DRAFT DETAILED REVIEW PAPER

ON

A FISH TWO-GENERATION TOXICITY TEST

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1.0 EXECUTIVE SUMMARY

The purpose of this Detailed Review Paper (DRP) is to define the basis and purpose of proposed reproductive two-generation tests for four species of fish (fathead minnow (Pimephales promelas), Japanese medaka (Oryzias latipes), zebrafish (Danio rerio) and sheepshead minnow (Cyprinodon variegatus)) for endocrine effects. The United States Environmental Protection Agency (EPA) plans to use the information contained in this DRP to produce a Fish Two-Generation Test (standardized transferable protocol) that will be used to identify potential endocrine-disrupting chemicals. The DRP summarizes, explains, and documents decisions regarding the relevant principles, methods, and techniques recommended for an initial protocol(s), and identifies issues that might require prevalidation studies to adequately address. This DRP critically evaluates the effect of disturbances in the endocrine system on sexual diffrentiation, development and reproduction in fish. A large portion of this document is focused on interpreting published data in the context of partial or multi-generation studies for the four species of fish, which are the most likely candidate species for use in regulatory testing.

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. In this DRP, the focus is on sexual differentiation, reproduction and paracrine signaling. However, the function of the endocrine system is much broader and contributes to the regulation of many physiological processes such as digestion, metabolism, growth and development. Size, sexual differentiation, sexual maturation, embryo fertilization, and maternal transfer are all examples of sensitive and critical phases in the life cycle of fish. Sensitivity to a toxicant will increase when more of these life-stages are included in a test such as the proposed two-generation fish test.

The culture of the four proposed species is well defined and has been well documented for many years. All four species also tolerate a wide range of water quality and water temperature conditions. These species are small enough to limit culture space and productive enough to continually produce the number of embryos needed for testing. Both the sheepshead minnow and the fathead minnow have a strong regulatory history in the U.S., and the zebrafish has a strong regulatory history in Europe.

The exposure duration of a test needs to encompass an appropriate time of exposure necessary to elicit an effect, but not beyond a necessary time to control costs and potential exposure interruptions. The utility of the partial and full life-cycle test and the multigenerational full life-cycle tests has been evaluated for use in testing EDCs. The partial life-cycle test is a relatively short exposure test designed to evaluate sexual reproduction in fish and the effects to the early life stages of their progeny from exposure to EDCs. A partial life-cycle exposure initiated with adults would have a duration of approximately 9 weeks. The multigenerational test is designed to be a definitive test for evaluating population-level effects of EDC in the environment. The long-term chronic exposure exposes fish through two complete life cycles, plus the option to evaluate the early life stages of the third generation. An alternative to a

multigenerational full life-cycle test, is a two-generation test in which the initial exposure is initiated with mature fish and the F1 generation is evaluated for embryo fertility, development, sexual maturation, reproduction and F2 viability is assessed. This test incorporates the maternal transfer from P to F1 and offers the advantage of a reduction in the time required to conduct the test. The endpoints included in this test allow inter-species extrapolation of the underlying mechanisms of toxicity and assess impacts upon fecundity and viability that address population level impacts integral to risk assessment.

A two-generation protocol that can be conducted with multiple species would be widely utilized, however, it will be necessary to demonstrate the reliability and reproducibility of the test with these species. For risk assessment, inter-comparison of the method employing the four species in this review must address issues such as differences in sensitivity among the species. Demonstration of the two-generation protocol will require significant cost and time commitments and trials with multiple species will required. Alternatively, the pre-selection of one of the four species, such as the fathead minnow, would limit the number of demonstration trials for full optimization of a method suitable for interlaboratory testing. Interlaboratory comparisons for protocol validation should be conducted with compounds that span the possible endocrine effects, including strong and weak androgen receptor agonists and antagonists, estrogen receptor agonists and antagonists, and thyroid agonists and antagonists.

2.0 INTRODUCTION

2.1 Developing and Implementing the Endocrine Disruptor Screening Program (EDSP)

Chemicals that are known or suspected of being endocrine disruptors (Kavlock et al., 1996), also referred to as hormonally active agents (NRC 1999), have received increased attention over the past decade. In 1996, the passage of the two laws, the Food Quality Protection Act (FQPA) and Amendments to the Safe Drinking Water Act (SDWA) mandated the United States Environmental Protection Agency (U.S. EPA) to screen substances found in drinking water sources of food to determine whether they possess estrogenic or other endocrine activity (Federal Register, 1998a, 1998b). Pursuant to this goal, the U.S. EPA is required to "develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or other such endocrine effect (FQPA, 1996).

In 21 U.S.C. §346a(p)(3), the FQPA also states that in carrying out its screening program, the EPA

> (A) shall provide for the testing of all pesticide chemicals and (B) may provide for the testing of any other substance that may have an effect that is cumulative to an effect of a pesticide chemical if the Administrator determines that a substantial population may be exposed to such a substance.

Additionally, Congress amended the Safe Drinking Water Act (SDWA) (42 U.S.C. §300j-17), authorizing the EPA

> to provide for the testing, under the FFDCA Screening Program . . . any other substance that may be found in sources of drinking water if the Administrator determines that a substantial population may be exposed to such substance.

Prior to the passage of the FQPA and the SDWA, the EPA initiated several endocrine disruptor investigations, including the development of a special report and effects assessment (EPA 1997a); a series of endocrine disruptor methods workshops funded by the World Wildlife Fund, Chemical Manufacturers Association (later known as the American Chemistry Council), and the EPA (Gray et al., 1997; EPA 1997b; Ankley et al., 1998); and co-sponsorship (with the National Institute of Environmental Health Sciences [NIEHS] and the Department of the Interior) of an independent critical literature analysis of hormone-active toxicants in the environment by the National Academy of Sciences (NRC 1999).

The U.S. EPA established the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), to provide recommendations regarding a strategy for developing a testing paradigm for compounds that may have activities similar to naturally-occurring hormones. Following the recommendations made by EDSTAC in its final report (EDSTAC, 1998), the U.S. EPA established the Endocrine Disruptor Screening Program (EDSP). The program's aim is to develop a two-tiered approach, e.g. a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial substances, and environmental contaminants (Federal Register 1998a, 1998b).

To date, the U.S. EPA has implemented the program on two fronts: (1) the development of the Endocrine Disruptor Priority Setting Database, and the approach that will be used to establish priorities for screening compounds, and (2) prevalidation and validation studies of some of the Tier 1 and Tier 2 assays that are likely to be included in the testing battery. The Endocrine Disruptor Methods Validation Subcommittee (EDMVS) has been set up to advise and review new and ongoing work in the validation of these assays.

The EDSP's proposed statement of policy, including public comments, was reviewed by a joint panel of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) and the EPA Science Advisory Board (SAB) in May 1999. Gray et al. (1997), EDSTAC (1998), and the National Research Council (NRC 1999) concluded that a tiered approach relying on a combination of *in vivo* and *in vitro* screens for Tier 1 was scientifically reasonable. This conclusion was based upon each group's assessment of the current state of the science on the evaluation of agents affecting the endocrine system. Another consistent conclusion was the need to validate the individual screens and tests in the EDSP. Validation and peer review are prerequisites to the development and approval of test guidelines for regulatory use. Many of the documents cited above and other EPA EDSP-related information may be found

at http:\\www.epa.gov/scipoly/oscpendo.

In addition to the EPA's domestic EDSP validation program, a separate effort to validate certain screening assays and tests for international use is being conducted by the Organization for Economic Cooperation and Development (OECD) Test Guidelines Program. The EPA actively participates as a member of the OECD test guidelines program and its Endocrine Disruptor Testing and Assessment Task Force. The EPA is relying on the OECD effort to serve as the mechanism for validation of some of the components of its EDSP. Separate domestic and international activities are necessary in that laws and regulatory procedures differ in various countries. Although international activities are distinct from domestic activities, overlapping membership on various committees ensures appropriate liaison and communication, eliminates duplication of effort, and facilitates international harmonization.

2.2 The Validation Process

The U.S. EPA (and EDMVS) chose to follow the validation process established by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), of which the U.S. EPA was a charter member, for validation of the EDSP screening and testing methods. ICCVAM was established by the National Institute of Environmental Health Sciences (NIEHS) as a standing interagency committee to aid in the validation, acceptance, and harmonization of test methods designed to reduce animal use, refine procedures involving the use of animals so that they would experience less stress, and to replace animal tests whenever appropriate (ICCVAM, 2000). To this end, ICCVAM defined a flexible, adaptable framework for test method validation that was applicable to conventional and alternate methods, and could be applied to the needs of different agencies and regulatory processes.

The purpose of the validation is to establish the reliability and relevance of a test method with respect to a specific use. The process is science-driven, and addresses the scientific principles of objectivity and experimental design (NIEHS, 1997). In addition, as stated in the ICCVAM report, "A test is considered validated when its performance characteristics, advantages, and limitations have been adequately determined for a specific purpose." (NIEHS, 1997).

The validation process consists of four discrete phases: (1) initial protocol development, (2) prevalidation studies, (3) validation studies, and (4) external scientific peer review. The initial protocol, developed from existing information and experience (past and current research), serves as the starting point for initiating the validation process. Prevalidation studies consist of further development and optimization of specific initial protocols through targeted investigations. Either before or during prevalidation, a detailed review paper (DRP) addressing all critical areas outlined in *Validation and Regulatory Acceptance of Toxicological Test Methods* (NIEHS, 1997) is prepared for each method to summarize, explain, and document decisions regarding the relevant principles, methods, and techniques recommended for the initial protocol. Targeted prevalidation investigations are designed to address questions necessary for

completing an optimized, transferrable protocol suitable for interlaboratory validation studies. Validation studies consist of comparative interlaboratory studies to establish the reliability and relevance of the protocols developed in the prevalidation stage. Validation requires the development of a detailed review paper to document what is known about the assay system proposed for validation.

A test is considered validated when its performance characteristics, advantages, and limitations have been adequately determined for a specific purpose. The measurement of a test's reliability and relevance are independent stages in the validation of a test method, and both are required. Reliability is an objective measure of a method's intra- and interlaboratory reproducibility. If the test is not sufficiently reliable, it cannot be used for its intended purpose. Alternatively, if the test is not relevant, of questionable relevance to the biological effect of interest, or if it is not an appropriate measure of the effect, its reliability is academic. The relevance of a test may be linked to the mechanism of the toxic effect it measures and to its proposed uses (NIEHS, 1997). The studies conducted will be used to develop, standardize, and validate methods, prepare appropriate documents for peer review of the methods, and develop technical guidance and test guidelines in support of the EDSP.

Following the validation studies, results of an external scientific peer review of the study and the optimized protocols will be used to develop the U.S. EPA test guidelines.

2.3 Purpose of the Review on Two-generation Fish Test

The purpose of this Detailed Review Paper (DRP), is to define the basis and purpose of the proposed reproductive two-generation tests for four species of fish (fathead minnow, zebrafish, and Japanese medaka, sheepshead minnow) for endocrine effects. The DRP summarizes, explains, and documents decisions regarding the relevant principles, methods, and techniques recommended for an initial protocol(s), and identifies issues that might require prevalidation studies to adequately address.

2.4 Objective of the Two-generation Fish Test

In 1996, the EPA formed the EDSTAC to provide guidance on how to design a screening and testing program to identify endocrine-disrupting chemicals. In its final report, the EDSTAC recommended a two-tiered approach, i.e., screening (Tier 1) and testing (Tier 2), for the identification of these compounds (EDSTAC 1998). The purpose of the testing is to determine whether a chemical or chemical mixture adversely affects the organism through endocrine-mediated pathways, and to evaluate those effects with respect to the estrogen, androgen, and thyroid systems. In addition, the tests must include exposure during the most sensitive lifestages, provide the opportunity for identification of dose-response effects, and encompass a variety of taxa.

Upon completion of Tier 1 screening and Tier 2 testing, the EPA and other stakeholders will accept, both scientifically and as a matter of policy, the assessment of chemical substances or mixtures according to whether a chemical has the potential or little or no potential for having estrogen, androgen, or thyroid endocrine-disruptive effects. Through the EDSP, individual tests and screens will be selected based upon completion of each topical DRP. In the case of this DRP, the recommended Fish Multigeneration (Tier 2) test, combined with other tests in the Tier 2 battery, will characterize the nature, likelihood, and dose-response relationship of the endocrine disruption of estrogen, androgen, and thyroid in humans and wildlife. The Tier 2 tests should complement Tier 1; however, results from Tier 2 supersede Tier 1 results. The Tier 2 is the final phase of the screening and testing program and, thus, should provide more detailed information regarding the endocrine disruption activity of a tested chemical or mixture. To fulfill this purpose, tests are often longer-term studies designed to encompass critical life states and processes, a broad range of doses, and administration by relevant route of exposure. In addition, the effects associated with EDC may be latent and not manifested until later in life or may not be apparent until reproductive processes occur in an organism's life history. Thus, tests for endocrine disruption will often encompass two generations, including effects on fertility and mating, embryonic development, sensitive neonatal growth and development, and transformation from the juvenile life state to sexual maturity. The results from the Tier 2 testing should be conclusive, and a discernible cause-effect relationship should be manifested during the test if one exists from the chemical exposure. In summary, the following points should be met when designing the Tier 2 test:

- determine whether effects are a primary or secondary disturbance of endocrine function
- establish exposure/concentrations/timing and effects relationships
- be sensitive and specific
- assess relevant endpoints
- include the life cycle of live-bearing and egg-laying species
- include a dose range for full characterization of effects
- be conducted in accordance with Good Laboratory Practices (GLP)
- be validated.

