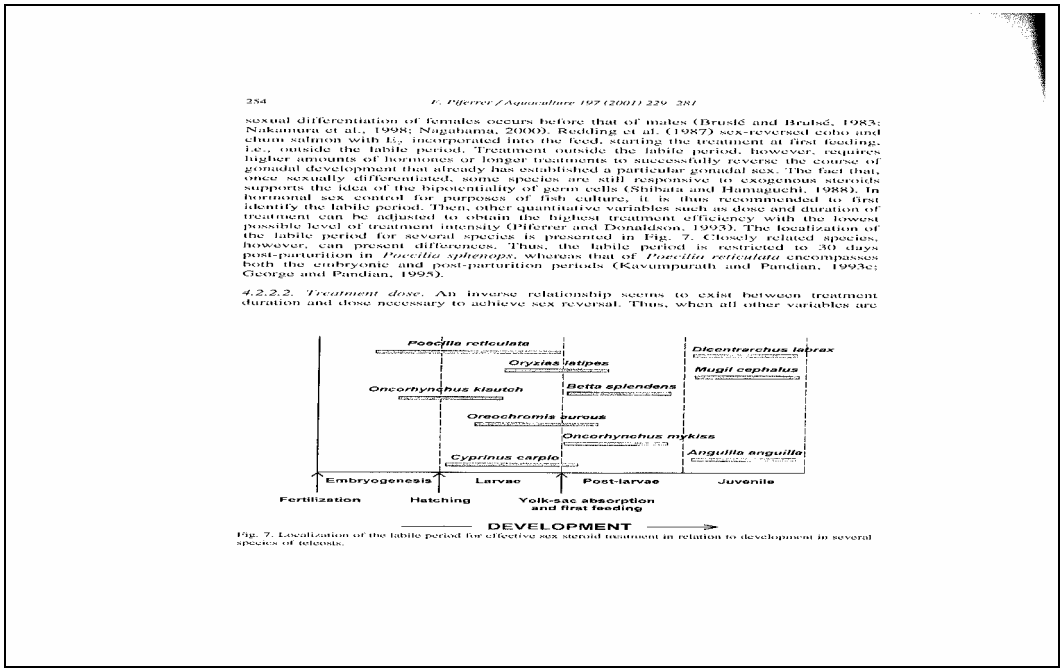


Figure 4.1 Localization of the Labile Period for Effective Sex Steroid Treatment in Relation to Development in Several Species of Teleosts (copied from Piferrer, 2001)

58. From previous results obtained by Andersen *et al*. (2003) the sex labile period in zebrafish seems to be closely related to the sex labile period determined for rainbow trout. This is supported by the fact that the gonadal development in both zebrafish and rainbow trout passes through a stage of juvenile hermaphroditism (Takahashi, 1977; Chan and Yenung, 1983). As seen from the figure the sex-labile period for medaka *(Oryzias latipes)* seem to occur earlier than for zebrafish. The sex-labile period for fathead minnow is during the embryo or very early life-stage (van Aerle et al, 2003). Significant effects on the sexual differentiation has been shown for zebrafish where Andersen et al. (2003) exposed zebrafish to 15.4 ng EE2/l in different critical life stages and observed 100% females after exposure from hatch to 60 days post hatch and from 20 to 60 days post hatch. Örn et al. (2003) found a sex ratio significantly skewing toward females after exposure of zebrafish from 20 to 60 days post hatch at only 0.6 ng EE2/l (lowest measured concentration). Finally Örn et al. (2003) found a sex ratio significantly skewing toward males (100%) after exposure of zebrafish from 20 to 60 days post hatch at only 26 ng/l Methyltestosterone (lowest measured concentration).

4.1.3 Gonad Histology (Techniques and Evaluation)

4.1.3.1 Histology Techniques

59. Histological assessment of gonadal tissue from fish exposed to endocrine disrupting chemicals affecting the HPG axis can be diagnostic (Leino et.al. 2004, Wester et al.2003). Several authors have described the methods used to prepare gonad and other tissues from small fish species for histological assessment (Orn et al., 2000; Wester & Canton 1986; Lange et al., 2001). It should be noted that although histological methods are widely practiced and are relatively routine, there are numerous variations employed. The specific methods used are influenced by many factors including the reagents available, specimen size, resolution required, and which staining protocol selected. However, for the purpose of OECD screening-assay protocols, histological methods need to be selected that are versatile and universal. That is, the method must provide reproducible results when performed by a reputable histopathology laboratory anywhere in the world. The first decision to be made regarding histological methods, or histotechnique, is what embedding medium should be used. Protocols for both paraffin-embedded and

glycol-methacrylate- embedded histology were described and compared by Jensen et al. (2001). The longer established paraffin methods, which are more commonly practiced, are well suited for histopathology assessments of large numbers of samples. Plastic embeddments, such as glycol-methacrylate, are appropriate for more specialized applications. Among several advantages offered by plastic embeddments is that plastics can be sectioned thinner than paraffin. Thin sections provide the pathologist with finer detail in both tissues and cells, which is sometimes necessary to discriminate subtle pathology. The principle disadvantages of plastic are that it is somewhat more difficult to produce large numbers of high quality slides in a routine histology lab, and it is difficult to produce slides of relatively large tissue samples, thus limiting the amount of tissue available for pathology analysis. It is not clear whether plastic embedment provide sufficient advantage to the pathologist to warrant their use in a screening assay. They may be necessary to diagnose subtle endocrine disruptor effects in the testis, but until more experience is gained, paraffin methods are preferred for a screening assay.

60. The choice of which embedment will be employed sets the stage for selecting the appropriate fixative. Tissue fixation is arguably the most important choice to be made once an embedment has been chosen because it impacts the necropsy procedure, tissue processing, and most importantly, the quality of slides for the pathologist to analyze. Most of the fixatives used today for routine tissue histopathology are formaldehyde-based, with the simplest formulation being 10% neutral-buffered formalin. Generally the selection of which formaldehyde-based fixatives to use is based on a set of attributes and undesirable side-effects deemed important by the pathologist. Examples include, superior fixation of a particular organelle-cell- or tissue-type, specimen size (fixative penetration rates), tissue decalcification properties, specimen staining quality, fixative handing qualities, etc. The use of routine fixatives such as neutral buffered formalin, Bouin's fixative, buffered glutaraldehyde-formaldehyde, and Davidson's fixatives in regard to fixation of fish reproductive tissues has been reported (Jensen et al., 2001; Schwaiger et al., 2000; Miles-Richardson et al., 1999b; Wester & Canton 1986). Of the above-mentioned fixatives, Bouin's and Davidson's fixatives appear to possess attributes desired by pathologists for routine gonad histopathology in small fish, based on the above-mentioned criteria (OECD 2003).

61. When considering the specific fixation methods to employ, it is critical to consider the size of the specimen being fixed. While different fixatives possess properties that affect how rapidly they penetrate the tissue, and thus, ultimately the maximum specimen size, there are practical limits. With smaller aquarium fishes that weigh 1 gram or less (e.g., medaka and zebrafish at all life-stages), and with small fathead minnows (e.g., early life stages and small adults), it is possible to fix entire individuals. These specimens, either whole or trimmed after fixation, can be sectioned to provide slides of the gonad within the abdominal cavity (i.e. *in situ).* These slides allow the pathologist to analyze the gonad, as well as other reproductive tissues that may be sensitive to endocrine disruption, such as the liver, kidney, and gonadal duct systems. For larger specimens, it is possible to obtain adequate gonadal fixation if the abdominal cavity is incised along the ventral midline followed by manual perfusion of the gonadal tissues before immersing the whole fish in the fixative solution. Variations on this protocol involve a short (1 to 5 minute) *in-situ* fixation as just described followed by micro-dissection of the gonad from the abdominal cavity, and finally placing the dissected gonad into the fixative. In all cases discussed previously, it is important to note that the fixative to tissue volume must be 20:1 or greater. Furthermore, to minimize autolysis and ensure satisfactory results, tissues must be in contact with the fixative within a few minutes following euthanasia, and they need to be fixed for the proper time prior to embedment.

62. Before fixed gonads can be evaluated for pathology, they must be embedded and sectioned. Regardless of the embedding medium used, before embedding and sectioning it is critical to consider the orientation of the sectioning plane that will provide the structural information required for the analysis. Determinants of which sectioning plane to use include a) the orientation of gamete development (if any) in the gonad (i.e., rostral to caudal, radial vs. random). The alternatives planes to consider include transverse (whole-body section, to assess the type of reproductive tract) , frontal, or sagittal. For example the ovaries

of Japanese medaka are oriented with their long axis roughly parallel to the longitudinal axis of the fish. In each lobe of the ovary, oocyte development progresses from the outer margin to the center. Unbiased staging of this ovary-type requires slides prepared from either transversely- or frontally-, but not sagittally-sectioned ovaries (Fournie, 2004). The testis of the medaka, like the ovary, cannot be successfully staged with sagittal sections. However, section orientation is not critical for unbiased staging of fathead minnow or zebrafish testis. Considering these issues it seems desirable and perhaps preferable to prepare slides from sections oriented in some fashion along the frontal/transverse planes from whole embedded medaka and zebrafish. For fathead minnows good sections for histopathology are obtained by sectioning along the longitudinal axis. Sections more or less aligned with the frontal plane (longitudinal but either frontal or transverse for the fathead minnow) also provide a representative sample of a large proportion of the gonad in only a few step-sections. For these small fishes, three parallel step-sections (50 micrometers apart for testis and 100 micrometers apart for ovary) are sufficient to assess the gonads for endocrine-disruptive effects.

63. Typically for routine histopathology, sections are stained with hematoxylin and eosin (H&E). This stain is extremely versatile in that it allows the pathologist to visualize cellular and tissues details at nearly any resolution afforded by the light microscope. Also, through tinctorial variation, this stain provides universally understood information about the structure and function of cells, tissues, and even organelles. This stain is preferred for routine pathology analysis of gonads for the effects of endocrine disruptors.

4.1.3.2 Interpretation of Histopathological Results.

64. The effects of endocrine disruptive agents on the tissues and cells of fish can be observed in several organs including the liver, pituitary, kidneys, circulatory system, and the gonads (Wester et al. 2003, Palace et al. 2002). However, for the purposes of screening chemicals for disruption of reproductive function in fish, gonadal histopathology appears to be diagnostic. The histopathological effects of EDCs on the gonadal tissues of the three small fish species of interest to the OECD have been determined for chemicals that exhibit three different modes of action; those that bind to the estrogen receptor or the androgen receptor, and those that inhibit aromatase, an enzyme in the steroid hormone synthesis pathway. In all of these pathways, histopathology effects can be observed in the gonadal tissues of one or both sexes of exposed fish. This review will not catalog the histopathology observed in gonads. Rather, it will describe how major effects identified to date have been observed by pathologists.

65. In general, the histopathological effects of EDCs via the three pathways described above, can be organized into three major groups; those that affect the sexual differentiation, those that effect the progression of gametogenesis, and those that cause cellular and subsequent tissue damage in the gonad. Effects on gametogenesis cause abnormal distributions of one or more stages of oogenesis or spermatogenesis. The histopathologist identifies these effects by staging the gonads. Cellular and tissue pathology is diagnosed by a number of structural hallmarks observable through the microscope such as the presence of necrosis, apoptosis, abnormal interstitial spaces, cellular hyperplasia and hypertrophy.

66. One of the major difficulties in histopathological analysis is that most effects evaluation are subjective. Because of this, the diagnosis from different pathologists or from the same pathologist analyzing effects at different times can and often varies. Various attempts to eliminate this bias, and consequent variance, in diagnosing effects have been discussed by histopathologists and others for a number of years. Perhaps the most common suggestion from non-pathologists is to analyze the slides *blind* (i.e. without knowing the treatment the specimen is from). Instead, pathologists have devised methods for reading slides that allow the pathologist to learn pathologies novel to them and still read the slides without bias. The method, described in the OECD Phase 1-B Histopathology Guidance Document (OECD, 2004), is to read the slides in a particular order, starting with control specimens followed by high-dose specimens,

then, reading the intermediate doses next. After the slides have been read once, apparent treatment-related effects will be confirmed by a *blind* re-reading of the slides. Then, to reach consensus of treatment effects (at least before an adequate body of knowledge of ED effects is developed), the slides are peer reviewed informally by convening a pathology working group. This informal peer-review will very much aid the consensus building around slide interpretation during the validation execises. The pathology working group reviews study-slides as a body, discussing the possible diagnoses, until consensus is reached. The group decision is considered definitive. As diagnostic characters and terminology are developed among the pathologists, a body of information coalesces that can be organized into a document to provide histopathology guidance for analysis of other potential EDCs. The guidance provides a glossary of appropriate terms and descriptions of the hallmarks of typical ED-induced pathology. Images help identify details for the pathologist to look for in diagnosing a particular effect. Few guidance documents exist describing the effects of EDCs in fish gonads. However, the task of pathologists will become easier as more definitive diagnoses develop as more chemicals are studied.

67. Another method to minimize the inherent subjectivity of histopathology is to quantify the salient morphology of a tissue or cells that respond to EDCs. This has been discussed especially as a possible method to quantify the gonad stage relative to gametogenesis as described above. A number of techniques can be used to quantify histomorphology. Two common methods suggested employ either stereological point-counting or image analysis techniques. For several reasons, neither of these suggestions is appropriate yet for ED histopathology in fishes. The most important reasons for reaching this conclusion are that both methods are quite time consuming. And, it is not clear yet what histomorphological features, if quantified would be diagnostic. Until these issues are resolved, histopathology analysis of fish gonads for ED-effects will have to be qualitative. Perhaps in several years there will be enough development, both technological and biological, to allow a transition from qualitative to quantitative assessments of ED-induced pathology in fishes. The presence of a male or female gonad duct can also be used as an unequivocal endpoint.

4.1.4 Secondary Sex Characteristics

68. Secondary sex characteristics in fish are hormonally controlled, making them viable endpoints for the evaluation of endocrine disruption. All of the species considered have some secondary sex characteristics, such as females having distinct genital papilla. The male fathead minnow has distinct breeding tubercles on the snout and dorsally located fatpad. The male zebrafish and medaka have larger or longer anal fins than the females, and the male medaka dorsal fin has a cleft. The mature male fathead minnow and medaka have distinct coloration. However, all characteristics may not always be used for the detection of endocrine active substances because the sex characteristic considered may show limited responsiveness to a given substance or because of the difficulty to measure a specific response in the context of a screening assay.

69. It is important to identify how the observation and measurement of secondary sex characteristics will be used. General observation or qualitative results can be made for the three species under consideration. Qualitative results would be supporting evidence of endocrine disruption and would be useful in a screening-type test. Actual measurements or quantitative results could reduce the candidate species to the medaka and fathead minnow, as both sexes in these species have strong secondary sex characteristics (eg. number of nuptial tubercles in fathead minnow, number of papillary processes in medaka). Quantitative analysis can be performed on semi-empirical observations of secondary sex attributes in chemically exposed fish (Ankley et al, 2002, Ankley et al, 2003; Seki et al, 2004). An excellent example is provided by Papoulias et al. (2000), who used a readily available statistical software package (SPSS, Chicago IL) to perform stepwise discriminant analysis to assess the predictability of secondary sex characteristics in identifying genotypic sex in the medaka. As regards zebrafish, one study showed that females have a well developed uro-genital papillae, who appears to be responsive to estrogenic

stimulation. A scoring system was established (Brion et al, 2004) to measure the development of the uro-genital papillae in adult males, following estrogenic exposure; results were significant.

4.2 Measures of Reproductive Performance

4.2.1 Fecundity

70. Female fecundity is the more common endpoint measured as part of an assessment of reproductive performance. This endpoint is an important one because of its direct relevance to fish populations dynamic. Fecundity integrates many potential modes of action in the reproductive endocrine system and therefore can be a powerful apical indicator of adverse effects, even though it may not be diagnostic of any one particular mode of action. Because the general spawning strategy for fish is to produce many eggs with limited or no parental protection, a large number of eggs can be produced by a female in a relatively short time.

71. More variability is seen in spawning data compared with data endpoints, such as fertilization success, hatching success, length, weight, and survival. Because of the limitations of replication, other endpoints might routinely be more sensitive than fecundity when measured based on the number of eggs deposited over time. Because all three species of fish being considered are fractional spawners, the time to deposit eggs might be an interesting parameter: for example, the number of eggs-per-female-per-day over a 4-week period might be similar, but higher treatment levels might induce spawning over a shorter time.

4.2.2 Gamete Viability

72. In addition to the formation of adequate numbers of gametes for reproduction, it is also important that the gametes possess high viability for a successful fertilization and larval survivability. Screening assays that expose both genders simultaneously and then determine fertilization rate will effectively measure gamete viability. However, it is also helpful to measure gender-specific effects on gamete viability, as this may provide important clues to the mode of action. Identifying a gender-specific mode of action would be particularly valuable for interpreting the significance of laboratory results using controlled fertilization trials and extending this to natural reproductive conditions. In this regard, specific measures of the viability of oocytes for fertilization and the ability of spermatozoa to fertilize eggs would be useful additions to a reproductive screen protocol. These endpoint are however very specific and need more guidance in general before they can be included in screening guidelines. Egg viability can be affected in a number of ways, including reduced VTG incorporation into the oocyte causing smaller sized eggs or disturbances in the levels of sex and thyroid hormones (reviewed in Kime & Nash 1999). Sperm viability is frequently assessed by motility, and a strong correlation can exist between sperm motility and fertilization success (Lahnsteiner et al., 1998). Accurate measurement of sperm motility has become easier in recent years with the advent of computer-assisted motion analysis. However, the specialized equipment for this procedure is not routinely available in most laboratories.

73. Male-specific effects on gamete viability are not routinely assayed. This endpoint is probably best measured using controlled fertilization trials that mate exposed males with non- exposed females (for example see Shioda et al., 2000). This added cost and complexity to screening assays limits its applicability but should be considered when reduced gamete viability is suspected to be the primary endpoint altered by treatment.

4.2.3 Changes in Spawning Behavior

74. General observations on spawning can be made for all three species being considered. Quantitative observations on spawning behavior require considerable effort in making observations, whether those observations are made directly by individuals or through the use of video equipment.

Special considerations must be made to ensure that the observation process does not impact the true spawning behavior of the fish. The observation of two or three spawning groups per replicate requires significant effort. The collection of eggs occurs at the same time and is also a very labor-intensive time during the exposure. Therefore, labor constraints at this time of the study may limit the amount of quantitative data that can be collected.

75. Quantitative spawning behavior is not routinely monitored nor widely published. In one study, male medaka spawning behavior was significantly altered by exposure to octylphenol, a weak estrogen agonist (Gray et al., 1999). However, other endpoints were as sensitive or more sensitive than the male spawning behavior. A more thorough description of spawning behavior has been made in the goldfish (*Carassius auratus*). In this species, discrete behavioral variables have been described, such as close following (both short and long duration), pushing, and courting activities, among other behavioral endpoints (Bjerselius et al., 2001). Measurement of these endpoints was used to demonstrate a reduction in male spawning behavior after exposure to E2 (Bjerselius et al., 2001).

76. All three species under consideration exhibit spawning behavior that could potentially be measured in a quantitative manner as has been demonstrated for the goldfish. However, more research effort is needed in this area, as most of the reported information is either anecdotal or based on suspect EACs. Additional studies with potent estrogen agonists and androgenic chemicals would be helpful in providing some context for interpreting changes in spawning behavior.

4.3 Biochemical Measures

4.3.1 Estrogen Responsive Gene Products: Vitellogenin Induction

77. Vitellogenin is an egg-yolk precursor protein that is synthesized in the liver of fish prior to its transport to the ovaries and incorporation into developing oocytes. Synthesis of VTG is under estrogen control mediated by ERs in the liver. Both male and female fish can be induced to synthesize VTG after estrogen exposure or after exposure to estrogen-mimics. Vitellogenin can also be induced in both juvenile and adult individuals, and detection of VTG synthesis has become the most widely studied biomarker of exposure to EACs. Yet, the endpoint can be equally as valuable in females for anti-estrogens, both direct (i.e., estrogen receptor agonists; Panter et al. 2000a) and indirect (e.g., inhibitor of aromatase) as well as some types of androgens. Fadrozole, an aromatase inhibitor, has been demonstrated to reduce the presence of vitellogenin in females at 2 µg/l, in a 21-day exposure study (Ankley et al. 2002). In another study, Ankley demonstrated the significant reduction of vitellogenin production by females in a 21-day exposure study at 0.5 µg/l (Ankley et al. 2003). In many fish, induction of VTG has been shown to be extremely sensitive to estrogen exposure. For example, in sexually mature male rainbow trout, a 28-day exposure to 0.3 ng/L EE2 caused a statistically significant increase in plasma VTG levels compared with those of control trout, with a predicted no observable effects level (NOEL) of less than 0.1 ng/L (Sheahan et al., 1994). For the test species under consideration as a reproductive screen, VTG synthesis has also been demonstrated to be sensitive to estrogen exposures, although somewhat less sensitive than that observed in rainbow trout. The reduced sensitivity may be due to difficulties in obtaining sufficient quantities of purified VTG protein and species-specific antibodies for use in enzyme-linked immunosorbent assay (ELISA)- or radioimmunoassay (RIA)-based measurement techniques (Appendix A) (Parks et al., 1999; Van den Belt et al., 2001). However, most likely the reduced sensitivity in different test species compared to e.g. rainbow trout is species specific and not due to difficulties in obtaining sufficient quantities of purified vitellogenin (VTG) protein and species-specific antibodies. Several specific and sensitive ELISA´s have been developed (Brion et al., 2002; Fenske et al., 2001; Holbech et al., 2001), which shows that differences in species sensitivity still exist.

78. Although there are a variety of methods to detect VTG in plasma, the most widely applied methods are the immunoassays, ELISA and RIA. These methods exploit the highly specific interaction of antibodies and the antigen VTG to detect the protein in a variety of samples, including plasma, tissue samples, and culture medium. Radioimmunoassay and ELISA typically use homologous polyclonal anti-VTG bodies to quantify VTG, although antibody cross-reactivity allows for VTG detection among related species. The methods offer similar sensitivities in the samples analyzed; however, ELISA typically requires the dilution of samples to reduce interferences in the assay, effectively reducing the sensitivity compared to RIA. The small volumes of plasma available from individual fish of the test species can be a limiting sensitivity factor, requiring dilution of the sample to perform ELISA. The ELISAs are used more frequently to measure VTG because, unlike RIA, it does not require radioactive isotopes, uses stable reagents, and is relatively easy to set up and use. Furthermore, methods are available to measure VTG mRNA levels in the plasma, the liver and whole bodies of the small species in question, i.e., the zebrafish and the medaka: Islinger et al., 2002; Ilsinger et al., 2003.Although these immunologically based methods have been developed and validated for a wide range of teleosts, more modern direct methods, such as mass spectrometry, may be applied in the future. The specifics of ELISA and RIA are discussed in Annex A.

79. In addition to the yolk-forming VTG, several eggshell envelope proteins, collectively called zona radiata proteins, are also induced upon exposure to estrogen. Zona radiata proteins are synthesized in the liver and ovaries and incorporated into the developing oocytes in a manner analogous to VTG. Although induction of both zona radiata proteins and VTG is estrogen- dependent, stimulation of zona radiata synthesis appears to require lower levels of estrogen compared with VTG (Sohoni et al., 2001). Furthermore, evidence suggests that zona radiata proteins are preferentially induced by weak estrogenic compounds, such as bisphenol-A and *o,p'*-DDT (Arukwe et al., 2000). This latter finding might be significant for a screening program, as chemicals with a weak estrogenic mode of action can be difficult to identify if VTG is not significantly induced. Thus, zona radiata proteins might be a more sensitive endpoint in this respect.

80. A third estrogen responsive gene product is the ER itself. Estrogen receptors are autoregulated in rainbow trout (Riehl et al., 1999), meaning that E2 is involved in a positive feedback loop to upregulate its own receptor. Therefore, ERs are estrogen responsive genes with regulatory estrogen responsive elements. Numerous studies have demonstrated in rainbow trout that liver ER mRNA and protein is upregulated by E2 treatment (Arcand-Hoy & Benson 1998; Gray et al., 1999; Riehl et al., 1999), and after in vivo exposure to an endocrine disruptor, nonylphenol (Cheek et al., 2000). The transcription rate of ER mRNA (or the lack thereof) could be a useful and sensitive measure of estrogenic endocrine disruptor activity in other fish species too. A number of xenobiotics have been shown to induce comparable effects on the synthesis of ER and VTG mRNA when added to rainbow trout liver cells *in vitro* (Skinner et al., 1999; Winn et al., 2000; Islinger et al., 1999; Pawlowski et al., 2000). Although the use of VTG as a biomarker in fish dominates the literature, future research could warrant the substitution of ER or zona radiata protein induction for VTG as a useful endpoint in screening assays.

4.3.2 Tissue Steroid Concentrations

81. The measurement of plasma levels of E2, 11-KT, and T can be used as endpoints to assess sex-steroid status in male and female fish, provided there is some knowledge about normal population levels when the samples are taken. In the context of a laboratory toxicology experiment, a control group of sufficient size is an absolute requirement. Plasma levels of E2 have limited utility in males, because levels of this steroid are usually low or non-detectable. The measurement of plasma E2 is most useful in sexually maturing females because of the gradual rise in this hormone during the period of vitellogenesis. A good example is the well-documented case for the rainbow trout, an iteroparous, seasonally breeding fish with group-synchronous ovarian development in which maximum levels are attained well before spawning (Scott et al., 1980a; Vanbohemen & Lambert 1981). In many fish, E2 begins to decline by the time of final

maturation, probably as a consequence of the completion of yolk synthesis by the ovary, and E2 levels drop significantly after that. A distinct switch is seen in fish ovarian steroidogenic pathways from estrogens to progestins at this time (Nagahama 1999).

82. Similar to E2 in female fishes, 11-KT is the sex steroid characteristic of the sexually maturing male. Very low levels (e.g., pg/mL range) are sometimes reported in adult female fish (Jensen et al., 2001; Simpson & Wright 1977), although the physiological significance, if any, is not understood. In toxicology studies 11-KT is occasionally measured in the blood of male fish to assess androgen status, with the expectation being that reduced levels of this hormone are synonymous with reproductive dysfunction. This correlation has not been conclusively established, although the necessity of 11-KT for fish spermatogenesis has emerged (Miura et al., 1991; Schulz et al., 2001). Testosterone levels are normally much lower than 11-KT in males at time of spawning (Jensen et al., 2001). However, plasma levels of T can approach E2 levels in females of some fish, such as the fathead minnow (Jensen et al., 2001), and would also be a useful endpoint to measure in assessing the endocrine status of sexually mature fish.

83. An important concern with single measurements of plasma sex steroids in mature fish is the relevance of one measurement to reproductive function or dysfunction. It is now well documented that circulating levels of sex steroids can vary seasonally, and in the case of fathead minnows, on a daily basis during the spawning cycle (Jensen et al., 2001). This would be expected to increase variability in the measurement of these parameters from groups of asynchronous spawning fish. An additional confounding problem is high inter-laboratory variability in the measurement of plasma steroids (McMaster et al., 2001), making it difficult to determine subtle effects on steroid levels and, hence, reducing the sensitivity of the endpoint. Despite these limitations, some authors have concluded that single measures of sex-steroid levels are useful in predicting reproductive dysfunction. For example, in a review of the effects of pulp-mill effluents on reproduction in fish, (Munkittrick et al., 1998) concluded that reduced levels of circulating sex steroids did indicate adverse effects on reproduction. These authors also pointed out, however, that a specific mechanistic relationship (e.g., mode of action) between depressed steroid levels and reproductive dysfunction is unclear (Munkittrick et al., 1998). As an aid in determining mode of action, steroid ratios, specifically E2/11-KT or E2/T in females, may be more useful. This approach was used to analyze steroid measurements made in carp collected from various sites in the United States (Goodbred et al., 1997). The basis for this analysis is the hypothesis that sex steroid ratios, as opposed to their absolute values, are more important in determining sexual differentiation and, perhaps, sexual development (Jalabert et al., 2000). A limiting factor with this approach and with sex steroid measurements in general is the paucity of baseline data on these parameters in the species of interest for a reproductive screen.

5. RESPONSE TO ESTROGEN AGONISTS AND ANTAGONISTS

84. Estrogen is the principal female reproductive hormone; in female fish, it is synthesized primarily by the granulosa cells of the ovarian follicle surrounding the oocyte. The major function of estrogen is in sex differentiation, development of the reproductive organs, and inducing female sexual behavior. A disturbance in normal estrogen signaling in sexually mature fish can produce changes in gametogenesis, gonad maturation, and spawning success.

85. A comparatively large number of environmental pollutants have been hypothesized to cause toxicity via binding to the ER (estrogenic compounds) (Hoare & Usdin 2001) or to produce responses

similar to estrogen without binding to ERs (estrogen-like) (Gillesby & Zacharewski 1998). As a result, experimental studies of the responses of fish exposure to natural or synthetic estrogens have received a higher degree of scrutiny than have studies on other fish hormonal systems. In this respect, relatively few studies have examined the response to treatment with androgenic compounds and thyroid hormone mimics. Similarly, studies of antagonistic effects of xenobiotics on hormone pathways have only recently been studied in fish.

86. A large number of experimental studies on EACs have used salmonid fish species or other synchronous annual spawning fishes. With respect to fish species used in screening tests, the preferred test species in the majority of studies are the fathead minnow, the medaka, and the zebrafish. The fathead minnow is typically used in reproductive screening assays and so does the medaka to a certain extent. Most studies with zebrafish focused on early life-stage tests or on longer term tests (e.g. full life-cycle test). Studies using medaka refer equally to reproduction assays as well as early life-stage tests.

87. Because of the importance in establishing the pattern of endpoint responses specific to estrogen agonists, the ensuing discussion largely focuses on studies that exposed fish to either E2 or synthetic analogues that are established estrogen agonists (e.g., EE2). Description of experimental results using environmental contaminants that are considered weak estrogen agonists have been grouped under a separate section to distinguish these studies from those using E2 or steroid analogues. There have been several recent reviews on this subject (Kime 1995, 1998; Gillesby & Zacharewski 1998; Kleinow et al., 1999) and so the focus in this paper will be on studies using better characterized weak estrogen agonists that have been evaluated for effects on reproduction in the test species of interest.

88. A brief overview of the molecular biology of the ER has been placed in Section 5.2.1 to provide a context for descriptions of anti-estrogen effects in fish. The reader may find it helpful to refer to that section for the ensuing discussion on estrogen agonists.

5.1 Endpoint Sensitivity to 17β –Estradiol or Synthetic Estrogen Exposure

5.1.1 Growth, Morphological Alterations, Reproduction and Biochemical measures (VTG)

89. **Morphological Alterations** Exposure to estrogens has been reported to cause both subtle and profound changes in growth and morphology in fish. In salmonids, sublethal, short-term exposure to estrogenic substances in mature individuals can increase liver mass (due in part to stimulation of VTG synthesis) and could have either no effect on gonad weight or cause increased ovarian mass and decreased testis size (Jobling et al., 1996). In addition, low-level exposure to an estrogenic substance can cause VTG induction without overt changes in liver weight (Sheahan et al., 1994). Because of the differential growth response of tissues to estrogenic exposure, the overall change in body weight after exposure to an estrogenic substance can potentially be quite varied.