This DRP considers the life-history regime associated with fathead minnow, zebrafish, Japanese medaka, and sheepshead minnow to apply toward the development of a multigenerational test that will achieve the above-stated goals in the most effective and efficient manner possible.

2.5 Methodology Used in the Analysis

Appendix A describes the methods employed for the literature search (i.e., key words, databases used, results, etc.). Briefly, after key papers were identified, retrieved, and read for content, pertinent information was extracted and synthesized to generate this DRP. In addition to the literature review, interviews with experts were conducted to obtain current views and

opinions regarding assays, methods, procedures, and measurement endpoints that hold promise for identifying and developing the most promising screening assay to identify substances that affect (i.e., inhibit or enhance) steroidogenesis. The results of the interviews are found in Appendix B. Finally, accompanying this report is a CD ROM that has the Reference Manager Database of all documents reviewed. This database includes the reference citation and abstract, in addition to summary information from each article.

2.6 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
EC_{50}	median effective concentration
ECD	electron capture detector
EDC	endocrine-disrupting chemical
EDMVS	Endocrine Disruptor Methods Validation Subcommittee
EDSP	Endocrine Disruptor Screening Program
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EE2	17α-ethynylestradiol
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EPA	United States Environmental Protection Agency
ER	estrogen receptor
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FQPA	Food Quality Protection Act
FSH, GTH I	follicle stimulating hormone
GC	gas chromatography

GSI	gonadosomatic index
GTH	gonadotropic hormones
HPLC	high performance liquid chromatography
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
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
MATC	maximum acceptable toxicant concentrations
MT	17 α-methyltestosterone
NIEHS	National Institute of Environmental Health Sciences
NOEL	no observed effects level
NRC	National Research Council
OECD	Organization for Economic Cooperation and Development
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
QRT-PCR	quantitative reverse transcription-polymerase chain reaction
RIA	radioimmunoassay
SAB	Scientific Advisory Board
SAP	Scientific Advisory Panel
SDWA	Safe Drinking Water Act
T	testosterone
T1S	Tier 1 Screening
T3	triiodothyronine
T4	thyroxine
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TSH	thyroid stimulating hormone
VTG	vitellogenin

3.0 OVERVIEW AND SCIENTIFIC BASIS OF ENDOCRINE CONTROL OF FISH REPRODUCTION

This detailed review paper will critically evaluate the effect of disturbances in the endocrine system on sexual diffrentiation, development and reproduction in fishes. A large portion of this document is focused on interpreting published data in the context of partial life-cycle or multi-generation studies in four species of fish (fathead minnow, zebrafish, Japanese medaka and sheepshead minnow), which for a variety of reasons discussed in section 4.0, are the most likely candidate species for use in regulatory testing. The concept of a life-cycle fish bioassay is not new with a number of early studies dating back to the mid-1970s. The emphasis in these studies was primarily on bioaccumulation of pesticides at different life history stages and growth and survival (see Schimmel et al. 1974; Hansen and Schimmel 1977; Jarvinen et al. 1977). More recently, emphasis has been placed on collection of additional biochemical and morphological endpoints that can aid in identifying the mode of action of a toxicant. Because endocrine control of sexual differentiation and development and the response to chemical exposure can vary across vertebrate taxa, multi-generation tests across the vertebrate taxa and including fish is needed. 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. Therefore, before discussion of the candidate species and their responses to endocrine disruptor exposure, it is pertinent to begin with an overview of the endocrine and reproductive systems in fish.

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 spawning) 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).

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 sexual differentiation, reproduction and paracrine signaling. However, the function of the endocrine system is much broader and contributes to the regulation of 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.

3.1 Morphology & Anatomy of the Neuroendocrine System

Neuroendocrine control of reproduction is exerted through actions of the brain, pituitary gland and the 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 turns 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).

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 compared to 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 Japanese 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 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, 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP; Janz 2000). The size of mature eggs from most freshwater fishes range between 0.4 and 3 mm (Redding and Patino 2000) and during ovulation are released into the visceral cavity or lumen of the ovary where they remain until time of spawning (Scott 1987).

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 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 other vertebrates in that they are anatomically distinct from the kidney (Redding and Patino 2000).

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). 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 (Wabuke-Bunoti and Firling 1983; Bonga 1993). In the Japanese medaka (*Oryzias latipes*), thyroid follicles appear next to the ventral aorta (Raine et al. 2001).

3.2 Reproductive and Thyroid Hormones

3.2.1 Hypothalamic, Pituitary and Thyroid Hormones

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. An additional type of hypothalamic secretagogue that is 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 GnRH's in fish are structurally conserved 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 GnRH's are present in the brain (Bently 1998). Apart from the lamprey, all fish appear to possess a common GnRH identical to that found in 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 GnRH's work in concert to regulate gonadotropin secretion by the pituitary (Bosma et al. 2000). There is one and possibly two distinct GnRH receptors 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 GnRH's 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 as a neurotransmitter and autocrine functions within certain tissues (reviewed in Habibi and Huggard 1998).

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

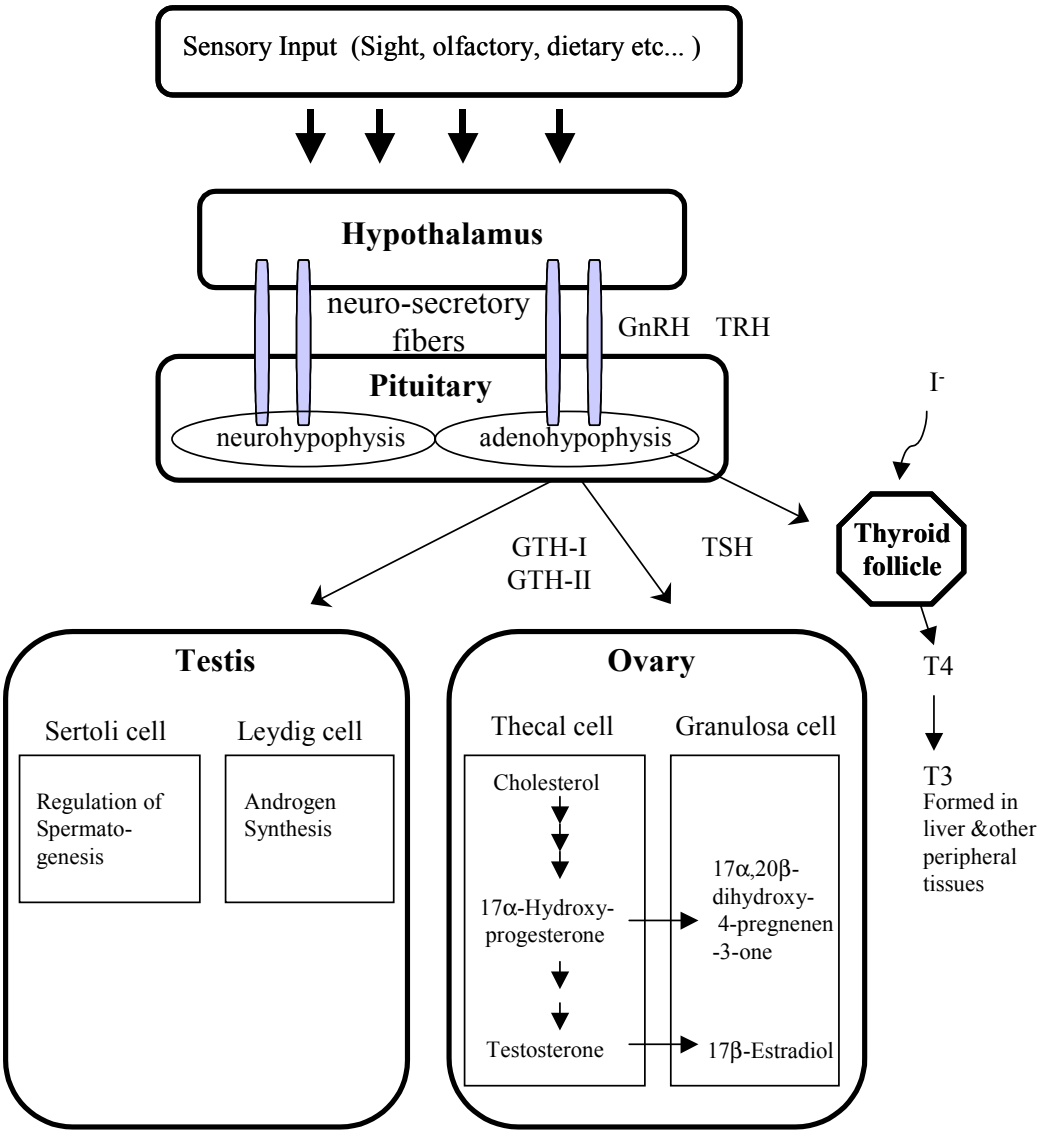

Figure 3-1. Outline of the Brain-Pituitary-Gonadal Axis and Thyroid System in Fish. The Two Cell Model for Steroid Synthesis is Shown for the Ovary

using pituitary cells isolated from the bighead carp (*Aristichthys nobilis*) indicated TRH exposure could upregulate TSH mRNA levels (Chatterjee et al. 2001). There have been two TRH pituitary receptors 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).

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 towards GTH-I and leydig cells 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 circulating levels GTH-I are much higher than 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 only expressed by granulosa cells (Nagahama et al. 1994).

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 than mammals in that thyroid follicles primarily secrete T4 and circulating levels of T3 are solely derived 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.

3.2.2 Gonadal Hormones

The biological consequences of simulation and inhibition of sex steroids forms the basis for most endocrine disruptor tests and is discussed in detail in sections 7, 8 and 9. In this section, the main sex steroids and their synthesis is described.

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 provide 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 suggest 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 testes 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 a male sex pheromone. 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). 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 7.2.2. 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).

A significant physicochemical difference between 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.

3.2.3 Feedback Control Mechanisms

As discussed in section 3.1, 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).

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 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 GnRH's occur within different regions of the hypothalamus (Parhar et al. 2000).

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).

3.3 Endocrine Disruption as a Target For Chemical Toxicity

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 pesticide DDT in wildlife (Bitman et al. 1969). Later observations of masculinization of female mosquitofish residing in near disposal sites for paper mill effluents indicated hormonal disturbances in fish can occur beyond that 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 was 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 1990's discussing the effects of chemicals on reproduction (Colburn 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, 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).

Although sufficient observations in wildlife support the endocrine disruptor hypothesis, it is nonetheless controversial due in part to difficulties in establishing links between environmental exposure to chemicals, changes in endocrine function and altered reproduction

(Van der Kraak 1998). 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 is more established. An excellent example of the latter is recent studies on wild fish living in U.K. rivers that indicate unnatural exposure to estrogen or chemicals with estrogen like activity causes impaired reproduction (Jobling et al. 1998; 2002; van Aerle et. al. 2001).

In conclusion, strong evidence exists that environmental concentrations of EDCs adversely affect sexual differentiation, development and reproduction in fish. 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 various aspects of life-cycle assays directed towards four species of fish (i.e., fathead minnow, zebrafish, Japanese medaka and Sheepshead minnow). This paper will outline the relevant principles, methods, and techniques needed for an initial protocol(s), and identifies issues that might require additional validation studies to adequately address. The final outcome will be a standardized and readily transferable multi-generation fish protocol that can be used to definitely test potential EDCs in a regulatory arena.

4.0 CULTURE AND HANDLING OF TEST SPECIES

This review paper will focus on four species of fish that are the most likely candidates for use in reproductive tests: fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*), zebrafish (*Danio rerio*) and sheepshead minnow (*Cyprinodon variegatus*). These fish share several attributes that make them ideal test species for multi-generation fish bioassays, including small size at maturity (which reduces maintenance costs) and overall ease of culture. All four species are asynchronous spawners, meaning the ovaries contain oocytes at all stages of maturity, allowing spawning to occur repeatedly over an extended time period. The time to sexual maturity is also relatively short (e.g. $\leq$6months) which reduces the time needed to complete testing. Also, these species are frequently used test species in toxicity studies providing important background information for which to design future studies. These advantages are the primary reasons for not considering other potential fish species such as the flagfish (*Jordanella floridae*) or salmonid species (high cost of maintenance).

4.1 Fathead Minnow (*Pimephales promelas*)

The fathead minnow (*Pimephales promelas*) 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 American (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 U.S. and a number of testing guidelines include detailed information on their laboratory culture (EPA/600/3-87/001) (Denny 1987).

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. 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 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 may 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 from the substrate and incubated in a container or in egg cups (glass cylinders with mesh bottoms). Fertilization can be immediately assessed with light magnification or it can be assessed 24 hours after spawning by counting the number of opaque (fertile) and white (nonfertile) embryos. Fertilization rates of 80% to nearly 100% are typical. Embryo incubation time is 4.5 to 6 days at 25ºC.

4.2 Medaka (*Oryzias latipes*)

The medaka (*Oryzias latipes*) is a freshwater fish belonging to the family of Asian rice fishes (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) http://biol1.bio.nagoya-u.ac.jp:8000/Yamabook.html, 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 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) (Yamamoto1975).

Medaka have a generation interval of 2 to 3 months, but can be induced to spawn throughout the year by controlling the temperature and photoperiod, and breeding groups with a ratio of 4 males per 6 females spawn 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 16-hour-light to 8-hour-dark cycle (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 simulating aquatic plants (Yamamoto1975). Even when spawning substrates such as a spawning sponge are provided, some females retain their embryos requiring manual stripping (Denny et al. 1991). The eggs should be collected as soon as possible after spawning to prevent their predation by adults. Fertilization can be easily assessed with low magnification because of the transparency of the egg chorion. The egg incubation period is approximately 1 week when kept at 28ºC (Yamamoto1975), and the embryos will tolerate a temperature range of 7ºC to 38ºC (Kirchen & West 1976).

4.3 Zebrafish (*Danio rerio*)

The zebrafish (*Danio rerio*) is a tropical minnow native to East India and Burma and like the fathead minnow is a member of the Cyprinidae family of fish. 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, are available from commercial suppliers, and detailed methods for their care in the laboratory are available (Westerfield 2000) http://zfin.org/zf_info/zfbook/zfbk.html. They can easily be maintained in aquaria at a temperature of 28ºC, with temperatures above 31ºC and below 25ºC resulting in abnormal breeding and development.

Adult zebrafish are vigorous swimmers reaching a length of 4 to 5 cm and should be fed a variety of food, including formulated dry or moist trout pellets, dry flake food, and live adult brine shrimp, a minimum of 2 times per day for optimal nutrition. Indistinct secondary sexual characteristics can make it difficult to sex zebrafish; however, mature female zebrafish typically have a rounded, fuller body contour, and males generally have larger anal fins and areas of gold coloration. Zebrafish reach sexual maturity in 10 to 12 weeks, and 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 (30 to 50 per tank) daily when paired in equal numbers of well-fed males and females under a 14-hour-light to 10-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 separate the eggs from consumption by the adult fish. Eggs and sperm can be collected from individual zebrafish for controlled fertilization studies(Westerfield 2000). The embryos normally hatch after approximately 3 days of development and begin to feed 1 day post-hatch. The larvae must be reared in separate aquaria that must be cleaned daily. Young larvae feed on live paramecia, and at 9 days post-hatch, freshly hatched brine shrimp can be added to their diet, followed by adult feed with increased growth. All life stages should be fed *ad libitum* 2 to 3 times per day.

The gonadal development in the zebrafish is unique when compared with other species, including the medaka and fathead minnow (Takahashi 1977a). The gonads of all juvenile zebrafish begin to differentiate into ovaries 10 to 12 days post-hatch. After 23 to 25 days, sex differentiation begins. Some fish continue to develop ovaries, whereas in others, the ovaries will begin to degenerate and testicular development will begin. The sex reversal is completed after 40 days, and the gonads will be fully developed in 60 days post-hatch.

4.4 Sheepshead Minnow (*Cyprinodon variegatus*)

The sheepshead minnow (*Cyprinodon variegatus*) is a killifish belonging to the family Cyprinodonitidae. They are commonly found in estuaries along the Atlantic and Gulf of Mexico coasts in North America. Sheepshead minnows can tolerate wide ranges in temperature (0 to 40ºC) and in salinity (0.1 ppt to 149 ppt) (USEPA 1983). The sheepshead minnow has been used routinely in regulatory aquatic toxicity testing in the U.S. Sheepshead minnows are easy to culture and are readily available from commercial sources. The continuous laboratory culture of the sheepshead minnow has been described in detail in a number of U.S. EPA documents (e.g., USEPA 1978; USEPA 1983).

Sheepshead minnows are small (35 mm to 50 mm in total length) and very easy to culture in laboratory aquaria. Adult males exhibit territorial behavior, but are still tolerant of a number of other adult male and female fish in an aquarium. They can tolerate low dissolved oxygen, a wide range of salinities, and a variety of water temperatures, but for optimal growth and reproduction, water temperatures should be within a range of 25ºC to 30ºC, and dissolved oxygen should remain above 60% of saturation. Salinities of 15 ppt to 30 ppt are recommended for laboratory cultures. However, sheepshead minnows live and reproduce in waters with much lower salinities, and maintaining viable laboratory cultures at much lower salinities should be possible. Adult sheepshead minnows are sexually dimorphic and can usually be sexed in the aquarium. The development of territorial behavior and secondary sex characteristics such as blue iridescent coloration along the dorsal region and vertical dark bands along the sides are signs of sexual maturity in males. Females are less colorful but typically have a black spot near

the base of the dorsal fin although males may also exhibit this trait. The generation time of sheepshead minnows is less than 2 months. They can be kept in breeding condition all year without manipulating light cycles and water temperature. Abundant high-quality food is important in maintaining a continuous spawning population. Better spawning synchronization can be obtained by separating the sexes within an aquarium for several days. The adults can also be held at 21ºC to 25ºC and then placed into 28ºC to 30ºC water to induce spawning (Overstreet et al. 2000). Each spawning female will produce 15 to 30 embryos per spawn; therefore, a large number of females will be needed to collect enough embryos to initiate an experiment. Spawning groups should be placed in 3- to 5-mm NITEX mesh baskets. The baskets should be placed in trays to collect the embryos that fall through the basket. Spawning should begin within 24 hours once the males and females are together. Embryos are collected from the trays holding the spawning baskets every 24 hours. The embryos can be incubated in shallow dishes or they can be incubated in egg cups (glass cylinders with mesh bottoms, or nylon mesh cylinders with Petri dish bottom). Fertilization can be immediately assessed with low magnification, or it can be assessed 24 hours after spawning by counting the number of opaque (fertile) and white (nonfertile) embryos. Fertilization rates can be low and variable for sheepshead minnows (40% to 60%). Embryo incubation time is 5 to 6 days at 28ºC.

4.5 Strength and Weaknesses of Test Species

The culture of all four species is well defined and has been well documented for many years. All four species also tolerate a wide range of water quality and water temperature conditions. These species are small enough to limit culture space and productive enough to continually produce the number of embryos needed for testing. Both the sheepshead minnow and the fathead minnow have a strong regulatory history in the U.S., and the zebrafish has a strong regulatory history in Europe. Some of the major strengths and weakness of the four species are summarized in Table 4-1.

Table 4-1. Strengths and Weaknesses of Species Evaluated for Testing

Species	Strengths	Weaknesses
Fathead minnow *(Pimephales promelas)*	- Large enough to collect individual blood plasma samples - Distinct secondary sex characteristics in both sexes - Large historical regulatory data - Many laboratories are familiar with culture and testing - Spawn on a substrate - High fertilization rate	- Relatively long life cycle - Relatively high variability in fecundity - Relative size of the fish require more space for culture and testing

Species	Strengths	Weaknesses
Medaka *(Oryzias latipes)*	- Relatively short life cycle - Relatively small fish making culture and testing possible in smaller space - Female sex determined during embryo stage vs male sex determined after hatch - Sex linked color strain	- Long sticky egg strands make handling eggs difficult - Small size yields small volumes of plasma - No regulatory data base - Limited testing experience in the U.S.
Zebrafish *(Brachydanio rerio)*	- Short life cycle - Small fish making culture and testing possible in smaller space - Male fish go through a hermaphroditic phase as juveniles in some stains - Widely used in other medical and genetic research - Commonly used in Europe for regulatory purposes	- Small size yields small volumes of plasma - Limited U.S. regulatory database - Minimal secondary sex characteristics - Limited testing experience in the U.S.
Sheepshead minnow *(Cyprinodon variegatus)*	- Very short life cycle (<60days to sexual maturity), seawater costs may be offset by shorter exposure times for testing - Relatively small fish making culture and testing possible in smaller space - At least males may by large enough for individual blood plasma samples - Distinct sexual dimorphism - Relatively low variability in fecundity - Relatively large historical regulatory database - Many laboratories are familiar with culture and testing	- Estuarine/marine species, salinity of 15 to 30 ppt recommended, however, lower salinity may be possible (5 ppt) - Culture requires a large number of females to produce enough eggs in a 24-hr period to initiate a life-cycle test - Limited information on reproductive endocrinology

5.0 EXPERIMENTAL DESIGN CONSIDERATIONS FOR MULTIPLE GENERATION FISH TESTS

5.1 Exposure Duration

An increase in the amount of exposure time typically results in quantification of effects at lower concentrations (McKim 1977; Bresch et al. 1990; Holcombe et al. 1995; Ensenbach & Nagel 1997; Nagel et al. 1998; Parrott et al. 2000). The age of the fish during an exposure can also impact the results of a study. Size, sexual differentiation, sexual maturation, embryo fertilization, and maternal transfer are all examples of sensitive and critical phases in the life cycle of fish. Sensitivity to a toxicant will increase when more of these endpoints are included in the experimental design. In general, early research with pesticides has shown that there is little difference in response in a variety of endpoints between the species of fish being considered (i.e., fathead minnow, medaka, sheepshead minnow, and zebrafish) (Shinomiya et al. 1997; Kawahara

& Yamashita 1997. However, developmental and reproductive toxicity might occur during larval development or the juvenile or adult stage, depending upon the species examined (Arcand-Hoy & Benson 1998). Macek and Sleight (1977) and Dionne and Kiamos (1994) found that the toxicity of approximately 20% of the chemicals and metals tested in full life-cycle studies in fish could not be predicted from the results of only an early life-stage exposure.

The maintenance of partial and full life-cycle exposures is costly and can result in unexpected interruptions in exposure as a result of test-substance behavior in 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 encompass an appropriate time of exposure necessary to elicit an effect, but not beyond a necessary time to control costs and potential exposure interruptions. As a result, the fish species with the shortest life cycle might be the preferred species for conducting partial and full life-cycle tests.

5.1.1 Partial Life Cycle: Exposure During P Phase

To minimize the exposure duration, partial life-cycle studies should be initiated with sexually mature fish. A partial life-cycle study exposes adult sexually mature fish (P) for a period of time prior to and during spawning, followed by a short-term exposure of F1 embryos and juvenile fish. Fertilization of the P eggs should occur in the exposure solutions. The F1 exposure can be a few days to a week if the endpoints of interest are limited to embryo viability and hatching success. If F1 survival and growth endpoints are included, the exposure should continued up to 4 weeks post hatch of the F1 larvae. This is consistent with a standard fish early life-stage test, with the exception that the eggs are fertilized in the exposure solution.

The induction of the egg-yolk precursor protein is under estrogen control mediated by estrogen receptors (ERs) in the liver of juvenile and adult fish. Detection of vitellogenin (VTG) has become the most widely studied biomarker of exposure to endocrine-active compounds. Vitellogenin induction in adult male fish can be quantified following an exposure of less than 7 days (Kishida et al. 2001; Elonen et al. 1998). However, longer exposures are needed to measure the effects of reproductive success, changes in secondary sex characteristics, behavioral changes, and gonadal changes. The exposure of the adult P fish should be sustained to allow incubation of at least three or four spawns per treatment level. For the fish species considered in this protocol, the typical exposure time for the P generation would be 21 days (OECD 2001)(Harries et al. 2000; Van den Belt et al. 2001). However, sheepshead minnow may only need a 7-day exposure to provide three to four spawnings. In addition to this exposure period, establishing a brief spawning history with P fish might be desirable prior to exposure; however, this would almost double the length of time that the P fish would be maintained in the exposure system (OECD 2001; Harries et al. 2000, Van den Belt et al 2001). The exposure duration will need to be lengthened if exposure begins with juvenile P rather than adults. The additional time

and effort that this would add to the exposure protocol depends upon the starting age of the juvenile P fish and husbandry practices. Because raising the species of fish under consideration in this protocol to sexual maturity in laboratory cultures can be performed with ease and in a cost-effective manner, there does not appear to be an advantage to initiating the exposure with juvenile P fish in a partial life-cycle exposure. In summary, a partial life-cycle exposure initiated with adults would have a duration of approximately 9 weeks, consisting of 14 days of pre-exposure spawning history, 21 days of exposure of P fish, and up to an additional 28 days of exposure for F1 fish.

5.1.2 Full Life Cycle: Continuous Exposure During F1 Phase

Full life-cycle studies have been conducted for over 25 years with sheepshead minnow, fathead minnow, and zebrafish (Carlson 1972; Nebeker, Puglisi, & DeFoe 1974; Shinomiya et al. 1997; Kawahara & Yamashita 1997; Yasuda et al. 2000; Metcalfe et al. 2000; Nakamura et al. 1998; Ensenbach & Nagel 1997). Many full life-cycle studies using the fathead minnow, and to a lesser extent the sheepshead minnow, have been conducted in the support of pesticide registrations. This required testing is a part of the registration requirements of the Federal, Insecticide, Fungicide and Rodenticide Act (FIFRA). A full life-cycle reproductive and developmental toxicity test should begin with <24-hour-old embryos (P) and should expose all life stages of the P and F1 generations. Sex determination is primarily governed by genetics; however, it can be strongly influenced by hormonal activity for critical periods of time during sexual maturation. These critical exposure times are not well defined for all species, resulting in increased importance of continuous exposures. Using good husbandry techniques, especially a balanced diet fed *ad libitum*, P fish can reach sexual maturity in 60 days (sheepshead minnow) to 120 days (fathead minnow). Allowing one to four weeks for spawning and an additional 28 days post hatch for the F1 generation for growth and survival endpoints, a fish full life-cycle study can be completed within 4.5 months (sheepshead minnow) to 7 months (fathead minnow).