90. An additional confounding factor that is problematic with asynchronous fractional spawning fishes such as those under consideration in this review is the daily fluctuation in gonad weight during the spawning cycle. This fluctuation is especially pronounced in females, where the GSI can vary as much as 45% over a 2-day period, depending on the sampling day during the spawning interval (Jensen et al., 2001). In contrast, the daily fluctuation in the GSI in males is usually less variable, and may be as little as 1% during the spawning cycle (Jensen et al., 2001). This latter observation tends to make GSI measurements in males a more sensitive and commonly reported parameter in reproductive studies of estrogenic substances. Another important male attribute in this regard is that the microscopic morphology of the testis is much more consistent (as compared with ovarian morphology) during the spawning cycle and is less influenced by environmental changes (Koger et al., 1999).

91. **Reproduction**. The ultimate measure of reproductive performance is successful spawning, or more specifically, the successful fertilization of sufficient numbers of eggs such that an acceptable number of larvae are produced that become yearling fish and ultimately recruited into the population. With regard to development of a reproductive screen, the experimental evaluation of estrogenic or estrogen-like compounds has primarily focused on measuring fecundity, fertilization rate, spawning frequency (duration between spawns), and hatching success as measures of reproductive performance. Also, several studies of reproduction in appropriate test species used exposure protocols significantly different from that suitable for a screening assay. For this reason, the ensuing discussion is restricted to those studies that initiated exposures to adult individuals.

92. **Biochemical measures (VTG).** The measurement of VTG induction in all three species considered has been hindered until recently by a lack of antibodies developed against the specific type of VTG produced in these fish species, which could be used in ELISA or RIA procedures. However several specific (homologous) and sensitive ELISAs for VTG have been developed and validated during the last years (Fenske et al., 2001; Holbech et al., 2001; Brion et al., 2002) for zebrafish, (Parks et al, 1999; Mylchreest et al, 2003) for fathead minnow, (Yokota et al, 2001; Nishi et al, 2002; Tatarazako et al, 2004) for medaka. All these homologous ELISA are now available as commercial kits.

5.1.1.1 Reported results with weak estrogen agonists.

93. Perhaps the best characterized class of contaminants considered to be weakly estrogenic are the alkylphenol ethoxylates and their environmental degradation products, alkylphenols. The basis for the presumption of an estrogenic mode of action for these and other contaminants stems from both in-vitro and in-vivo studies measuring induction of VTG, typically in salmonid species (Jobling & Sumpter 1993; White et al., 1994; Lech et al., 1996). With respect to growth and morphological endpoints in proposed test species, the effects of these compounds can be ambiguous.

Table 5.1: Reported Results with Weak Estrogen Agonists

Chemical [conc. Range]	Species [Assay]	Days of exposure	Effect parameter	NOEC / LOEC	Reference
4-Nonylphenol [0.05 to 3.4 µg/L]	Fathead minnows (adults)	42	Increased Sertoli cell numbers and necrotic spermatozoa Secondary sex characteristics in males	NOEC: 0.4 µg/L LOEC: 1.6 µg/L NOEC: 3.4 µg/L	Miles-Richardson et al., 1999
4-Nonylphenol [0.62 to 58.6 µg/L]	Pair breeding fathead minnows [21-day reproductive assay]	21	Reduced fecundity to 60%, 45% and < 5% of control values respectively at 0.65, 8.1 and 58.6 µg/L. GSI males and appearance of nuptial tubercles in males GSI females Vtg induction Thickness of the dorsal fat pad Decrease in spawning frequency	 NOEC: 8.1 µg/L LOEC: 48.1µg/L NOEC: 0.62 µg/L LOEC: 8.1µg/L NOEC: 8.1 µg/L LOEC: 48.1µg/L NOEC: 0.62 µg/L LOEC: 8.1 µg/L NOEC: 0.62 µg/L LOEC: 8.1 µg/L	Harries et al., 2000
4-nonylphenol [0.05 to 3.4 µg/L]	Fathead minnows (adults)	42	Fecundity VTG	NOEC: 3.4 µg/L NOEC: 3.4 µg/L	Giesy 2000
Nonylethoxylate [0.15 to 5.5 µg/L]	Fathead minnows (adults)	42	Secondary sex characteristics in males	NOEC: 0.15 µg/L	Miles-Richardson et al., 1999
Nonylphenol ethoxylates [0.21 µg/L to 7.9 µg/L]	Fathead minnows (adults)	42	Survival and fecundity VTG	NOEC: 7.9 µg/L NOEC: 7.9 µg/L	Nichols et al., 2001

Chemical [conc. Range]	Species [Assay]	Days of exposure	Effect parameter	NOEC / LOEC	Reference
Nonylphenol [20 µg/L]	Medaka (adult)	4	VTG	LOEC: 20 µg/L	Foran et al., 2000
Nonylphenol [0.03 to 0.3 µmol/L]	Medaka (adult) exposure followed by spawning	14	Hatching rate	NOEC: 0.1 µmol/L LOEC: 0.3 µmol/L	Shioda & Wakabayashi 2000
4-nonylphenol [10 to 100 µg/L]	Medaka larvae (hatch to 3 month ph) [FPLCT]	3 month	Observation of testis ova Sex ratio	NOEC: 10 µg/L LOEC: 50 µg/L (50%) NOEC: 50 µg/L LOEC: 100 µg/L	Gray & Metcalfe 1997
Nonylphenol [0.5 to 1.9 µg/L]	Medaka larvae (hatch to 30 dph)	30 days followed a period of 28 days in clean water	Sex ratio Reproductive capability	NOEC: 1.9 µg/L NOEC: 1.9 µg/L	Nimrod & Benson 1998
4-nonylphenol [20 to 50 µg/L	Male medaka	7	Vtg induction	NOEC: ≤ 20 µg/L LOEC: 20 µg/L	Islinger et al., 2002
Mixtures of nonylphenol mono- and di-ethoxylates [25 to 100 µg/L]	Medaka juvenile life stages (egg to 100 dph) [FPLCT]	100	Decreased growth Sex ratio	NOEC: 50 µg/L LOEC: 100 µg/L NOEC 100 µg/L	Metcalfe et al. 2001

Chemical [conc. Range]	Species [Assay]	Days of exposure	Effect parameter	NOEC / LOEC	Reference
Methoxychlor [0.55 µg/L and 3.56 µg/L]	Fathead minnows [21-day reproductive assay]	21	GSI males and females Increase in the numbers of atretic follicles in the ovaries Decreased fecundity rate VTG induction (males) VTG (females)	NOEC: 3.56 µg/L NOEC: 0.55 µg/L LOEC: 3.56 µg/L NOEC: 0.55 µg/L LOEC: 3.56 µg/L NOEC: 0.55 µg/L LOEC: 3.56 µg/L NOEC: 3.56 µg/L	Ankley et al., 2001
Methoxychlor [0.2 to 2.3 µg/L]	Medaka larvae (hatch to 30 dph)	30 days followed a period of 28 days in clean water	Sex ratio Reproductive capability	NOEC: 2.3 µg/L NOEC: 2.3 µg/L	Nimrod & Benson 1998
Butyl benzyl phthalate [69 to 82 µg/L]	Pair breeding fathead minnows [21-day reproductive assay]	21	No effects	NOEC: 82 µg/L	Harries et al., 2000
Bisphenol A [10 to 200 µg/L]	Medaka juvenile life stages (egg to 100 dph) [FPLCT]	100	Sex ratio Observation of testis ova (2 males) Morphological changes in male testis Growth	NOEC: 200 µg/L ≥ 10 µg/L NOEC: 10 µg/L LOEC: 50 µg/L NOEC: 200 µg/L	Metcalfe et al. 2001

Chemical [conc. Range]	Species [Assay]	Days of exposure	Effect parameter	NOEC / LOEC	Reference
Bisphenol A [0.3 to 10 µmol/L]	Medaka (adult) exposure followed by spawning	14	Hatching rate	NOEC: 3 µmol/L LOEC: 10 µmol/L	Shioda & Wakabayashi 2000
Bisphenol A [100 to 600 µg/L]	Zebrafish [FPLCT]	40	VTG Sex ratio	NOEC: 600 µg/l 85% females and 10% males at 600 µg/L	Holbech et al., 2004 (unpublished results)
Di-2-ethylhexylphthlate [500 to 5,000 µg/L]	Medaka juvenile life stages (egg to 100 dph) [FPLCT]	100	Growth Sex ratio	NOEC: 5,000 µg/L NOEC 5,000 µg/L	Metcalfe et al. 2001
DEHP [0.1 to 1 µmol/L]	Medaka (adult) exposure followed by spawning	14	Hatching rate	NOEC: 1 µmol/L	Shioda & Wakabayashi 2000
4-tert-octylphenol [20 and 230 µg/L]	Male medaka	21 followed by mating	Significant correlation between VTG in serum (males) and percent fertilized eggs Number of eggs	NOEC: < 20 µg/L LOEC: 20 µg/L	Gronen et al., 1999
Octylphenol [12.5 to 100 µg/L]	Zebrafish Pair breeding	21	Spawning and fertilization success OSI VTG	NOEC: 100 µg/L NOEC: 12.5 µg/L LOEC: 25 µg/L NOEC: 100 µg/L	Van den Belt et al., 2001
Octylphenol [1.2 to 97.5 µg/L]	Zebrafish [FPLCT]	40	VTG Sex ratio	NOEC: 97.2 µg/l 100% females at 97.2 µg/L	Holbech et al., 2004 (unpublished results)

5.1.1.2 Reported results with Estradiol and Synthetic Estrogen Agonists.

94. Several studies have been conducted on the effects of short-term exposure to estrogenic chemicals on the growth, reproduction and induction of vitellogenin of fathead minnows, medaka and zebrafish.

Table 5. 2: Reported Results with Estradiol and Synthetic Estrogen Agonists

Chemical [conc. Range]	Species [Assay]	Days of exposure	Effect parameter	NOEC / LOEC	Reference
Estriol [0.01 to 10 µg/L]	Medaka juvenile life stages (egg to 100 dph) [FPLCT]	100	Sex ratio Observation of testis ova (35 males) Growth	NOEC: 10 µg/L 10 µg/L NOEC: 10 µg/L	Metcalfe et al. 2001
Estriol [0.09 to 22 µ/L]	Zebrafish [FPLCT]	40	VTG Sex ratio	NOEC: 0.3 µg/l LOEC: 0.6 µg/l NOEC: 6.7 µg/l LOEC 22 µg/l	Holbech et al., 2004
Estrone [9.9 to 993 ng/L]	Fathead minnow (adult male)	21	VTG GSI (testis)	NOEC: 9.9 ng/L LOEC: 31.8 ng/L NOEC: 99.3 ng/L LOEC: 317.7 ng/L	Panter et al., 1998
Estrone [0.01 to 10 µg/L]	Medaka juvenile life stages (egg to 100 dph) [FPLCT]	100	Sex ratio Observation of testis ova (2 males) Growth	NOEC: 0.1 µg/L LOEC: 1 µg/L ≥ 0.01 µg/L NOEC: 0.1 µg/L LOEC: 1 µg/L	Metcalfe et al. 2001
Estrone [14 to 98 ng/L]	Zebrafish [FPLCT]	40	VTG Sex ratio	NOEC: 97 ng/l NOEC: 36 ng/l LOEC: 49 ng/l	Holbech et al., 2004 (in prep.)
E2 [25 ng/L to 100 ng/L]	Fathead minnows (24h post fertilisation to 30 dph)	30	VTG	NOEC: 25 ng/L LOEC: 50 ng/L	Tyler et al. 1999

Chemical [conc. Range]	Species [Assay]	Days of exposure	Effect parameter	NOEC / LOEC	Reference
E2 [31 to 1470 ng/L]	Fathead minnow (par breeding adults)	19	Inhibition of egg production VTG induction males	EC50: 120 ng/l EC50: 251 ng/l	Kramer et al., 1998
E2 [0.0625 to 1000 nM]	Fathead minnow (par breeding adults)	19	Sec. sex characteristics Histological alterations in male gonads	NOEC: 1 nM LOEC: 2 nM NOEC: 0.25 nM LOEC: 0.5 nM	Miles-Richardson et al., 1999b
E2 [10 to 1000 ng/L]	Fathead minnow (adult male)	21	VTG GSI (testis)	NOEC: 100 ng/L LOEC: 320 ng/L NOEC: 100 ng/L LOEC: 320 ng/L	Panter et al., 1998
E2 [30 to 120 ng/L]	Fathead minnow (adult male)	21	VTG GSI (testis)	NOEC: ≤ 30 ng/L LOEC: 30 ng/L NOEC: 60 ng/L LOEC: 120 ng/L	Panter et al., 2000
E2 [10^{-10} to 10^{-8} M]	Fathead minnow (adult male)	21	VTG	NOEC: ≤ 10^{-10} M LOEC: 10^{-10} M	Parks et al.1999
E2 [1 to 100 nmol/L]	Medaka (adult) exposure followed by spawning	14	Number of eggs Hatching rate	NOEC: 1 nmol/L LOEC: 3 nmol/L NOEC: 1 nmol/L LOEC: 3 nmol/L	Shioda & Wakabayashi 2000
E2 [20 µg/L]	Medaka (adult)	4	VTG	LOEC: 20 µg/L	Foran et al., 2000
E2 [10 to 100,000 ng/L]	Male medaka	21	VTG	EC50: 200 ng/L	Thompson et al., 2000
E2 [0.1 to 100 µg/L]	Medaka (embryo to sexual maturity)	230	Sex ratio	NOEC: 0.1 µg/L LOEC: 1 µg/L	Tabata et al., 2001

Chemical [conc. Range]	Species [Assay]	Days of exposure	Effect parameter	NOEC / LOEC	Reference
E2 [0.001 to 1 µg/L]	Medaka juvenile life stages (egg to 100 dph) [FPLCT]	100	Sex ratio Observation of testis ova (3 males) Growth	NOEC: 0.01 µg/L LOEC: 0.1 µg/L ≥ 0.01 µg/L NOEC: 1 µg/L	Metcalfe et al. 2001
E2 [0.01 to 1.66 µg/L]	Medaka larvae (hatch to 30 dph)	30 days + 28 days unexposed	Sex ratio Reproductive capability	NOEC: 1.66 µg/L NOEC: 0.12 µg/L LOEC: 1.66 µg/L	Nimrod & Benson 1998
E2 [0.2 to 6.9 ng/L]	Zebrafish Adult male	10	VTG	NOEC: 0.8 ng/l LOEC: 1.1 ng/l	Duis et al., 2003
E2 [12.9 to 616 ng/L]	Zebrafish Adult male	8	VTG	NOEC: 12.9 ng/l LOEC: 21.0 ng/l EC50: 41.2 ng/l	Rose et al. 2002
E2 [5 to 100 ng/l]	Zebrafish Embryo-larvae Zebrafish Juvenile Zebrafish Adult	21 21 21	VTG, Sex ratio (histology) VTG, Sex ratio (histology) VTG, 2y sex characteristic Histology	LOEC: 100 ng/l LOEC : 100 ng/l LOEC : 100 ng/l LOEC : 100 ng/l LOEC : 25 ng/l LOEC : 25 ng/l LOEC : 25 ng/l NOEC : 100 ng/l	Brion et al, 2004
E2 [4 to 250 ng/L]	Zebrafish [FPLCT]	40	VTG Sex ratio	NOEC: 24 ng/l LOEC: 54 ng/l NOEC: 24 ng/l LOEC: 54 ng/l	Holbech et al., 2004 (in prep.)
EE2 [5 to 50 ng/L]	Zebrafish Pair breeding	21	Spawning and fertilization success OSI VTG (females)	NOEC: 5 ng/L LOEC: 10 ng/L NOEC: 5 ng/L LOEC: 10 ng/L NOEC: 5 ng/L LOEC: 10 ng/L	Van den Belt et al., 2001

Chemical [conc. Range]	Species [Assay]	Days of exposure	Effect parameter	NOEC / LOEC	Reference
EE2 [5 ng/L]	Zebrafish Adults	14	Survival of eggs and larvae VTG	LOEC: 5 ng/L LOEC: 5 ng/L	Kime & Nash 1999
EE2 [0.06 to 23 ng/L]	Zebrafish [FPLCT]	40	Sex ratio VTG	NOEC: < 0.6 ng/l LOEC: 0.6 ng/l NOEC: 0.6 ng/l LOEC: 1.5 ng/l	Örn et al 2003
EE2 [0.72 to 90.1 ng/L]	Zebrafish Adult male	8	VTG	NOEC: 2.2 ng/l LOEC: 3.6 ng/l EC50: 2.51 ng/l	Rose et al. 2002
EE2 [2.9 to 22.1 ng/L]	Fathead minnow (juvenile)	21	Body weight VTG	NOEC: 22.1 ng/L NOEC: 2.9 ng/L LOEC: 4 ng/L	Panter et al., 2000a
EE2 [50 ng/L]	Fathead minnow (males)	35	VTG	LOEC: 50 ng/L	Schmid et al., 2002
EE2 [1 to 100 ng/L]	Medaka (Newly hatched larvae to sexual maturity) [FPLCT]	60 followed by 6-weeks recovery	Sex ratio Egg production and GSI	NOEC: 10 ng/L LOEC: 100 ng/L NOEC: 1 ng/L LOEC: 10 ng/L	Scholz and Gutzeit 2000
EE2 [0.0001 to 1 µg/L]	Medaka juvenile life stages (egg to 100 dph) [FPLCT]	100	Sex ratio Observation of testis ova (1 male) Growth	NOEC: 0.01 µg/L LOEC: 0.1 µg/L ≥ 0.0001 µg/L NOEC: 0.001 µg/L LOEC: 0.01 µg/L	Metcalfe et al. 2001
Phytoestrogen, genistein [280 to 920 µg/L]	Fathead minnow (juvenile)	21	Body weight	NOEC: ≤ 280 µg/L LOEC: 280 µg/L	Panter et al., 2000a

5.1.2 Biochemical Measures: Tissue Steroid Levels

95. In addition to measuring VTG levels following chemical exposures, it may also be helpful to measure plasma concentrations of endogenous E2 and the androgens, 11-KT and T. In contrast to

salmonids, for which an extensive database is available on seasonal cycles in circulating plasma concentrations of reproductive hormones, relatively little information is available for the proposed test species. Furthermore, the effect of chemical exposure on circulating hormone levels in these species is largely untested, and the ensuing discussion is largely limited to a review of reported values in control fish.

96. The most thorough study available for review in a relevant species measured reproductive hormone levels during a 4-day spawning cycle in fathead minnows. In this species, plasma E2 concentrations in actively spawning females reached a maximum value of 10 ng/mL one day after spawning and then steadily declined until time of next spawning, at which point the concentration was approximately 4 ng/mL (Jensen et al., 2001). In contrast, 11-KT concentrations were undetectable, whereas T levels were reported to be on average 3.1 ng/mL and remained constant during the spawning cycle (Jensen et al., 2001). In male fathead minnows, little fluctuation in plasma concentrations of 11-KT or T was observed during the spawning cycle, with mean values reported to be 33.1 and 9.1 ng/mL respectively (Jensen et al., 2001). Interestingly, detectable concentrations of E2 were present in plasma from male fathead minnows, with a mean value of 0.4 ng/mL (Jensen et al., 2001). The results of the aforementioned study are in general agreement with two previous studies of E2 and T concentrations in fathead minnows.

97. In a prior study, the plasma concentrations of E2 and T were measured in mature fathead minnows that were caged in either a reference site or in wastewater treatment effluents. Estradiol concentrations in both male and female minnows from the reference site were considered similar and varied between 1.4 ng/mL and 11.1 ng/mL (Nichols et al., 1999). Similarly, T concentrations were similar between sexes, varying only minimally between 6 ng/mL and 7 ng/mL (Nichols et al., 1999). In minnows that were exposed to various wastewater treatment effluents, a general decrease in both E2 and T was observed in both male and female individuals (Nichols et al., 1999).

98. Giesy et al. (2000) reported that concentrations of circulating E2 in male and female flathead minnows ranged between 1 ng/mL to 5 ng/mL. Concentrations of circulating E2 were elevated to a similar degree in male and female fish exposed for 21 days to 4-nonylphenol at nominal exposure concentrations between 50 ng/L and 3400 ng/L.

99. A more recent study evaluating exposure to methoxychlor reported that 21-day exposures to measured concentrations of 3.56 µg/L significantly decreased E2 plasma levels in females while causing a significant decrease in T and 11-KT in male individuals (Ankley et al., 2001). With regard to medaka and zebrafish, the limited data make it difficult to make comparisons with fathead minnows, other than evidence for cyclic levels of E2 in females. For example, in the medaka, E2 plasma concentrations appear to oscillate during the spawning cycle, with peak concentrations occurring approximately 8 hours before spawning (Soyano et al., 1993). No published data on E2, T, or 11-KT levels in zebrafish could be found.

5.2 Estrogen Antagonists

100. The biological effects of anti-estrogens in fish reproduction have only recently been studied, and relatively few studies of anti-estrogens are reported. Consequently, the following discussion has been broadened to include additional fish species beyond those under consideration as a reproductive screen. For purposes of this review, anti-estrogens have been separated into two groups based on their mechanism of action. Pollutants that interfere with estrogen signaling through competitive inhibition of estrogen binding to the ER are considered direct-acting anti-estrogens. Contaminants that alter the normal turnover of estrogen in the fish (e.g., alter synthesis or elimination rates of estrogen) are considered indirect acting anti- estrogens. This latter category includes aromatase inhibitors.

5.2.1 Direct-Acting Anti-Estrogens

101. To provide a background for descriptions of anti-estrogen effects in fish, it is helpful to briefly review the molecular biology of estrogen signaling. In mammalian systems the predominant effects of estrogens are regulated by intracellular Ers. There are two distinct ER subtypes, ER-α and ER-β (Nilsson et al., 2001). Estrogen receptors are transcription factors that, once bound to E2 in the cell nucleus, form dimer complexes that interact with specific DNA regions called estrogen response elements. The binding to estrogen response elements appears to be facilitated by several additional proteins, and eventually leads to the transcription of specific genes (Tsai & Omalley 1994; reviewed in MacGregor & Jordan 1998). This is the classical ligand-dependent mechanism of action, but two other genomic pathways are known, ligand-independent and DNA-binding dependent, as well as a non-genomic pathway utilizing cell- surface signaling (Hall et al., 2001). Little information is available on these latter three pathways in fish and therefore, for the purposes of this review, estrogen signaling will be confined to the ligand-dependent mechanism of action. However, it should be noted that endocrine disruptors may antagonize ERs in fish by different mechanisms.

102. In mammals, the two ER subtypes have been demonstrated to possess different affinities for several agonists and antagonists (Sun et al., 1999). Thus, some chemicals are antagonistic only towards a specific ER subtype. For example, tetrahydrochrysene is a selective inhibitor of ER-β with little activity towards ER-α (Sun et al., 1999). In fish, similar to mammals, there are also two ER subtypes (Thornton 2001), but due to duplication events within the genomes of fishes (Robinson-Rechavi et al., 2001), more than one form of the ER-β subtype (i.e., ER-β1, ER-β2) has been reported in some fish species (Hawkins et al., 2000; Ma et al., 2000). At this time, the biological function(s) of different ER subtypes or forms is not known for any fish species. Ligand-binding differences between ER subtypes have been less characterized in fish (Thomas & Smith 1993) compared with mammals, although the existence of distinct ER subtypes in fish suggests the potential for ER-specific antagonists to elicit a tissue- or cell- specific response. The amino acid sequence differences in ER subtypes between mammals and fish creates the possibility of important differences in biological activity and/or sensitivities. However, the few studies available for review have tended to use ER antagonists with broad specificity towards all ERs. Vitellogenin is perhaps the best studied gene product of ER activation, although numerous other gene products, such as the zona radiata proteins or ER itself, are also regulated by the ER in fish (Arukwe & Goksoyr 1998; Skinner et al., 1999). Therefore, one expected outcome of anti-estrogen exposure in fish is the diminished synthesis of ER- responsive gene products such as VTG, either expressed at basal levels or in response to a challenge dose of E2. Other potential effects of anti-estrogens might be delayed or reduced oogenesis that could also be manifested as lowered fecundity.

103. Based on studies using mammalian animal models, direct anti-estrogens can be classified into two broad categories: 1) analogs of tamoxifen or its metabolites (Type I), which are actually weak ER agonists at low concentrations and antagonists at high exposure concentrations, and 2) pure anti-estrogens (Type II), which have no agonist-like properties. Both types of anti- estrogens are competitive inhibitors of E2 binding to the ER, but differ in their effectiveness at blocking ER mediated transcription. Tamoxifen analogs form a receptor complex that is only partially active in initiating gene activation and may cause a single round of gene transcription, although high concentrations are completely inhibitory (MacGregor & Jordan 1998). Pure anti- estrogens bind to the ER immediately after synthesis in the cytoplasm prior to nuclear localization (Dauvois et al., 1992). The inactivated receptor complex appears to be rapidly degraded, preventing estrogen-mediated gene transcription from occurring (Dauvois et al., 1992).

104. The few experimental studies of anti-estrogens in fish appear to have tested, with one exception, Type I anti-estrogens. An interesting study by Kawahara and Yamashita (2000) used the shift in phenotype from male to female after E2 exposure to assess the in-vivo activity of tamoxifen in the medaka. In this study, juvenile medaka were exposed either to E2 alone or co- exposed with tamoxifen and then

raised to sexual maturity. The results indicated that co- exposure with tamoxifen blocked the shift in phenotype from male to female caused by E2 exposure (Kawahara & Yamashita 2000).

105. Further evidence that classic anti-estrogens are active in fish comes from a study by Panter et al. (2000a), in which juvenile fathead minnows were exposed to the Type II anti- estrogen, ZM189,154, for 21 days at measured concentrations of 7.9, 33.7, and 95.3 µg/L. A significant decrease in VTG formation was observed at all exposure levels after 14 days of exposure without causing significant changes in body weight or total length of the fish (Panter et al., 2000a). This finding is consistent with observations made using female carp primary hepatocytes treated with tamoxifen (Smeets et al., 1999). When hepatocytes were incubated with 1 µM tamoxifen, a decreased synthesis of VTG was observed (Smeets et al., 1999).

106. In addition to the three cited studies using established Type I anti-estrogens, additional studies with fish models suggest that certain polycyclic aromatic hydrocarbons (PAHs) may also be anti-estrogenic in a manner similar to Type I anti-estrogens. A series of experiments using cultured rainbow trout hepatocytes treated with either α-naphthoflavone or β-naphthoflavone suggest this compound can interfere with ER activation by E2 (Navas & Segner 2000). In this study, hepatocytes were co-incubated with 1 µM E2 and either 3.1 µM or 6.25 µM µ- orβ- naphthoflavone and the VTG response was measured. When the hepatocytes were co-incubated with either naphthoflavone, a decrease in VTG synthesis was observed (Navas & Segner 2000). Similar findings have been reported by in-vivo studies that administered E2 and various doses of β-naphthoflavone. When rainbow trout were administered 0.5 mg/kg E2 and 25 mg/kg or 50 mg/kg β-naphthoflavone, a decrease in VTG synthesis was observed (Anderson et al., 1996a). Conversely, when the β-naphthoflavone dose was reduced to 12.5 mg/kg, a seemingly paradoxical stimulation in VTG synthesis was observed (Anderson et al., 1996a). This mixed stimulatory and inhibitory effect on VTG synthesis is consistent with a Type I anti-estrogenic mode of action.

107. Other xenobiotics, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Smeets et al., 1999) and 2,3,4,7,8-pentachloro-dibenzofuran (Anderson et al., 1996a), have also been shown to inhibit *in-vitro* VTG synthesis. Although a Type I anti-estrogen mechanism is indicated with naphthoflavones and related compounds, an alternative mechanism has been proposed involving the Ah receptor and inhibitory or rather, disruptive interactions between the activated ER complex and recognition of estrogen-response elements (reviewed in Nicolas 1999). Although this latter mode of action is an intriguing hypothesis, further evaluation is required before a Type I action can be completely ruled out.

108. Regardless of the specific mechanism of action of direct anti-estrogens, a common endpoint measured in most studies is the effect on VTG synthesis. Only limited information is available in the literature on the specific effects of anti-estrogens on reproduction in fish. Thus, it is difficult to state what effect, if any, anti-estrogens may have on reproductive success. Based on the results indicating partial suppression of VTG synthesis, some effect on oogenesis would be expected but this remains to be demonstrated. The recent study of fadrozole in fathead minnows suggest that low reproduction can occur (Ankley et al., 2002).

109. An additional, notable gap is the lack of studies using pure anti-estrogens, which would be helpful in clarifying the specific pattern of effects that may occur through competitive inhibition of E2 binding to the ER. Other types of anti-estrogens produce mixed agonist and antagonist responses. Some, like the naphthoflavones, produce a variety of effects mediated through the Ah receptor, including cyp 1A1, 1A2 induction, that further complicate interpretation of anti-estrogen specific effects (see discussion in Gillesby & Zacharewski 1998). Experiments using a pure anti-estrogen would provide a better indication of the effects on reproduction caused by inhibition of E2 binding to the ER. A particularly useful fish model would be the development of a transgenic fish lacking an ER. This approach has been used to successfully develop ER- null mice (Couse & Korach 1999), which has helped elucidate potential target tissue and cell-specific effects of anti-estrogens in rodents (MacGregor & Jordan 1998).

5.2.2 Indirect-Acting Anti-Estrogens

110. For purposes of this review, an indirect-acting anti-estrogen is considered to be a xenobiotic that significantly lowers the unbound or free E2 concentration in blood plasma. Because E2 freely diffuses into cells prior to binding with the ER (Rao 1981), intracellular levels of E2 will depend on the concentration of circulating E2 that is not bound to steroid-binding proteins in plasma. Based on this definition, there are at least three separate mechanisms by which indirect anti-estrogens could reduce intracellular E2 levels: 1) decreasing the rate of synthesis of E2; 2) increasing the binding of E2 to sex hormone binding proteins (thereby reducing the free fraction of E2); and 3) increasing the rate of elimination of E2 through increases in oxidative (Phase I) or conjugative (Phase II) metabolism. Of these three possible modes of action, decreased synthesis of E2 through aromatase inhibition appears to be the most biologically significant, and this review is focused on this class of anti-estrogens.

111. In fish, aromatase activity is due to at least two separate enzymes encoded by multiple CYP19 genes (Tchoudakova & Callard 1998) and is the critical enzyme responsible for the final, irreversible step in estrogen synthesis from androgen precursors (Simpson et al., 1994). The existence of multiple P450-aromatase isoenzymes suggests tissue-specific, differential expression, which could explain the exceptionally high activity found in the teleost brain (Tchoudakova & Callard1998). In any case, inhibition of this enzyme has been demonstrated to adversely affect sexual differentiation and reproduction.

112. The potential for aromatase inhibitors to alter sex differentiation was demonstrated in an early study by Piferrer et al. (1994), in which a group of genetically female, juvenile Chinook salmon were treated with fadrozole, a well-characterized aromatase inhibitor used in the treatment of breast cancer. When fish were raised to sexual maturity, all fadrozole-treated individuals had developed as fertile males (Piferrer et al., 1994). Similar results have been reported in other species as well, such as the Japanese flounder, in which fadrozole treatment to larvae resulted in the development of predominantly male populations (Kitano et al., 2000).