The full life-cycle protocol with fathead minnows is well established and has been successfully conducted by a number of laboratories. This protocol was first developed in 1970 and has changed little since that time. Better understanding of animal husbandry, especially dietary requirements, has resulted in reduced time to sexual maturation. The fathead minnow protocol currently includes a 4-month spawning period, which produces thousands of embryos. Based on the results of the partial life-cycle work with the fathead minnow, sufficient spawning data for the fathead minnow study can be collected in 3 or 4 weeks, which would significantly reduce the overall time and effort associated with this full life-cycle exposure. In addition, it is possible to maintain fathead minnows on a 16-hr-light to 8-hr-dark light cycle throughout their life cycle, thereby further reducing the time to maturation. However, the sheepshead minnow, zebrafish, and medaka offer the advantage of faster maturation compared with the fathead minnow.

5.1.3 Continuous Multiple Generation Exposures

Testing beyond the reproductive phase of the F1 generation would be difficult for many laboratories. Multiple generation exposures have been conducted; however, continuous exposure of P, F1, and juvenile F2 generations has not been reported (Bresch et al. 1990; Patyna et al. 1999; White et al. 1999). The long-term maintenance of an exposure system requires extensive experience that is not widely available. However, the value of studying the effects of long-term exposure on the reproductive system in fish is extremely valuable for determining population-level effects. The value of a multigenerational exposure has been recognized by advocates of zebrafish, medaka, and fathead minnow fish models (Bresch et al. 1990; Arcand-Hoy & Benson 1998; Patyna et al. 1999; White et al. 1999). In addition to these fish models, the sheepshead minnow is a valid model for a multigenerational exposure and offers specific advantages. In considering a reasonable time frame in which to conduct a multigenerational study, it is worth noting that life-cycle tests with exposure durations of over 260 continuous days are currently conducted with fathead minnows. Therefore, it is reasonable to consider a multigenerational test that could be completed in an 8- to 10-month timeframe. The sheepshead minnow, zebrafish, and medaka reach sexual maturity within 60 to 90 days post hatch, making all three species viable candidates for a multigenerational exposure within this timeframe. The fathead minnow has an extensive testing history, can reach sexual maturity in 4 months, and should be considered a candidate for a multigenerational exposure.

The key to a feasible multigenerational exposure is early maturation and the efficient collection of spawning data. The sheepshead minnow is a continuous spawning species. Each female can produce 15 to 30 eggs per day. Assuming that a study design would have 10 to 16 females per treatment level, the number of eggs collected in a week from each treatment level can approach 2000. High reproductive potential in a short period of time would result in a study with P spawning data collected within 2 months and F1 spawning data collected within 4 months. In contrast, by reducing the number of spawns collected from fathead minnows, a multigenerational test can be completed in 10 months.

5.1.4 Strength and Weaknesses of Partial versus Full Life-cycle Exposures

The strength of a partial life-cycle exposure is the reduced time it takes to conduct the test compared with a full life-cycle test. A multigenerational full life-cycle test would reach the beginning of P spawning in the time a partial life-cycle study would be completed. The partial life cycle is the exposure most suited to the fathead minnow. The fathead minnow is relatively larger in size when compared with the sheepshead minnow, medaka, and zebrafish, which allows the collection of more blood plasma, an important consideration for protein and sex-steroid analysis. The presence of VTG in male fish is a reliable biomarker of exposure to an estrogenic compound; however, the interpretation of sex-steroid levels in fish plasma is not as clear. The goal of a partial life-cycle study is to identify potential EDCs. Thus, only two or three widely spaced treatment levels are necessary to identify an EDC.

The weakness of the partial life-cycle test is its inability to expose all life stages of the fish. Therefore, the partial life-cycle study does not provide data on potential population-level effects. The full life-cycle and multigenerational exposures ensure that all life stages are exposed. Therefore, population-level effects can be ascertained, and potentially of more importance, concentrations that do not cause population-level effects can be determined. If a partial life-cycle study has assessed VTG levels and sex-steroid levels, then the life-cycle study can focus on reproduction, growth, and survival endpoints. Histopathology might be a necessary endpoint for life-cycle studies. If the partial life-cycle study is conducted with the fathead minnow, then the life-cycle study can be conducted with a different species. This allows for the use of a smaller and faster maturing species, which reduces the study duration. It also allows for evaluating the effects of an EDC on another species.

The weakness of the life-cycle study is the difficulty in maintaining long-term exposure systems and the cost of running long-term studies. However, long-term exposures are the only reliable way to evaluate the chronic effects of EDCs on fish.

5.2 Ontogenic Period of Exposure

Based on the life-history model, fish can be broadly divided into two ontogenic groupings: those with an indirect life cycle and those with direct life cycles (Balon 1975). In the case of the indirect life cycle, five distinct life-history periods are found: embryonic, larval, juvenile, adult, and senescent. Fish with an indirect life cycle tend to be oviparous. The direct life cycle is characterized by four life-history periods: embryonic, juvenile, adult, and senescent, the larval period being absent. Fish with a direct life cycle are typically viviparous, with internal fertilization and gestation. Once a fish has attained sexual maturity for the first time and entered the adult period of its life history, two patterns are observed: gonochorism or hermaphroditism (Chan & Yeung 1983). In the case of gonochoristic fish, the sex, either male or female, is fixed; whereas in hermaphroditic fish, the sex may change from male to female (protandry) or from female to male (protogyny) as adults, or both sexes can be simultaneously and functionally present. Because all the test species being considered in this review are gonochoristic, the hermaphrodite pattern of sexual development will not be further discussed.

Not surprisingly, there is considerable variability amongst different species of gonochoristic fish with respect to when during the life cycle the gonads first differentiate and sex can be determined. A generality that is emerging from fish studied to date is that ovaries differentiate in females before testes differentiate in males (Nakamura et al. 1998). Therefore, in a given species, the female gonad may begin to differentiate in a different life-history period (e.g., embryonic) from that of the male (e.g., larval or juvenile). Two pathways for gonad differentiation in fish have been documented (Yamamoto 1969). In the undifferentiated-type, the indifferent gonads pass through a female phase (i.e., initially appear as ovaries) before either taking a male path or continuing as a female. In the differentiated-type, the indifferent gonad directly develops into an ovary or testis. The zebrafish has the former, undifferentiated-type of gonadal development, in which ovaries appear around Day 10 in all juveniles and by Day 25

begin to transform into testes in genetic males (Takahashi 1977b). Alternatively, the medaka has the differentiated-type, in which the ovaries are distinguishable during the embryonic period, and the testes in males appear later during the larval period (http://biol1.bio.nagoya-u.ac.jp:8000/). This phenomenon is probably explained by the mechanism of sex determination in these two fish in which the medaka has a clear genetic mechanism that uses sex chromosomes with male heterogamety (i.e., XY), whereas that for the zebrafish does not and likely uses a polygenic mechanism involving autosomes.

The significance of the ontogenic period of exposure to EDCs is that, depending on the species and sex, different life-history periods may be more or less susceptible to significant reproductive effects. The sexually differentiating gonad in gonochoristic fish, although under genetic control, can be dramatically influenced by sex steroids (Hunter & Donaldson 1983). Male salmonids treated with estrogens can be sex reversed to fully functional females, and females treated with androgens can be sex reversed to males. Endocrine disruptors, with estrogenic or androgenic modes of action, are expected to have similar effects if threshold concentrations are achieved. The review by Piferrer (Piferrer 2001b) describes the powerful effects of estrogenic compounds to cause complete sex reversal or intersex conditions in males of a variety of fish, depending on when treatments are given. This shows the developmental timing of exposure is critical and important in terms of the expected effects.

5.3 Route of Administration

5.3.1 Water

Water exposure is the most common route to expose fish to EDCs, and a water-exposure concentration can be correlated with water concentrations found in the aquatic environment. The delivery of a toxicant in water at different concentrations is well established. Water exposures have been successfully conducted by laboratories around the world, using a variety of pumps, valves and gravity. The biggest challenge in a water exposure is solubilizing the test substance in the water. Organic solvents are often used as carriers to assist in delivering a test substance in water, which requires the maintenance of a solvent control. Organic solvents also enhance bacterial growth in the test system, which increases maintenance time during the exposure. In some cases, saturator columns have been used to eliminate the use of solvents.

5.3.2 Oral (Food)

Patyna et al. (1999) recommends that hydrophobic compounds with a K_{ow} >5 should be administered via food. Dosing some commercial artificial fish food is relatively simple, but dosing food items such as live or frozen foods may not be possible. Because a balanced diet is important in the rearing of fish in an exposure system during many life stages, dosing via a variety of prepared and live foods would present a significant challenge. In addition to the need to provide a variety of chemically prepared feeds, the major problem with oral dosing is the inability to determine the dose that each fish receives. Another confounding factor of oral dosing

occurs with uneaten food that remains in the tank, releasing chemical into the water, thereby combining the oral uptake with water exposure.

5.3.3 Intra-Ova Injection

Intra-ova injection is a highly specialized technique. This exposure route has been used to directly simulate maternal transfer of a toxicant and the resultant effects upon sexual differentiation (Papoulias et al. 2000a, 2000b). However, the small egg size of fathead minnow, sheepshead minnow, zebrafish, and medaka makes this task even more difficult. Practical application of intra-ova concentrations to environmental concentrations is also difficult. Similar to oral dosing, intra-ova injection has limited application in a large-scale screening and testing program.

5.4 Dose Selection

Dose levels for both partial and full life-cycle studies should be selected with the use of range-finding data, unless reliable data are readily available. Selected concentrations should be less than lethal levels and less than the level of water solubility. Exposure concentrations should be measured weekly during the exposure. The concentration of some test substances will decrease over time in mature life-cycle systems. If this occurs, nominal concentrations should be increased in an effort to maintain constant exposure levels in the aquaria.

Two or three widely spaced treatment levels would be appropriate for partial life-cycle studies if they are used as a screening tool. The treatment levels can be separated by up to an order of magnitude. It is not necessary for a partial life-cycle study to have a treatment level that causes no effect. A life-cycle study should be conducted with at least five treatment levels, and the treatment levels should be separated by approximately a factor of two. At least one of the treatment levels should be below the no-observed-effect level.

5.5 Statistical Considerations

The objective of the multiple generation fish test is to provide the most precise and accurate estimate of toxicity associated with endocrine disruption and reproductive fitness for an identified potential EDC. Thus, the assay must be biologically sensitive, have minimal variability associated with dose exposure throughout the duration of the test, and have a statistically powerful inference. Biological sensitivity is a function of the choice of species tested, the relevance of the endpoints measured to species survival, and the route and duration of the chemical exposure. Design-associated variability in dose exposure is a function of exposure route and duration, chemical stability and purity within the testing environment, and the testing protocol. The power of a statistical inference is a function of the inherent variability in response; design-associated variability; the degrees of freedom and the source of variability for testing; and the estimation process and decision criteria. Other areas in this section have discussed biological sensitivity; this subsection will focus on design-associated variability and statistical power.

Design-associated variability can be reduced by minimizing the variability in the exposure dose and chemical purity through the route and duration of exposure. Chemical analysis of water and/or food samples over time from the exposure tanks must be performed. Species with shorter life cycles reduce the time on test and by default reduce the variability in the exposure. Oral exposure could reduce food intake, thus affecting the exposure dose for several days of testing. Alternatively, a water route for a flow-through system produces difficulties in maintaining a constant dose over time. Both exposure routes could be affected by a change of purity and/or dose throughout the time on test.

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 1) experimental containers within a testing environment, 2) treatment application to experimental containers, and 3) application of organisms to experimental containers allows one to incorporate the variability associated with the environmental conditions, the containers, and the organism equally across all treatments. 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.

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 inferences associated with the treatment response are 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 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. The variability between replicate experimental units may also include noise that was not randomized out due to a poor randomization or variable measurement error. These sources of variability can be reduced without loss to inference.

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. Thus, the choice of which species should be tested and the relevant endpoints measured should include a comparison of inherent variability or CVs (standard deviation/mean x 100%). In terms of power, high CVs have low power for detecting small-scale differences. For example, control data from experiments with fathead and sheepshead minnows with only 2 replicates per treatment provide estimates of CVs for a variety of endpoints (Table 5-1) that can be used to evaluate power. The measured response with a CV greater than or equal to 15% will be unlikely to detect differences smaller than 50% between the test and reference treatment response at a type I error rate of α= 0.05 (Figure 5-1). 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, requires the least replication for a given level of power and, thus, are more cost effective.