113. Although these studies demonstrate the pronounced effects of aromatase inhibitors on sexual differentiation, extremely high doses are typically employed and, in the case of fadrozole, dose rates of up to 500 mg/kg have been used to achieve sex reversal (Kwon et al., 2000). Application of these high doses may cause a sustained loss in aromatase activity through decreased expression of the CYP19 gene (Kitano et al., 2000).

114. At lower treatment rates, fadrozole and other aromatase inhibitors can transiently lower circulating levels of E2. For example, pre-spawning Coho salmon administered fadrozole by intraperitoneal injection at doses down to 0.1 mg/kg had significantly lower E2 plasma levels three to six hours after dosing (Afonso et al., 1999b). Additional studies in Coho salmon demonstrated that fadrozole treatment inhibited oocyte development and overall ovarian growth (Afonso et al., 1999a). Interestingly, other established aromatase inhibitors, letrozole and clotrimazole, have been shown to be sensitive inhibitors of aromatase activity in vitro using trout ovarian microsomes, but could not lower circulating E2 levels or suppress VTG synthesis in juvenile rainbow trout after dietary administration for two weeks at a dose rate of 1 mg/kg (Shilling et al., 1999).

115. With respect to fish species under consideration, studies that examined aromatase inhibition are presented in Table 5.3.

Table 5.3: Indirect Acting Anti-Estrogens (Aromatase Inhibitors)

Chemical [conc. Range]	Species [Assay]	Days of exposure	Effect parameter	NOEC / LOEC	Reference
Fadrozole [1.4 to 57 µg/l]	Fathead minnow [21-day reproductive assay]	21	Decreased fecundity VTG reduction in females E2 decrease, T-KT increase Gonad histology	NOEC: < 1.4 µg/l LOEC: 1.4 µg/l	Ankley et al.,2002
Fadrozole [37.7 to 114.6 µg/l]	Fathead minnow Juvenile	21	VTG reduction	NOEC: 37.7 µg/l LOEC: 87.4 µg/l	Zerulla et al. 2002
Fadrozole [25 to 100 µg/l]	Fathead minnow Juvenile		VTG	NOEC: 100 µg/l	Panter et al. 2000a
Fadrozole [10 to 100 µg/L]	Zebrafish [FPLCT]	40	Sex ratio (decreased number of females) VTG reduction	NOEC: < 10 µg/l LOEC: 10 µg/l NOEC: 100 µg/l	Andersen, 2004. (unpublished results)

116. Aromatase inhibition can redirect sexual differentiation in juvenile fish if high dosages are employed. At lower treatment rates, aromatase inhibition has been demonstrated to lower E2 levels and gametogenesis in females. One study using adult fathead minnows (Ankley et al, 2002) demonstrated a concentration-dependant reduction of fecundity, a decrease of plasma estradiol and of vitellogenin levels in females, observed after 21 days of exposure. Histological observations of ovaries showed a decreased in mature oocytes and an increase in preovulatory atretic follicles. Exposure of males induced significant increase of testosterone and keto-testosterone in plasma; assessment of testes revealed an accumulation of sperm. A recent study of perch collected from a Swedish lake receiving drainage from a nearby landfill reported that many fish had a lowered ovarian somatic index and reduced levels of circulating E2 that were associated with decreased aromatase activity (Noaksson et al., 2001). This pattern of effects is consistent with those observed with aromatase inhibitors and suggests that inhibition of E2 synthesis could be an environmentally relevant mode of action for EACs.

5.3 Gender Differences

117. There does not appear to be significant gender differences in overall growth (body weight, total length) among the test species in response to estrogenic or anti-estrogenic chemicals. However, the complexity of E2's role in sexual differentiation, vitellogenesis, and overall gametogenesis makes it clear that male and female individuals can have differential sensitivities to several of the more specific endpoints measured during performance of a screening assay. Male individuals appear to be more sensitive to estrogenic compounds based on gonad morphology and assessment of testis-ova formation. The GSI is likely to be more sensitive to estrogen exposure in males, as the relative testis mass is a less variable parameter in asynchronous spawners, making this parameter more sensitive as a result of improved statistical power. Estrogen-induced changes in secondary sex characteristics are also a more sensitive parameter in male fish (particularly fathead minnows). An additional endpoint in which male fish appear

to be more sensitive is the induction of VTG. Because of the naturally lower background levels of VTG in males, even slight (<10-fold) increases in plasma concentrations can be statistically significant. Similarly, VTG synthesis after an E2 challenge has been shown to be sensitive to anti-estrogen exposure in males. In regards to reproduction, most endpoints either directly assess female effects (fecundity) or combined gender effects (fertilization success). This makes it difficult to assess specific gender sensitivities. A few studies have assessed male- specific fertility endpoints, which appear to be very sensitive to estrogenic exposure (Schultz et al., 2002). However, more research is needed to determine whether male fertility is more sensitive to estrogenic exposures. A specific endpoint for which females appear to be more sensitive is changes in circulating steroid levels, which tend to be more dynamic during the spawning cycle. In addition, morphological and reproductive responses to aromatase inhibitors and, to a lesser extent, direct acting anti-estrogens appear to be more sensitive endpoints in female fish.

5.4 Strengths and Weaknesses of Test Species

118. A weakness common to all species is the small size at maturity, which limits the quantity of blood plasma available for simultaneous analysis of VTG and sex steroids in individual animals. However, part of the efforts to develop a standard screening assay valid for all three species, is the description of standard operating procedures (SOP) for the efficient sampling of tissue (blood in zebrafish and fathead minnow and liver in medaka) for VTG measurement. Such SOP will be necessary for the validation of the assay to ensure minimal inter-laboratory variations.

119. Despite this limitation, several endpoints appear to be equally sensitive in all test species. Similar sensitivities were observed for growth and gonad morphology and VTG induction. All three species appear capable of VTG induction in response to E2 or EE2 exposures as low as 1.5 ng/L. In addition, all three species appear capable of induced spawning in a short- term assay, and effects on fecundity have been documented after exposures to E2 or EE2.

6. RESPONSE TO ANDROGEN AGONISTS AND ANTAGONISTS

120. In contrast to the predominant role E2 plays in the development of feminine characteristics, several androgenic steroids appear to be involved with male differentiation and reproduction. In male teleost fishes, 11-oxygenated androgens, especially 11-KT, appear to be the most important endogenous androgenic steroids, although T and androstendione have also been shown to be important in male sexual development (Borg 1994; Fostier et al., 1983). In females, 11-KT plasma concentrations are very low, often below 1 ng/mL, whereas T levels may approach E2 concentrations during portions of the spawning cycle in some fractional spawners, such as the fathead minnow (Jensen et al., 2001). The elevated T levels in females are assumed to be attributable to T serving as the immediate precursor in the biosynthesis of E2 (Afonso et al., 2000). High T levels might also be due to its importance in exerting positive or negative feedback toward gonadotropin secretion by the hypothalamus in both male and female fish (Redding & Patino 1993).

121. The biosynthesis of androgens primarily occurs in the testes and ovaries, although extra- gonadal synthesis has been demonstrated in several tissues including the liver and brain (Fostier et al., 1983). In the testis, synthesis of 11-KT and T primarily occurs in the Leydig and Sertoli cells (Redding & Patino 1993). Testosterone may serve as a precursor to the synthesis of the 11- oxygenated androgens (Shibata et

al., 2000), and administration of T to both male and female fathead minnows results in the formation and excretion of 11-KT (Parks & LeBlanc 1998).

122. Although 11-KT is a more biologically potent androgen than T in fish, early studies of androgen binding in cytosolic and nuclear extracts of fish tissues (reviewed in Borg 1994) suggested that T is preferentially bound to tissue extracts compared with 11-KT. This discrepancy between biological activity and tissue binding might in part be explained by the recent discoveries of multiple androgen receptors, each with different tissue distributions, binding affinities, and binding capacities for various androgens (Sperry & Thomas 1999a; Sperry & Thomas 1999b). Importantly, multiple androgen receptor subtypes appear to be expressed in Leydig and Sertoli cells and also in developing spermatocytes (Takeo & Yamashita 2001).

123. The variety of androgenic substances naturally occurring in fish, combined with the potential for cell- and androgen-receptor-specific responses, makes it difficult to generalize about the biological effects of xeno-androgens to an extent comparable for estrogenic substances. Relatively little information is available on the effects of exposure to androgenic or anti- androgenic substances in fish, which is perhaps partly due to the complexity of the biological activity of androgens. An additional limitation for the characterization of androgenic substances is the lack of a suitable biochemical biomarker that is as broadly applicable as VTG is for estrogenic substances. An encouraging development in this area is the recent molecular characterization of the adhesive protein spiggin, which is produced by the kidney of male three-spined sticklebacks (*Gasterosteus aculeatus*) for use in nest building during the breeding season (Jones et al., 2001.Both 17a-MT and DHT have been shown to be strong inducers of spiggin production (Katsiadaki et al, 2002a), furthermore, pulp mill effluent has been shown to induce spigging production (Katsiadaki et al, 2002b).

6.1 Endpoint Sensitivity to Natural and Synthetic Androgen Exposure

124. It is well established that exposure of juvenile fish to potent androgenic substances can lead to formation of testis-ova in females or a complete shift in phenotype from female to male (Nakamura et al., 1998; Koger et al., 2000). The effects of androgen exposure to adult or sexually differentiating individuals has received less study, however, a few studies are available:

Table 6.1. Reported Results Obtained with Androgenic Substances

Chemical [conc. Range]	Species [Assay]	Days of exposure	Endpoint	NOEC / LOEC	Reference
Methyltestosterone [120 µg/L and 1700 µg/L]	Fathead minnow Adult	12	GSI males and females Decrease in male plasma T and K-ketotestosterone conc. Secondary sex characteristics VTG induction (males and females)	LOEC: 120 µg/l LOEC: 120 µg/l LOEC: 120 µg/l LOEC: 120 µg/l	Ankley et al., 2001
Methyltestosterone [22-930 ng/l]	Zebrafish [FPLCT]	40	Sex ratio (based on type of gonadal tissue) VTG reduction	NOEC: < 22 ng/l LOEC: 22 ng/l NOEC: 42 ng/l LOEC: 70 ng/l	Örn et al 2003
Trenbolone [0.005 to 50 µg/L]	Fathead minnow Adult	21	Fecundity VTG 2y sex characteristics plasma steroid concent.	0.027 µg/l	Ankley et al, 2003
Trenbolone [0.05 to 5 µg/L]	Zebrafish [FPLCT]	40	Sex ratio (based on type of gonadal tissue) VTG reduction	NOEC: <0.05 µg/l LOEC: 0.05 µg/l NOEC: 0.05 µg/l LOEC: 0.5 µg/l	Petersen et al., 2004 (unpublished results)

125. Observation of androgen-stimulated nuptial tubercle growth in female fathead minnows was made over 25 years ago by Smith (1974). Additional studies in other fish species support the conclusion that formation of secondary male characteristics in females during androgenic exposure can be diagnostic for this mode of action. For example, in the adult medaka, a 15-day dietary exposure to 11-KT at dose rates ranging from 25 µg/g to 500 µg/g-food increased the number of papillary processes (growths) on anal fin rays in females in a dose- responsive manner (Hishida & Kawamoto 1970). In a series of related studies, appearance of papillary processes on the anal or dorsal fin ray in female medakas was observed after oral dosing with 19-*nor*-testosterone, MT, ethisterone, T, and androstenedione (Kawamoto 1973; Kawamoto 1969; Uwa 1975). In a study using 17-β trenbolone at concentrations ranging from 0.005 to 50 µg/l (Ankley et al, 2003), adult fathead minnows exposed for 21 days showed de novo production of nuptial tubercles (normally present in mature males only) from 0.05 µg/l. Of these androgenic substances, 19-*nor*-testosterone was the most potent at stimulating formation of papillary processes, with a dose rate as low as 0.6 µg/g-food producing a measurable effect (Kawamoto 1969). In the male mosquitofish

(*Gambusia affinis*), the anal fin develops into a gonopodium, which serves as an intromissive organ. This development is under androgen control and female mosquitofish administered 11-KT in their diet at dose rates as low as 20 µg/g-food will develop a gonopodium or exhibit a modified anal fin suggestive of gonopodium formation within 20 days of exposure (Angus et al., 2001). This phenomenon appears to be environmentally relevant, as masculinization of mosquitofish has been observed in the wild populations (Howell et al., 1980) and appears to be the result of androgenic substances, such as androstenedione found in pulp-mill effluents released into surface waters (Jenkins et al., 2001; Parks et al., 2001). Other observations following 17-β trenbolone relate to a decrease in VTG production in females (in a nonmonotonic way), a clear decrease in fecundity from 0.5 µg/l, significant changes in steroid concentrations in plasma in both males and females and histological evidence in ovaries consistent with decrease in VTG and impaired spawning (Ankley et al, 2003).

126. A final, less characterized effect of androgen exposure in fish is on courtship behavior. In many fish species, the males exhibit either pronounced territorial behavior or courting activity during spawning, which may be stimulated by androgen exposure. In some species, such as the white perch (*Morone americana*), courtship behavior in castrated males could be restored by administration of 11-KT (Salek et al., 2001). In this regard, induction of courtship behavior in females might be an additional response to androgen exposure. This latter possibility is a research area that needs further study.

127. In summary, androgen exposure to juvenile fish that are sexually undifferentiated can lead to phenotypic reversal. In adult fish, androgen exposure decreases gonad growth (size) and circulating androgen levels in plasma and severely reduces reproduction in females. The available data indicate that formation of male secondary sex characteristics in females is the most useful endpoint for detecting an androgenic substance. Because some androgenic substances (e.g. methyltestosterone at high concentrations (Örn et al., 2003) may be aromatized, formation of VTG may occur.

6.2 Androgen Antagonists

6.2.1 Direct-Acting Anti-Androgens

128. A well-characterized environmental contaminant known to antagonize androgenic steroid action is vinclozolin, a chlorinated fungicide widely used as a pesticide for over 20 years. The anti-androgenic activity of vinclozolin was first described in developing rats exposed in-utero or lactationally via oral dosing of vinclozolin to the pregnant mother (Gray et al., 1994; Kelce et al., 1994). When exposure occurred during gestational day 13 through postnatal day 3, a variety of defects in male offspring was observed that reflected an overall feminization of the rat (Kelce et al., 1994). The anti-androgen effect of vinclozolin was attributed to the competitive inhibition of T binding to the androgen receptor by two main metabolites of vinclozolin: 2-[(3,5- dichlorophenyl)]-carbamoyloxy-2-methyl-3-butenoic acid, abbreviated as M1, and 3',5'-dichloro- 2-hydroxy-2-methylbut-3-enanilide (M2) (Kelce et al., 1994). Recently, a thorough evaluation of vinclozolin exposure to sexually mature fathead minnows was described by Makynen et al. (2000). In this study, a 21-day exposure to measured concentrations of vinclozolin of 200 µg/L or 700 µg/L had little effect on males other than an increase in E2 plasma concentrations. In female minnows, vinclozolin treatment was more toxic, causing a decrease in the GSI and reduced oocyte maturation (Makynen et al., 2000). The important vinclozolin metabolites M1 and M2 were measured in whole fish homogenates after the 21-day exposure and were approximately 2% to 6% (M1) or 0% to 1.2 % (M2) of the total vinclozolin body burden. Interestingly, vinclozolin, M1 and M2 were not observed to compete with T binding sites in brain and ovary cytosolic extracts from fathead minnows (Makynen et al., 2000). Similar results for these compounds have also been reported using trout and goldfish tissues (Wells & Van der Kraak 2000). The lack of antagonism toward T binding suggests vinclozolin and metabolites may not be anti-androgenic in fish, although further competitive binding studies are needed, particularly with 11-KT, before this conclusion is established.

129. In contrast to findings with male fathead minnows, oral exposure of vinclozolin at nominal levels up to 100 µg/g food appears capable of demasculinizing adult male guppies (Baatrup & Junge 2001). After a 30-day exposure, male guppies fed vinclozolin exhibited a decreased GSI and number of ejaculated sperm cells, along with a partial loss of orange-yellow coloration, a male secondary sex characteristic of this species (Baatrup & Junge2001). In an earlier study using the medaka, the potent anti-androgen cyproterone acetate (CPA) was also shown to retard development of male secondary sex characteristics. A 21 day exposure to nominal CPA concentrations ranging from 100 µg/L to 2000 µg/L reduced the number of papillary processes on anal fin rays in male medakas (Hamaguchi 1978). This effect was even more pronounced in female medakas co-exposed to 20 µg/L MT. In this latter experiment, CPA suppressed the MT stimulated formation of papillary processes in a dose-dependent manner (Hamaguchi 1978).

6.2.2 Indirect-Acting Anti-Androgens

130. As discussed previously for indirect anti-estrogens, there would conceivably be a minimum of three mechanisms by which indirect anti-androgens could reduce intracellular steroid levels: 1) decreasing the rate of synthesis; 2) reducing the plasma free fraction; and 3) increasing the rate of elimination. Unfortunately, there is only very limited information available to assess the biological consequences of these modes of action. In contrast to the biosynthesis of E2, which can be blocked using aromatase inhibitors, no equivalent androgen-specific inhibitors have been identified. However, it is known that E2 administration to juvenile or post-differentiating fish decreases androgen secretion by the testes, and this effect is due to diminished expression of enzymes involved in their biosynthesis (Fitzpatrick et al., 1993; Govoroun et al., 2001). These results imply that xenobiotics capable of inhibiting androgen steroidogenesis could be effective at lowering circulating T or 11-KT levels. However, demonstration of this mode of action has not yet been made.

131. It is established that sex steroids in general, and androgens in particular, are highly bound to specific plasma proteins in fish (Hobby et al., 2000). However, it appears that exceptionally high concentrations of suspect EACs are required to displace sex steroids from these binding proteins (Milligan et al., 1998). Thus it is doubtful that, by itself, reduced plasma binding is a pertinent mode of action for endocrine disruptors.

132. The biotransformation of androgens is complex, and a variety of metabolites and conjugates may be formed and eliminated (reviewed in Shibata et al., 2000). Both T and 11-KT excretion have been well studied in trout, where it appears conjugation to glucuronide is the most important elimination pathway (Yeoh et al., 1996). However, adult fathead minnows briefly exposed to T (4-hour exposure; measured concentration of 288.4 µg/L) formed 11-KT, which appeared to be excreted directly, i.e., no conjugates could be isolated (Parks & LeBlanc 1998). Regardless of the potential for species differences in conjugation, it remains to be established whether changes in the rate of androgen elimination have any important biological effects in fish.

6.3 Gender Differences

133. The available data suggest (anti-) androgen activity is best identified in the test species based on the appearance of secondary sex characteristics and shift in sex ratio. In the case of androgen agonists, the development of male external characteristics in females and the shift towards males appears to be diagnostic for this mode of action. As for anti-androgens, a reduced display of secondary sex characteristics in males is suggestive of this mode of action, although it may not be as diagnostic as with agonistic androgen activity on females.

6.4 Strengths and Weaknesses of Test Species

134. The complexity of androgen activity and the similarity of many endpoints to other modes of action indicate a single test species may not provide sufficient diagnostic power for adequately identifying chemicals that disturb androgen activity. An obvious weakness of the proposed test species is the lack of a suitable biochemical biomarker sensitive to androgen agonists. In this respect, inclusion of the three spine stickleback in a testing program would be beneficial, as the 11-KT-induced synthesis of spiggin appears to be a very sensitive indicator of androgen exposure in this species. In addition, fish species possessing a gonopodium, such as the guppy and mosquitofish, appear to be superior in identifying both androgen agonists and anti-androgens through changes in the overall extent of masculinization of males. All three species appear although capable of responding to (anti-)androgens by changes in secondary sex characteristics: medaka and fathead minnow (Ankley et al, 2001; Ankley et al, 2003) and shift in sex ratio: zebrafish (Orn et al, 2003).

7. RESPONSE TO THYROID HORMONE AGONISTS AND ANTAGONISTS

135. Thyroid hormones play an important role in the maintenance of a “normal” physiological state in vertebrates. In fact, it has been said that thyroid hormones influence the activity of a wider variety of tissues and biological functions than any other hormone (Janz 2000). Therefore, it is prudent of any endocrine disruptor screening assay to include some measure of effect on thyroid tissue. However this may not be a simple task in most fishes, which differ from higher vertebrates in that a discrete or compact thyroid organ is not normally formed. Rather, thyroid follicles (the basic unit of all thyroid tissue) are typically distributed in a diffuse manner throughout the ventral pharyngeal region (Bonga 1993; Janz 2000). In addition, thyroid follicles may develop at secondary locations within the fish such as in the ovary, head kidney and pericardium (Janz 2000). The dispersed nature of thyroid tissue in fish makes certain morphological assessments of thyroid tissue such as determination of a thyroid tissue-somatic index impractical.

136. Teleost fish resemble higher vertebrates in that the primary thyroid hormone produced by thyroid follicles is thyroxine (T4). The biosynthesis of T4 requires iodide, which is absorbed from the water by the gills (Hunn & From 1966). Normally, iodide levels are sufficiently high in both fresh and saltwater such that a deficiency is rare in fish, although there have been occasional reports of goitre in wild fish (reviewed in Eales & Brown 1993). Secretion of T4 by thyroid follicles is regulated by the hypothalamic-pituitary axis through the action of thyroid stimulating hormone (TSH). Secreted T4 is bound to a variety of plasma proteins (Power et. al. 2000; Babin 1992) and only the free or unbound fraction can diffuse into peripheral tissues (Eales and Brown 1993). The biological activity of T4 is low, and it is generally considered that the de- iodinated form of T4, triiodothyronine (T3), is the most important thyroid hormone (Cyr and Eales 1988). Conversion of T4 to T3 is via the enzyme, iodothyronine 5’-monodeiodinase, and inhibition of this enzyme have been shown to diminish thyroid hormone activity (Cyr & Eales 1988).

137. Thyroid hormone activity is mediated through binding to specific cytosolic receptors. Two distinct thyroid receptors have been characterized, termed _ and _ (Yamano and Inui 1995). These thyroid hormone receptors are considered to be part of the steroid receptor superfamily and as such, are believed to operate in a similar manner as estrogen and androgen receptors (e.g., as transcription factors for specific genes; Power et al. 2001).

138. In addition to reproduction, thyroid hormones have been linked to a multitude of important functions in fish that are conveniently grouped under growth, metabolism, and osmoregulation (see reviews in Cyr & Eales 1996; Power et al. 2001). Interference with thyroid hormone function could be expected to have wide-ranging effects on proper growth and development of gonadal tissues and to affect estrogen synthesis in the ovary (Cyr & Eales 1988a, 1988b, 1989; Soyano et al., 1993; Legler et al., 2000; Siwik et al., 2000). Conversely, E2 administration has been recently reported to lower circulating T3 levels in immature trout (Alestrom et al., 1994). In the medaka, MT exposure was reported to stimulate thyroid activity (Nishikawa 1976). These studies would suggest the possibility that sex-steroid agonists may alter thyroid function. Despite these intriguing results, relatively few studies in fish have investigated whether environmental contaminants alter thyroid hormone status or directly interact with thyroid receptors to impair reproduction. However, one group of environmental contaminants, the polychlorinated biphenyls (PCBs), is of particular concern relative to thyroid hormones because of their structural similarity. Studies in the American plaice (*Hippoglossoides platessoides*) have demonstrated that exposure to select PCB congeners can alter the turnover of T4 and T3 (Iwamatsu et al., 2000). Given that PCBs affect thyroid hormone function in fish, a fruitful area of investigation might be to explore the mode of action of PCBs in the test species and establish whether thyroid hormones could be used as significant endpoints for reproductive dysfunction. Additionally, TSH activity is another research area warranting additional study in the context of reproductive performance. Plasma levels of TSH are rarely reported in fish reproductive studies, although the TSH receptor(s) are highly expressed in oocytes and testicular cysts of some teleosts (Kumar et al., 2000), which indirectly suggests that gonadal secretion of thyroid hormones may be important in gametogenesis (Huang et al., 2001).

7.1 Endpoint Sensitivity to Thyroid Stimulation

139. As alluded to in the introductory comments, it has been proposed that certain PCB congeners or their metabolites may bind to vertebrate thyroid receptors (Fentress et al., 2000; Kovriznych & Urbancikova 2001). This potential would suggest that stimulation of the thyroid system is possible through a direct mode of action (e.g., receptor mediated). However, the available data in fish regarding PCB exposure and thyroid status are contradictory (Schnurstein & Braunbeck 2001), and no generalizations can be made regarding the environmental significance of this mode of action. Many studies in fish evaluate thyroid status by measuring circulating plasma T4 and T3 levels. A good example of this approach was a study of hydrogen cyanide exposure to rainbow trout reported by Ruby et al. (Creech et al., 1998). Trout exposed for 12 days to 10 _g/L cyanide had significantly reduced E2 and T3 plasma concentrations, which corresponded to a lower GSI and oocyte diameters in females (Creech et al., 1998). Single measurements (or point estimates) of thyroid hormone levels may not be a good indicator of mode of action, as a complicating factor is the strong feedback control of T4 and T3 levels. For example, Iwamatsu et al. (2000) reported that a 5 _g/kg or 25 _g/kg intra-peritoneal injection of PCB congener 77 lowered T3 levels after one week in the American plaice. However, the same treatment stimulated conversion of T4 to T3 (specifically, T4 outer-ring deiodination) in liver microsomes (Schnurstein & Braunbeck 2001). These seemingly paradoxical results were hypothesized by the authors to be the result of increased clearance of T3, which triggered the compensatory action of stimulating its biosynthesis in peripheral tissues (Schnurstein & Braunbeck 2001). These results illustrate the difficulty in assessing xenobiotic effects on thyroid status and the unlikelihood that point estimates of circulating thyroid hormones measured as part of a reproductive screen will be useful by themselves in identifying thyroid agonist activity.

7.2 Inhibition of Thyroid Function

140. In contrast to the difficulties in identifying thyroid agonists, there is evidence that certain environmental contaminants can act specifically as anti-thyroidal agents. A study of the effects of thiocyanate on thyroid function and reproduction in fathead minnows was reported by Lanno and Dixon

(1994). In this study, sexually differentiated but immature fathead minnows were exposed to measured concentrations of thiocyanate ranging from 0.06 mg/L to 32.6 mg/L for 21 days and then an additional 103 days, during which time spawning activity was monitored. The results indicated that fathead minnows exposed to 16.6 mg/L and 32.6 mg/L thiocyanate completely lacked or underwent incomplete development of secondary sex characteristics. These fish also made no attempt to reproduce (Lanno & Dixon 1994). However, toxicity was quite high at these exposure levels, with reported mortalities during the exposure to be 30% and 63% at the 16.6-mg/L and 32.6-mg/L exposure levels respectively (Lanno & Dixon 1994). A lower exposure rate of 7.3 mg/L was non-toxic but still impaired reproduction, as measured by the delay in time of first spawning and decreased fecundity (Lanno & Dixon 1994). Antagonism of thyroid function was evidenced at 7.3 mg/L to 32.6 mg/L thiocyanate exposure rates by the development of overt goitrous nodules along the branchial region of the lower jaw. Histopathological examination of these fish indicated a clear dose-response relationship between thiocyanate exposure rate and the extent of the hyperplastic and colloidal goitrous follicles (Lanno & Dixon 1994). The time to first appearance of the goiters or gender-specific differences of goiter formation were not discussed in this study.

141. Consistent with the findings of the aforementioned study, other anti-thyroidal agents have been shown to cause hyperplasia of thyroid follicles and decreased development of secondary sex characteristics. For example, adult catfish (*Clarias batrachus*) exposed for one year to 100 mg/L ammonium sulphate or three months to 300 mg/L thiourea (nominal levels) developed complex histopathology of the thyroid follicles suggestive of overall hyperemia and hyperplasia (Sathyanesan et al., 1978). In the medaka, an intra-peritoneal injection of thiourea reduced the MT-stimulated growth of papillary processes of the anal fin (Fujiwara 1980). A related study (Jensen et al., 2001) noted that medaka exposed for several months to various sodium bromide water concentrations had reduced secondary sex characteristics. A recent study in sexually mature medaka measured T4 and T3 plasma levels before and after a 10-day exposure to various nominal water concentrations of thiourea. Exposure to 300 mg/L thiourea lowered plasma levels of T4 and T3 from 8 ng/mL and 5 ng/mL respectively, to less than 2 ng/mL within 24 hrs (Tagawa & Hirano 1991). The effects of thiourea exposure on fecundity were not reported; however, fertilization success and time to hatching were unaffected by the exposure (Tagawa & Hirano 1991).

7.3 Gender Differences

142. Because of the paucity of studies available for review, little discussion of gender differences to thyroid stimulation or inhibition can be made. Certainly, more research is needed focusing on thyroid agonists or direct stimulation by T3 and T4 and subsequent effects on reproduction. The limited data on anti-thyroid compounds suggest both male medaka and fathead minnows may be a more sensitive model. In these species, impairment of the appearance of secondary sex characteristics, such as papillary processes on the anal fin in medaka or nuptial tubercles in minnows, may become apparent after short-term exposures. However, it is unclear whether significant goiter formation can occur in these species over the relatively short exposure periods (14 to 21 days) used in screening assays. In this respect, histopathological analysis of the thyroid follicles would be particularly helpful in identifying anti-thyroidal chemicals.

7.4 Strengths and Weaknesses of Test Species

143. The limited data serve only to underscore the broader issue of the scarcity of information on basic thyroid function in fish. Clearly, more basic information on thyroid function during reproduction in the test species is needed. However, in lieu of this, both the fathead minnow and medaka appear equally capable of serving as an adequate test species for identifying thyroid- hormone disrupting chemicals.

8. EXPERIMENTAL DESIGN CONSIDERATIONS FOR FISH SCREENING ASSAYS

8.1 Route of Administration

144. A variety of exposure pathways have been used in endocrine studies with fish. In addition to aqueous and dietary exposures, direct injection techniques such as intravascular and intraperitoneal administrations have also been applied.

8.1.1 Water

145. Besides being ecologically relevant, water is the most common laboratory route for exposing fish to EACs. The delivery of a toxicant to water at different concentrations is well established. Laboratories around the world, using a variety of pumps, valves and gravity, have successfully conducted water exposures. A water exposure concentration is more readily correlated with water concentrations found in the aquatic environment. The biggest challenge with water exposure is preparing aqueous solutions of high concentration relative to solubility limitations of the test substances in the water.

8.1.2 Use of Solvent Carrier

146. Organic solvents are often used as carriers to assist in delivering a test substance in water. If an organic solvent is used a solvent control should always be included when testing a range of concentrations and the solvent concentration should be equal in all concentrations of the chemical tested. The use of an organic solvent requires the maintenance of a solvent control. Organic solvents also enhance bacterial growth, which increases maintenance time during the exposure. Especially with acetone and ethanol, bacterial – and occasionally – fungal growth may be excessive and may compromise the entire experiment.