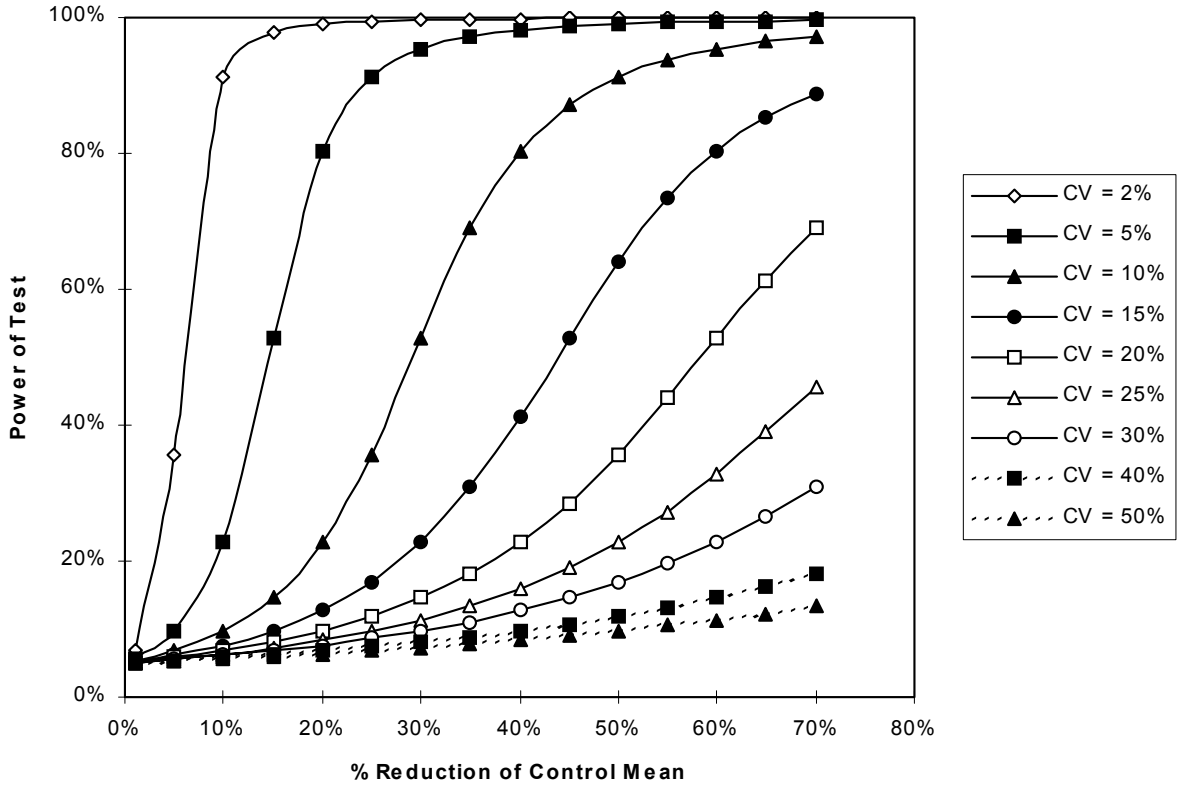

Figure 5-1. Power of a one-sided independent-samples t-test as a function of the percent reduction detected between the test and reference means with 2 replicates per treatment, a = 0.05

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. 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.

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 to 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).

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.

Hurlbert's (1984) classic paper on pseudoreplication provides an excellent discussion of the relationship between inference and replication in experimental design for which tests of significance are desired. Pseudoreplication is defined as the use of inferential statistics to test for treatment effects with data from experiments in which either treatments are not replicated (though samples may be) or replicates are not statistically independent. For either type of pseudoreplication, one would obtain an error term that is invalid for testing the hypothesis associated with the mean responses from the population of fish over time and space. Without true treatment replication, the effect of the treatment is confounded with the variability of the response. It is possible that the response is mainly due to a poor random sample of fish exposed to the treatment rather than the treatment. Thus, the strength of the inference of causality is diminished.

5.5.1 Sample Size: Ensuring Adequate Offspring for Testing of Successive Generations

Typical considerations for sample size are based on the number of endpoints to be collected and whether a fish has to be sacrificed to collect the data. With chronic dosing protocols, a dose response is expected (i.e., over some specified range of doses there will be varying intensity of endpoint response that is significantly different from un-dosed or control fish). Additionally, it is assumed that at some dose there will be no difference between the dosed and un-dosed fish. A specific concern related to multigeneration tests is the need for adequate F1 offspring to be produced to allow for sufficient replication of adult F1 responses. If at high doses complete or substantial embryo-larval lethality occurs, the assay, in effect, approximates a screening test, as only P reproductive effects can be assessed. To guard against this possibility, prior results on fertilization and hatching success obtained as part of a screening test should be carefully scrutinized to avoid excessively high exposure rates. Furthermore, it may be necessary to increase the sample size of P spawners at high exposure rates to ensure adequate collection of surviving F1 larvae. However, due to the size of the system needed to culture and expose fish through a (partial) life cycle and the need for multiple treatment levels, the number of replicates that can be maintained will be limited. Regulatory testing and studies from the literature routinely use a number of treatment levels with two true replicates per treatment level. A dilution water control and a carrier control (if used) are always required. Subreplicates for spawning groups and monitoring the hatch or survival and growth of embryos and larval fish from unique spawning groups within a replicate are advisable if uncertainty regarding F1 larval toxicity exists.

As a useful guide, 100 embryos per replicate has been a standard sample size for starting a life-cycle exposure used for regulatory purposes. This number is twice the number previously recommended by regulatory agencies (Rexrode & Armitage 1986), but ensures adequate numbers of larvae can be procured for continuation of the exposure to maturity. The literature generally has smaller starting sample sizes ranging from approximately 20 embryos or larval fish per replicate (Carlson1972; Nebeker et al. 1974; White et al. 1999) to 240 larval fish (Nimrod & Benson 1998). One hundred embryos allows for a good examination of hatchability, fry survival, and growth of the P generation. It also allows for adequate numbers of fish to set up spawning groups and collect sexually mature but never spawned fish for GSI, blood biomarkers, and histopathology. Fewer P post-spawn fish will be available, with more females than males for most species. Collecting 50 to 100 F1 embryos per P spawning group would also be adequate for the F1 exposure. Additional embryos from the P spawning groups can be collected for other endpoints, e.g., eggs per female and fertilization or hatching success. Finally, 50 to 100 F2 embryos per F1 spawning group will be sufficient for evaluating growth and survival of the third generation.

5.5.2 No Observed Effect Concentration (NOEC) and Maximum Acceptable Toxicant Concentration (MATC): Limitations for Use in Multigeneration Studies

The no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) have been used to evaluate data from a multigeneration study typically when the

differences between exposure levels are no greater that a factor of approximately 2 to 2.5. A multigeneration study will generate approximately 20 to 30 NOEC/LOEC pairs of endpoints for both the P and F1 generations, plus an additional three or four endpoints for the F2 generation. This would provide a total of 60+ NOEC/LOEC pairs for evaluation of a NOEC for fish chronic toxicity. These endpoints are life-stage specific, sex specific, and generation specific.

There has been much debate over the use of the NOAEL in toxicity assessment and the associated risk analysis (Crane & Newman 2000)(Chapman et al. 1996). The debate stems from the perceived goal of the reproductive test. One goal is to detect effects on the reproduction of the test population at the lowest concentrations that produce biologically significant effects. The desire to detect effects implies a comparison of means; analysis of variance (ANOVA) methods are appropriate to compare means (e.g., are treatment means statistically different from the control) such as in a screening test or a validation test. However, ANOVA methods are not appropriate when a precise and accurate estimate of toxicity and the pattern of response are required. Regression techniques provide an estimate of the level of effect as a function of exposure (nominal or actual concentration) and the functional relationship between dose and response. Further, by analyzing the different dose-response relationships, one can compare the sensitivity and potential thresholds of effect for different endpoints.

Even though the NOEC is widely used, it should not be relied on as the sole indicator of low toxicity. The largest dose for which statistical differences have failed to be detected is a direct function of the power of the test. For certain fathead minnow endpoints, such as P eggs/female/day, it is unlikely that any effects will be detected with only two replicates per treatment. It is also conceivable that short-term range finding experiments will have difficulty in predicting the location of an NOEC in a multigeneration test. Using effects concentration (EC_x) calculations are an appropriate alternate for estimating doses associated with low toxicity. However, care must be taken not to estimate an EC_x value that is more sensitive than the data and what the experimental design will allow. The difficulty in interpreting the results from a multigeneration study might lie in whether a statistically significant or not significant result will have true population-level effects. Precision and accuracy of the EC_x is a function of the spread between treatment concentrations and the number of concentrations tested (Chapman et al. 1996).

The design and analysis requirements for estimating the NOEC are different than those for fitting a dose-response model (Chapman et al. 1996); (Stephan & Rogers 1985). ANOVA methods require experimental unit replication and achieve greater power in testing as a function of the number of replicates. As shown in Figure 5-1 and Table 5-1, the different endpoints would require different amounts of replication to achieve the same level of power. Transformation of the data to satisfy homogeneity of variance is required for the parametric test and the regression approach. Estimation of the NOEC does not require the assumption of a specific model, such as a lognormal, and ANOVA methods such as the t-test and Dunnett's test are robust to non-normal errors (Sheffé 1959).

The design of a study intended for dose-response modeling does not require replication of the treatments (Snedecor & Cochran1980). Individual responses are assumed to be a random response from a normal population of responses for a given dose. The variance is assumed to be equal for each population. Replication of doses provides a test of equal variance and lack-of-fit (Draper and Smith 1981). Further, due to the unpredictable nature of survival and fertility in the multi-generation test and the large variability in specific endpoints, it is desirable to have some level of treatment replication in order to provide a more accurate estimate of the mean population response for a given dose. The number of replicates would depend on the maximum expected variability in response for each dose. The variability in response may be a function of the dose. In this case, either a weighted analysis should be conducted or a data transformation applied that satisfies the assumption of homogeneity of variance.

Benefits of the regression approach include 1) estimation of the pattern (e.g., slope) of toxicity as a function of dose; 2) estimation of the distance between effect concentrations and environmental concentrations; 3) estimation of ED_x and their associated confidence intervals for x equal to a low to medium effect; 4) the ED_x estimates are not limited to doses on test; 5) both measured and nominal concentrations can be used; and 6) the ability to compare dose-response curves across endpoints (Chapman et al. 1996). The size of the resulting confidence intervals (i.e., precision of the estimated ED_x) is a function of the inherent variability in the response and the number and spacing of the concentrations tested. Guidelines often require five concentrations that are geometrically spaced and sub lethal plus a no-dose control. Thus, a range-finding test would be required to determine appropriate concentrations. Regression modeling is flexible enough to handle a wide range of dose-response patterns including non-monotonic. If there are only one or two responses that are not either 0 or 100% affected and at least one greater than 50% affected, the Spearman-Karber non-parametric method can be used to estimate an ED_{50}. Finally, the regression approach can handle a wide range of responses including continuous responses, counts, and quantal data by re-expressing or transforming the data (e.g., log (y+c; $y+c)^{1/2}$, and probit respectively).

6.0 DESCRIPTION OF ASSAY ENDPOINTS REFLECTIVE OF REPRODUCTIVE DYSFUNCTION

6.1 Growth and Morphological Alterations

6.1.1 Gonadal Development

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).

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 endocrine-active compounds. Although frequently reported, the appropriateness for comparison of GSIs 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 (Wall et al. 2000). 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.

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 × 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); zebrafish: females 6.7% ±1.6%; males 0.98% ±0.2 % (Van den Belt et al. 2001); medaka: females 8.5%; males 1.5% (Scholz & Gutzeit 2000).

Although measurement of the GSI is a simple procedure, its determination and application as a useful indicator of reproductive status has been questioned. It has been frequently noted in fish that differences in mass of the reproductive organs are not always attributable to changes in overall gamete production or gamete size. For example, a reproductive study of Gizzard shad (*Dorosoma cepedianum*) indicated that only 50% of the variability in ovarian weight could be explained by differences in fecundity or mean egg volume (Jons & Miranda 1997). Other authors have suggested that a better predictor of gonad maturation in females is measuring oocyte diameter (Wall et al. 2000).

An additional problem with the use of the GSI separate from the issue of comparing fish at different stages of development is the general criticism of calculating ratio-based indices of fitness. This criticism is based on several factors, including the assumption of an isometric relationship between tissue mass and body weight (Raubenheimer & Simpson 1992; Packard & Boardman 1999). However, in fish as in other vertebrates, most tissues exhibit either positive or negative allometry with respect to overall body weight (Weatherley 1990);(Schultz et al. 1999). Normalizing gonad mass to body weight may introduce biases into the analyses that could potentially mask or cause misinterpretation of the effect of a toxicant on the size of the reproductive organs.

As a more statistically sound alternative to the measurement of GSI, several authors have recommended the use of multivariate analysis of covariance of the directly measured gonad and body weights (Packard & Boardman 1999; Raubenheimer & Simpson 1992). In this approach, the raw, untransformed data for gonad and body weights from control and exposed fish are plotted graphically and a regression analysis is performed to each dataset to determine the slope. Assuming the slopes are similar, a weighted average is calculated by multiplying gonad weight by the average of the slopes for each treatment group. After adjustment of the gonad weight to an average body weight, ANOVA can be performed to assess the differences between treatment groups (Packard & Boardman 1999).

6.1.1.1 Histopathology in Juveniles. Although the normal cycle of reproductive development cannot be observed in sexually immature individuals, the lack of development or abnormalities in the immature ovarian and testicular tissue may be assessed in juvenile fish including the failure to develop any sexually differentiated tissue. For example, (Lange et al. 2001) reported that in male fathead minnows exposed to 4 ng/L of a synthetic estrogen, no testicular tissue was found 172 days post-hatching. In addition, other organs systems may be affected in juveniles such as liver. For example, (Schwaiger et al. 2000) described alterations to the liver, spleen and kidney of juvenile carp (*Cyprinus carpio*) receiving chronic exposure to ethynylestradiol (EE2). In these organs, marked pathological changes were observed including hypertrophy and degeneration of epithelium and endothelium, hemorrhage and accumulation of eosinophilic material. In another study, larval zebrafish exposed to 17β-estradiol (E2) were reported to show evidence of fragmenting and disintegrating renal tubular cells but details were not provided (Olsson et al. 1999). The same authors reported other pathological manifestations, including craniofacial malformations, and bile stasis, following exposure to E2.