147. It is emphasised that any use of solvents should be avoided whenever possible. Saturator columns have been used in some cases to eliminate the use of solvents (Ota et al., 2000; Kahl et al, 1999). For further information of how to handle difficult substances see the OECD Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. This document (Guidance Document No. 23) can be found at the following URL:

http://www.oecd.org/document/30/0,2340,en_2649_34377_1916638_1_1_1_1,00.html.

148. Although Guidance Documents indicates that solvent concentrations below 100 mg/l (100 µl/l) can be used, it has been proposed that if solvents had to be used in fish tests for the detection of endocrine active substances, an interim recommendation is to keep concentration below or equal to 20 mg/l (20 µl/l). Any test where a solvent is used will include a solvent control as well. However, the Fish Draftig Group of the Validation Management Group recommended avoiding the use of solvents as much as possible.

149. Accompanied by discussion in the OECD Fish Drafting Group, the following information on the use of different solvents (DMSO; Triethylene glycol; Acetone; Ethanol; DMF) has been compiled based on experience in working groups of several fish experts:

150. The use of DMSO as a solvent has shown promising results in long-term toxicity studies with zebrafish (Raoul Kuiper/University Utrecht, pers. comm.). No bacterial growth was observed and it was shown that concentrations up to 3.2 % during 4 weeks of exposure did have no adverse effects on growth and behaviour, furthermore the histopathology evaluation at the end of the study showed no additional effects. Long term exposures performed by Braunbeck and co-workers also showed no adverse effects by the use of DMSO in concentrations up to 0.1% (Thomas Braunbeck/University Heidelberg, pers. comm.).

151. Triethylene glycol has been used as a solvent in a 293-day study with Fathead minnows with a concentration of 0.09 ml/l (Hans Rufli/ Ecotoxsolutions, pers. comm.). In this study no effects were seen on hatchability, survival rate and growth in neither the F0 nor the F1 generation. Also the reproduction in the F0 generation (number of eggs and percent-fertilised eggs) was not influenced. Measuring the VTG content in the plasma of the adult fish (F0 generation) it was seen that a significant elevation was obtained in the vehicle control group compared to the control group. This occurred both in the males and the females. Also a significant increase of VTG in the whole body homogenate of the F1 generation was seen.

152. Several scientists have commonly used acetone and ethanol: e.g., provided information shows that the use of both acetone and ethanol induces bacterial growth and thus higher oxygen consumption in the test aquaria (Christoph Schaefers/Fraunhofer IME, pers. comm).

153. Dimethylformamid (DMF) was used in another study at a concentration of 31 mg/l (Reinhard Laenge/Schering AG, pers. comm). Endpoints studied in the 21-day test were mortality, growth, morphological and behavioural changes and VTG levels. None of these parameters were affected by the solvent (Zerulla et al., 2002).

8.1.3 Oral (Food)

154. Oral exposure is also ecologically relevant and can be an advantageous route of exposure with compounds of poor water solubility or high instability in aqueous solutions. Some researchers have recommended that hydrophobic compounds with a log K_{ow} >5 should be administered via food (Patyna et al., 1999). Incorporating the test chemical into commercially prepared fish food is relatively simple, but dosing food items such as live or frozen foods may be difficult if not impossible. Another consideration is the estimation of the administered dose. With small aquarium fish such as those being considered, estimation of the amount of food consumed each day can be difficult. These considerations, in addition to the need for a balanced diet in the maintenance of fish during an exposure, limit the utility of the oral route of exposure.

8.1.4 Direct Injections (Intraperitoneal / Intramuscular)

155. There are several alternatives to the water and oral routes of exposure in fish. These alternative routes are more invasive and involve direct injection of the test substance into the fish body. Administrative routes suitable for smaller sized fish are direct injection into the peritoneal cavity (intraperitoneal), intramuscular (best performed midway between the leading edge of the dorsal fin and lateral line), or the dorsal sinus (part of the secondary or lymphatic circulation; Olson 1996). All of these exposure routes have been used on smaller sized fish in the course of the evaluation of endocrine toxicants (for example, see Schwaiger et al., 2000; Korte et al., 2000; Zaroogian et al., 2001). These exposure routes may be considered when the quantity of test chemical is very limited or of low water solubility. To perform direct injections, the test chemical needs to be concentrated in a non-toxic solvent (termed the dosing vehicle) that is also unreactive towards the endocrine system. In this regard, a suitable dosing vehicle for many compounds is DMSO (Schultz et al., 2001) or ethanol-water, ethanol-vegetable oil mixtures (Schwaiger et al., 2000; Kahl et al., 2001; Olsson et al., 1999). The injection can be accomplished using small syringes (0.5 mL is best) and a 30 G needle. To reduce stress during the injection, fish should be lightly anesthetized. Care needs to be exercised during the injection to avoid injuring vital tissues. In this respect, intramuscular and dorsal sinus injections offer an advantage over intraperitoneal administration.

156. Direct injection procedures impose greater stress on the fish because of the extra handling, anesthetic exposure, and trauma inflicted by the injection (Heming 1989). The tolerance of the injections may vary across species. For example, a recent study in fathead minnows indicated repetitive

intraperitoneal injections did not alter plasma sex steroid levels or reproductive performance in this species (Kahl et al., 2001). Regardless of the actual level of stress imposed on a fish, direct injection methods should be considered as a final option after water and oral exposures. An occasional problem with direct injection procedures is loss of delivered dose through leakage of the injectate through the entry wound (Horsberg 1994). Depending on the toxicokinetic behavior of the test compound, multiple injections would likely be needed to maintain some type of consistency in tissue concentrations during the assay and the required frequency could be challenging to estimate. These problems would make it difficult to reproduce the effective administered dose (e.g., internally absorbed dose x test duration) across individuals, which can add to the variability in the measurement of toxicological endpoints.

157. It is suggested that ip injection may not be used in a routine screening programmes because of the skills, time and resources involved; it may be more relevant for research purpose, only.

8.2 Dose Selection

158. The basic considerations for dose selections are that a sub-lethal exposure is used and in the case of water exposures, water solubility limits are not exceeded. Ideally, the dose level used should not alter the growth or the survival of the test fish.

159. A potentially serious problem is the frequently large discrepancy between measured and nominal concentrations of test chemicals. This problem has been particularly noticeable with exposures using steroid analogues. For example, Nimrod and Benson (1998) used direct delivery of estradiol (E2) (dissolved in acetone) to exposure tanks via a peristaltic pump and observed that measured concentrations of E2 in the tanks ranged from 11% to 17% of the predicted nominal concentration. In a recent study of E2 and ethynylestradiol (EE2) stability in water over a 48-hr time period, measured concentrations were on average 43% and 29%, respectively, of nominal concentrations (Metcalfe et al., 2001). The difference between measured and nominal levels in exposure chambers tends to be especially noticeable at both high (>50% water solubility) and low-test concentrations. Instability at low-test concentrations has been attributed to biodegradation of added steroids (Metcalfe et al., 2001). These observations reinforce the need to experimentally measure test chemical levels in the exposure chambers or exposure media and the need to cautiously interpret experimental results obtained from studies that do not directly measure exposure levels of test chemicals.

160. At the OECD expert consultation meeting on endocrine disrupters testing in fish (OECD, 2000) the following recommendations concerning dose selection were made:

> It was agreed in general that the top concentration must not cause lethality. Furthermore, the spacing between concentrations will depend on a number of factors including the slope of the LC50 response curve, and will generally lie between factors of 2 and 10. The following recommendation was also made: in every case, actual exposure concentrations should be measured in both screening and testing assays.

8.3 Fish Life Stage

161. The utility of juvenile fish in short term screening assays has recently been demonstrated for (anti)-estrogens (Panter et al., 2002). The primary advantage of using juvenile fish in an endocrine screen is the reduction in costs associated with maintenance and performance of the assay. The use of juvenile fish also allows for a corresponding reduction in the quantity of water and test chemical required for the test. This factor could be especially important when using test chemicals that are expensive to produce or difficult to obtain in sufficient quantities and with high chemical purity. The use of juvenile life stages would also allow the inclusion of additional fish species that are too large at sexual maturity for practical

use in a screening assay (e.g., salmonids). The application of a juvenile salmonid species as a test organism has been explored with estrogen mimics and shown to be responsive to induction of VTG and changes in other biomarkers indicative of steroid mimic exposure (Thorpe et al., 2001). Changes in blood plasma steroid concentrations as a result of chemical treatment can also be detected using juvenile fish.

162. The main disadvantage of using juvenile fish lies in the inability to directly assess reproductive performance. Other features of juvenile assays that are limiting include the lack of sexual differentiation, maturation, and the absence of secondary sex characteristics. However when the exposure is performed during the sexual development of the fish as in the fish partial life cycle assay endpoints as sex differentiation and VTG response are possible to determine. The application of early and juvenile fish as test organism has been explored with estrogen and androgen substances and shown to be responsive to induction and reduction of VTG and significant changes in sex ratios in zebrafish (Andersen et al., 2001; Van den Belt et al., 2001; Örn et al., 2003; Holbech et al., 2001) and fathead minnows (Zerulla et al., 2002).

8.4 Exposure Duration

163. Overall the exposure duration should be sufficient to ensure that changes, if they occur, can be observed during the exposure period, and this depends on the endpoints measured. However, a variety of concerns may need to be considered in the determination of the duration of the toxicant exposure. An underlying principle of toxicology is that an increase in the amount of exposure time typically results in identification of effects at lower concentrations (Ensenbach & Nagel 1997; Holcombe et al., 1995; McKim 1977; Nagel et al., 1998; Parrott et al., 2000). Thus, the longer the duration of the exposure, the more likely an effect will be observed. With respect to a screening assay, the use of a high toxicant concentration might allow for a shorter exposure period to be used. The exposure duration should be sufficiently long to allow for extensive absorption of the toxicant and potential biotransformation events (e.g., disposition) to occur to a significant extent. As a useful approximation, an exposure duration lasting sufficiently long to achieve a pseudo-equilibrium between toxicant concentrations in the exposure media and those in fish tissues would be acceptable. For most chemicals, this equilibrium would be expected to occur within 30 days of exposure (Veith et al., 1979). However, for lipophilic chemicals, a pseudo-equilibrium might be difficult to achieve in this time span.

164. The above considerations suggest an exposure duration of at least 12 and up to 30 days would be adequate for screening purposes. However, an additional consideration not previously mentioned is the cost of performing the screening assay. The maintenance of an exposure system can be costly. In addition, longer exposure durations can increase the possibility of unexpected interruptions in exposure as a result of test-substance behavior in the water or equipment malfunction. Chemical analysis of the exposure solutions and cleaning the exposure system to maintain high dissolved oxygen concentrations, especially in the presence of organic solvents used as carriers, add significantly to the time and effort in maintaining a long-term exposure. Therefore, the exposure duration of a study needs to be a balance between adequate time to elicit an effect and the need to minimize costs.

8.5 Statistical Considerations

165. The purpose of the screening assay is to detect potential EDCs and their likely mode of action in fish. Thus, a screening assay must be (a) biologically sensitive while providing a mechanistic link between changes in endpoints and reproductive dysfunction, (b) statistically powerful, and (c) cost effective (Munkittrick et al., 1998). The screening assay is meant to detect potential EDCs and not to produce a precise estimate of toxicity. The statistical considerations here are restricted to the demands of the screening assay. The amount of information obtained from the screening assay can be limited to detecting a series of specific differences in reproductive traits when both genders are exposed or can be used to

determine whether or not gender-specific differences can be detected when gender-selective exposure is used.

166. The most biologically pertinent endpoints measured in an endocrine screening assay are those directly associated with reproduction: fecundity, fertilization success, and embryo hatching. These endpoints are ideally quantified before and during the exposure period. Additional endpoints collected at termination of the exposure might include GSI, morphology, gonad histology, and biochemical endpoints (VTG and sex steroids). For those endpoints measured during pre- and post-exposure, the data for statistical testing are the differences between the paired pre- and post- exposure rates calculated for each replicate of the exposure treatments if it has been established that there is no effect associated with time. Alternatively, the post-exposure response is compared with a no-dose control. For those endpoints measured only post-exposure, statistical significance is evaluated based on the difference in the mean characteristics between the treated and control groups using Dunnett's test when there are greater than two doses above zero (Dunnett 1955) (Chapman et al., 1996) and Fisher's Exact test or a t-test when only one dose is used (Chapman et al., 1996). The OECD draft Guidance Document on the Statistical Analysis of Ecotoxicity Data (OECD, 2003) provides further advice on the existing statistical tests that can be performed in given circumstances.

167. The time series of daily fitness measurements taken pre- and post-exposure can also be used to evaluate the toxicity response. For example, a regression of the rate of egg production against time elapsed (0 to 14 days for pre-exposure and 0 to 21 days for post-exposure) can be used to assess the shape of the response (linear or curvilinear), the daily within-class variation in response, a potential time lag between exposure and response, and the appropriateness of the exposure duration.

8.5.1 Experimental Design and Statistical Power

168. Ideally, an experimental design incorporates randomness, independence, and replication (Cochran & Cox 1957). Randomness is used to remove noise, independence is used to extend the inferences made, and replication provides a measure of variability for testing (Chapman, et al., 1996). Randomization of experimental containers within a testing environment allows one to incorporate the variability associated with the environmental conditions equally across all containers. Randomization of treatment application to experimental containers allows one to incorporate the variability associated with the containers equally across all treatments. Randomization of the application of organisms to experimental containers (conducted blind and in a random order) and a random sample of organisms from a population of organisms (or at least haphazard and without bias) allows one to incorporate the variability associated with the organisms equally across all containers. Thus, when one evaluates the difference between treatment means, the variability associated with experimental environment, experimental containers, and organisms being treated is removed and only the effect of the treatment remains.

169. Independence of treatment application, including the creation of the treatment, incorporates the variability associated with someone else, somewhere else making and applying the same treatment. Thus, the inference made associated with the treatments under test is extended to someone else repeating the experiment. The random sample of organisms from a given population actually limits the inference to that population. However, one can evaluate the stability of the inherent variability of the population over time. An experimental unit is defined as the group of material to which a treatment is applied independently in a single trial of the experiment (Cochran & Cox 1957). Replication of experimental units for each treatment provides a measure of all the necessary sources of variability needed to extend the inference across time and space. A reduction in the sources of variability that are truly independent constrains the inference (Hurlbert 1984). Thus, if only one solution of each treatment is made and then divided between replicates, the source of variation associated with making the treatment is not included in the variability for testing, and the inference is limited. Some would say that this variability is also nuisance noise, too small to be of

concern, and costly to include. Therefore, if this source of variability is not included, it should at least be acknowledged. This source of variation is commonly encountered through the use of a single diluter mixing chamber to deliver replicate concentrations. The variability between replicate experimental units may also include noise that was not randomized out because of a poor randomization or variable measurement error. These sources of variability can be reduced without loss to inference.

170. Statistical power is the probability of rejecting the null hypothesis (of equal means) when the alternative is true (i.e., detecting a difference when there is a difference). Statistical power is a function of the variability between replicate experimental units (i.e., within a treatment), the number of replicate experimental units, the size of the type I error, and the percentage of difference one wishes to detect. One can control the latter three components; however, the variability in response is inherent in the organism being tested, the endpoint being assessed, the method used to measure the endpoint (in some cases), and the uniformity of experimental conditions. Thus, the choice of which species should be tested and the relevant endpoints measured should include a comparison of inherent variability or coefficients of variation (CVs) (standard deviation/mean x 100%) and of the relative size of the response.

- In terms of power, high CVs have low power for detecting small-scale differences. Experiments with the standard number of 5 replicates per treatment and CVs for the measured response greater than 50% will be unlikely to detect differences smaller than 70% between the test and reference treatment response at a type I error rate of $\alpha = 0.05$. For a given CV, one can increase power by increasing the number of replicates. The choice of the test species and endpoints with the least inherent variability, by default, require the least replication for a given level of power and thus are more cost effective.

- One must also consider the relative size of the response. For example, if we suppose that the exposure to a given concentration of some compound causes a 100 fold increase in vitellogenin, and measurements of vitellogenin have a CV of 30%; the measurement of another endpoint shows a 20% change relative to controls and a CV of 20%. Under these conditions, statistical tests involving vitellogenin will have the higher power to detect the relevant amount of change, even though the CV for vitellogenin measurements is higher. This would imply that the number of replicates required to detect the effects on vitellogenin from a given level of exposure of the substance would be smaller than the number to detect effects on the second endpoint.

- Also the statistical test used and the method for controlling the experiment-wise error rate associated with multiple comparisons can have considerable effects on power to detect changes of a given size. Depending on the statistical method used, the pattern of responses with increasing dose can affect power (e.g. power of the test could depend on whether there is a gradual increase in response with increasing dose or whether there is a response at only the highest dose).

- Finally, power increase can also be reached by a slight increase in the rate of false positives (e.g. $\alpha=0.10$ instead of the usual $\alpha=0.05$).

8.5.2 Decision Criteria

171. In some cases, individual responses would be diagnostic of a specific endocrine mode of action (e.g., induction of vitellogenin in males caused by estrogen receptor agonists), but in most cases the pattern of responses of the various endpoints would have to be considered to assess which (if any) endocrine pathway has been affected. In general, each endpoint will be evaluated to determine if at least one concentration of a test material produces a significantly different mean response (greater or lesser response depending on the endpoint) from the control mean response using Dunnett's test when there are at least two doses greater than zero being tested (Dunnett 1955; ARS 1977; Chapman et al., 1996). This procedure

uses an experiment-wise error rate. The degrees of freedom for testing are the number of experimental units (not sub-replicates) minus one times the number of doses plus the no-dose controls. When the variance for testing is based on the variance among fish in a treatment chamber (i.e., sub-replicates) instead of between true replicates, the inference of repeatability through time and space as described above is invalid (Hurlbert 1984). If only one dose and one control are tested, then a t-test or Fisher's Exact test can be used (Chapman et al., 1996).

172. Before conducting statistical tests, the assumptions required for a particular inference should be evaluated. Analyses should not be conducted blindly, and often a simple plot of the data will provide as much or more insight as a statistical test. Quartile plots and box-and- whisker plots are useful qualitative tools for evaluating normality and homogeneity of variance, respectively, among classes. Analysis of variance (ANOVA) methods such as the *t*-test and Dunnett's test are robust to non-normal errors (Scheffé 1959). A rule of thumb used to evaluate homogeneity of variance is that the largest class standard deviation should be no greater than three times the smallest class standard deviation. Following conclusions by Scheffé (1959), qualitative forms of evaluation are appropriate and eliminate the concern related to conducting conditional analyses that result from multiple tests of assumptions, transformations, and final analyses.

173. In the case where homogeneity variance is not verified, appropriate data transformations will be applied to maintain homogeneity of the within-class variances (i.e., data expressed as a percentage will be arcsine-square root transformed, counts will be square root transformed, and continuous data will be transformed to the natural logarithm) (Snedecor & Cochran 1980). A rank transformation or nonparametric statistic will be used when the common data transformation is not successful in controlling heterogeneity (Daniel 1978). Steel's rank sum test (Steel 1959) is a nonparametric alternative for comparing a control with at least two doses greater than zero. This procedure uses an experiment-wise error rate. Fisher's Exact Test for binomial data (e.g., the number hatched divided by the number fertile) can be used when there is only one dose and one control being tested (Steel & Torrie 1980; Chapman et al., 1996).

174. Analysis can be conducted both with and without suspected outliers (Chapman et al., 1996). Potential outliers can be identified by values that exceed the median plus 3 times the inter-quartile range (i.e., the difference between the 75th and 25th percentiles). If an explanation cannot be made as to why the data diverge from the rest, then both analyses should be presented, assuming that the results differ. If there are no changes to the results, then the analysis including the outliers should be presented. If differences occur, then the implications of removing the outliers should be carefully documented. If an explanation can be made as to why there are outliers, the analysis excluding outliers may be sufficient.

175. For chemical concentration data, analytical results that are below the detection limit can be evaluated in several ways. If all of the results from one or more treatments are less than the detection limit, then there is no transformation of the data that will satisfy the parametric analysis assumption of equal within-class variance. If those treatments are not the control, they can be removed from analysis. However, if the control (zero dose) has all values less-than-detected and there is no variation in the detection limit, then ideally, one should revert to testing each treatment compared with the detection limit. Alternatively, the nonparametric analysis using ranks (Steel 1961) can be used without requiring values such as zero, one-half the detection limit, or the detection limit substituted for less-than-detected results. If there is variation in the detection limit due to variable sample weights, one can substitute the detection limit for the less- than value and proceed as usual. The latter procedure has the undesirable property that the assumption of homogeneity may not be met, even after data transformation. Often analysis can proceed as normal if only a few values are less than detected. In this case, less-than values can be replaced with zero, one-half the detection limit, the detection limit, or a random number from zero to the detection limit. The latter substitution provides variation that is not provided with a constant substitution.

176. Finally, statistical significance and biological significance should not be confused. Unfortunately, statistical significance is often used in place of biological significance when little biological knowledge is available. If the sample size of a test is very large, the probability of detecting a statistically significant difference between means becomes large. A decision will be made for the number of replicates, number of concentrations, and number of fish per treatment level based on expected variabililty of endpoints measured, their biological significance and the costs associated with the optimal test design, meant to be used routinely. It should be verified through validation of the selected test design that the power of the assay and the need to detect biologically meaningful differences both address the objective of the screening assay.

9. CANDIDATE PROTOCOLS

177. In the following sections, four protocols have been chosen to be discussed in detail and in context with the fathead minnow, medaka and zebrafish as the test species. Two protocols use reproductively active fish and one protocol uses mature but non-reproductively active fishes while the last one uses immature fish which are exposed during the sexual differentiation. The candidate protocols considered here are 1) 21-day Fish Screening Assay (OECD Draft - 2003 which has been experimentaly validated during 2003 and 2004), 2) a 14-day reproductive assay (an abbreviated version of the Ankley et al. 2001 protocol), 3) a short-term (21-day) reproduction assay (USEPA 2002) and 4) a Fish Partial Life Cycle (developmental) Assay (submitted to the OECD by the Nordic countries 2001). In addition to the protocols considered here as promising, several other fish screening protocols have been described elsewhere using other fish species such as mosquitofish, stickleback, carp, and zebrafish (Angus et al., 2001; Gimeno, et al., 1998a and 1998b; Jakobsson, et al., 1999).

178. Two additional assays were considered but ultimately were not selected for detailed description here. These additional assays are a 14- day juvenile fish protocol that is primarily designed to detect (anti)-estrogens (Panter et al., 2002) and an assay that uses monosex carp that are genetic males. This latter assay has been used with both juvenile and adult carp in tests with weak estrogenic chemicals (Gimeno et al., 1998a; Gimeno et al., 1998b). It also should be pointed out that although some of the ensuing descriptions are discussed specifically for fathead minnows, they are also adaptable for the medaka and zebrafish.

179. A brief overwiew of the four candidate protocols is presented in Table 9.1. The underlying protocols are attached as Annexes B, C, D, and E, respectively.

Table 9.1: Overview of Candidate Protocols

Protocol	Species	Exposure period [d]	Pre-exposure period [d]	End points	Number of animals/treatment	Age at start of the test
FSA	**FH, JM, ZF**	21	0	• Gross morphology • Spawning status • Vitellogenin (blood) • Gonadal histology	2 x 5F + 5M	Reproductively mature females
RA-14d	**FH**, JM, ZF, SM	14	14 (observation of spawning)	• Adult survival • Reproductive behaviour • Gross morphology • Vitellogenin (blood) • Sex steroids concentrations • GSI • Gonadal histology • Fecundity • Fertility • Embryo hatch and larval survival • Hatching success and post-hatch viability	4 x 4F + 2M	Reproductively mature females
RA-21d	**FH**, JM, ZF, SM	21	21 (observation of spawning)	• Adult survival • Reproductive behaviour • Gross morphology • Vitellogenin (blood) • Sex steroids concentrations • GSI • Gonadal histology • Fecundity • Fertility • Embryo hatch and larval survival • Hatching success and post-hatch viability	4 x 4F + 2M	Reproductively mature females
FPLCT	**FH, JM, ZF**	60	0	• Hatching success • Larvae survival • Gross morphology • VTG (whole body) • Gonadal histology • Sex ratio	2 x 80	Newly fertilised eggs

10. ADDITIONAL DATA NEEDS AND IMPLEMENTATION CONSIDERATIONS

10.1 Animal Welfare

180. It is expected that the optimized protocol for the fish screening assay will obtain the maximum amount of information from the minimum number of animals in the least amount of time necessary to detect a potential endocrine active substance. The incorporation and the combination of several viable endpoints to detect disruption of male and/or female reproductive systems ensure that the most information possible is obtained from the test. As with most *in vivo* animal tests, the fish employed in this assay must be humanely sacrificed at the end of the test.

10.1.1 Rationale for the Need to Use Animals

181. To date, there is no validated test to replace the use of the whole animal model in the study of fish reproduction. Although there are several non-whole animal assays that are critical to identifying the mechanism and site of action of an EDC, exposure of the whole animal is necessary to evaluate the effect of a suspected EDC on the intricately related processes that define sexual development and reproduction in fish.

10.1.2 Relative Pain or Distress for Animals

182. It is not anticipated that the animals would suffer prolonged pain or distress. The study protocols necessitate that great care be given to test animals. Because the goal of the assay is to evaluate the effects of EDCs on reproduction, it is anticipated that the doses will be chosen such that there is little overt parental toxicity and mortality. Animals will be sacrificed humanely by using MS-222, a proven and effective fish anesthetic.

10.2 Information on Facilities and Major Fixed Equipment Needed to Conduct the Test

183. Any facility that conducts testing following government-approved guidelines has the necessary equipment to conduct the fish screening assay. However, it should be noted that those facilities that have in-house fish culture would be advantageous but not a prerequisite to running the test.

10.3 Additional Data Needs

184. Considerable efforts are being made to achieve standardisation of endpoint measurement and to have better knowledge about the natural variability of these endpoints with a view to facilitate test results interpretation. Standardisation of endpoint measurement is particularly crucial for *i)* biomarkers, which future use in regulatory work is conditioned by sensitivity and reproducibility, and *ii)* gonadal histology, for which no harmonised severity grading system exists yet.

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ANNEXES:

ANNEX A:

TECHNICAL ASPECTS OF MEASUREMENT OF BIOCHEMICAL ENDPOINTS

1.0 MEASUREMENTS OF SEX STEROIDS IN TISSUES

1. Sex-steroid hormones are primarily synthesized by somatic, and in some cases germ cells, within the gonads of bony fish (Ozon 1972a; Ozon 1972b; Vizziano et al., 1996). Other tissues, such as adrenal/interrenal or brain (Pasmanik et al., 1988), have the ability to synthesize specific sex-steroid products if provided with the appropriate precursors. Because of their lipophilic nature, steroids pass freely between cellular compartments. Sex steroids travel throughout the body most efficiently bound to plasma-binding proteins in the blood, with a small fraction present as free steroids (Petra 1991). Therefore, blood is a useful tissue to monitor to determine the endocrine status of sex steroids in fish.

2. In the intact animal, the blood vascular system is the most convenient and popular source to sample via heart puncture or caudal vessels with a needle and syringe for sex-steroid measurements in fish. It should be noted that toxicant exposure can alter red-blood-cell volume, resulting in a change in concentration of plasma constituents; therefore, hematocrit analysis is recommended as a preliminary step before quantification of steroids in plasma is initiated. A 100 µL blood sample will yield sufficient plasma (~40 µL) to permit detection of most of the common sex steroids with currently available assays, provided the fish has begun to sexually mature. In the case of small fish, lethal blood sampling, in which the tail is excised, might be required. In addition, when blood volumes are limited from small fish, pooling from a number of individuals might be necessary. Sex steroids have been measured in homogenates of small, whole fish with mixed results, depending on the assay used (e.g., fish embryos) (Feist et al., 1990; Okimoto et al., 1991). For sex-steroid measurement, considerations of body size, the number of individuals in each treatment group, and state of sexual maturity need to be incorporated into experimental and statistical designs.

3. A number of methods for quantification of sex steroids in body fluids of fish have been developed that differ in sensitivity, specificity, and technical difficulty. Currently, assays for individual steroids based on competitive binding with a specific antibody (e.g., RIA, ELISA) are most widely used. Depending on the specificity of the antisera, and lipids and proteins in the biological fluid being tested, some form of sample preparation might be required to remove interfering substances. Typically, solvent extraction (Scott et al., 1980a; Simpson & Wright 1977; http://www.shef.ac.uk/~spider/method_1.html) or some type of chromatography (e.g., HPLC, Sephadex LH-20, thin layer, or paper partition; Abraham et al., 1977) is used to prepare the sample. The reagents for sex-steroid RIA have been commercially available either in complete kit form or as individual components for over 30 years, whereas ELISA is a more recent development. Current gas chromatography-mass spectrometry (GC-MS) protocols that do not rely on ligand-antibody reactions hold promise for simultaneous quantification of steroid mixtures in small sample volumes.

1.1. Estrogens

4. Estrogens belong to the estrane (i.e., C_{18}) series of steroid hormones, with a phenolic group at Position 3. The ovaries of sexually maturing bony fish have a high synthetic capacity for estrogen

production (Vanbohemen & Lambert 1981). The principal estrogen in bony fish is 17_-E2, although estrone and estriol have also been detected (Ozon 1972a). In the female, estrogens are significant for their involvement with oocyte development and female sexual behavior (Fostier et al., 1983). The highest concentrations of E2 are found in body fluids, particularly blood plasma, of sexually maturing females. In bony fish, plasma concentrations of E2 range up to and exceed 100 ng E2/mL plasma, depending on the species and state of sexual maturity (Scott et al., 1980). Much lower E2 levels, with a maximum of usually not more than 1.0 ng E2/mL plasma, have been reported in the males of some species (Truscott et al., 1986; Makynen et al., 2000; Nichols et al., 1999). The adrenal/interrenal tissues or testes in the male might be responsible for this biosynthesis.

1.1.1. Radioimmunoassay (RIA

5. The principle of RIA is based on the reversible reaction between the antigen (the steroid hormone of interest) and a specific antibody that has been raised against the antigen. In the assay, a limited amount of specific antibody is reacted with the corresponding steroid labeled with a radioisotope (tritium or iodine) and differing amounts of unlabeled steroid in solution. Increasing the amount of unlabeled steroid competing for the antibody results in progressively less radiolabeled steroid bound to the antibody. The free steroid and that bound to the antibody can be separated, quantified with a radioactive detector, and these two fractions used to construct a standard curve against which unknown samples can be compared.

6. In the majority of studies to date on estrogens in fish, E2 has been quantified by RIA. These assays, when properly validated for the species under study, are sensitive (detection limit = 10 pg E2/mL plasma) and specific, with minimum cross-reactivity with other related steroids. The main consideration in the application of RIA is the volume of blood necessary to obtain sufficient plasma (i.e., 5 _L to 10 _L) to work with. When plasma E2 levels are low, the volume of blood (>50 _L) required may necessitate lethal blood sampling or pooling of blood from a number of individuals if the fish are small (i.e., <50 g).