Pathological changes are expected in the developing but not yet mature reproductive tissues of juvenile fish from compounds that affect reproductive development in adult fish. Gonadogenesis is reported to be greatly retarded in both sexes but particularly in female medaka receiving 17α-methyltestosterone (MT) (http://biol1.bio.nagoya-u.ac.jp:8000/KobayashiH85.html). In the medaka, genetic females 7 to 8 mm in length had a smaller gonad, and fewer developing oogonia and oocytes than untreated females. Some germ cells formed clumps of distinct acini typical of early testicular structures. Subsequently, oocyte development was interrupted prior to vitellogenesis, and various abnormal structures were observed. The degenerative process, observable by histopathological evaluation, was described in some detail in the medaka but is not well studied in most species. This effect in females is known to be dose-dependant in fish such as rainbow trout, in which lower dose ranges of MT are used to produce phenotypic males from genetic females by dosing eggs or first feeding fry. Higher doses result in sterility (i.e., retarded development; (Solar, et al.1984)). In the medaka, the investigators reported that the first evidence of sex reversal seen was the appearance of several clumps of acini in the gonad of androgenized female larvae with a 9-10mm body length (http://biol1.bio.nagoya-u.ac.jp:8000/KobayashiH85.html).

6.1.1.2 Histopathology in Sexually Mature Individuals. The primary structural effect of EDCs on mature fish is observed in reproductive follicles. In female fish, ovarian development can be staged by enumerating the proportion of a fixed number of ova in either normal or degenerative stages of development. In regard to the normal development of oocytes West (1990) reviewed histological staging in fish and notes the following developmental sequence: 1) chromatin nucleolar stage, 2) perinucleolar stage, 3) yolk vesicle (cortical alveoli) formation, 4) vitellogenic (yolk) stage, and 5) ripe (mature) stage. Although West (1990) concludes that histology is the most sensitive and appropriate method for staging ovaries, he also notes that the final stages of oocyte maturation are often difficult to follow in histological material because of the shrinkage and distortion of the cells during processing and the loss of ovulated cells during histological processing.

In fathead minnows exposed to E2, Miles-Richardson et al. (1999) reported an increased number of atretic follicles and fewer secondary and Graffian follicles in comparison to unexposed female fish. Other authors have also reported that the ovaries in female fish exposed to estrogenic chemicals show fewer mature follicles and a greater number of atretic follicles (Ankley et al. 2001; Lange et al. 2001).

Effects on male reproductive follicles are reported to include degeneration of spermatocytes, generalized atrophy, the development of ova-testes and a proliferation of Sertoli cells related to the concentration of exposure (Miles-Richardson et al. 1999). These authors evaluated testicular lesions based on the degree or severity of relative or absolute Sertoli cell proliferation and the percentage of seminiferous tubules affected. The semi-quantitative method of Sertoli cell proliferation rated the proportion of affected Sertoli cells from 0 to 4, corresponding to the quartile percentage of affected cells. Degenerative changes included germ cell syncytia, mineralization of spermatozoa and necrotic spermatozoa.

6.1.2 Histopathology of Non-Reproductive Tissues

Direct effects of estrogen or androgen (anti-) agonists may occur in tissues that express receptors for these compounds leading to histologically detectable changes, particularly when exposure causes excessive or unnatural stimulation of these pathways. In addition, indirect effects may occur in peripheral tissues resulting from excessive stimulation of receptor-gene products such as VTG. In this latter example, unnaturally high VTG levels may be responsible for the accumulation of excess protein in various organs and the frequently noted accumulation of eosinophilic fluid in tissues during prolonged exposure to high E2 levels (Herman & Kincaid 1988; Metcalfe et al. 2001). More specific histopathological effects reported from exposure to estrogenic compounds include vacuolization of hepatocytes and degeneration of renal glomeruli (Metcalfe et al. 2001).

Effects of chemicals acting on the thyroid gland are also detectable by histological analysis. Histological changes in the thyroid gland have been found to be the most sensitive parameter observed in fathead minnows exposed to a anti-thyroidal chemical (Lanno & Dixon

1994). This study showed that a sufficiently high dose results in hyperplasia of the thyroid causing a visible goiter. Clearly, at lower doses, physiologically significant thyroid hyperplasia can occur but will only be detected histologically. Other thyroid active compounds can produce complex effects such as thyroid hypertrophy and hyperplasia of follicular epithelium that are only detectable through histological analysis (Sathyanesan et al. 1978). Other toxicant induced effects detected by histological means in the medaka include lipomatosis (development of adipose tissue) in the liver and kidney, vacuolation of liver cells and buildup of amorphous eosinophilic precipitate in renal glomeruli, liver sinusoids and around the splenic capsule (Wester & Canton 1986).

These examples are mentioned to illustrate the utility of histological assessments of non-reproductive organs. Histological analysis of non-reproductive tissues can be particularly valuable in juvenile fish, as the less-developed gonads may not allow a thorough characterization of the mode of action of the test chemical.

6.1.3 Sexual Development

Once 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. In oviparous female fish, 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-ketotestosterone (11-KT) in the male is 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 and zonagenesis or production of egg membrane. Therefore, endocrine-active compounds that affect any aspect of the reproductive endocrine system causing reduced synthesis or release of gonadotropins, or 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 GSI 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 life-cycle studies of fish that presumably result from this mode of action. Fertility may also be affected in both sexes when fewer germ cells develop and mature. This can be detected by quantifying the number of eggs produced in females or assessing sperm number in males.

The effects of alterations on sexual development are technically easy to measure and any of the four test species proposed would be amenable to the types of analysis described above. Indeed, many of these endpoints have been previously reported in a number of studies with these species (Harries et al. 2000; Maack et al. 1999).

6.1.4 Secondary Sex Characteristics

The development of secondary sex characteristics in fish is hormonally controlled, making them viable endpoints for the evaluation of endocrine disruption. All of the species considered have some secondary sex characteristics. The female zebrafish and fathead have distinct genital papilla. The male fathead minnows have 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, medaka and sheepshead minnow have distinct coloration. The male sheepshead minnow has a distinct vertical band along the posterior edge of the caudal fin. The female sheepshead minnow has a distinct spot located on the anterior portion of the dorsal fin. Other species specific secondary sex characteristics are discussed in Section 4.1 to 4.4.

It is important to identify how the observation and measurement of secondary sex characteristics will be utilized. General observation or qualitative results can be made for the four species under consideration. Qualitative results would be supporting evidence of endocrine disruption and would be useful in an assay. The fathead minnow, medaka and sheepshead minnow have strong secondary sex characteristics, however, actual measurements or quantitative results may reduce the candidate species to the fathead minnow and medaka, which have a history of this endpoint in endocrine disruptor studies. Quantitative results would be necessary to link secondary sex characteristic biometrics to population level effects (e.g. relationship between secondary sex characteristics and fecundity / fertilization success). Qualitative evaluation of secondary sex characteristics has not been a standard endpoint in life-cycle studies. (Parrott et al. 2000) found that ovipositor index and male secondary sex characteristics were sensitive endpoints for EE2. However, they were not the most sensitive endpoints and there were other endpoints with equal sensitivity.

6.2 Measures of Reproductive Performance

6.2.1 Fecundity

Fecundity can be measured as GSI by sacrificing fish or by counting eggs and spawns from spawning groups of fish. Since 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. The collection of eggs is a very time consuming phase in the study especially if the eggs are to be evaluated for fertilization or hatching success.

There is more variability in spawning data when compared with data endpoints like fertilization success, hatching success, length, weight and survival. GSI is also less variable, but requires sacrificing females. Due to the limitations of replication, other endpoints may routinely be more sensitive than fecundity, when measured based on the number of eggs deposited over time. Since all four species of fish being considered are continuous spawners, the time to deposit eggs may be the most critical endpoint. For example, the number of eggs per female per day

over a four-week period may be similar, but higher treatment levels may induce spawning over a shorter period of time. However, if eggs are released from the ovary before they are mature, fertilization success may also be affected.

6.2.2 Gamete Viability and Fertilization Rate

In addition to the formation of adequate numbers of gametes for reproduction, it is also important that the gametes possess high viability for successful fertilization and larval survivability. Screening assays that expose both genders simultaneously and then determine fertilization success 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 could be useful additions to a multigeneration protocol. The subsequent two sections briefly discuss possible mechanisms for impairment of gamete viability and approaches to assess viability.

6.2.2.1 Sperm Viability. Although the true measure of sperm quality is the ability to fertilize, sperm viability is frequently assessed by motility and a strong correlation can exist between sperm motility and fertilization success (Kime & Nash 1999; Lahnsteiner et al. 1998). Accurate measurement of sperm motility has become easier in recent years with the advent of computer assisted motion analysis. This method generates quantitative measures of sperm movement independent of the variation found among individuals measuring movement with manual observation and ranking systems. However, computer-assisted sperm analysis (CASA) requires expensive specialized equipment that may be outside the scope of a multi-generational toxicity test.

Decreased motility of sperm can result from the loss of mitochondrial function, cytological damage, or from structural developmental abnormalities. These effects can result from exposure of developing sperm, or from abnormal testicular development resulting from early life-stage or maternal exposure, to pollutants or hormones (Cheek et al. 2000). Detailed sperm structure can be observed with scanning or transmission microscopy, however, changes in sperm morphology, including length and width of the sperm head and flagella, can be measured with light microscopy and computer assisted analysis (Cheek et al. 2000). Preliminary work has detected changes in flagellar movement through stroboscopic observation in sperm exposed to levels of mercury affecting motility (reported in Kime & Nash1999). This technique may prove useful in measuring the motility of sperm affected by endocrine disruptors.

An alternative mechanism separate from reduced motility might involve failure of spermatozoa to locate the micropyle of the egg or failure to induce egg activation after reaching the egg surface. This latter mechanism may involve recognition of sperm guidance substances on the egg surface, as has been recently described for the medaka (Iwamatsu et al. 1997).

6.2.2.2 Egg Viability. Contaminants can gain entry into fish eggs through maternal transfer or during a brief permeable period after ovulation. The effects of endocrine active substances on egg viability may prevent fertilization or inhibit the development of the embryo. For example, some chemicals may deplete thyroid hormones in the egg which could affect hatching success (Tagawa & Hirano 1991). Damage to the micropyle can prevent entry of the sperm into the egg. This effect can be documented through scanning electron microscopy, although there are significant costs associated with this measure of egg viability (Schaefers et al. 2001). Egg viability can be affected by reduced VTG incorporation into the oocyte, resulting in smaller sized eggs, or disturbances in the levels of sex hormones (reviewed in Kime & Nash 1999). Changes in spawning frequency can result with chemical exposure. Delayed egg release has resulted in a decrease in the number of viable eggs, which may be due to deterioration of the egg (reviewed in Kishida et al. 2001).

6.2.3 Fertility and Embryonic Development

The formation of the zygote heralds the beginning of the embryonic period in animal life history. The embryonic period in the life history model for fish ends with "swim-up", the transition from the endogenous yolk supply to exogenous feeding (Balon 1975). In oviparous fish the embryonic period will encompass all development within the egg and can extend after hatching until the yolk supply is exhausted (sac-fry). All four test species proposed in this review are oviparous, which are advantageous for the study of EDCs and early development as the eggs are released by the female and develop external to the parents. Therefore, they can be easily studied after spawning and fertilization without invasive procedures on the adults.

The early development is rapid, in all four test species proposed, as a consequence of their small size and short life cycle. All are typically raised at warm water temperatures, which aids in accelerating their development. The embryonic development of the zebrafish (Kimmel et al. 1995) and medaka (Iwamatsu 1994) are very well characterized and the web sites (zebrafish- http://zfin.org/; medaka- http://biol1.bio.nagoya-u.ac.jp) for each provide detailed explanations and diagrams of all distinguishable stages (e.g., 128-cell, 256-cell, etc.). The utility of measuring embryonic development in the zebrafish or medaka is the transparent characteristics of the vitelline envelope (i.e., eggshell) such that every stage during development can be visually assessed in the living animal. This allows periodic data collection over time from the same individual. In addition, these fish embryos develop at water temperatures (25ºC to 28ºC) near that of normal room temperature and the eggs are small and easily studied using conventional light microscopy. These features, among others, have made the study of zebrafish embryos an important model for toxicant effects on development (Tanaka et al. 2001). For example, a study by Blader and Strahle (1998) described the effects of ethanol on prechordal plate formation in the zebrafish embryo using conventional microscopic techniques. Unfortunately, there are no similar information sources on stages of embryonic development for the fathead minnow (USEPA 1996)or sheepshead minnow. This is a major negative in the use of these species in a multi-generational test, especially when many effects of endocrine disruptors occur during early development. However, a number of studies have been performed with these

latter two species in which endpoints such as hatching success or growth during embryological development have been reported (Hansen et al. 1983) (Creech et al. 1998; Lange et al. 2001). While these endpoints are useful, the greater resolution and detail afforded by the zebrafish or medaka is superior for assessing changes during early development.

6.2.4 Changes in Spawning Behavior

General observations on spawning can be made for all four species being considered. Once again these observations would only be qualitative in nature. Quantitative observations on spawning behavior requires considerable effort in making observations, whether those observations are made directly by individuals or through the use of video equipment. Direct quantitative observations of spawning behavior has been successfully performed in some species such as goldfish (Sorensen et al. 1989). However, special considerations must be made to insure that the true spawning behavior of the fish is not impacted by the observation process. The observation of two or three spawning groups per replicate with up to seven treatment levels will require a significant effort. The collection of eggs occurs at the same time and is also a very labor-intensive time during the exposure. Therefore, labor restraints at this time of the study may limit the amount of quantitative data that can be collected.

Quantitative spawning behavior is not routinely collected in life-cycle studies and is not widely published. In one study male medaka spawning behavior was significantly altered by exposure to octylphenol, a known estrogen agonist (Gray et al. 1999). However, other endpoints were as sensitive or more sensitive than the male spawning behavior.

6.2.5 Strength and Weaknesses of Partial versus Full Life-cycle Exposures

The same reproductive performance endpoints can be measured in both a partial and a multigenerational or full life-cycle study. However, there are two key differences between the two studies, which may direct the choice of study method. Reproductive end points are the obvious and critical endpoints when evaluating EDC. Reproductive end points also happen to be the most variable endpoints, thus the statistical power to detect differences in these endpoints is often low. The statistical power can be improved by increasing replication and/or sampling frequency. While increasing replication in a multigenerational or full life-cycle study can be prohibitive, the shortened duration and reduced number of treatment levels in a partial life cycle are conducive to increasing the number of replicates. Thus, the strength of a partial life cycle is the potential for more replication, which can provide for greater statistical power for the naturally variable reproductive endpoints. The weakness of the partial life-cycle study is not being able to evaluate the exposure of all life stages and the effects of this long-term exposure on reproduction. However, the strength of the multigenerational or full life-cycle study is that it does expose all fish life stages and evaluates the effects of a long-term exposure on reproduction.

6.3 Biochemical Measures

6.3.1 Vitellogenin Induction

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 estrogen receptors in the liver. Juvenile and adult fish, both male and female, can be induced to synthesize VTG after estrogen exposure or after exposure to estrogen-mimics. Detection of VTG synthesis has become the most widely studied biomarker of exposure to endocrine-active compounds. Although there are a variety of methods to detect VTG in fish, the most widely applied methods are the immunoassays, enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (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. ELISA is used more frequently to measure VTG because, unlike RIA, it does not require radioactive isotopes, uses stable reagents, and offers ease in set up and use.

6.3.1.1. Induction During Juvenile Life Stages. The induction of VTG can be measured through whole-body sampling of juvenile fish, as early as 2-4 days post-hatch in some fishes from which samples of plasma are not available (Todorov et al. 2002). This measure of induction during juvenile life stages has been shown to be a sensitive measure of exposure to estrogens. For example, in a study of mixed-sex juvenile fathead minnows aged 45 days-post-hatch, VTG levels showed a statistically significant increase after a 4-day exposure to 2 ng/L of the synthetic steroid EE2 (Panter et al. 2002). When juvenile fathead minnows were exposed to 2.9 µg/L of the synthetic estrogen diethylstilbestrol (DES), VTG was induced to a level 100 times greater than control fish after 4 days (Panter et al. 2002). In addition to rapid detection of induction, the VTG levels in the EE2 and DES exposed fish remained elevated during the 21-day exposure period and reached maximal levels reported for cyprinids (Panter et al. 2002). In addition to synthetic estrogens, the natural phytoestrogen, genistein, and the weak estrogen mimic, 4-tert-pentylphenol (TPP), induced VTG in juvenile fish (Panter et al. 2002). Although potent estrogens can elicit a measurable response in several days, a minimum of a 14-day exposure was necessary to measure induction in the majority of the chemicals tested in this study. In addition to measuring estrogenic activity in juvenile fathead minnows through VTG induction, anti-estrogenic activity of some substances has been demonstrated through a measured reduction in VTG synthesis relative to control fish. For example, in the Panter et al. (2002) study, the pharmaceutical anti-estrogen ZM 189,154 caused a statistically significant reduction in VTG levels in juvenile fathead minnows relative to control fish.

6.3.1.2 Induction During Adult Life Stages. In adult 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 in multi-genreation tests, VTG synthesis has also been demonstrated to be sensitive to estrogen exposures, although somewhat less sensitive than that observed in rainbow trout (discussion of VTG induction in candidate species is presented in section 7.2.1.3). The reduced sensitivity appears to be due in part to difficulties in obtaining sufficient quantities of purified VTG protein and species-specific antibodies for use in ELISA- or RIA-based measurement techniques (Section 6.3.1) (Parks et al. 1999; Van den Belt et al. 2001).

This section has focused on VTG as this protein is the best studied and most commonly measured estrogen responsive gene product in fish. However, other proteins such as the 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. 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 reproductive 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.

6.3.2 Tissue Steroid Concentrations

6.3.2.1 17β-estradiol Concentrations During Juvenile and Adult Life Stages. The measurement of plasma levels of E2 can be used as an endpoint to assess estrogen sex steroid status in 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. An exception may be the male fathead minnow, which is discussed below. The measurement of plasma E2 is most useful in sexually maturing females due to 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 (reproducing over more than one reproductive or spawning period), 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). Therefore, in juvenile fish that have immature gonads and have not begun active vitellogenesis ovarian synthesis of E2 will be negligible and plasma levels are consequently very low. Since many toxicants or environmental contamination scenarios typically affect sex steroids by depressing plasma titers it may be difficult to discern an effect in juvenile fish that already have very low levels of these hormones.

In the four test species proposed, there are no data on plasma E2 levels in juveniles. There are limited data for adult fathead minnow and medaka; none exist for sheepshead minnow or zebrafish. One study on female fathead minnow reported mean plasma levels peaking at 10 ng E2/mL one day after spawning before decreasing to ~4 ng E2/mL (Jensen et al. 2001). Another reported mean plasma levels in the range of 3 ng E2/mL to 11 ng E2/mL for adult female fathead minnows, that were unaffected by three-week exposure to sewage treatment effluent (Sternberg & Moav 1999). There are three reports of the detection of E2 in the plasma of male fathead minnows; these values fall in the range of 0.5 ng E2/mL to 5 ng E2/mL (Jensen et al. 2001; Makynen et al. 2000; Sternberg & Moav 1999). In a study on the effect of vinclozolin (an anti-androgen) in male fathead minnows plasma E2 levels were significantly elevated in a group treated for three weeks with 700 µg/L, compared with the control (Makynen et al. 2000). Measuring E2 plasma levels in male fathead minnow may be a useful endpoint dependent on the toxicant used. One study reports plasma E2 levels in adult 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. At least in fathead minnow and medaka it can be concluded that it will be important to have appropriate controls (and adequate sample sizes) to compare to treated fish because plasma E2 levels fluctuate considerably over narrow temporal periods due to their short reproductive cycle.

6.3.2.2 11-ketotestosterone Concentrations During Juvenile and Adult Life Stages. Similar to E2 in female fish, 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 routinely 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). Plasma levels of 11-KT are very low in juvenile fish that are not sexually maturing (Simpson & Wright 1977). Once spermatogenesis is under way in the testes, and later spermiation, considerable amounts of 11-KT appear in the blood (Fostier et al. 1982; Scott et al. 1980b; Simpson & Wright 1977). Therefore, provided an appropriate control group is available point measures of 11-KT could be used in sexually maturing males to assess the effects of EDs.

There are two studies that report plasma concentrations of 11-KT in adult male fathead minnow, one in which mean plasma levels of ~33 ng /mL were found throughout the spawning period (Jensen et al. 2001)and another reporting much lower levels of 7 ng/mL (Makynen et al. 2000). The differences in these studies may be explained by the reproductive state of the fish; in one case, fish were actively spawning while in the other, although sexually mature, all individuals may not have been breeding. Caution is suggested in interpreting 11-KT levels in adult male fathead minnows until more data are collected. There is no available plasma 11-KT

data for juveniles of any of the four test species, or adult medaka, zebrafish, or sheepshead minnow.

7.0 RESPONSE TO ESTROGEN AGONISTS AND ANTAGONISTS

The majority of endpoints measured in multigeneration tests are either directly measured in sexually mature individuals or are measures of reproductive performance. As reviewed under Section 5.2 (Ontogenic Period of Exposure), fish pass through four or five broad life-history stages. Because the time of sexual differentiation varies between gender and species, the potential for variable life-stage sensitivities to (anti-) estrogenic compounds may exist. Furthermore, many of the endpoints measured in adults will undoubtedly reflect exposure during immature life stages. Thus, endpoints reflective of gonadal recrudescence or fertilization and hatching success could be linked to changes in normal gonad development caused by exposures occurring prior to sexual maturity. For this reason, the section on juvenile responses to (anti-) estrogenic chemicals is discussed from two separate viewpoints: 1) effects manifested in adults that result from exposures during a specific life stage (e.g., embryonic, larval, and juvenile); and 2) sublethal effects that are manifested during juvenile life stages. In the former viewpoint, exposures can occur over two life stages (e.g., larval – juvenile). In this case, the discussion will be based on the life stage when exposures were initiated. With respect to sexually mature life stages, a similar approach will be used in the discussion: 1) effects observed in adults resulting from exposures occurring subsequent to sexual maturity; and 2) effects manifested in adults during a continuous full life-cycle exposure. The purpose of this approach is an attempt to clarify the biological and ontogenic significance of experimental results obtained from multigeneration fish tests.

7.1 Juvenile Life Stages

The classic studies in the medaka during the 1950s and 1960s established that exposure of potent sex steroids during juvenile life stages can cause sex reversal in fish (Yamamoto 1969). This area of fish research has now become an important aspect of aquaculture in which the cultivation of monosex populations is commonly practiced and used to increase productivity (Piferrer 2001a). Although well characterized from an aquacultural perspective, the environmental significance of sex reversal or other less pronounced changes in gonad morphology resulting from juvenile exposure to endocrine-active compounds is less established. However, an interesting study in Chinook salmon (*Oncorhynchus tshawytscha*) suggests sex reversal in wild fish might be more extensive than previously thought (Nagler et al. 2001).

7.1.1 Sensitivity to E2 or Synthetic Estrogen Exposure

Embryonic Exposure

Embryos can be exposed to endocrine-active compounds through two main pathways:

1) maternal transfer of the chemical to the developing oocyte prior to ovulation; and 2) direct uptake from the water after fertilization and during subsequent embryogenesis. An interesting experimental approach to simulate these exposure pathways is the direct or nano-injection of embryos with the test chemical. This approach was used to study the effects of embryo exposure to EE2 on the subsequent phenotype and gonad morphology in the medaka. Medaka embryos of the d-rR strain (a mutant strain possessing sex-linked pigmentation; males have orange-red coloration and females have white coloration) were injected with graded doses of EE2 ranging from 0.005 ng/egg to 5.0 ng/egg and then cultivated to sexual maturity (Papoulias et al. 2000b). The survival of the injected embryos at doses up to 2.5 ng/egg was comparable to uninjected or dosing vehicle injected embryos (62% to 67% survival). After attaining sexual maturity, the results indicated that embryo exposure to 0.5 ng or 2.5 ng EE2 caused sex reversal in 25% and 80% of the fish examined (Papoulias et al. 2000b). The sex-reversed fish appeared to have functional ovaries, although some histopathological effects, such as increased numbers of atretic oocytes were noted (Papoulias et al. 2000b).

Similar findings have been observed when medaka embryos were incubated in saline solutions of E2 until hatching. When embryos were incubated at 1 µg/mL and then raised to maturity, complete sex reversal of the genetic males (d-rR strain) was observed (Iwamatsu 1999). This study also noted that incubation of embryos at E2 concentrations of 5 µg/mL and 10 µg/mL either hatched out prematurely and died soon after or hatched normally and died before sexual maturity (Iwamatsu 1999).

An interesting study of maternal transfer of E2 in zebrafish was described by Olsson et al. (1999), in which sexually mature female zebrafish were injected i.p. with E2 at 0.27 µg/kg or 272 µg/kg and mated to unexposed males. Mortality of embryos produced from the pairings was high at both treatment doses. The surviving larvae were reared to maturity and observed to be 90% female at the high E2 exposure. Additional histopathological effects noted were indications of bile stasis in the livers of both treatment groups (Olsson et al. 1999).

Larval Exposure

Exposure of larval fish to estrogenic chemicals has received the most scrutiny of the different life stages. A wide variety of exposure protocols have been used varying in dose, duration, and route of administration (typically water or oral). This makes it somewhat challenging to review the literature in an organized manner, and preference is given to those studies using E2 or EE2 (or other accepted potent ER agonists) and providing detailed description of pertinent endpoints.

A thorough study was described by Metcalfe et al. (2001), in which newly hatched medaka fry were exposed to E2 and EE2 concentrations ranging logarithmically from 0.0001 µg/L to 1.0 µg/L for 90 days. The results indicated both total length and body weight increased at the highest concentration of E2, whereas body weight decreased at similar concentrations of EE2 (Metcalfe et al. 2001). A number of histopathological changes were observed in exposed

medaka, including accumulation of an eosinophilic fluid in the body cavity in general, and in the kidney and liver specifically, after E2 or EE2 exposures at or above 0.1 µg/L (Metcalfe et al. 2001). The sex ratio of exposed fish was also shifted toward females at exposure rates above 0.1 µg/L, whereas formation of testis-ova in male individuals became noticeable in E2 treatments as low as 0.01 µg/L (Metcalfe et al. 2001). The authors also noted that when testis-ova occurred, a gradient appeared to exist with oocytes in the posterior region and testes in the anterior region of the gonads (Metcalfe et al. 2001).