7. Quantification of E2 by RIA has been done primarily to determine the sex of the fish when this cannot be easily deduced from external morphology, and/or to assess the state of sexual maturity in known females. Schulz (1984) showed that increasing concentrations of plasma E2 in female rainbow trout correlates well with a histological staging scheme based on increasing oocyte size, histological appearance of the ovaries, and GSI. The available data on plasma E2 measurements by RIA in fathead minnow in relation to a reproductive cycle are limited. Jensen et al. (2001) reported mean plasma levels peaking at 10 ng E2/mL one day after spawning before decreasing to ~4 ng E2/mL. Average plasma E2 concentrations are in the range of 6 ng E2/mL and 0.5 ng E2/mL in mature females and males, respectively. One study reports plasma E2 levels measured by RIA in mature female medaka during the daily period of vitellogenesis and spawning (Soyano et al., 1993). These data show low levels of 4 ng E2/mL plasma at 16 hours before spawning, with a peak of 16 ng E2/mL plasma at 8 hours prior to spawning before levels decrease again. There are no available plasma E2 data measured by RIA for zebrafish.

1.1.2 Enzyme-linked Immunosorbent Assay

8. The principle of ELISA is also based on the reaction between an antigen (the steroid of interest) and a specific antibody, one of which is passively adsorbed to a solid surface (e.g., the wells of a microtiter plate) (Crowther 1995). There are a variety of schemes for ELISA, but the one used most widely for sex-steroid measurements is a modification of the direct-labeled antigen method (Varadaraj 1990). In this ELISA, the antibody is attached to the solid phase via a secondary antibody, and the antigen, both non-isotopically labeled and unlabeled steroid, is added to compete for the antibody. The labeled steroid is modified by addition of an enzyme moiety (e.g., acetylcholinesterase). After a suitable incubation period, the steroids are washed out of the well, and color development reagents are added to react with the bound

labeled steroid remaining. Provided the amount of labeled steroid is held constant, increasing amounts of unlabeled steroid will reduce the amount of labeled steroid that is bound, resulting in a decrease in the amount of color development detected. Therefore, the degree of color development will be inversely proportional to the amount of unlabeled steroid added. The specific wavelength of the color produced is detected by a spectrophotometer. A standard curve can be developed with known amounts of unlabeled steroid, with which unknown samples can be compared.

9. Commercially developed ELISA kits for E2 (e.g., Cayman Chemical) are available, but have not been applied extensively to measurement of E2 levels in fishes. A limited number of individual research laboratories have reported E2 ELISA methods used on fish plasma with in- house developed reagents (Asahina et al., 1989; Okimoto et al., 1991; Varadaraj 1990). These assays are sensitive (detection limit = 10 pg E2/mL plasma) and specific, with minimum cross- reactivity with other related steroids. It appears that ELISAs are not suitable for quantifying E2 in whole-body homogenates of fish because of protein or lipid interference in the assay (Okimoto et al., 1991). Similar to RIA, the principal consideration in the application of ELISA is the volume of blood necessary in order to obtain sufficient plasma (i.e., 5 _L to 10 _L) with which to work, in conjunction with the reproductive status of the test fish.

10. There are no studies that have used ELISA to measure plasma E2 levels over a reproductive cycle in any fish species. However, based on the suitability of ELISA for quantification of E2 in fish plasma samples, it could be used for the same purposes for which RIA has been applied. Available data on plasma E2 measurements by ELISA in fathead minnow are reported in studies using sexually mature fathead minnows (Kramer et al, 1998; Sternberg & Moav 1999). The reported mean plasma levels are in the range of 3 ng E2/mL to 11 ng E2/mL in females and 1 ng E2/mL to 6 ng E2/mL in males. The E2 levels in female fathead minnows measured by ELISA are comparable to data determined by RIA in this species, but levels assayed by ELISA in males are higher than those reported by RIA in this species (Jensen et al., 2001). Further study is needed to elucidate whether this is a methodological discrepancy or experimental variation. There are no available plasma E2 data measured by ELISA for medaka or zebrafish.

1.1.3 Liquid/Gas Chromatography with Mass Selective Detection (LC/GC-MS)

11. Analysis of sex steroids using gas-liquid chromatographic techniques and mass selective detection has been performed since the mid 1960s (see review of early studies by Sjovall & Axelson 1982). Routine analysis of sex steroids using mass spectrometry is a more recent occurrence with the advent of capillary chromatography columns and improvements in mass spectroscopy. Both liquid chromatography (LC) and GC methods have been applied, although GC-MS is more commonly used. As a result, the ensuing discussion is primarily focused towards GC-MS techniques. The basic principle behind GC/LC-MS detection is to rely on chromatography to separate the steroid from contaminating substances, allowing the steroid to enter the ion source of the spectrometer in a purified form. The MS detector then measures ion fragments characteristic of the particular steroid being analyzed. The configuration of MS instrumentation varies as continued advances in MS occur, although electron impact, positive and negative chemical ionization are the three principle ionization techniques (Wolthers & Kraan 1999). Regardless of the type of sex steroid to be analyzed (estrogens, androgens or progestins), four basic steps are involved in quantitative GC-MS (and to a lesser extent LC-MS) analysis of steroids in biological matrices: 1) Selection of internal standard, 2) Extraction from blood plasma (or tissue homogenates) and sample clean-up, 3) Chemical derivatization of the non-volatile steroids (primarily used in GC-MS only), 4) MS analysis.

12. As with any quantitative analytical method utilizing chromatography, an internal standard(s) is added to account for sample loss during subsequent steps in the analysis and fluctuations in detector response. Preferred internal standards for MS analysis are isotopically labeled steroid analogues, especially those synthesized with carbon-13, which typically contain minimal quantities of unlabeled

steroid (Wolthers & Kraan 1999). The internal standard is added directly to the sample prior to extraction and later during the MS detection, a minimum of two fragment ions are simultaneously measured that originate from the endogenous steroid and labeled analogue. The ratio of the respective peak heights (or peak areas) is then used for quantitation. For some sex steroids such as E2 and T, ^{13}C-labeled analogues are available at a reasonable cost. However, for other reproductive hormones such as 11-KT, this may not be the case and structural analogues, as opposed to isotopic analogues, are used as internal standards. In the case of 11-KT, non-endogenous anabolic steroids such as 19-*nor*-testosterone can be used as an internal standard (Schultz et al., 2001).

13. Extraction of sex steroids from biological matrices is normally accomplished using a combination of liquid-liquid extraction and/or solid phase extraction (SPE) procedures. For liquid-liquid extractions, non-polar solvents such as hexane, dichloromethane or methyl-tert- butyl ether are used (Wolthers & Kraan 1999; Schultz et al., 2001). For extraction from tissue homogenates, diethyl ether is preferred over dichloromethane (Zerulla et al., 2000). The solvent extract is then evaporated to dryness prior to derivatization or further purified using solid phase extraction (SPE) procedures after reconstituting in an aqueous solution. The preferred SPE matrix is C-18 (for silica based stationary phases) in addition to several types of polymeric resins that have been demonstrated to posses excellent retention for estrogens and progestins (de Alda & Barcelo 2001).

14. Analysis of steroids by GC-MS requires derivatization of the steroid molecule. A variety of derivatizing agents have been developed for steroid quantification and the selection of the appropriate agent is often the critical step for the analysis procedure. Important considerations for any derivatizing procedure are stability of the derivative and generation of a single derivative with at least one fragment ion greater than 400 *m/z* (Wolthers & Kraan 1999). Commonly used derivatizing agents for analysis of estrogens, androgens and progestins are silylating agents such as N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) and trimethylsilyl-imidazole (Ferchaud et al., 1998; LeBizec et al., 1993) in addition to fluoroacyl derivatives such as pentafluoropropionic acid (LeGrand et al., 1995) or heptafluorobutyric anhydride (Dikkeschei et al., 1991). The MSTFA derivatizing agent is also suitable as a GC solvent allowing direct injection of the derivatization mixture onto the GC without additional clean-up or extraction. Derivatization with fluoroacyl agents is more labor intensive as post-derivatization clean-up / extraction steps are typically required (Croley et al., 2000). However, fluoroacyl agents may offer advantages over silylating agents due to a lack of interference caused by the high natural abundance of ^{29}Si and ^{30}Si (Wolthers & Kraan 1999).

15. After derivatization the sample can be analyzed by GC-MS. For highest sensitivity the MS is operated in the selected ion-monitoring (SIM) mode. Important considerations for increasing reproducibility and sensitivity are maintaining a signal to noise ratio > 200 and, when using isotopically labeled steroid analogues, determination of the upper and lower limits of linearity for the ratio of the fragment ion(s) of interest (Wolthers & Kraan 1999). In the latter case the ratio can deviate from linearity at extreme ranges in concentration due to contaminating labeled steroid naturally occurring in samples and/or unlabeled steroid that may be present in the synthesized internal standard.

16. In conclusion, MS detection of sex steroids has become a widely used technique. Because of the requirement of more specialized and expensive equipment (e.g., an MS), GC/LC-MS is less used in endocrinology studies compared to immunoassays. However, MS measurements can be particularly valuable as a reference method for sex steroid measurements. Properly validated MS protocols are highly reproducible during inter-laboratory comparisons, an important requirement of any reference analytical procedure (Wolthers & Kraan 1999). This latter point may be important for screening assays because interlaboratory studies of sex hormones in fish tissues can be highly variable (McMaster et al., 2001).

1.2 Androgens

17. Androgens belong to the androstane (i.e., C_{19}) series of steroid hormones. A number of androgens have been reported from bony fish tissues, including T, 11-KT, androstenedione, dehydroepiandrosterone, and androsterone (Ozon 1972b). The most prominent and reproductively important androgens appear to be 11-KT and T in the male (Idler, Horne, & Sangalang 1971) and T in the female (Schmidt & Idler 1962). In the male, these androgens are mainly synthesized in the testes and have been shown to affect germ-cell development, male sexual behavior, and secondary sexual characteristics (Fostier et al., 1983; Fostier et al., 1987). The ovary of the female synthesizes androgens, primarily T, as a precursor that is aromatized to E2. The plasma concentrations of 11-KT in the sexually maturing male can range up to 200 ng 11-KT/ml plasma and are higher than the levels of T, which are usually about 10-fold lower in a given species. Plasma T levels in sexually maturing females can be considerable, with levels up to 100 ng T/ml plasma (Scott et al., 1980a; Nagler & Idler 1992), whereas 11-KT is usually non-detectable. These levels will vary considerably depending on the species and state of sexual maturity.

1.2.1 Radioimmunoassay

18. The principal for RIA of androgens is similar to that used for E2. The reagents for T RIAs are commercially available either in complete kit form or as individual components. Testosterone RIAs are sensitive (detection limit = 10 pg T/mL plasma) and specific, with minimal cross-reactivity to related androgens. An RIA in complete kit form or individual reagents (i.e., radioligand, antibodies) for 11-KT is not available commercially. However, 11-KT radioligand and antisera have been prepared by a number of individual laboratories (Idler & Ng 1979; Schulz 1984; Scott et al., 1980a; Simpson & Wright 1977). A survey of 11-KT RIAs developed by individual laboratories is comparable in sensitivity and specificity with those for T.

19. Androgen measurements by RIA in fish, principally 11-KT, are done to determine the sex of the fish and/or assess the state of sexual maturity in known males. Similar to what has been done in the female, a correlation between the concentration of plasma 11-KT and a histological staging scheme based on histological appearance of the testis and GSI has been produced for the male rainbow trout (Schulz 1984). It shows distinctive variations in plasma 11-KT levels with different phases of testicular development. In the mature male fathead minnow, mean plasma levels of 33 ng 11-KT/mL and 9 ng T/mL were found during the spawning period, with no distinct variations observed (Jensen et al., 2001). In mature female fathead minnows the mean T level was 3 ng T/mL, with 11-KT occasionally detectable with a mean level of 0.3 ng 11- KT/mL. There are no available plasma androgen data measured by RIA for medaka or zebrafish.

1.2.2 Enzyme-linked Immunosorbent Assay

20. The principle of ELISA for the measurement of androgens is similar to that used for E2. Reagents for T or 11-KT ELISAs are commercially available in complete kit form (e.g., Cayman Chemical), or as individual components from independent research laboratories. Both androgen ELISAs appear sensitive (detection limit = 10 pg/mL plasma) and specific, with minimal cross-reactivity to related androgens.

21. There are no studies that have used ELISA to measure plasma T or 11-KT levels over a reproductive cycle in any fish species. However, based on the suitability of ELISA for quantification of T and 11-KT in fish plasma samples, it could be used for the same purposes for which RIAs have been applied. Measurements by ELISA for androgens in fathead minnow plasma are restricted to one study in which the state of sexual maturity is reported. Nichols et al. (Sternberg & Moav 1999) showed mean plasma T levels in mature females to be 0.95 ng T/mL to 6.9 ng T/mL, and 0.77 ng T/mL to 6.9 ng T/mL

in mature males. There are no reports of 11-KT measured by ELISA from fathead minnow, or either androgen (T or 11-KT) in medaka or zebrafish.

1.3 Progestins

22. Progestins belong to the pregnane (i.e., C_{21}) series of steroid hormones. A number of progestins have been detected in bony fish, including progesterone, pregnenolone, 17á- hydroxyprogesterone, 17,20-DHP, 17,20,21-THP, and 3â,17á,20á-trihydroxy-5â-pregnane (Canario & Scott 1991; Idler, Fagerlund, & Ronald 1960; Petrino et al., 1989; Scott et al., 1982; So et al., 1985; Trant et al., 1986; Truscott et al., 1986; Truscott et al., 1992). Progestins usually appear suddenly in the blood and in high concentration during the final stage of reproductive development in the female and male (Scott et al., 1983; Truscott et al., 1986). They have been shown to be critical for the final maturation of the gametes from both sexes, and are often referred to as the maturation-inducing steroid. Good evidence suggests that significant species specificity exists with respect to the predominant maturational progestin. In salmonids, 17,20-DHP is the major progestin, whereas in some perciforms, such as the Atlantic croaker, 17,20,21-THP is the major form. Studies on the medaka (Iwamatsu et al., 1987; Sakal et al., 1987) and zebrafish (Selman et al., 1993) suggest that 17,20-DHP is produced by ovarian follicles and is the most effective progestin for initiating final maturation. Detailed studies of plasma levels of 17,20-DHP in both sexes are lacking. It is not known in the fathead minnow which progestin(s) is most important or would best serve as an indicator of progestin status.

1.3.1 Radioimmunoassay and Enzyme-linked Immunosorbent Assay

23. The principles of RIA and ELISA for progestin measurement are similar to those used for estrogens and androgens. Radioimmunoassays for any of the progestins considered to be maturation-inducing steroids in fishes (e.g., 17,20-DHP or 17,20,21-THP) are not commercially available. Individual reagents for some progestins in the form of radioligand or antibodies can be obtained from individual investigators (Scott et al., 1997; Scott et al., 1982). Plasma progestin data measured by RIA are not available for fathead minnow, medaka, or zebrafish. An ELISA for 17,20-DHP is commercially available (e.g., Cayman Chemical), and a 17,20-DHP ELISA has been applied to measuring plasma levels in a single female rainbow trout (Asahina et al., 1989). However, this ELISA has not been used to quantify plasma levels of 17,20-DHP in fathead minnow, medaka, or zebrafish.

1.4 Summary

24. Currently, the most popular and durable assays available for measuring sex steroids in fish body fluids are antibody-based, either RIA or ELISA. Both are equally suitable, sensitive, and specific for detecting the common sex steroids of interest (E2, T, 11-KT) in fish blood plasma. With respect to the three test species advocated in this paper, an important consideration for both methods is the minimum volume of plasma required for the assay (5 _L to 10 _L). The size of the fish test species and corresponding blood sample size possible (2.5 times plasma volume) might necessitate lethal blood sampling and/or pooling of plasma samples from a number of individuals. A larger volume of plasma might be required to detect these sex steroids if the fish is sexually immature or steroid production has been depressed as a result of exposure to an endocrine disruptor. These factors need to be incorporated into experimental and statistical designs.

25. In comparing the two antibody-based methods, RIA and ELISA, a major disadvantage for the RIA emerges involving radioisotopes. The handling of radioisotopes in RIA, the equipment required for radioactive detection, and problems with radioisotope disposal are less desirable compared with ELISA. Future trends will likely see the ELISA replace the RIA in most instances.

26. Regardless of the assay used, there is a major lack of baseline information on plasma E2 and androgen levels throughout the period of sexual development in medaka and zebrafish. Until this information is obtained, the usefulness of estrogen or androgen measurements as an endpoint for endocrine disruptor screening in these species will be limited. Although plasma estrogen and androgen data exist for sexually mature fathead minnow of both sexes, data are limited to a few studies and have not been related to histological development of the gonads. No information exists for plasma sex steroid levels in juvenile (i.e., sexually immature) fathead minnows. The situation is more serious for progestins (i.e., maturation-inducing steroid) in all four species. Although 17,20-DHP appears to be an important progestin during final maturation in medaka and zebrafish, plasma levels during this latter phase of reproductive development are not available for these species. Which progestin is the major steroid form in fathead minnow is not known, although 17,20-DHP is a likely candidate, because it is prevalent in the zebrafish and other cyprinids (e.g., goldfish). This lack of information is a serious impediment to the use of progestin status as an endpoint for endocrine-disruptor screening in these fish.

27. Besides the value of individual plasma concentrations of sex steroids as indicators of sexual maturity or biomarkers of endocrine disruption, estrogen and androgen measurements from the same fish can be used in concert as an estrogen:androgen ratio. This type of analysis has been correlated to pesticide content of the water from which fish were sampled (USGS 1997), and a significantly negative correlation has been demonstrated in female carp with increasing pesticide exposure. This type of sex-steroid analysis could be of value for endocrine- disruptor screening and should be explored in the future.

2.0 MEASUREMENT OF VITELLOGENIN

28. A number of methods have been developed for the quantification of VTG in blood plasma, liver tissue, or whole-body homogenates. As discussed previously for sex steroid measurement, the various methods differ in sensitivity, specificity, and technical difficulty. Currently, the most popular approach to measure VTG is some form of an ELISA, although RIAs may occasionally be employed. These antibody-based procedures for VTG quantification may use antisera prepared against VTG obtained from a fish species different from the species under study. Although antisera can cross react with VTG from multiple fish species, the affinity can vary substantially (Silversand et al., 1993). The small body size of fish species used in screening assays restricts the quantity of VTG that can be obtained for use in antisera preparation. Difficulties in obtaining adequate quantities of VTG is a concern for the development of a

29. Standard procedure for VTG measurement, as batch differences in binding affinity for VTG could be excessive. The difficulties in obtaining adequate quantities of VTG from the small test species can however be solved e.g. by using lipovitellin as antigen and standards. Lipovitellin is immunologically identical to VTG and has been implemented successfully in VTG ELISA´s (Holbech et al., 2001) or by VTG purification from whole body homogenate from adults exposed to estrogen (Brion et al., 2002).

30. In the ensuing sections, various methods of VTG quantification are discussed in detail Initially, a brief review of indirect methods of VTG measurement are discussed, although these procedures are no longer in use because of the wide availability of VTG antisera. To complete this section, we briefly discuss the potential for mass spectroscopy as an analytical tool for VTG quantification, with an emphasis on its ability to offer a specific and highly quantitative alternative to antibody-based procedures.

2.1 Indirect Quantification of Vitellogenin Protein: Alkaline-labile Phosphate Assay

31. A variety of techniques have been used to quantify plasma VTG in naturally reproducing females or to measure induction in fish exposed to estrogens or xenoestrogens. In addition to applying the immunologically-based RIA or ELISA for direct quantification of plasma VTG, the measurement of alkali-labile phosphoprotein phosphorus (ALPP) has been used to indirectly quantify VTG. Vitellogenin is a glycolipophosphoprotein complex that contains significant amounts of phosphorus. For example, in rainbow trout, the phosphoprotein phosphorous content of VTG is 0.6% (Sumpter 1985). Therefore, increasing levels of VTG in plasma can be indirectly measured through increases in phosphoprotein phosphorous content. To measure ALPP plasma proteins are precipitated, delipidated, and hydrolyzed, and the phosphorous content is measured colorimetrically relative to a phosphorous standard curve. When ALPP and VTG (RIA) were simultaneously measured in plasma from individual mature female rainbow trout, a linear relationship between the two measures was established (Nagler et al., 1987). Although this relationship allows the indirect measure of VTG in the later stages of vitellogenesis, the authors did not find experimental evidence that ALPP could be reliably used to indicate the lower levels of VTG found in early vitellogenesis. Direct measurement of VTG, by RIA or ELISA, is recommended to quantify the lower levels of VTG in fish plasma found at the onset of vitellogenesis.

2.2 Direct Quantification of Vitellogenin Protein: RIA

32. The principle for RIA of fish VTG is similar to that used for sex steroids. In this instance, the antigen is VTG from the test species of interest, and an antibody against this antigen is used. The VTG ligand is usually isotopically labeled with iodine. A number of VTG RIAs for different fish species have been reported from individual research laboratories (So et al., 1985; Diamond & Oris 1995; Goolish et al., 1998; Kim & Cooper 1999), based on the first VTG RIA developed for rainbow trout (Sumpter 1985). A commercial fish VTG RIA has never been marketed.

33. The measurement of plasma levels of VTG by RIA in fish was first done to understand the process of vitellogenesis and ovarian development in oviparous species (Scott & Sumpter 1983). It has been used to determine the sex of immature fish when this cannot be determined from external morphology, or to assess the state of sexual development in known females. Fish VTG RIAs, when properly validated, are sensitive with detection limits down to 10 ng VTG/mL plasma. Although VTG antisera is species-specific (So et al., 1985), VTG antibodies from some fish have shown sufficient immunological cross-reactivity to be able to quantify plasma VTG in a wide variety of related species (Guram & Boatwright 1989; Wiegand et al., 1999). Similar to sex-steroid RIAs, blood volume is a consideration to obtain sufficient plasma. However, plasma VTG levels can be in the mg VTG/mL plasma range in actively vitellogenic females, and plasma samples routinely need to be diluted with assay buffer before they will fall within the range of the standard curve. Therefore, plasma sample volumes of 5 _L would be sufficient in instances when VTG levels are expected to be elevated.

34. Plasma VTG levels measured by RIA have been used in the past as an indicator of reproductive impairment in fish toxicological studies (Creech et al., 1998; Nagler et al., 1987; Suedel Burton 1997). Because the endocrine regulation of VTG synthesis in the liver is estrogen-dependent, and because the male also carries the VTG gene, VTG induction in male fish became the obvious model to apply to situations in which environmental estrogens were suspected. The landmark study by Purdom et al. in 1994 exemplified the utility of measuring plasma VTG levels in male fish exposed *in situ* in rivers contaminated with endocrine disruptors. The induction of VTG has been shown to be a sensitive and reproducible indicator of estrogen exposure in fish, and measurement of this endpoint by RIA has been used in laboratory studies (Thorpe et al., 2001) and field experiments (Shinomiya 1995). An RIA suitable for quantification of cyprinid plasma VTG, such as fathead minnow and zebrafish, has been published (Guram & Boatwright 1989), although levels throughout a reproductive cycle in either species have not

been reported. Plasma VTG data measured by RIA are not available for zebrafish. The use of RIAs to measure fish VTGs will likely be superceded by ELISAs, which obviates the need for radiolabeled ligand.

2.3 Direct Quantification of Vitellogenin Protein: Enzyme-linked Immunosorbent Assay

35. In addition to the RIA, VTG has been measured by other immunoassay techniques including ELISA, immunodiffusion, and immunoblotting. The ELISA employs enzyme-linked antibodies and an adsorbent surface to detect specific antigens in solution. The ELISA has been widely used to quantify VTG in teleosts due to the ease in use and, unlike the RIA, ELISA does not require the use of radioactive isotopes. When developing and validating an ELISA, researchers seek to achieve sensitivity comparable to the low detection levels achieved with RIA, while minimizing the time and steps necessary to perform a robust ELISA. There are a variety of ELISA designs that typically fall into three general assay formats. These formats are described in the following sections and include competitive, sandwich, and direct ELISAs. Variations of each of these methods have been applied to the test species considered in this review. It should be noted that there is conflict in ELISA terminology in the literature. For the purposes of this review, the antibody-capture ELISAs employ antigen bound to the test plate. Similarly, the antigen-capture ELISAs quantify the amount of antigen captured by antibodies bound to the test plate. Within each of these three ELISA formats the resulting signal can be directly linked to the target analyte, sometimes referred to as a direct ELISA, or the signal can be amplified with additional interactions in an indirect ELISA. In this review, the term direct will be used to denote ELISAs that apply the VTG sample directly to the test plate. An informative overview of ELISA and the approach used to develop an assay is presented in Specker and Anderson (1994).

2.3.1 Competitive Enzyme-linked Immunosorbent Assay

36. Competitive ELISAs incorporate a step in which the samples and antibody (antibody-capture) or labeled antigen (antigen-capture) are incubated together prior to adding the sample on the test plate. This non- equilibrium design is often used to enhance sensitivity and counteracts potential preferential binding (Edmunds et al., 2000). For example, in the antibody-capture ELISA, the incubation period allows for affinity differences of the antibody to the antigen bound to the test plate compared with the antigen in solution in the sample. To achieve high sensitivity, this pre- incubation step is typically conducted for 16 hours at 4ºC (Blader & Strahle 1998; Edmunds et al., 2000; Giesy et al., 2000; Maack et al., 1999; Rodina & Horvath 1999; Shepherd et al., 2001), and the amount of antibody or labeled antigen captured on the test plate is inversely proportional to the amount of antigen present in the sample.

37. *Antibody-capture.* In an antibody-capture competitive ELISA, a known amount of antigen is coated onto the surface of the microwell plate, and the binding sites that remain free of antigen are blocked. Known amounts of the primary antibody are pre-incubated with samples, or standards, containing variable amounts of antigen. The antigen binds to the antibody such that if a sample has a high level of antigen, only a small amount of antibody will be free to bind to the antigen coating the plate. Following this pre-incubation, the samples are added to the wells of the plate, and the antibodies that did not bind to the antigen in the sample bind to the test plate. In the next step, a known amount of the enzyme-linked secondary antibody specific to the primary antibody is added to the wells. An enzyme substrate is added and the color change is measured by a microwell plate reader. Because of the competitive nature of this ELISA, the color change is inversely proportional to the antigen present in the sample.

38. In the late 1980s, Nunez Rodriguez et al. (1989) developed and validated an antibody- capture competitive ELISA for sole (*Solea vulgaris*) VTG. The assay used a polyclonal antibody to circulating VTG and was based upon the competition between soluble VTG in the samples and standards and the VTG adsorbed on the microwell plates. The time-course of the assay was relatively brief, the reagents were deemed stable and inexpensive when compared with other methods (RIA), the intra- and inter-assay

reproducibility provided comparable values, parallelism of the competition curves demonstrated the integrity of the assay, and the sensitivity was 2.5 ng/mL VTG in the sample assayed (125 ng/mL plasma). Following this protocol, antibody- capture competitive ELISAs were developed and validated for a wide range of teleosts, including fathead minnow (Nakamura 2000), and carp (Edmunds et al., 2000). The reported sensitivity of these homologous antibody-capture competitive ELISAs expressed as the lowest detectable standard in the working range of the assay (approximately 80% to 90% of binding) range from 1 ng/mL to 33 ng/mL in the sample analyzed, which is comparable to the sensitivity achieved with RIA and sandwich ELISA (Blader & Strahle1998; Edmunds et al., 2000; Giesy et al., 2000; Hatakeyama et al., 1999; Maack et al., 1999; Nakamura 2000; Rodina & Horvath 1999; Scholz & Gutzeit 2000; Shepherd et al., 2001; Tanaka et al., 2001; Tyler et al., 1999). It should be noted that although ELISA detection limits in the low ng/mL range are reported, the practical detection limit for accurate quantification of low levels of VTG in samples is significantly higher. This is because of the required dilution of the sample resulting from small plasma volumes available from individual test fish, or because of the need to eliminate interferences in the ELISA.

39. The homologous fathead minnow antibody-capture competitive ELISA developed by Parks et al. (1999) can be conducted within a relatively brief period of time and offers sensitivity on the lower end of the range of similar ELISAs. As demonstrated in other competitive, antibody capture ELISAs, the sensitivity could be increased at least 3-fold, to 1 ng/mL, by conducting the pre-incubation step overnight at 4ºC instead of briefly at 37ºC (Rodina & Horvath 1999).

40. In addition to developing and validating antibody-capture competitive ELISA for a specific species, this type of ELISA has been applied to detecting VTG in other species (Edmunds et al., 2000; Hatakeyama et al., 1999; Scholz & Gutzeit 2000). The homologous ELISA protocol established and validated for carp VTG (Edmunds et al., 2000) incorporates materials and methods that provide a robust assay. The working range of this assay is 1 ng/mL to 50 ng/mL analyzed and was applied to carp plasma and fathead minnow plasma and whole-body homogenates in this study. The authors note, however, that the absolute concentrations of VTG in fathead minnows using the carp ELISA might not be entirely accurate. The carp antibody was immunoreactive to a single major protein band in carp plasma and cross-reacted with two protein bands in fathead minnow plasma. The slopes of carp and fathead minnow curves showed differences that could reflect differences in binding affinity of the carp antibody to carp VTG, used in the assay for coating the plates and for standards, compared with the VTG in fathead minnow samples. The carp ELISA is offered as a valid method to quantify changes in concentrations of VTG in fathead minnow plasma and whole-body homogenates that can be used to discern males from immature females, the degree of sexual maturity of females, and response to estrogen. An inter-laboratory assessment of this carp ELISA applied to fathead minnow plasma and juvenile whole-body homogenates of fish exposed to estrogenic substances demonstrated VTG induction (Pickering 1983). The application of the assay to measure VTG induction in fathead minnows was further evaluated with nine synthetic and natural endocrine disruptors (Panter et al., 2000a).

41. *Antigen-capture.* Antigen-capture competitive ELISA follows a format similar to the antibody-capture competitive ELISA except that a known amount of antibody is immobilized on the plate. The samples are pre-incubated with a known amount of enzyme-labeled antigen prior to adding them to the coated plate. The amount of labeled antigen that binds to the plate is inversely proportional to the amount of antigen in the sample.

42. Reports of antibody capture competitive ELISA to measure VTG in fish were not found in the literature. This type of ELISA requires the conjugation of antigen for use in the competitive pre-incubation step. However, the conjugation of antigen does not offer the advantage of requiring less purified antigen to perform the assay, as necessary for the sandwich ELISA, and a labeled antigen introduces stability and antigen-recognition issues. The use of antibodies bound to the surface of a well plate to capture VTG

present in fish samples has been exploited with specificity and sensitivity in the sandwich ELISA detailed in a following section.

2.3.2 Direct Enzyme-linked Immunosorbent Assay

43. In a direct antibody-capture ELISA, the sample and standards are adsorbed directly on the surface of the microwell plate. After incubation, the wells are blocked and anti-VTG antibody is added to bind to the VTG attached to the well. As with other ELISAs, subsequent steps culminate in the development of color reflective of the amount of antigen present in the sample. Folmar et al. (2000) developed a direct ELISA for sheepshead minnow (*Cyprinodon variegates*) that was subsequently used in a study examining the induction of mRNA and VTG in fish exposed to E2 (Yaezawa et al., 2000). Although this direct ELISA used secondary antibodies and avidin-biotin complex to amplify the signal, the effective detection limit for plasma, with a required minimum dilution, was 2,000 ng/mL. The authors controlled for interferences in the binding of VTG in the plasma samples to the plate, which could result in an underestimation of the VTG present in the sample, by adding male plasma to the standards to create similar interference for the standard curve. This type of ELISA has been applied in semi-quantitative measurement of VTG in zebrafish (Van den Belt et al., 2001). Plasma samples were coated onto the plates and incubated with carp anti-VTG antibody, with a standard curve generated with plasma from E2 VTG-induced zebrafish, the concentration of which was determined by SDS page, western blotting, and densitometry.

44. Although direct ELISAs can offer fewer steps than do other ELISAs, they are subject to interferences in antigen binding to the microwell plate that can be difficult to discern and control. The problems of non-specific binding and incomplete antigen binding are more readily controlled in other antibody capture ELISAs, such as the competitive ELISA.

2.3.3 Sandwich Enzyme-linked Immunosorbent Assay

45. Sandwich ELISAs employ two antibody preparations to detect the antigen. The antigens can recognize different epitopes on the target analyte, thereby providing a large degree of specificity and sensitivity; however, this ELISA can require significant amounts of multiple antibodies (Cooke & Hinton 1999). In this ELISA, the wells of a microwell test plate are coated with the primary antibody and samples, or known amounts of antigen to generate a standard curve, are then added to the wells. The primary antibody on the well captures the antigen in proportion to the amount present in the sample. The test plate is washed to remove any unbound antigen and an enzyme-labeled secondary antibody is added to the wells. This secondary antibody binds to the antigen captured by the primary antibody in the well, thus creating a sandwich of antigen and two antibodies. The wells are washed to remove unbound secondary antibody, and a substrate for the enzyme bound to the secondary antibody and a chromogen are added; the change in color in the individual wells is measured by an automated microwell plate reader.

46. Aspects of the materials and methods for the teleost sandwich ELISA are similar to the competitive antibody-capture ELISA with the following exceptions. For the secondary antibody, Fab' fragment is prepared from the rabbit IgG and conjugated with enzyme (e.g., horseradish peroxidase). The plates are coated with primary antibody, typically 200 _L of 40 _g/mL, overnight at 4ºC. The plates are blocked (e.g., BSA), and the samples are diluted and are added directly to the antibody wells. The remaining steps are similar to the competitive antibody- capture ELISA.

47. In the late 1980s, with the recent application of ELISA to measure VTG in fish-blood plasma with a competitive antibody-capture ELISA (Kiparissis & Metcalfe 1997; Kwon et al., 2000; Shinomiya et al., 1997) developed a homologous sandwich ELISA to detect VTG in the plasma of whitespotted charr (*Salvelinus leucomaenis*). Subsequently, this method was applied to developing and validating similar

ELISAs (Bauer & Goetz 1998; Grant 1995; Miles- Richardson et al., 1999a; Wester & Van 2000). In developing the sandwich ELISA for charr, Kwon et al. (2000) prepared antibodies in this ELISA against lipovitellin. Similarly, Okumura et al. (1995) developed a sandwich ELISA for the Japanese eel with anti-lipovitellin antibodies, and the secondary antibody was biotinylated (with steptavidin-HRP steps) to magnify the signal with a detection limit of 0.8 ng/mL. Korsgaard and Pedersen (1998) developed a sandwich ELISA and achieved a detection limit of 1 ng/mL VTG with affinity purification applied to increase the specificity of the anti-VTG antibodies and careful blocking procedures.

48. A homologous zebrafish sandwich ELISA using anti-lipovitellin antibodies and lipovitellin to generate a standard curve with a detection limit of 0.2 ng/mL sample analyzed has recently been developed and applied to whole body homogenates (Holbech et al. 2001). Purified carp VTG and anti-VTG carp antibodies, or a complete carp VTG sandwich ELISA kit, are commercially available (Biosense). This carp-based sandwich ELISA can be used to measure fathead minnow VTG, and sandwich-based kits to detect zebrafish and medaka VTG are under development. The carp VTG sandwich ELISA kit has a detection limit of 0.2 ng/mL sample analyzed, with intra- and inter-assay variation of 3% to 8%, and this ELISA can be applied to the fathead minnow to measure VTG induction.

49. In summary, the homologous competitive antibody-capture has been developed for the fathead minnow (Nakamura 2000) and for carp (Edmunds et al., 2000), which has been applied to a variety of species, including the fathead minnow. An inter-laboratory assessment of this carp ELISA applied to fathead minnow plasma and juvenile whole-body homogenates of fish exposed to estrogenic substances demonstrated VTG induction (Pickering 1983). The application of this assay to measure VTG induction in fathead minnows was further evaluated with nine synthetic and natural endocrine disruptors (Panter et al., 2000a). A semi-quantitative direct homologous antibody-capture ELISA (Van den Belt et al., 2001) and a quantitative homologous sandwich ELISA have been developed and validated for zebrafish (Brion et al., 2002; Fenske et al., 2001; Holbech et al., 2001). A carp VTG-based sandwich ELISA kit is commercially available for measuring fathead minnow VTG; similar kits are available or in development for zebrafish and medaka. In addition, an ELISA using an anti-carp VTG antibody has been applied to zebrafish; however, full details of the ELISA method were not given (Kime & Nash 1999). To date, direct inter-comparisons of these ELISA methods by measuring VTG in plasma and tissue samples of known concentrations have not been conducted.

50. The ELISAs that offer ease of use, sensitivity, and specificity for measuring VTG in the test species include the competitive antibody-capture and sandwich ELISA. The sensitivity of the competitive antibody-capture and sandwich ELISAs are similar, with slightly greater sensitivity achieved with sandwich ELISAs. These ELISA methods have been successfully applied to plasma and tissue homogenates for the test species. The homologous competitive antibody-capture ELISA for carp (Edmunds et al., 2000) has been tested in an inter-laboratory assessment to measure VTG induction in fathead minnows (Pickering 1983) and was further evaluated with natural and synthetic endocrine disruptors (Panter et al., 2000a). The use of purified VTG homologous to the test species to produce antibodies and as standards offers direct quantification and specificity. However, methods based on the cross-reactivity of anti-VTG carp antibodies can be used to measure VTG induction in the test species. Purified carp VTG and anti-VTG carp antibodies are available commercially. Accurate quantification of VTG can be achieved with the sensitive carp sandwich ELISA through the use of homologous VTG to prepare the standard curve in the assay.

2.4 Quantifying Vitellogenin mRNA

51. An alternative to measuring the VTG protein is to quantify the messenger ribonucleic acid (mRNA) for VTG that codes for the protein. In oviparous fishes, the liver of both sexes contain VTG gene(s) that are responsive to E2 (Carvan et al., 2000b; Mallett 1997; Riehl et al., 1999). Estrogen mimics

will upregulate VTG mRNA transcription, which has been shown to be a sensitive indicator of exposure to these compounds (Skinner et al., 1999). There is also evidence that physiological levels of androgen alone can increase VTG mRNA expression by male rainbow trout hepatocytes in vitro (Kawahara & Yamashita 2000). Vitellogenin mRNA expression might not, however, result in translation of protein, because plasma VTG levels in male trout are typically very low (Copeland et al., 1986).

52. Two major requirements for the quantification of VTG mRNA need to be realized. First, it is required that some knowledge of the DNA (or RNA) sequence corresponding to the protein coding region of the VTG gene be known for the fish species of interest. This understanding is needed to design specific probes or primers to detect the VTG sequence. Secondly, fresh liver tissue needs to be harvested from the test animal and, if not extracted immediately for RNA, the tissue must be frozen quickly to prevent mRNA degradation. If the fish species is small in size, sample procurement will necessitate killing the animal, although technically the minimum mass of the liver required for mRNA isolation is modest (i.e., 5 mg to 10 mg). Therefore, individual measurements from any of the three test species should be possible. Larger fish (i.e., >100 g) could be biopsied for liver tissue non-lethally under anesthesia, but this has not been reported. Two methods for quantifying fish VTG mRNA have emerged, the ribonuclease protection assay (RPA) and quantitative reverse transcription-polymerase chain reaction (QRT-PCR), although other methods exist (e.g., Northern blot, slot-blot) that have drawbacks relative to sample throughput or sensitivity. All methods can be used for absolute or relative quantification of mRNA.

2.4.1 Ribonuclease Protection Assay (RPA)

53. The quantification of VTG mRNA by RPA relies on hybridization in solution of a homologous antisense RNA probe (either radiolabeled or non-isotopically labeled) with sample RNA extracted from the liver of the test species. Probes are designed to be 200 to 400 nucleotides long. After the hybridization step, any RNA that has not hybridized to the probe (i.e., any single-stranded RNA) is degraded using ribonucleases. The remaining hybridized RNA fragments (target + probe) are electrophoresed on an acrylamide gel and the amount of radioactivity quantified. In the case of a non-isotopically labeled probe, the samples are transferred from the gel to a membrane and measured with a secondary detection method (e.g., chemoluminescence). Molecular biological kits to conduct RPA are commercially available (e.g., Ambion) and have been adapted for fish VTG mRNA measurement (Kishida & Callard 2001) or developed in-house from published methods on the technique (Spitsbergen et al., 2000).

54. The advantages of RPA are sensitivity (e.g., 10- to 100-fold greater than a Northern blot), and that partial RNA sample degradation is not a problem because the fragment size (200 to 400 nucleotides) is small. The major drawback of RPA is the need to electrophorese each sample on a gel and, when a non-isotopically labeled probe is used, transfer it onto a membrane. This increases sample-to-sample variation and could limit this method for high-sample throughput. Care must be taken when handling RNA probes to prevent degradation. The option of non- isotopic labeling of the probe will circumvent the use of radioisotopes.

55. In response to E2 injection, fathead minnow VTG mRNA has been measured in liver samples by RPA and shown to be very sensitive, 1000-fold more so than VTG protein detected by ELISA (Kishida & Callard 2001). The measurement of medaka or zebrafish VTG mRNA has not been attempted with RPA, although zebrafish VTG sequence information is available (Kuwahara et al., 2000) such that probes could be designed.

2.4.2 Quantitative Reverse Transcription-Polymerase Chain Reaction (QRT-PCR)

56. In QRT-PCR, all the mRNAs in a liver RNA sample from the test fish are copied into complementary DNAs (cDNAs) using oligo-dT primer and reverse transcriptase (RT). Alternatively, a

species-specific VTG 3'-primer can be used in the RT reaction to yield only VTG cDNA. Independent of the RT method used, the next step uses polymerase chain reaction (PCR) to preferentially amplify the cDNA using VTG sequence-specific primers. Subsequent detection of VTG PCR products currently follows two routes. The first involves electrophoresis of the DNA products on an agarose gel. If radiolabeled nucleotides or primers were used, the gel is dried and some means of detection (e.g., phosphoimager, X-ray film) is used to quantify the radioactivity associated with the DNA products. If not isotopically labeled, the DNA products are detected using DNA-binding dyes (e.g., ethidium bromide, SYBR green) (Van Den et al., 1987). The other detection method uses real-time PCR in which a fluorescent probe, specific to the amplified DNA sequence, is used to detect the amount of product produced (Yasuda et al., 2000). The amount of fluorescence is monitored at the end of each cycle of PCR (i.e., real-time), and the amplification curve is recorded by computer. With either detection scheme, the amount of DNA product measured is assumed to be directly proportional to the amount of starting mRNA. This measurement is compared with known amounts of VTG mRNA that have been included in the assay to determine the amount of starting mRNA target. A number of commercially available QRT-PCR kits (e.g., Life Technologies; Applied Biosystems) are currently on the market that could be adapted for species-specific VTG mRNA measurements.

57. Quantitative reverse transcription-polymerase chain reaction is the most sensitive technique for mRNA quantification and, similar to RPA, slight sample degradation is not a problem because the DNA product can be quite small (e.g., ~200 nucleotides in real-time PCR). For the detection of DNA products using QRT-PCR, real-time PCR will replace detection on agarose gels, since it obviates the need for electrophoresis and handling radiolabeled DNA. It has the added features of automation and high sample throughput (e.g., 384-well sample plates). The disadvantages of QRT-PCR are the initial optimization experiments necessary to develop suitable primers and probes.

58. The application of QRT-PCR has been tested for measuring the VTG responses in rainbow trout exposed to estrogenic compounds. Celius et al. (2000) showed that E2 and _-zearalenol, when injected into juveniles, elevated liver VTG mRNA levels, and that this method was more sensitive than quantifying plasma VTG by ELISA. In vivo exposure to nonylphenol during early life-history periods in rainbow trout also elevated VTG mRNA, as measured by QRT-PCR (Powell 1996). The measurement of fathead minnow, medaka, or zebrafish VTG mRNA has not been attempted with QRT-PCR, but could be applied to fathead minnow and zebrafish because the VTG sequence information is available (Kishida & Callard 2001; Kuwahara et al., 2000).

2.5 MS Approaches to Quantification of Vitellogenin

59. The characterization of protein-based biomarkers such as VTG has become an integral part of a reproductive-screening program. As reviewed in the preceding sections, immunoassays are the most commonly used approach in VTG analysis. Although not without considerable merit, immunoassays can suffer from problems related to antibody specificity and the limitation that only a single specific protein can be assayed during each RIA or ELISA. Thus, development of a secondary detection procedure for VTG suitable for use as a reference method in external quality assessments would be desirable. Additionally, if at some point in the future other biomarker proteins were to be added to the measured endpoints, additional immunoassays need to be developed. In these respects, mass spectrometry (MS) offers the potential for becoming a reference method for VTG (analogous to its use in steroid quantification) and for combining multiple protein analysis from a single tissue sample. However, the application of MS techniques towards protein quantification in general is a relatively new field of study (Leonil et al., 2000) and has been applied to VTG or structurally related proteins in only a few instances (Chen & Kuo 1998; Reith et al., 2001).

60. In general, MS approaches to protein quantification attempt to measure the protein largely in its intact form or rely on digestion procedures (chemical or enzymatic) to reduce the size of the protein into smaller fragments. A major obstacle with large proteins is the ionization efficiency, which tends to be poor relative to their smaller counterparts, and so relatively pure samples are required before they can be observed. A commonly used approach for increasing the ionization efficiency of macromolecules is matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Although MALDI-MS is capable of producing ions from very large molecules, the appropriate choice of matrix and solvent conditions is critical and can have a dramatic effect on the ability to measure large proteins (Leonil et al., 2000). An additional consideration for analysis of proteins like VTG is that they are heavily glycosylated, which may reduce the crystallization and/or ionization efficiency in MALDI (Kim et al., 2001). Besides difficulties related to ionization, glycosylation can also interfere with proteolytic digestion of the protein, which is frequently used to augment MS analysis of the intact protein. With a protein like VTG, the extensive glycosylation will interfere with digestion efficiency and peptide yield, complicating the analysis of the resulting peptide mixture. For these reasons, some attempt at de- glycosylation of the VTG protein will likely be necessary prior to MALDI-MS analysis.

61. The effect of de-glycosylation on protein mass and ionization efficiency is demonstrated in Figure 11-1, which shows the mass spectra of rainbow trout VTG before and after de- glycoslyation with N-Glycanase (Wunschel & Wahl 2002). In this example, three charge states (1+ to 3+) can be observed for the VTG sample. The de-glycosylated protein appears to be approximately 1 kDa smaller in size at 188 kDa, versus 189 kDa for the untreated protein. This size is still significantly larger than the average mass predicted from the amino acid sequence of trout VTG which is roughly 182 kDa (NCBI Sequence gi|3123011|ref|Q92093).

62. Although the broadness of the peaks prevents definitive identification of the size of the oligosaccharide removed, the de-glycosylated VTG sample appears smaller and with better signal to noise. For a comparison, a Western Blot of the same VTG sample used in the MS analysis is included in Figure 11-1 (inset figure). This Western Blot was made using a polyclonal anti-body against rainbow trout VTG (Schultz et al., 2001). In comparing the Western Blot to the Mass spectra of VTG, it is interesting to note that the three charge states identified for VTG appear to correspond in mass to the top three bands visible on the blot.

63. From this example it is evident that MALDI-MS is capable of directly measuring the VTG protein. The ability to generate quantitative results using VTG from species of interest for screening assays remains to be explored. Although it is extremely unlikely that MS will one day replace immunoassays as the preferred approach for quantifying VTG, MALDI-MS does offer potential to serve as a reference method for judging the accuracy of immunoassays and perhaps other protein based biomarkers as well.

ANNEX B

NON-SPAWNING FISH SCREENING ASSAY (21 days)

INTRODUCTION

1. This protocol describes an *in vivo* screening assay for identifying endocrine active chemicals in sexually dimorphic fish. The fish screening assay normally exposes fish to chemicals for up to 21 days. The *in vivo* fish screening assay is intended to detect chemicals that affect androgenic or estrogenic activity in fish exposed during a limited part of their life-cycle, including reproduction. The assay is not designed to identify specific mechanisms of hormonal disruption (such as hormone receptor agonistic or antagonistic effects, or disturbance of hormone synthesis or metabolism). The concept for this protocol is derived from work on the fathead minnow *(Pimephales promelas)* (1)(2)(3)(37), the Japanese medaka (*Oryzias latipes*) (4) (5) (6) and the zebrafish (*Danio rerio*) (7) (8) (9) (10). There is limited evidence that the protocol could also be used for the three spined sticleback (*Gasterosteus aculatus*) (11).

2. This protocol does not require *in situ* pre-exposure period. Four core endpoints as indicators of endocrine disrupter activity, namely: i) gross morphology (eg secondary sexual characteristics), ii) spawning status of fish, iii) vitellogenin levels and iv) gonadal histology. To address all core endpoints, sexually dimorphic adult fish in spawning conditions are normally used.

PRINCIPLE OF THE ASSAY

3. Adult fish as test organisms. Overviews of the relevant bioassay conditions are provided in Appendix 1 to this protocol. The assay is normally initiated with fish sampled from populations that are in spawning condition, senescent animals should not be used. Guidance on the age of fish is provided in the section on selection of test organisms. The assay is conducted using three chemical exposure concentrations for each test substance, as well as a water control. The use of a solvent carrier is not needed. However, in case a solvent would be used for any of the three test substances, a solvent control should be added, using the same solvent concentration as in the chemical treatments. A positive control is included (1 concentration) for each test substance. Two vessels (replicate) per treatment will be used (each vessel containing 5 males and 5 females). The exposure is conducted for 21-days and sampling of fish is performed at day-21 of exposure. Daily observations of the spawning status in each test vessel will be recorded as a Yes/No type of answer, and eggs will be removed daily from the test chamber. Laboratories may wish to add quantitative measures such as daily egg counts, but this is not required.

4. On sampling at day 21, 20 fish (10 males and 10 females) per treatment level are killed humanely and blood samples are collected for determination of vitellogenin (note - as an alternative to plasma collection, liver will be sampled for VTG analysis in medaka). The fish is then sectioned in a way that the gonads and gonoducts can be fixed and embedded without being removed from the body cavity. Gonads are then processed for subsequent histological analyses (see Draft Guidance Document, under preparation, for the details on preparation, sectioning of gonads and subsequent histological analysis).

INFORMATION ON THE TEST SUBSTANCE

5. Characterisation of the test substances is the responsibility of the original chemical supplier.

TEST ACCEPTANCE CRITERIA

6. For the test results to be acceptable the following conditions apply:

- the mortality in the control(s) does not exceed 10 per cent at the end of the exposure period;
- the dissolved oxygen concentration has been at least 60 per cent of the air saturation value (ASV) throughout the exposure period.
- the water temperature did not differ by more than ± 1 °C between test vessels at any one time during the exposure period and was maintained within a range of 2°C within the temperature ranges specified for the test species (Appendix 1).

DESCRIPTION OF THE METHOD

Apparatus

7. Normal laboratory equipment and especially the following:

(a) oxygen and pH meters;
(b) equipment for determination of water hardness and alkalinity;
(c) adequate apparatus for temperature control and preferably continuous monitoring;
(d) tanks made of chemically inert material and of a suitable capacity in relation to the recommended loading and stocking density (see Appendix 1);
(e) spawning substrate for fathead minnow and medaka; the spawning substrate for fathead minnow may consists of five half tiles constructed of PVC pipe in each test chamber. For zebrafish, Appendix 5 gives the necessary details.
(f) suitably accurate balance (i.e. accurate to ± 0.5%).

Water

8. Any water in which the test species shows suitable long-term survival and growth may be used as test water. It should be of constant quality during the period of the test. The pH of the water should be within the range 6.5 to 8.5, but during a given test it should be within a range of ± 0.5 pH units. In order to ensure that the dilution water will not unduly influence the test result (for example by complexion of test substance), samples should be taken at intervals for analysis. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl, SO_4), pesticides (e.g. total organophosphorus and total organochlorine pesticides), total organic carbon and suspended solids should be made, for example, every three months where a dilution water is known to be relatively constant in quality. If water quality has been demonstrated to be constant over at least one year, determinations can be less frequent and intervals extended (e.g. every six months). Some chemical characteristics of acceptable dilution water are listed in Appendix 2.

Test solutions

9. Test solutions of the chosen concentrations are prepared by dilution of a stock solution. The stock solution should preferably be prepared by simply mixing or agitating the test substance in the dilution water by using mechanical means (e.g. stirring or ultrasonication). Saturation columns (solubility columns) can be used for achieving a suitable concentrated stock solution.

10. A flow-through test system should be used. Such a system continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) in order to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water should be checked at intervals, preferably daily, during the test and should not vary by more than 10% throughout the test. Care should be taken to avoid the use of low-grade plastic tubing or other materials that may contain biologically active substances.

Selection of test organisms

11. The exposure phase will be started with sexually dimorphic adult fish from a laboratory supply of reproductively mature animals, in spawning conditions. As a general guidance for phase 1B, based on the technical judgement of experienced laboratory personnel, it is recommended that fish used should be reproductively mature (namely, with clear secondary sexual characteristics visible) and actively spawning. For general guidance only (and not to be considered in isolation from observing the actual reproductive status of a given batch of fish), fathead minnows should be approximately 20 (± 2) weeks of age, assuming they have been cultured at 25 ± 2°C throughout their lifespan. Medaka should be approximately 16 (± 2) weeks of age, assuming they have been cultured at 25 ± 2°C throughout their lifespan. Zebrafish should be approximately 15 (± 2) weeks of age, assuming they have been cultured at 25 ± 2°C throughout their lifespan.

12. Test fish shall be selected from a laboratory population of a single stock, which has been acclimated for at least two weeks prior to the test under conditions of water quality and illumination similar to those used in the test (note, this acclimation period is not an *in situ* pre-exposure period). Fish should be fed *ad libitum* throughout the holding period and during the exposure phase. Note- feed should not be fed within 12 hours of necropsy.

13. Following a 48-hour settling-in period, mortalities are recorded and the following criteria applied:

- mortalities of greater than 10% of population in seven days: reject the entire batch;
- mortalities of between 5% and 10% of population: acclimation for seven additional days; if more than 5% mortality during second seven days, reject the entire batch;
- mortalities of less than 5% of population in seven days: accept the batch.

14. Fish should not receive treatment for disease in the two week acclimation period preceding the test, or during the exposure period.

TEST DESIGN

15. Three concentrations of the test substance and one concentration of the positive control are used per experiment. The data may be analyzed in order to define the Lowest Observed Effect Concentration or the No-Observed Effect Concentration based on endocrine sensitive endpoints (LOEC and NOEC respectively). Calculation of these statistical parameters will be useful in order to establish whether any further longer term testing for adverse effects (namely, survival, development, growth and reproduction) is required for the chemical.

16. At initiation of the experiment on day-0, 10 males and 10 females from the non-exposed population are sampled for the measurement of the three core endpoints. On day-21 of the experiment, 10 males and 10 females from each treatment level (5 males and 5 females for each of the two replicates) and from the control are sampled again for the measurement of the three endpoints. (see Appendix 3 for diagram of test design).

PROCEDURE

Selection and weighing of test fish

17. It is important to minimise variation in weight of the fish at the beginning of the assay. Suitable size ranges for the different species recommended for use in this test are given in Appendix 1 to this protocol. For the whole batch of fish used in the test, the range in individual weights at the start of the test should be kept, if possible, within ± 20% of the arithmetic mean weight. It is recommended to weigh a subsample of fish before the test in order to estimate the mean weight.

Conditions of exposure

Duration

18. The test duration is 21 days.

Loading rates and stocking densities

19. It is important that the loading rate and stocking density (for definitions, see Appendix 4) is appropriate for the test species used (see Appendix 1). If the stocking density is too high, then overcrowding stress will occur leading to reduced growth rates and possibly to disease. If it is too low, territorial behavior may be induced which could also affect growth. In any case, the loading rate should be low enough in order that a dissolved oxygen concentration of at least 60% ASV can be maintained without aeration.

Feeding

20. The fish should be fed *ad libitum* with an appropriate food (Appendix 1) at a sufficient rate to maintain body condition (2). Care should be taken to avoid microbial growth and water turbidity. The daily ration may be divided into two or three equal portions for multiple feeds per day, separated by at least three hours between each feed. Food should be withheld from the fish for 12 hours prior to the day of sampling to aid in histology processing of small fish.

21. Fish food, other than the one recommended in Appendix 1, should be evaluated for the presence of contaminants such as organochlorine pesticides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and any estrogenic activity.

22. Uneaten food and fecal material should be removed from the test vessels each day by carefully cleaning the bottom of each tank using suction.

Light and temperature

23. The photoperiod and water temperature should be appropriate for the test species (see Appendix 1).

Frequency of analytical determinations and measurements

24. During the test, the concentrations of the test substance are determined at regular intervals, as follows: the flow rates of diluent and toxicant stock solution should be checked at intervals, at least twice per week, and should not vary by more than 15% throughout the test. It is recommended that the actual test chemical concentrations be measured in all vessels at the start of the test and at weekly intervals thereafter.

25. Results should always be based on measured concentrations and included in reporting.

~~26.~~ Samples may need to be filtered (e.g. using a 0.45 μm pore size) or centrifuged. If needed, then centrifugation is the recommended procedure. However, if the test material does not adsorb to filters, filtration may also be acceptable.

27. During the test, dissolved oxygen and temperature should be measured in all test vessels daily and pH should be measured at least once per week. Total hardness and alkalinity should be measured in the controls and one vessel at the highest concentration at least once per week. Temperature should preferably be monitored continuously in at least one test vessel.

Observations

28. A number of general (e.g. survival) and core biological responses (e.g. vitellogenin levels) are assessed over the course of the assay. Collection of these endpoints and their utility are described below:

Survival

29. Fish should be examined daily during the test period and any external abnormalities (such as hemorrhage, discoloration) noted. Any mortality should be recorded and the dead fish removed as soon as possible. Dead fish should not be replaced in either the control or treatment vessels.

Behaviour

30. Any abnormal behavior (relative to controls) should be noted; this might include signs of general toxicity including hyperventilation, uncoordinated swimming, loss of equilibrium, and atypical quiescence or feeding. Such behavioral observations may provide useful qualitative information to inform potential future fish testing requirements (for example, territorial aggressiveness in normal males or masculinized females has been observed in fathead minnows under androgenic exposure).

Spawning status

31. Daily qualitative observations of spawning in each test vessel will be recorded as Yes for the presence of eggs, and No in the absence of eggs. Optionally, laboratories may count the eggs to add a quantitative component to the observation. Eggs will be removed daily from the test chambers. A spawning substrate should be placed in the test chamber for the fathead minnow and zebrafish to enable fish to spawn in normal conditions. For fathead minnow, the spawning substrate may consists of five half tiles constructed of PVC pipe. For zebrafish, Appendix 5 gives further details.

Appearance and observation of secondary sex characteristics

32. Secondary sexual characteristics are under endocrine control; therefore observations of physical appearance of the fish should be made over the course of the test, and at conclusion of the study (Appendix 6a and 6b). Experience to date with fathead minnows suggests that some endocrine active chemicals may initially induce changes in the following external characteristics: body color (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and specialized secondary sex characteristics (size of dorsal nape pad, number of nuptial tubercles in male fathead minnow, ovipositor size in females, papillary processes in male medaka). Notably, chemicals with certain modes of action may cause abnormal occurrence of secondary sex characteristic in animals of the opposite sex; for example, androgen receptor agonists, such as methyltestosterone and dihydrotestosterone, can cause female fathead minnows to develop pronounced nuptial tubercles (3)(12) (13). It also has been reported that estrogen receptor agonists can decrease nuptial tubercle numbers and size of the dorsal nape pad in adult males (14)

(15). Such gross morphological observations may provide useful qualitative and quantitative information to inform potential future fish testing requirements.

33. Because some aspects of appearance (primarily color) can change quickly with handling, it is important that qualitative observations be made prior to removal of animals from the test system. Other endpoints, such as the number and size of nuptial tubercles in fathead minnow and papillary processes in medaka, can be quantified directly or in preserved specimens (3). Standard operating procedures for the evaluation of secondary sex characters in fathead minnow and medaka are available as Appendix 6a) and 6b).

Humane killing of fish

34. At day 0 and day 21 at conclusion of the exposure, the fish should be anaesthetized with MS-222 (100 mg per L buffered with 200 mg $NaHCO_3$ per L) or with FA-100 and ice-cold water, individually weighed as wet weights (blotted dry) and blood collected from the caudal artery/vein with a heparinised microhematocrit capillary tubule. Depending upon the size of the fish, collectable blood volumes generally range from 20 to 80 µl per individual for fathead minnows (1) (16) and 5-15 µl per individual for zebrafish (17). Plasma is separated from the blood via centrifugation (3 min; 15,000 g; room temperature), and stored with protease inhibitors at -80^{o}C, until analyzed for vitellogenin. Alternatively, in medaka the liver will be used as a tissue-source for vitellogenin determination. Further details on vitellogenin determination for each species will be available before the start of Phase 1B in paragraphs 41 to 43 and in Appendix 7 (under preparation) for each species.

Gonad Histology (*to be supplemented by the Guidance Document under preparation***)**

35. The first step of gonad histological analysis is necropsy and rapid fixation of the gonad to prevent autolysis and cellular deterioration. Immediately after humane killing of an individual fish (plus length and weight measurements and collection of fresh tissues – eg blood or liver for VTG analysis – the abdomen should be sectioned and the paired gonads fixed. Suitable fixatives are 4% formaldehyde / 1% glutaraldehyde (Bouin's fixative may also be used), all of which are ideal for maintaining the structural integrity of the gonad.

36. The fixed gonads should be processed and embedded in paraffin wax. Using standard histological techniques (20), microtome cut histological sections should be taken along the longitudinal axis of the gonads in a serial-step fashion (for example, at 10-20 µm intervals). Two longitudinal sections per gonad should be collected from each fish.

37. After staining with haematoxylin and eosin, histological sections should be evaluated by an experienced pathologist. The Guidance Document for the preparation of gonads and evaluation of gonadal histology should be used for gonad staging and scoring severity of lesions and other abnormalities. In general, evaluation of the testis is based on the amount of germinal epithelium present, and the degree of spermatogenic activity. The ovary is evaluated based upon relative numbers of perinucleolar, cortical alveolar, and vitellogenic oocytes (21). Classification schemes potentially useful for assessing fish gonads are summarized elsewhere (3)(22). Recent studies have documented a variety of alterations in fathead minnow gonadal histology associated with exposure to endocrine active chemicals with oestrogenic or androgenic properties (3) (14) (23) (24) (25) (26).

38. The gonadal histology observations will be interpreted based on the expert judgement of a professional pathologist. It is not necessary to evaluate the histological sections using extensive image-analysis techniques with associated statistical analyses. The pathologist's interpretation of the material should be fully documented in the study report.

Vitellogenin (VTG)

39. VTG is a phospholipoglycoprotein precursor to egg yolk protein that normally occurs in sexually-active females of all oviparous species; the production of VTG is controlled by interaction of oestrogens with the estrogen receptor. Significantly, males maintain the capacity to produce VTG in response to stimulation with estrogen receptor agonists; as such, induction of VTG in males and immature females has been successfully exploited as a biomarker specific for oestrogenic compounds in a variety of OECD fish species (1)(2) (3) (28) (29)(30) (31) (32) (33)

40. The measurement of VTG should be based upon a validated homologous ELISA method. Information should be available upon the intra-assay and inter-assay variability and on the lower detection limit of the method. It is recommended to use method capable to detect VTG levels as low as few ng/ml plasma (or liver for medaka). It is required that laboratories using the same species in Phase 1B also use the same measurement method.

41. A complete transparency of the ELISA method is needed to understand potential sources of variability. The technical lead laboratory will select the most suitable method for the species considered, prepare standard operating procedures for blood or liver treatment and VTG determination, and make them available to other participating laboratories (Appendix 7, under preparation). The outcome of the comparative studies of vitellogenin methods should be used in selecting the most appropriate one for each species (43). Polyclonal fathead minnow vitellogenin antibody and purified vitellogenin protein also from the fathead minnow are utilized (28) (29) for the determination of vitellogenin in that species. The same applies for medaka and zebrafish (use of homologous VTG antibodies and standard). To calibrate results obtained, a common VTG standard for each species will be provided to each laboratory. This is to level-off possible differences across laboratories using the same method and species.

Optional endpoint: measurement of gonadal steroids

42. Laboratories willing to measure sex steroids concentration on plasma can do so.

DATA AND REPORTING

Treatment of results

43. Excel Spreadsheets for the collection of test results will be made available to participating laboratories before the start of Phase 1B. All laboratories will use the same template for data collection. It is the responsibility of each laboratory to check the data for accuracy before submitting the completed Excel Spreadsheets to the Lead laboratory. The Lead laboratory CERI will collect all test results for all three species and test substances and make the statistical analysis of data. The Lead laboratory will also prepare a draft report.

Evaluation of Biomarker Responses by Analysis of Variance (ANOVA) or by Regression Analysis

44. To identify potential endocrine activity by a chemical, responses are compared between treatments versus controls groups using analysis of variance (ANOVA). All biological response data should be analyzed and reported separately by sex (either females or males). If the required assumptions for parametric methods are not met - non-normal distribution (e.g. Shapiro-Wilk's test) or heterogeneous variance (Bartlett's test or Levene’s test), consideration should be given to transforming the data to homogenize variances prior to performing the ANOVA, or to carrying out a weighted ANOVA. Dunnett’s test (parametric) on multiple

pair-wise comparisons or a Mann-Whitney with Bonferroni adjustment (non-parametric) may be used. Additional information can be obtained from the OECD Draft Guidance Document on Statistical Analysis of Ecotoxicity Data, available on the OECD public website (47).

45. To identify potential endocrine activity following chemical exposure, responses across the different treatment concentrations could also be examined based on regression analysis (based on three exposure concentrations per chemical). All biological response data should be analyzed and reported separately by sex (either females or males). Direct observation of concentration-related trends in biological responses can also usefully inform the outcome of such a study for the intrinsic endocrine activity of a test chemical.

Interpretation of results

46. The results should be interpreted with caution where measured toxicant concentrations in test solutions occur at levels near the detection limit of the analytical method.

Test report

47. The test report must include the following information:

Test substance: physical nature and relevant physical-chemical properties, chemical identification data including purity and analytical method for quantification of the test substance where appropriate.

Test species: at a minimum scientific name, supplier and any pretreatment.

Test conditions: test procedure used (test-type, loading rate, stocking density, etc.); method of preparation of stock solutions and flow-rate; the nominal test concentrations, the means of the measured values and standard deviations in the test vessels and the method by which these were attained and evidence that the measurements refer to the concentrations of the test substance in true solution; dilution water characteristics (including pH, hardness, alkalinity, temperature, dissolved oxygen concentration, residual chlorine levels, total organic carbon, suspended solids and any other measurements made); water quality within test vessels: pH, hardness, temperature and dissolved oxygen concentration; detailed information on feeding (e.g. type of food(s), source, amount given and frequency and analyses for relevant contaminants if necessary (e.g. PCBs, PAHs and organochlorine pesticides);

Results: evidence that controls met the validity criterion for survival, and data on mortalities occurring in any of the test concentrations; statistical analytical techniques used, statistics based on fish, treatment of data and justification of techniques used; tabulated data on biological observations of gross morphology (including secondary sex characteristics) and vitellogenin; detailed report on gonadal histology; results of the statistical analysis preferably in tabular and graphical form; incidence of any unusual reactions by the fish and any visible effects produced by the test substance.

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APPENDIX 1: EXPERIMENTAL CONDITIONS FOR THE NON-SPAWNING FISH ENDOCRINE SCREENING PROTOCOL

1. Recommended species	Fathead minnow (*Pimephales promelas*)	Medaka (*Oryzias latipes*)	Zebrafish (*Danio rerio*)
2. Test type	Flow-through	Flow-through	Flow-through
3. Water temperature	25 ± 2°C	24± 2°C	26 ± 2°C
4. Illumination quality	Fluorescent bulbs (wide spectrum)	Fluorescent bulbs (wide spectrum)	Fluorescent bulbs (wide spectrum)
5. Light intensity	10-20 µE/M^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)	10-20 µE/M^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)	10-20 µE/M^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h dark	16 h light, 8 h dark	16 h light, 8 h dark
7. Loading rate	<5 g per L	<5 g per L	<5 g per L
8. Test chamber size	10 L (minimum)	2 L (minimum)	4 L (minimum)
9. Test solution volume	8 L (minimum)	1.5 L (minimum)	4 L (minimum)
10. Volume exchanges of test solutions	Minimum of 6 daily	Minimum of 5 daily	Minimum of 5 daily
11. Age of test organisms	Reproductively mature fish (not senescent)	Reproductively mature fish (not senescent)	Reproductively mature fish (not senescent)
12. Wet weight of adult fish (g)	Females: 1.5 ± 20% Males: 2.5 ± 20%	Females: 0.35 ± 20% Males: 0.35 ± 20%	Females: 0.65 ± 20% Males: 0.5 ± 20%
13. No. of fish per test vessel	10	10	10
14. No. of treatments	= 3 (plus appropriate controls)	= 3 (plus appropriate controls)	= 3 (plus appropriate controls)

APPENDIX 1 (continued)

15. No. vessels per treatment	2 minimum	2 minimum	2 minimum
16. No. of fish per test concentration	10 adult females and 10 males (in separate vessels)	10 adult females and 10 males (in separate vessels)	10 adult females and 10 males (in separate vessels)
17. Feeding regime	Frozen adult brine shrimp twice daily (*ad libitum*)	Brine shrimp nauplii twice daily (*ad libitum*)	Frozen adult brine shrimp twice daily (*ad libitum*)
18. Aeration	None unless DO concentration falls below 4.9 mg per L	None unless DO concentration falls below 4.9 mg per L	None unless DO concentration falls below 4.9 mg per L
19. Dilution water	Clean surface, well or reconstituted water	Clean surface, well or reconstituted water	Clean surface, well or reconstituted water
20. Dilution factor	max 10	max 10	max 10
21. Pre- exposure period	none	None	None
22. Chemical exposure duration	21-d	21-d	21-d
23. Biological endpoints	Non-specific: - survival - behavior Endocrine biomarkers: - gross morphology (incl. 2y sex characteristics) - VTG - gonadal histology	Non-specific: - survival - behavior Endocrine biomarkers: - gross morphology (incl. 2y sex characteristics), - VTG - gonadal histology	Non-specific: - survival - behavior Endocrine biomarkers: - gross morphology, - VTG - gonadal histology
24. Test acceptability	Dissolved oxygen $\geq$60% of saturation; mean temperature of 25 ± 2^{o}C; 90% survival of fish in the controls	Dissolved oxygen $\geq$60% of saturation; mean temperature of 24 ± 2^{o}C; 90% survival of fish in the controls	Dissolved oxygen $\geq$60% of saturation; mean temperature of 26 ± 2^{o}C; 90% survival of fish in the controls

APPENDIX 2: SOME CHEMICAL CHARACTERISTICS OF ACCEPTABLE DILUTION WATER

SUBSTANCE	**CONCENTRATIONS**
Particulate matter	< 20 mg/l
Total organic carbon	< 2 mg/l
Unionised ammonia	< 1 □g/l
Residual chlorine	< 10 □g/l
Total organophosphorus pesticides	< 50 ng/l
Total organochlorine pesticides plus polychlorinated biphenyls	< 50 ng/l
Total organic chlorine	< 25 ng/l

APPENDIX 3: ABBREVIATIONS & DEFINITIONS

CV – coefficient of variation

Loading rate - the wet weight of fish per volume of water.

Stocking density - is the number of fish per volume of water.

VTG - vitellogenin is a phospholipoglycoprotein precursor to egg yolk protein that normally occurs in sexually active females of all oviparous species.

ANNEX C

REPRODUCTIVE ASSAY, 14-day version

14-day Fish Assay for Identification of Potential Endocrine Disrupting Chemicals

Note: The protocol described below is modified from the full description of a short-term (21-day) assay method described in U.S. EPA (2002) and Ankley et al. (2001) for fathead minnow. This method should also be adaptable to other small fish species, such as zebrafish, medaka, and sheepshead minnow. Recent updates to this protocol can be found in OECD Draft (November 2002) that includes considerations for zebrafish and medaka and that places sexes together in tanks separated by a mesh barrier.

INTRODUCTION

1. This protocol describes a 14-day reproduction assay using the fathead minnow (*Pimephales promelas*). This test is a14-day short-term assay that measures the reproductive performance of groups of fathead minnows as the primary indicator for endocrine disruption. Additional measurements of morphology, histopathology, and biochemical endpoints are performed to aid identification of the specific toxicological mode of action of the test chemical.

2. The assay is started with mature male and female fish previously observed for spawning activity. During the subsequent 14-day chemical exposure, survival, reproductive behavior, and secondary sexual characteristics are observed; and daily fecundity and fertilization success measurements are recorded. Successful embryological development is measured based on the hatching success of fertilized eggs. At termination of the assay, measurements are made of external morphology, GSI, gonadal histology, and plasma concentrations of VTG and sex steroids (E2, T, 11-KT).

PRINCIPLE OF THE ASSAY

3. An overview of the assay and relevant test conditions is provided in Table 12-1. The assay is initiated with mature adults that appear to be reproductively active as determined by observations of spawning that are made during the 14-day pre-exposure phase. No quantitative measures of fecundity or embryo viability (e.g., hatchability) are made during the pre-exposure phase. As with the 21-day assay described below, the 14-day assay is conducted using two chemical concentrations, as well as a control with four experimental units (replicates) per treatment. Each replicate tank contains four female and two male fish. The test chemical is delivered to the exposure chamber using a proportional diluter. The exposure is conducted for 14 days, during which time the appearance of the fish, behavior, and fecundity are assessed daily. Hatching success and post-hatch viability of larvae is monitored for 24 hours in control water. At termination of the exposure, blood samples are removed from adults and analyzed for sex steroids and VTG. The gonads are also removed for GSI determination and later histological analyses.

DESCRIPTION OF THE METHOD

Test Animals and Assay System

4. **Test Animals:** The procedure described here is initiated with adults, as opposed to embryos or larvae. The test should be started with newly-mature fish (typically 4-6 months old), as opposed to older animals that have been actively reproducing for some period of time, for example, in a culture setting.

Thus, to maintain a ready supply of known-quality animals at the desired age for routine testing, it is preferable to maintain a fathead minnow culture, as opposed to purchasing the animals prior to testing. Field collected fathead minnows generally should not be used to initiate cultures or for testing. Information on general culture and testing requirements for the fathead minnow are provided in Table 12-2.

Table 12-1. Test Conditions for the 14-day or 21-day Fathead Minnow EDC Screening Assay (with Measurement of Reproductive Performance)

Test type	Flow-through
Water temperature	25 Å1ºC
Illumination	Quality Fluorescent bulbs (wide spectrum) or gold fluorescent lighting
Light intensity	10-20 ìE/M2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)
Photoperiod	16 h light, 8 h dark
Test chamber size	18 L (40 x 20 x 20 cm) (minimum)
Test solution vol.	10 L
Volume exchanges of test solutions	Minimum of six daily
Flow rate	Approx 3.5L/g fish/day
Age of test organisms	Reproducing adults (120 day minimum)
No. of fish per test chamber	4 females and 2 males
No. of treatments	2 (plus appropriate controls)
No. of replicate test chambers per treatment	4
No. of fish per test concentration	16 females and 8 males
Feeding regime	Frozen adult brine shrimp twice daily
Aeration	None unless DO concentration falls below 4.9 mg/L
Dilution water	Clean surface, well, or reconstituted water
Dilution factor	37385
Chemical exposure duration	21-day test; 14-day test
Endpoints	Adult survival, reproductive behavior, secondary sexual characteristics, GSI and gonadal histology, plasma VTG and sex steroids (E2, T, 11-KT) concentrations, fecundity, fertility, embryo hatch and larval survival.
Test acceptability	Dissolved oxygen >60% of saturation; mean temperature of 25 Å1ºC; 90% survival of in the controls; successful egg production in controls

5. **Water:** The fathead minnow can reproduce successfully over a wide range of water quality. Therefore, no specific water type is required for this test. Any uncontaminated surface, well, or reconstituted water in which the fish can be cultured successfully should be acceptable. Minimal recommended water quality characteristics are listed in Table 12-2. The animals should be tested using a flow-through water renewal system that enables maintenance of adequate water quality (temperature, dissolved oxygen, low ammonia, etc.), as well as ensuring a consistent exposure to the parent chemical (for those tests where water is the route of exposure).

Table 12-2. Recommended Ranges of Water-quality Characteristics for Culturing and Testing the Fathead Minnow

Water characteristic	Preferred range	Method of measurement
Temperature (ºC)	24.0-26.0	Alcohol or electronic thermometer
Dissolved oxygen (mg/L)	>4.9 mg/L (>60% saturation)	Iodometric or membrane electrode
PH	6.5-9.0	Electronic meter
Total alkalinity (mg/L as CaCO3)	>20	Acid titration
Total organic carbon (mg/L)	<5	TOC analyzer
Unionized ammonia (_g/L)	<35	Nesslerization with pH and temperature adjustments

6. **Assay System:** Glass, stainless steel, or other chemically inert material should be used for construction of the test system. The dimensions of the test chambers must be such that the animals can interact in a fashion conducive with successful spawning. The test chamber contains 10 L of test solution, which is renewed at least once every four hours. This particular animal loading/water renewal rate is within recommended guidelines and in studies conducted according to this method, has maintained acceptable water quality (Tables 12-1, 12-2), while not utilizing an excessive amount of test material.

Experimental Design

7. The range-finding test should be conducted under conditions (water quality, test system, animal loading) similar to those used for the reproduction test. It should utilize adult fish and focus on lethality over the course of at least a 4- to 7-day assay. If the route of exposure is via water, the highest concentration utilized should be at water solubility; in general, a logarithmic dilution series should suffice for the range-finding assay.

8. For the 14-day reproduction test, the highest concentration used should not have caused significant mortality in the initial range-finding assay (this may be limited to water solubility), and the lower concentration/dose used should be a factor of 5- to 10-times lower than the highest test concentration. At present, a minimum of four replicate tanks (each containing four females and two males) is recommended per treatment. Based on this design, a minimum of 72 fish is required per assay (six fish in each of four replicates for two treatments, plus one control).

Exposure via Water

9. Water exposures can be conducted either with or without a solvent carrier for the test chemical of concern. When a chemical is relatively soluble in water (ionic compounds) a solvent is not required to enhance water solubility for preparation of stock solutions; however, much of the toxicity testing historically conducted with aquatic animals and sparingly soluble, nonionic chemicals has utilized carrier solvents.

10. On occasion it may be necessary to utilize solvents to generate stock solutions for aqueous testing; this could occur when a chemical is very insoluble, unstable in a saturator system, or so expensive/limited in availability that the use of saturators is not practical. It is essential that any test utilizing a carrier solvent include both solvent-exposed and non-exposed controls.

Analytical Determination

11. Regardless of the exposure technique utilized for this assay, supporting analytical chemistry is critical to 1) ensure chemical purity, 2) document that the test chemical is reaching the fish, and 3) confirm system performance. In the water exposures, concentrations of the (parent) chemical should be measured at the start of the assay, at least weekly in the stock solution(s) and, ideally, in a subset of the test tanks as well.

PERFORMANCE OF THE ASSAY

Assay Initiation and Conduct

12. **Pre-exposure:** The 14-day pre-exposure phase of the assay should use five to six months-old minnows, previously maintained in communal culture tanks. Four females and two males should be randomly assigned to the replicate exposure chambers at each treatment concentration. In addition to the number of replicates actually exposed at each concentration (e.g. four), a number of additional tanks also should be started; these can serve as "replacement" units for tanks in which pre-exposure spawning is not observed, and/or mortality occurs before initiation of the chemical exposure. Identification of gender may be difficult to resolve for some fish; these animals should not be used for the assay. At this stage in development, males will exhibit nuptial tubercles, while females possess an ovipositor; in addition, males tend to be larger and darker than females from the same cohort.

13. The pre-exposure phase of the assay is conducted under conditions (temperature, photoperiod, feeding, etc.) identical to those used during the chemical exposure (Table 12-2). The animals are fed frozen *Artemia* twice daily ad libitum. The fish will be observed daily for spawning activity.

14. **Chemical Exposure:** Once spawning activity has been observed, the chemical exposure can be initiated. The target exposure duration is 14 days, which is sufficient for healthy females to produce several clutches of eggs.

Observations

15. **Survival:** Daily assessment of survival is made to provide a basis for expression and interpretation of reproductive output, that is, number of eggs/female/d. In animals exposed to the test chemical, overt lethality may occur, particularly in later portions of the assay not reflective of the initial (shorter) range-finding test.

16. **Behavior of Adults:** Any abnormal behavior (relative to controls) should be noted; this might include signs of general toxicity including hyperventilation, uncoordinated swimming, loss of equilibrium, and atypical quiescence or feeding. From the standpoint of EDC screening, alterations in reproductive behavior, particularly loss of territorial aggressiveness by males, may be affected by chemicals which interact with estrogen and/or androgen pathways. Because of the relative subjectivity of this endpoint, it may be necessary to document behavioral alterations via photographs or videotape.

17. **Fecundity:** Egg production should be determined daily. Fathead minnows usually spawn in the early morning (before 10:00 am) so, except for feeding, they should not be disturbed until late morning. This allows time for spawning and fertilization to be completed, and for eggs to water-harden. The spawning substrates can be removed from the tanks to enumerate any eggs which are present. One spawn typically will be comprised of 50 to 150 eggs, however smaller clutches are not uncommon. If no embryos are present, the substrate is left in the tank; new substrates should be added to replace any that are removed. Fecundity should be expressed on the basis of surviving females per reproductive (test) day per replicate. Therefore, if all four females survived the treatment in a given replicate for the duration of 14 days, there would be 56 female reproductive days.

18. **Fertilization Success:** After the spawning substrate has been removed from the tank, the embryos should be carefully rolled off it with a gentle circular motion of an index finger and visually inspected under appropriate magnification. If spawning occurred that morning, embryos typically will be undergoing late cleavage, and determination of the fertility rate (number embryos/number of eggs x 100) is easily achieved. Infertile eggs are opaque or clear with a white dot where the yolk has precipitated; viable embryos remain clear for 36 to 48 hours until reaching the eyed stage. An alternative to the microscopic approach to determining fertilization success is to enumerate eyed embryos at this time. If the latter approach is used for fertility determination, the embryos should be placed in "incubation" chambers and held in a system apart from the adults to avoid possible predation.

19. Regardless of the method used to determine fertility, if information concerning hatching success and/or subsequent larval development is desired, the embryos will need to be maintained for up to an additional 7 days in incubation chambers. Depending upon study objectives, water in the embryo holding system could either be clean or contain concentrations of the test chemical comparable to those used in the adult exposure. To maintain adequate water quality, the incubation system should either provide a continuous flow of water, or the test solution must be renewed daily. During this period of time, if desired, alterations in normal embryologic development can be assessed.

20. **Hatchability, Larvae Appearance, and Survival:** At 25_C, untreated animals will hatch in 4.5 days to 6 days. Each incubation chamber should be evaluated weekly for newly hatched embryos; this endpoint should be expressed as a relative percentage of those eggs deemed fertile. The hatching rate of control animals typically is in the range of 95% to 98%.

21. The appearance and behavior of hatched larvae can be evaluated, and results described either qualitatively or quantitatively (e.g., malformation rate). Gross morphological anomalies that may be observed include lordosis, scoliosis, kyphosis, retarded swim bladder development, and craniofacial abnormalities. Survival of the larvae may be assessed through yolk sac absorption (ca., 96 h at 25_C); if estimates of survival are required after this, the animals must be fed (generally live *Artemia*).

22. **Appearance of Adults:** Observations of physical appearance of the adults should be made over the course of the test, and at conclusion of the study. From the perspective of screening EDCs, characteristics of particular importance include: body color (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and specialized secondary sex characteristics (size of dorsal nape pad, number of nuptial tubercles in males; ovipositor size in females). Notably, chemicals with certain MOA cause abnormal occurrence of secondary sex characteristic in animals of the opposite sex.

23. **Blood Sampling:** At the conclusion of the exposure, the fish will be anesthetized by transfer to an oxygenated solution of MS-222 (100 mg/L buffered with 200 mg NaHCO3/L), and weighed. Blood will be collected from the caudal artery/vein with a heparinized microhematocrit capillary tubule. Depending upon the size of the fathead minnow (which usually is sex- dependent), blood volumes

generally range from 30 to 80 _l. Plasma is separated from the blood via centrifugation (3 minutes at 15,000 x g) and stored with protease inhibitors at -80ºC, until analyzed for VTG and sex steroids.

Gonad Size and Morphology

24. The gonads should be fixed in situ and then removed and weighed (to the nearest 0.1 mg) for determination of the GSI (GSI=100 x gonad wt/body wt). Typical GSI values for reproductively active fathead minnows range from 8% to 13% for females and from 1% to 2% for males. Many chemicals that reduce fecundity also will reduce the GSI in one or both sexes. After removal of the gonads, the remainder of the carcass of the fish may be discarded, or preserved for further measurements (e.g., frozen for chemical analysis).

25. Routine histological procedures can be used to assess condition of testes and ovaries from the fish. Gonads should be fixed in situ in an appropriate fixative, such as 4% formaldehyde/1% glutaraldehyde, and embedded in paraffin or plastic. Serial sections 4 µm to 5 µm thick should be cut along the long axis of the gonad. At a minimum, two serial sections should be collected from at least three steps equally spaced between the leading edge of the tissue and the midline of the gonad, for a total of six-tissue sections/sample. Sections can be stained with hematoxylin and eosin, and should be evaluated by an experienced histologist without prior knowledge of the treatment regime associated with specific samples. In general, evaluation of the testis is based on the amount of germinal epithelium present, and the degree of spermatogenic activity. The ovary is evaluated based upon relative numbers of perinucleolar, cortical alveolar, and vitellogenic oocytes. It should be explained in what cases histopathology would add valuable information to the test (since this is an expensive and time consuming effort), as it is done on p81, last para, for the non-spawning fish screen. In cases, where reproductive effects have been detected by other means, routine histopathology may be superfluous. It may only add to sensitivity, when no other reproductive endpoints are affected. (In this case, the histopathological examination should start with a comparison of the control and the highest concentration in order to make the assay more economical.)

Vitellogenin

26. Different methods are available to assess VTG production in fish; a measurement technique that is both relatively sensitive and specific is determination of protein concentrations in plasma via ELISA. For ELISA, polyclonal fathead minnow VTG antibody and purified VTG protein also from the fathead minnow are utilized. Polyclonal and/or monoclonal VTG antibodies prepared using protein from other fish species may cross-react with fathead minnow VTG and, hence, also could be useful for assessing this endpoint.

Sex Steroids

27. Plasma concentrations of E2, T, and 11-KT can be determined using RIA or related enzyme immunoassays (EIA) techniques optimized for the relatively small sample volumes obtained from the fathead minnow.

PERFORMANCE CRITERIA

28. Water quality characteristics should remain within the limits of tolerance depicted in Tables 1 and 2.

29. There should be documentation (via appropriate analytical chemistry) of purity of the test material, as well as delivery of chemical to the fish (e.g., concentrations of the chemical in test water).

30. There should be more than 90% survival of control animals over the duration of the chemical exposure, and the control fish in each replicate should spawn, at a minimum, every three to four days.

31. There should be greater than 95% fertility and hatchability of eggs and embryos, respectively, from the control animals.

DATA REPORTING

Treatment and Interpretation of Results

32. Any endpoints that are significantly affected by the test chemical should be reported as such. This information will then be used in a weight-of-evidence analysis to assess the need for further testing.

Test Report

33. The test report must include the following:

Test Substance:

34. The report must include a detailed description of the test substance, including information on its CAS number, source, lot number, and purity.

35. Additional information should be provided, when available, such as its solubility in water, octanol:water partition coefficient, vapor pressure, and toxicity to fathead minnows.

Test Species:

36. Information must be provided on the fathead minnows used in the test. This information must include the source of the fish, age and condition of the fish at the initiation of the test, and the pre-exposure reproductive performance.

37. Any observed abnormalities in reproductive behavior or performance of control fish must also be reported.

Test Conditions: The report must specify the conditions under which the test was performed, this includes:

38. Information on the source, treatment of, and basic chemical characteristics of the dilution water.

39. Means and ranges for water temperature, dissolved oxygen, pH, hardness, alkalinity and conductivity.

40. The photoperiod and light intensity used during the exposure.

41. The chamber size, number of females and males per replicate, and number and composition of spawning substrates.

42. Information on food used to feed the fish during the exposure, including supplier and lot number.

43. The basic nature of exposure (i.e. flow-through, ip injection, or dietary) in addition to specific information related to the exposure type (e.g. whether flow-through water delivery type, daily number of volume exchanges of dilution water).

44. Use of solvent or dispersant if any, the specific solvent or dispersant and the concentrations to which the fish were exposed must be specified.

Results:

45. The results must include data for the control (plus solvent control when used) and the treatment fish.

46. The table of results must include the mean, standard deviation and range for each test endpoint from the replicates employed in the test. Statistical significance of means should be indicated.

ANNEX D:

REPRODUCTIVE ASSAY, 21-day version

SHORT-TERM (21-DAY) REPRODUCTION ASSAY WITH THE FATHEAD MINNOW FOR IDENTIFICATION OF ENDOCRINE DISRUPTING CHEMICALS

Note: The protocol description below is a summary of the full description detailed in U.S. EPA (2002).

INTRODUCTION

1. This guideline describes a 21-day reproduction assay with the fathead minnow *(Pimephales promelas)* that considers reproductive fitness as an integrated measure of toxicant effects. It also enables measurement of a suite of histological and biochemical endpoints that potentially are directly reflective of effects associated with the classes of EDCs of concern. The test is a short-term assay that utilizes reproductively active fish to assess endocrine-sensitive endpoints. The assay may also be adapted for most endpoints with other small fish species such as zebrafish, medaka, and sheepshead minnow.

2. The assay is initiated with mature male and female fish; during a 21-day chemical exposure, survival, reproductive behavior, and secondary sexual characteristics are observed, and fecundity monitored. Assessments of fertility and F1 development can be made, if desired. At the end of the test, measurements are made of a number of endpoints reflective of the status of the reproductive endocrine system, including the gonadal-somatic index (GSI), gonadal histology, and plasma concentrations of VTG and sex steroids (E2, T, 11-KT).

PRINCIPLE OF THE TEST

3. An overview of the test and relevant test conditions are provided in Table 12-1. The test is initiated with mature adults that have a record of reproductive success as measured both by fecundity (number of eggs) and embryo viability (e.g., hatchability). This is established during a "pre-exposure" period of 14 days to 21 days in the same system/tanks as will be utilized for the chemical test. The test is conducted at a minimum of two chemical concentrations, as well as appropriate controls, with a minimum of four experimental units (replicates) per treatment. Each replicate tank contains four female and two male fish. Chemical delivery can be via an aqueous route (with or without carrier solvents). The exposure is conducted for up to 21 days, during which appearance of the fish, behavior, and fecundity are assessed daily. Viability of resultant embryos (e.g., hatching success, developmental rate, occurrence of malformations, etc.) can be assessed in animals held either in clean water, or the same treatment regime to which the adults were exposed. At conclusion of the test, blood samples are collected from the adults for determination of sex steroids and VTG, and the gonads sampled for measurement of the GSI and histological analyses.

DESCRIPTION OF THE METHOD

Test animals and assay system

4. **Test animals:** The procedure described herein is unique to most fathead minnow tests to date in that it is initiated with adults, as opposed to embryos or larvae. The test should be started with newly-mature fish (typically 4 to 6 months old), as opposed to older animals that have been actively reproducing for some period of time, for example, in a culture setting. Thus, to maintain a ready supply of known-quality animals at the desired age for routine testing, it is preferable to maintain a fathead minnow culture,

as opposed to purchasing the animals prior to testing. Field collected fathead minnows generally should not be used to initiate cultures or for testing. Information on general culture and testing requirements for the fathead minnow are provided in Table 12-2.

5. **Water:** The fathead minnow can reproduce successfully over a wide range of water quality. Therefore, no specific water type is required for this test. Any uncontaminated surface, well, or reconstituted water in which the fish can be cultured successfully should be acceptable. Minimal recommended water quality characteristics are listed in Table 12-2. The animals should be tested using a flow-through water renewal system that enables maintenance of adequate water quality (temperature, dissolved oxygen, low ammonia, etc.), as well as ensuring a consistent exposure to the parent chemical (for those tests where water is the route of exposure).

6. **Assay System:** Glass, stainless steel, or other chemically inert material should be used for construction of the test system. The dimensions of the test chambers must be such that the animals can interact in a fashion conducive with successful spawning. The test chamber contains 10 L of test solution, which is renewed at least once every four hours. This particular animal loading/water renewal rate is within recommended guidelines and, in studies conducted according to this method and has maintained acceptable water quality (Tables 12-1 and 12-2) while not utilizing an excessive amount of test material.

Experimental Design

7. The range-finding test should be conducted under the same conditions (water quality, test system, animal loading) as those used for the reproduction test (Table 12-1). It should utilize adult fish, and focus on lethality over the course of at least a 4- to 7-day assay. If the route of exposure is via water, the highest concentration utilized should be at water solubility; in general, a logarithmic dilution series should suffice for the range-finding assay.

8. For the 21-day reproduction test, the highest concentration used should not have caused significant mortality in the initial range-finding assay (note, this may be at water solubility), and the lower concentration/dose used should be a factor of 5- to 10-times lower than the highest test concentration. At present, a minimum of four replicate tanks (each containing four females and two males) is recommended per treatment. It has been demonstrated that a sample size of four enables detection of statistically-significant differences for a majority of the endpoints in control fish versus animals treated with “model” EDCs, including E2, vinclozolin, methoxychlor and MT. Based on this design, a minimum of 72 fish is required per assay (six fish in each of four replicates for two treatments, plus one control).

Exposure via Water

9. Water exposures can be conducted either with or without a solvent carrier for the test chemical of concern. When a chemical is relatively soluble in water (ionic compounds) a solvent is not required to enhance water solubility for preparation of stock solutions; however, much of the toxicity testing historically conducted with aquatic animals and sparingly soluble, nonionic chemicals has utilized carrier solvents.

10. On occasion it may be necessary to utilize solvents to generate stock solutions for aqueous testing; this could occur when a chemical is very insoluble, unstable in a saturator system, or so expensive/limited in availability that the use of saturators is not practical. Indicated in Table 12-2 is the acute (96-h) toxicity of several commonly used carrier solvents to the fathead minnow. The toxicity of these has not been evaluated in 21-day tests, nor have effects of the solvent on the fish endocrine system been evaluated. Hence, it is essential that any test utilizing a carrier solvent include both solvent-exposed and non-exposed controls.

Analytical Determination

11. Regardless of the exposure technique utilized for this assay, supporting analytical chemistry is critical to 1) ensure chemical purity, 2) document that the test chemical is reaching the fish, and 3) confirm system performance. In the water exposures, concentrations of the (parent) chemical should be measured at the start of the assay, at least weekly in the stock solution(s) and, ideally, in a subset of the test tanks as well.

PERFORMANCE OF THE ASSAY

Assay Initiation and Conduct

12. **Pre-exposure:** The pre-exposure phase of the assay should be started with animals that have achieved reproductive maturity, as evidenced by initial development of secondary sex characteristics, but have not been held in a culture/test situation conducive to routine spawning. These animals, which typically are four to six months old, are held in a "mass" culture tank that corresponds to the date the animals were hatched. They typically represent the pooled offspring of 10 to 20 pairs of adult fish from the culture facility. Four females and two males should be randomly assigned to the replicate exposure chambers at each anticipated treatment concentration. In addition to the number of replicates actually exposed at each concentration (e.g., four), a number of additional tanks also should be started; these can serve as "replacement" units for tanks in which pre-exposure spawning is not observed, and/or mortality occurs before initiation of the chemical exposure. Identification of gender may be difficult to resolve for some fish; these animals should not be used for the assay. At this stage in development, males will exhibit nuptial tubercles, while females possess an ovipositor; in addition, males tend to be larger and darker than females from the same cohort.

13. The pre-exposure phase of the assay is conducted under conditions (temperature, photoperiod, feeding, etc.) identical to those used during the chemical exposure (Table 12-2). The animals are fed *Artemia* twice daily ad libitum for water and injection exposures. The fish should be monitored daily for obvious alterations in secondary sex characteristics (breeding tubercles in males, ovipositor in females), reproductive behavior, and spawning activity. It also would be desirable to assess aspects of development of resultant embryos, such as hatching success and rate, and gross appearance of newly hatched larvae. This monitoring phase establishes both reproductive capacity of the test animals, and provides tank-specific baseline data for potential statistical comparison after initiation of chemical exposure.

14. The pre-exposure phase of the assay should last at least 14 days; if acceptable spawning has not occurred within 28 days, an assessment should be made as to why satisfactory biological performance had not been achieved. This might entail examination of water quality or condition of the fish. Minimal criteria for acceptable pre-exposure performance include 1) 100% survival of all adults, 2) presence of eggs in each replicate tank every 3 to 4 days, and 3) >90% fertility of the spawned embryos.

15. **Chemical Exposure:** Once successful spawning has been established, the chemical exposure can be initiated. The target exposure duration is 21 days, which is sufficient for healthy females to produce several clutches of eggs. This allows for a robust data set for assessments of sexual development (e.g., associated with egg maturation), fecundity and fertilization success, embryo development, and hatching success. In addition, the 21-day test period should help optimize exposure of the fish to relatively hydrophobic chemicals, which require a period of time to reach steady-state concentrations in the animal.

Observations

16. A number of endpoints are assessed over the course of, and/or at conclusion of the 21-day assay. Below are described collection of these endpoints and their utility, particularly in the context of the test as an EDC screen.

17. **Survival:** Daily assessment of survival is necessary to provide a basis for expression and interpretation of reproductive output, that is, number of eggs/female/d. In animals exposed to the test chemical, overt lethality may occur, particularly in later portions of the assay not reflective of the initial (shorter) range-finding test.

18. **Behavior of Adults:** Any abnormal behavior (relative to controls) should be noted; this might include signs of general toxicity including hyperventilation, uncoordinated swimming, loss of equilibrium, and atypical quiescence or feeding. From the standpoint of EDC screening, alterations in reproductive behavior, particularly loss of territorial aggressiveness by males, may be affected by chemicals which interact with estrogen and/or androgen pathways. Because of the relative subjectivity of this endpoint, it may be necessary to document behavioral alterations via photographs or videotape.

19. **Fecundity:** Egg production should be determined daily. Fathead minnows usually spawn in the early morning (before 10:00 am) so, except for feeding, they should not be disturbed until late morning. This allows time for spawning and fertilization to be completed, and for eggs to water-harden. The spawning substrates can be removed from the tanks to enumerate any eggs, which are present. One spawn typically will be comprised of 50 to 150 eggs, however, smaller clutches are not uncommon. If no embryos are present, the substrate is left in the tank; new substrates should be added to replace any that are removed. Fecundity should be expressed on the basis of surviving females per reproductive (test) day per replicate. Therefore, if all four females survived the treatment in a given replicate for the duration of 21 days, there would be 84 female reproductive days.

20. **Fertilization Success:** After the spawning substrate has been removed from the tank, the embryos should be carefully rolled off it with a gentle circular motion of an index finger and visually inspected under appropriate magnification. If spawning occurred that morning, embryos typically will be undergoing late cleavage, and determination of the fertility rate (number embryos/number of eggs x 100) is easily achieved. Infertile eggs are opaque or clear with a white dot where the yolk has precipitated; viable embryos remain clear for 36 to 48 hours until reaching the eyed stage. An alternative to the microscopic approach to determining fertilization success is to enumerate eyed embryos at this time. If the latter approach is used for fertility determination, the embryos should be placed in “incubation” chambers and held in a system apart from the adults to avoid possible predation.

21. Regardless of the method used to determine fertility, if information concerning hatching success and/or subsequent larval development is desired, the embryos will need to be maintained for up to an additional 7 days in incubation chambers. Depending upon study objectives, water in the embryo holding system could either be clean or contain concentrations of the test chemical comparable to those used in the adult exposure. To maintain adequate water quality, the incubation system should either provide a continuous flow of water, or the test solution must be renewed daily. During this period of time, if desired, alterations in normal embryologic development can be assessed.

22. **Hatchability, Larvae Appearance, and Survival:** At 25_C, untreated animals will hatch in 4.5 days to 6 days. Each incubation chamber should be evaluated daily for newly hatched embryos; this endpoint should be expressed as a relative percentage of those eggs deemed fertile. The hatching rate of control animals typically is in the range of 95% to 98%.

23. The appearance and behavior of hatched larvae can be evaluated, and results described either qualitatively or quantitatively (e.g., malformation rate). Gross morphological anomalies that may be observed include lordosis, scoliosis, kyphosis, retarded swim bladder development, and craniofacial abnormalities. Survival of the larvae may be assessed through yolk sac absorption (ca., 96 h at 25_C); if estimates of survival are required after this, the animals must be fed (generally live *Artemia*).

24. **Appearance of Adults:** Observations of physical appearance of the adults should be made over the course of the test, and at conclusion of the study. From the perspective of screening EDCs, characteristics of particular importance include: body color (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and specialized secondary sex characteristics (size of dorsal nape pad, number of nuptial tubercles in males; ovipositor size in females). Notably, chemicals with certain MOA cause abnormal occurrence of secondary sex characteristic in animals of the opposite sex.

25. **Gonad Size and Morphology, and Biochemical Endpoints (VTG, Steroids):** At conclusion of the exposure, the fish should be anaesthetized with MS-222 (100 mg/L buffered with 200 mg NaHCO3/L), weighed, and blood collected from the caudal artery/vein with a heparinized microhematocrit capillary tubule. Depending upon size of the fathead minnow (which usually is sex-dependent), blood volumes generally range from 30 to 80 _l. Plasma is separated from the blood via centrifugation (3 min at 15,000 x g), and stored with protease inhibitors at -80ºC, until analyzed for VTG and steroids.

GONAD SIZE AND MORPHOLOGY

26. After sampling the blood, fish should be weighed, and the gonads fixed in situ and then removed and weighed (to the nearest 0.1 mg) for determination of the GSI (GSI = 100 x gonad wt/body wt). Typical GSI values for reproductively active fathead minnows range from 8% to 13% for females and from 1% to 2% for males. Many chemicals that reduce fecundity also will reduce the GSI in one or both sexes. After removal of the gonads, the remainder of the carcass of the fish may be discarded, or preserved for further measurements (e.g., frozen for chemical analysis).

27. Routine histological procedures can be used to assess condition of testes and ovaries from the fish. Gonads should be placed in an appropriate fixative, such as 4% formaldehyde/1% glutaraldehyde, and embedded in paraffin or plastic. Serial sections 4 µm to 5 µm thick should be cut along the long axis of the gonad. At a minimum, two serial sections should be collected from at least three steps equally spaced between the leading edge of the tissue and the midline of the gonad, for a total of six-tissue sections/sample. Sections can be stained with hematoxylin and eosin, and should be evaluated by an experienced histologist without prior knowledge of the treatment regime associated with specific samples. In general, evaluation of the testis is based on the amount of germinal epithelium present, and the degree of spermatogenic activity. The ovary is evaluated based upon relative numbers of perinucleolar, cortical alveolar, and vitellogenic oocytes.

VITELLOGENIN

28. Different methods are available to assess VTG production in fish; a measurement technique that is both relatively sensitive and specific is determination of protein concentrations in plasma via ELISA. For ELISA, polyclonal fathead minnow VTG antibody and purified VTG protein also from the fathead minnow are utilized. Polyclonal and/or monoclonal VTG antibodies prepared using protein from other fish species may cross-react with fathead minnow VTG and, hence, also could be useful for assessing this endpoint.

SEX STEROIDS

29. Plasma concentrations of E2, T, and 11-KT can be determined using RIA techniques optimized for the relatively small sample volumes obtained from the fathead minnow.

PERFORMANCE CRITERIA

30. Water quality characteristics should remain within the limits of tolerance depicted in Tables 12-1 and 12-2.

31. There should be documentation (via appropriate analytical chemistry) of purity of the test material, as well as delivery of chemical to the fish (e.g., concentrations of the chemical in test water).

32. There should be more than 90% survival of control animals over the duration of the chemical exposure, and the control fish in each replicate should spawn, at a minimum, every three to four days.

33. There should be greater than 95% fertility and hatchability of eggs and embryos, respectively, from the control animals.

DATA REPORTING

Treatment and Interpretation of Results

34. Any endpoints that are significantly affected by the test chemical should be reported as such. This information will then be used in a weight-of-evidence analysis to assess the need for further testing.

Test report

35. The test report must include the following:

Test substance:

36. The report must include a detailed description of the test substance, including information on its CAS number, source, lot number, and purity.

37. Additional information should be provided, when available, such as its solubility in water, octanol:water partition coefficient, vapor pressure, and toxicity to fathead minnows.

Test species:

38. Information must be provided on the fathead minnows used in the test. This information must include the source of the fish, age and condition of the fish at the initiation of the test, and the pre-exposure reproductive performance.

39. Any observed abnormalities in reproductive behavior or performance of control fish must also be reported.

Test conditions: The report must specify the conditions under which the test was performed, this includes:

40. Information on the source, treatment of, and basic chemical characteristics of the dilution water.

41. Means and ranges for water temperature, dissolved oxygen, pH, hardness, alkalinity and conductivity.

42. The photoperiod and light intensity used during the exposure.

43. The chamber size, number of females and males per replicate, and number and composition of spawning substrates.

44. Information on food used to feed the fish during the exposure, including supplier and lot number.

45. The basic nature of exposure (i.e. flow-through, ip injection, or dietary) in addition to specific information related to the exposure type (e.g. whether flow-through water delivery type, daily number of volume exchanges of dilution water).

46. Use of solvent or dispersant if any, the specific solvent or dispersant and the concentrations to which the fish were exposed must be specified.

Results:

47. The results must include data for the control (plus solvent control when used) and the treatment fish.

48. The table of results must include the mean, standard deviation and range for each test endpoint from the replicates employed in the test. Statistical significance of means should be indicated.

ANNEX E

PARTIAL LIFE CYCLE TEST (OR EXTENDED EARLY LIFE-STAGE TEST)

DRAFT PROPOSAL

Fish Partial Life Cycle Test Guideline

INTRODUCTION

1. This protocol describes a partial life cycle test for identifying endocrine disrupting chemicals in fish. The protocol is in principal a further development of OECD Guideline No. 210 'Fish, Early Life Stage Toxicity Test' (25), where the exposure is continued until the fish reach sexual maturity. The *in vivo* partial life cycle assay is intended to detect chemicals both with androgenic or estrogenic properties. The concept for this protocol is derived from work on the fathead minnow *(Pimephales promelas)* (1)(2)(3), the Japanese medaka (*Oryzias latipes*) (4) (5) (6) (24) and the zebrafish (*Danio rerio*) (7) (8) (9) (10) (26) (27).

2. The protocol is based on chemical exposure during the sex labile period during which the fish is expected to be most sensitive towards endocrine disrupting chemicals. Four core biomarker endpoints are measured as indicators of endocrine activity, namely: i) gross morphology (e.g. secondary sexual characteristics); ii) vitellogenin levels iii), gonadal histology and iv) sex ratio.

PRINCIPLE OF THE BIOASSAY

3. An overview of relevant test conditions specific for the three species: Fathead minnow; Japanese medaka and zebrafish is provided in Annex 1.

4. The test is initiated with healthy parental fish not older than 6 months, which are kept in spawning tanks. Fertilised eggs are collected and transferred to the exposure beakers. Exposure is initiated on newly fertilised eggs (see Annex 1).

5. The assay is based on a minimum of five test concentrations as well as appropriate controls, with approx. 60 individuals per treatment. The choice of the test design should be justified and the test results analysed with appropriate statistical methods.

6. Exposure to the test chemical is via the aqueous route with or without carrier solvent. Monitoring continues for up to 60-dph (Zebrafish and Japanese medaka); 90-dph (Fathead minnow) and includes hatching rate, development, survival, growth (total length and body weight), sexual differentiation, secondary sex differentiation, gonadal development, histology and VTG levels.

INFORMATION ON THE TEST SUBSTANCE

7. As a minimum, results of an acute toxicity test (see Guideline 203 (11)) should be available. This implies that the water solubility of the test substance is known and a reliable analytical method for the quantification of the substance in the test solutions with known and reported accuracy and limit of detection is available.

8. The following information should normally be available prior to undertaking this bioassay: the structural formula, chemical purity, stability in water and light, pK_a and P_{ow}. Regarding chemical stability, it would be useful to have available results from a ready biodegradability test (see Guideline 301 (12)).

TEST ACCEPTANCE CRITERIA

9. For the test to be valid the following conditions apply:

- Post hatch survival in the control(s) should be ≥ 70%.
- The dissolved oxygen concentration must have been at least 60 per cent of the air saturation value (ASV) throughout the exposure period;
- The water temperature must not differ by more than ± 1°C between test vessels at any one time during the exposure period and should be maintained within a range of 25±2°C;
- If a solvent or dispersant is used, they should not exert any significant effect on fish.

DESCRIPTION OF THE METHOD

Apparatus

10. Normal laboratory equipment and especially the following:

(a) oxygen and pH meters;
(b) equipment for determination of water hardness and alkalinity;
(c) adequate apparatus for temperature control and preferably continuous monitoring;
(d) tanks made of chemically inert material and of a suitable capacity in relation to the loading and stocking density
(e) suitably accurate balance (i.e. accurate to ± 0.5%).

Water

11. Any water in which the test species shows suitable long-term survival and growth may be used as test water. It should be of constant quality during the period of the test. The pH of the water should be within the range 6.5 to 8.5, but during a given test it should be within a range of ± 0.5 pH units. In order to ensure that the dilution water will not unduly influence the test result (for example by complexion of test substance), samples should be taken at intervals for analysis. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl, SO_4), pesticides (e.g. total organophosphorus and total organochlorine pesticides), total organic carbon and suspended solids should be made, for example, every three months where a dilution water is known to be relatively constant in quality. If water quality has been demonstrated to be constant over at least one year, determinations can be less frequent and intervals extended (e.g. every six months). Some chemical characteristics of acceptable dilution water are listed in Annex 2.

Test solutions

12. Test solutions of the chosen concentrations are prepared by dilution of a stock solution. The stock solution should preferably be prepared by simply mixing or agitating the test substance in the dilution water by using mechanical means (e.g. stirring or ultrasonication). Saturation columns (solubility columns) can be used for achieving a suitable concentrated stock solution. The use of solvents or dispersants (solubilising agents) may be required in some cases in order to produce a suitably concentrated stock solution. Examples of suitable solvents are acetone, ethanol, methanol, dimethylsulfoxide, dimethylformamide, isopropanol and triethyleneglycol. Examples of dispersants are given in (13). Care should be taken when using readily

biodegradable agents (e.g. acetone) and/or highly volatile compounds as these can cause problems with bacterial build-up. Where a solubilising agent is used to assist in stock solution preparation, its final concentration should not be greater than 0.1 mL/L and should preferably be the same in all test vessels.

13. For flow-through tests, a system which continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) is required to deliver a series of concentrations to the test chambers. Care should be taken to avoid the use of plastic tubing or other materials, which may contain biologically active substances.

14. For semi-static tests, the frequency of medium renewal will depend on the stability of the test substance. There must be evidence that the concentration of the test substance being tested has been satisfactorily maintained (see paragraph 24-26).

Test animals

15. The exposure phase will be started with newly fertilised embryos. Candidate species for this assay include fathead minnow *(Pimephales promelas)*, Japanese medaka (*Oryzias latipes)* and zebrafish *(Danio rerio)* (Annex 1).

16. Parental fish for the individuals subjected to exposure should be selected from a population of a single stock. The fish should be paired and acclimatized for at least two weeks prior to the test under conditions of water quality and illumination similar to those used in the test (Annex 1). Parental fish should be fed with *Artemia* nauplii minimum twice a day. For avoiding genetic bias eggs are collected from several breading pairs, mixed and randomly selected for initiation of the test.

Conditions of exposure

Age of test organism at start of exposure and duration of test

17. Medaka and zebrafish are exposed from embryos and until 60 dph, while fathead minnow are exposed from embryos until 100 dph (see Annex 1).

To be discussed further: Detailed description is needed for the difference of exposure duration for each test species.

Feeding

18. Throughout the test the fish should be fed adequate amount (food taken up within one hour) of *Artemia* nauplii, exclusively. Care should be taken to avoid microbial growth and water turbidity. The fish should be fed at least 2 times per day, separated by at least three hours between each feeding.

Maintenance of aquaria

19. Excess bacterial or algal growth should be removed by appropriate measures.

20. Uneaten food and faecal material should be removed from the test vessels each day by carefully cleaning the bottom of each tank using suction.

Light and temperature

21. The photoperiod should be 16 h light: 8 h dark and water temperature should be 25 ± 2°C.

Test concentrations and controls

22. It is recommended to use a minimum of five chemical concentrations. The highest test concentrations should be sublethal and should not exceed 10% of the LC50 value of the test substance determined in an early life stage test (OECD 210). A control group is exposed in rearing medium. If a solvent or dispersant is used an additional solvent or dispersant, control is included.

23. As estrogen and androgen control 17α-ethinylestradiol (10 ng/L) and methyldihydrotestosteron (25 ng/L), respectively is recommended. Other potential model substances should discussed further.

Frequency of analytical determinations and measurements

24. During the partial life cycle assay, the concentrations of the test substance are determined at regular intervals, as follows: the flow rates of diluent and toxicant stock solution should be checked at intervals, at least twice per week, and should not vary by more than 10% throughout the assay. It is essential that the actual test chemical concentrations be measured in all vessels at the start of the assay and at weekly intervals thereafter. The test substance concentration is expected to remain within 20% of nominal values.

25. In semi-static (renewal) tests where the concentration of the test substance is expected to remain within ±20% of the nominal values, it is recommended that, as a minimum, the highest and lowest test concentrations and positive controls be analysed when freshly prepared and immediately prior to renewal at the start of the study and weekly thereafter. For tests where the concentration of the test substance is not expected to remain within ±20% of nominal, all test concentrations must be analysed following the same regime as for more stable substances.

26. It is recommended that results are based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance in solution has been satisfactorily maintained within ±20% of the nominal or measured initial concentration throughout the test, then the results can be based on either nominal or measured values.

27. During the test, dissolved oxygen, pH and temperature should be measured in all test vessels at least once per week. Total hardness and alkalinity should be measured in the controls and one vessel at the highest concentration at least once per week. Temperature should preferably be monitored continuously in at least one test vessel. In semi-static tests, it is recommended that dissolved oxygen be measured more frequently, preferably before and after each water renewal or at least once a week.

Observations

28. In the beginning of the test the embryo survival and hatching success should be recorded. Throughout the test fish should be inspected daily and any external abnormalities (such as haemorrhages, malformations, head and skeletal deformities) noted. Any mortality should be recorded and dead eggs or fish removed as soon as possible.

29. Any abnormal behaviour (relative to controls) should be noted; this might include signs of general toxicity including hyperventilation, uncoordinated swimming, loss of equilibrium, change in pigmentation and atypical quiescence or feeding.

Sampling of fish

30. Sampling scheme for the individual species is given in Annex 1. Sampled fish should be anaesthetised with MS-222 (500 mg per L buffered with 200 mg $NaHCO_3$ per L) or FA-100 (4-allyl-2-methoxyphenol: eugenol), individually measured (total length) and weighed as wet weight (blotted dry).

1. Vitellogenin (VTG)

31. The measurement of VTG should be based upon a quantitative and validated method. Information should be available upon the intra-assay and inter-assay variability of the method used in a given laboratory. Different methods are available to assess VTG production in different fish species considered in this assay; a measurement technique that is both relatively sensitive and specific is determination of protein concentrations in plasma via enzyme-linked immunosorbant assay (ELISA). It is preferable to use homologous antibodies (raised against VTG of the same species) and most important homologues standards. Validation of a quantitative method is essential to obtain meaningful results. One challenge to the implementation of the fish endocrine screening assay for routine use will be standardization of the measurement of VTG; ideally, both a standard (perhaps monoclonal) antibody and protein (VTG) standards should be broadly available (1) (2) (14) (15) (16) (17) (18) (19) (20) (21) (22) (23)

32. Fish sampled for VTG measurements should be individually frozen in liquid nitrogen. Due to the small size of the fish VTG should be measured in whole body homogenate samples, e.g., by use of ELISA (3) (21). The minimum weight of the individual fish for whole body VTG measurement should be 10 mg. Maximum weight shall be considered.

To be discussed further: With regard to medaka, the livers should be removed and weighed for measurement of hepatic VTG concentration. The liver removed should be stored at –70 to –80 °C until the VTG measurements. For the assay, they should be individually homogenized with ELISA buffer, centrifuged and the collected supernatants are used for VTG determination.

Gonadal histology

33. At termination of the exposure the remaining fish are fixed in an appropriate fixative for morphological evaluation. Proposed fixatives include phosphate buffered formalin or Bouin's solution. For histological evaluation the fish are processed and embedded in paraffin or plastics. Fish should be sectioned longitudinally at 3-5 µm in thickness. An appropriate number of whole body sections showing different levels of the gonads are cut. Sections are stained with haematoxylin and eosin or other suitable alternative stains and examined by light-microscopy with respect to the gonadal development including sex, intersexuality (simultaneous presence of oocytes and sperm). It should be noted that some fish naturally lack a fully developed pair of gonads and only one gonad may be present (eg. medaka). All such observations must be recorded.

To be discussed further: With regard to medaka and fathead minnow, after sampling the liver, fish should be ventrally opened and viscera gently separated to reach the gonads. The gonads should ideally be fixed in situ for approximately 10 minutes, then removed and weighed (to the nearest 0.0001 g) for determination of the gonado-somatic index (GSI).

Secondary sexual characteristics

34. Secondary sexual characters are under endocrine control; therefore observations of physical appearance of the fish should be made at the end of the exposure. E.g. in medaka, papillary process on the posterior part of the anal fin in females is androgen sensitive. The shape of dorsal and anal fins is also sensitive to endocrine active substances.

DATA AND REPORTING

Treatment of results

35. The primary goal of the data analyses are to calculate the No-Observed Effect Concentration and the Lowest Observed Effect Concentration based on potentially adverse population effects ($NOEC_{adverse}$ and $LOEC_{adverse}$, respectively). These calculations should address the effects on survival, gross development, growth and sex ratio. Secondarily, the data for VTG and gonadal histology may be used to calculate NOEC and LOEC values based on mechanistic biomarker response ($NOEC_{biomarker}$ and $LOEC_{biomarker}$, respectively).

36. If a carrier solvent is used in the test, appropriate analytical method should be used prior to data analysis to determine whether there are differences between the solvent control and control group. If no differences are found, these groups are pooled for subsequent analysis. If differences are found, the control group without solvent is excluded from the subsequent analysis. To identify potential endocrine activity by a chemical, biological responses except for sex ratios may be compared using analysis of variance (ANOVA) followed by multiple comparison test (e.g. Dunnett's multiple comparison test). If the assumptions for parametric methods are not met, non-parametric test should be conducted. The data on sex ratios should be assessed by chi-squared analysis.

Test report

37. The test report must include the following information:

Test substance: Relevant physical-chemical properties; chemical identification data including purity and analytical method for quantification of the test substance where appropriate.

Test conditions: Test procedure used (e.g. semi-static/renewal, flow-through); test design including test concentrations, method of preparation of stock solutions, frequency of renewal (the solubilising agent and its concentration must be given, when used); the nominal test concentrations, the means of the measured values and their standard deviations in the test vessels and the method by which these were attained and evidence that the measurements refer to the concentrations of the test substance in true solution; dilution water characteristics (including pH, hardness, alkalinity, temperature, dissolved oxygen concentration, residual chlorine levels, total organic carbon, suspended solids and any other measurements made); water quality within test vessels: pH, hardness, temperature and dissolved oxygen concentration; detailed information on feeding (e.g. type of food(s), source, amount given and frequency and analyses for relevant contaminants (eg PCBs, PAHs and organochlorine pesticides);

Results: Evidence that controls met the validity criteria, data on mortalities occurring in any of the exposed groups; statistical analytical techniques used, treatment of data and justification of techniques used; tabulated data on biological observations of gross morphology, vitellogenin and gonad histology; results of the statistical analysis preferably in tabular and

graphical form; incidence of any unusual reactions by the fish and any visible effects produced by the test substance.

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APPENDIX 1:

EXPERIMENTAL CONDITIONS FOR THE FISH PARTIAL LIFE CYCLE TEST GUIDELINE

1. Recommended species	Fathead minnow (*Pimephales promelas*)	Medaka (*Oryzias latipes*)	Zebrafish (*Danio rerio*)
2. Test type	Flow-through or semi-static	Flow-through or semi-static	Flow-through or semi-static
3. Water temperature	25 ± 2°C	25± 2°C	25 ± 2°C
4. Illumination quality	Fluorescent bulbs (wide spectrum)	Fluorescent bulbs (wide spectrum)	Fluorescent bulbs (wide spectrum)
5. Light intensity	10-20 µE/M^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)	10-20 µE/M^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)	10-20 µE/M^2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)
6. Photoperiod	16 h light, 8 h dark	16 h light, 8 h dark	16 h light, 8 h dark
7. Loading rate	<5 g per L	<5 g per L	<5 g per L
8. Test chamber size	12 L (until 34 dph) 54 L (from 34 to 90 dph)	2 L (minimum)	6 L (minimum)
9. Test solution volume	9.5 L (until 34 dph) 45 L (from 34 to 90 dph)	1.5 L (minimum)	5 L (minimum)
10. Volume exchanges of test solutions	Minimum of 3 daily	Minimum of 3 daily	Minimum of 3 daily
11. Age of test organisms at start of exposure	Newly fertilised eggs	Newly fertilised eggs	Newly fertilised eggs
12. No. of eggs per test vessel	80	80	80
13. No. of treatments	Minimum 5 (plus appropriate controls)	Minimum 5 (plus appropriate controls)	Minimum 5 (plus appropriate controls)
14. No. vessels per treatment	2 (minimum)	2 (minimum)	2 (minimum)
15. Feeding regime	Live *Artemia* minimum twice daily (*ad libitum*)	Live *Artemia* minimum twice daily (*ad libitum*)	Live *Artemia* minimum twice daily (*ad libitum*)

16. Aeration	None unless DO concentration falls below 4.9 mg per L	None unless DO concentration falls below 4.9 mg per L	None unless DO concentration falls below 4.9 mg per L
17. Dilution water	Clean surface, well or reconstituted water	Clean surface, well or reconstituted water	Clean surface, well or reconstituted water
18. Dilution factor	≤ 3.2	≤ 3.2	≤ 3.2
19. Pre- exposure period	None	None	None
20. Chemical exposure duration	Approximately 100-d	Approximately 70-d	Approximately 65-d
21. Biological endpoints	Non-specific: - hatching success - survival - behavior Endocrine biomarkers: - gross morphology - VTG - gonadal histology Sex ratio	Non-specific: - hatching success - survival - behavior Endocrine biomarkers: - gross morphology - VTG - gonadal histology Sex ratio	Non-specific: - hatching success - survival - behavior Endocrine biomarkers: - gross morphology - VTG - gonadal histology Sex ratio
22. Sampling for VTG analysis	34 days post hatch larvae	34 days post hatch larvae	34 days post hatch larvae
Needs to be discussed further			
23. Number of fish sampled for VTG measurement	Approximately 20	Approximately 20	Approximately 20
24. Sampling for gonadal histology and sex ratio examination	End of exposure	End of exposure	End of exposure
25. Number of fish sampled for histology and sex ratio determination	Approximately 40	Approximately 40	Approximately 40
26. Test acceptability	- Hatching success > 66% - Post hatch survival ≥ 70% post hatch survival of fish in the controls	- Hatching success > 80% - Post hatch survival ≥ 80% post hatch survival of fish in the controls	- Hatching success > 80% - Post hatch survival ≥ 70% post hatch survival of fish in the controls

APPENDIX 2:

SOME CHEMICAL CHARACTERISTICS OF ACCEPTABLE DILUTION WATER

SUBSTANCE	CONCENTRATIONS
Particulate matter	< 20 mg/l
Total organic carbon	< 2 mg/l
Unionised ammonia	< 1 □g/l
Residual chlorine	< 10 □g/l
Total organophosphorus pesticides	< 50 ng/l
Total organochlorine pesticides plus polychlorinated biphenyls	< 50 ng/l
Total organic chlorine	< 25 ng/l