Another study in the medaka exposed freshly hatched larvae for 2 months to nominal EE2 concentrations up to 0.1 µg/L, followed by a 6-week grow-out period (Scholz & Gutzeit 2000). Similar growth rates (total body weight and length) in both male and female individuals were observed compared with control fish, although the GSI of female fish was decreased at EE2 exposure levels of 0.01 µg/L and 0.1 µg/L (Scholz & Gutzeit 2000). This study also observed a shift in phenotype from male to female in all fish exposed to 0.1 µg/L of EE2 (Scholz & Gutzeit 2000). Interestingly, EE2 exposure rates of 0.001 and 0.01 µg/L did not cause formation of testis-ova in male medaka (Scholz & Gutzeit 2000). In a similarly designed study, newly hatched medaka fry were exposed to measured E2 concentrations ranging from 0.01 µg/L to 1.66 µg/L for 28 days followed by a post-dosing recovery period of 28 days. At the end of the recovery period, both the total length and body weight appeared to decrease with increased E2 exposure levels, although the authors concluded that these differences were not significant (Nimrod & Benson 1998).

An alternative approach used by some investigators to study morphological changes after E2 exposure involves short duration exposures to very high concentrations. For example, when newly hatched medaka larvae were given a 48-hr exposure to high E2 concentrations of 4, 29.4, or 115.6 µg/L, and then allowed a 14-day grow-out period, both a shift in phenotype and a high incidence of testis-ova in genetic male medaka were observed (Hartley et al. 1998). In a study using zebrafish, both embryos and larvae exposed to E2 at 2,720 µg/L for up to 120-hr post-fertilization developed several deformities, such as an enlarged pericardium and a curved tail phenotype (Kishida et al. 2001). These types of studies demonstrate phenotype reversal (among other changes) can occur over a short period of exposure. However, the requirement of exceptionally high E2 exposure rates raises questions regarding the environmental significance of these findings. More specifically, the biological significance is uncertain as steroidogenesis is believed to be low during this exposure period, and in some species, such as the zebrafish, significant expression of ER-α and ER-β does not appear to occur until 28 to 35 days post-hatch (Legler et al. 2000).

Biochemical measures have been infrequently reported for larval life stages. However, one study exposed newly hatched fathead minnows for 30 days to nominal concentrations of E2 ranging from 25 ng/L to 100 ng/L and observed dose-dependent increases in whole-body concentrations of VTG with peak values reported to be 12,000 µg/g to 15,000 µg/g (Tyler et al. 1999).

Juvenile Exposure

Exposure studies using juvenile life stages have received less attention compared with larval life stages. A detailed study using juvenile fathead minnows was reported by Panter et al. (Panter et al. 2000a) as part of the development of a juvenile fish-screening assay. In this study, juvenile minnows were exposed for 21 days to the synthetic estrogens, EE2 and diethylstilbestrol (DES), at measured concentrations ranging from 2.9 µg/L to 22.1 µg/L for DES and 1 ng/L to 20 ng/L for EE2 (Panter et al. 2000a). At these concentrations, neither chemical altered body weight or total length as compared with control fish (Panter et al. 2000a). However, this same study reported that a 21-day exposure to the phytoestrogen, genistein, at measured concentrations of 280 µg/L and 920 µg/L significantly increased the body weight of juvenile fathead minnows (Panter et al. 2000a). Exposure to EE2 concentrations as low as 4 ng/L did, however, cause an approximately 5- to 10-fold increase in VTG levels compared with control fish (Panter et al. 2000a).

7.1.2 Anti-estrogens

The biological effects of anti-estrogens in fish reproduction have only recently been studied, and relatively few studies of anti-estrogens are reported. For purposes of discussion, a direct anti-estrogen is a chemical known to interfere with signaling through competitive inhibition of binding established to the ER(s). Chemicals 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.

Embryo-Larval Exposure

Only one study could be found that exposed embryos or larvae to anti-estrogens. In this study, several exposure protocols were used to study the effect of tamoxifen and ICI182780 (a pure anti-estrogen) on the E2-induced shift in phenotype in d-rR medaka (Kawahara & Yamashita 2000). In one experiment, embryos were exposed until hatching to 0.2 µg/L E2 and reared to maturity on a normal diet or a diet containing 2000 µg/g tamoxifen or ICI182780. When medaka were raised on a normal diet, 97% of the genetic males were phenotypically female. However, the anti-estrogen diets were able to partially block the shift in phenotype and only 66% and 70% (tamoxifen and ICI182780, respectively) of the males were phenotypically female (Kawahara & Yamashita 2000). In a second experiment, newly hatched larvae were placed on a diet containing graded E2 concentrations (5 µg/g to 50 µg/g) or E2 + tamoxifen (100 µg/g to 5000 µg/g) or ICI82780 (2000 µg/g). Genetic male fish reared on diets containing only 20 µg/g or 50 µg/g E2 were all phenotypically female at maturity. However, this phenotypic shift could be completely blocked at higher exposure rates of tamoxifen and partially blocked with ICI182780. Female medaka reared on the diets containing only the anti-estrogens underwent normal sexual development (Kawahara & Yamashita 2000).

Juvenile Exposure

As with earlier life stages, only a single study could be found that exposed anti-estrogens to a juvenile life stage. In this study, juvenile fathead minnows were exposed to the pure 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).

Indirect Anti-estrogens

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. The best-characterized, indirect-acting anti-estrogens are the aromatase inhibitors. 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 & Callard 1998). In any case, inhibition of this enzyme has been demonstrated to adversely affect sexual differentiation and reproduction.

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 juvenile Chinook salmon that were genetically female 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). With respect to fish species under consideration as a reproductive screen, only a single study was found that examined aromatase inhibition. In this study, a 21-day exposure to nominal fadrozole concentrations of 25, 50, and 100 µg/L did not alter growth or reduce VTG synthesis in juvenile fathead minnows (Panter et al. 2000a). Based on published studies in salmonids reviewed above, this latter study appears to have used insufficient exposure levels for meaningful conclusions to be made.

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 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 continued suppression of the CYP19 gene (Kitano et al. 2000).

7.1.3 Strength and Weaknesses of Test Species

Only limited comparisons between species can be made here. No published studies using juvenile sheepshead minnows could be found and a relatively few used the zebrafish and fathead minnow. Therefore, until additional information becomes available, the medaka is clearly the preferred species for juvenile studies because of the greater emphasis placed on this species in early life-stage studies of (anti-) estrogenic compounds.

7.2 Sexually Mature Life Stages

A comparatively large number of studies have used sexually mature fish in endocrine disruptor studies. 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. Occasionally, results from studies using weak estrogenic substances have been included but only in areas for which specific information on E2 or EE2 exposures is limited. As stated previously in Section 7.0, the discussion is divided between exposures only during sexual maturity and full life-cycle exposures. Because of the quantity of information available for review for exposures using mature fish, this section is grouped according to the type of endpoint discussed: growth and morphological, reproductive performance, and biochemical measures.

7.2.1 Sensitivity to E2 or Synthetic Estrogen Exposure

7.2.1.1 Adult Exposure: Growth and Morphological Alterations. A confounding factor that is particularly problematic with asynchronous fractional spawning fish 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, in which 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 statistically sensitive 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).

Several studies have been conducted on the effects of short-term exposure to estrogenic chemicals on the growth of fathead minnows. In fathead minnows, a 14 to 19-day exposure to measured E2 concentrations at or above 870 ng/L appears to begin to induce lethality, particularly in male individuals (Kramer et al. 1998; Miles-Richardson et al. 1999). In male fathead minnows, a 21-day exposure to nominal concentrations of E2 at 320 or 1000 ng/L significantly decreased the GSI compared with control fish (Panter et al. 1998). This study also reported that exposure to lower E2 concentrations of 10, 32, and 100 ng/L did not alter the GSI in male fathead minnows (Panter et al. 1998).

A more recent study by the authors confirmed these observations and reported that the GSI in male fathead minnows is significantly decreased during a 21-day exposure only when the nominal concentration of E2 is greater than 60 ng/L (Panter et al. 2000b). Thus, a reduction in testis mass (or GSI) and lethality caused by short-term exposure to E2 in adult fathead minnows occurs between an exposure window of 100 ng/L and 800 ng/L to 1000 ng/L.

These exposure rates to E2 have also been documented to cause severe testicular abnormalities described as a loss of germinal cells and presence of degenerate spermatozoa (Miles-Richardson et al. 1999). Additional testicular histopathology noted proliferation of Sertoli cells that in extreme cases led to complete occlusion of seminiferous tubules (Miles-Richardson et al. 1999). E2-induced ovarian histopathology is, in general, less pronounced when compared with testicular lesions, but appears to cause a significant increase in primary follicles, with diminished numbers of secondary follicles at nominal concentrations of 17 ng/L (Miles-Richardson et al. 1999).

Several of the aforementioned studies using fathead minnows also reported changes in growth and development of secondary sex characteristics. A very thorough examination of E2 treatment and development of secondary sex characteristics was reported by Miles-Richardson et al. (1999). In this study, external female characteristics assessed by measuring the ovipositor length were unaltered after 19-day E2 treatments up to 2.72 µg/L (Miles-Richardson et al. 1999). In contrast, male individuals exhibited a more feminized appearance resulting from atrophy of the nuptial tubercles after nominal E2 exposures at or above 545 ng/L and atrophy of the fat pad at 27.24 µg/L (Miles-Richardson et al. 1999). In other species, such as the zebrafish, exposure of mature adults to EE2 for 21 days at nominal concentrations of 10 ng/L and 25 ng/L decreased the GSI in both males and females (Van den Belt et al. 2001). This study also noted that besides reducing the overall size of the ovaries, exposure to 10 ng/L and 25 ng/L EE2, greatly reduced the number of mature, yolk-filled oocytes (Van den Belt et al. 2001).

Not surprisingly, routine histopathological examination of the gonads reveals toxicological effects at lower exposure rates than those needed to produce changes in tissue mass. These histopathological findings tend to be manifested as a general accumulation of eosinophilic staining material and perhaps formation of testis-ova in genetically male individuals. Some authors have suggested the buildup of eosinophilic staining material in male fish is a toxic consequence of VTG induction (Herman & Kincaid 1988).

7.2.1.2 Reproductive Performance. A thorough study of reproductive performance was reported by Ankley et al. (2001), in which adult fathead minnows were exposed to methoxychlor, a weak estrogenic chemical, for 21 days at measured concentrations of 0.55 µg/L and 3.56 µg/L. The results from this study indicated the mean fecundity rate decreased from 20.5 to 8.3 eggs/female/day (control versus 3.56 µg/L treatment), and was attributed to both a decrease in egg numbers per spawn and less frequent spawning (increased spawning interval) (Ankley et al. 2001). An earlier study exposed adult fathead minnows to various E2 exposure rates for 19 days and observed a decrease in fecundity with a calculated E2 median effective

concentration (EC50) of 120 ng/L for decreased egg production (Kramer et al. 1998). An additional study of note exposed pair breeding fathead minnows to the weak estrogenic pollutant, 4-nonylphenol, at measured concentrations between 60 µg/L and 80 µg/L for 21 days. At this water concentration, 4-nonylphenol caused a significant reduction in both fecundity and spawning frequency (Harries et al. 2000).

An interesting study by Shioda and Wakabayashi (2000) measured the effects of E2 exposure on reproductive performance in male and female medaka exposed separately and then bred to control medaka of the opposite sex. The results indicated that nominal exposures to E2 of 817 ng/L or higher significantly decreased fertilization success of eggs fertilized by exposed males (Shioda & Wakabayashi 2000). In females, E2 treatments at or above 27.2 ng/L decreased both fecundity and fertilization success (Shioda & Wakabayashi 2000).

In a similarly designed study, the reproductive performance of pair breeding zebrafish was evaluated after 21-day exposures to measured EE2 concentrations of 5, 10, and 25 ng/L (Van den Belt et al. 2001). Exposure to the two higher EE2 concentrations significantly decreased the percentage of spawning females (defined as the number of breeding pairs producing viable offspring) when mated to control males (Van den Belt et al. 2001). This study also reported that in exposed male zebrafish mated to control females, all EE2 treatments reduced fertilization success to below 70%, which is the historical reference value reported by the authors for their laboratory (Van den Belt et al. 2001). A previous study of EE2 exposure in zebrafish also observed decreased reproduction (measured from hatching success) after a 12-day exposure of sexually mature adults to a nominal concentration of 5 ng/L (Kime & Nash 1999). The decrease in hatching success of fertilized eggs was attributed to an arrest in development at the early blastula stage (Kime & Nash 1999).

7.2.1.3 Biochemical Measures: VTG. Several exposure pathways have been used to study VTG induction and elimination, although the more common exposure pathway is through waterborne exposure. This exposure route has been used in several studies of VTG induction in fathead minnows using E2 or synthetic estrogens, such as EE2. When adult fathead minnows were exposed to nominal E2 concentrations as low as 30 ng/L for 21 days, an approximately 10-fold increase in VTG plasma concentrations was observed (Panter et al. 2000b). Similar findings were also reported in a study using adult male fathead minnows exposed to nominal E2 concentrations down to 27 ng/L, which caused an approximately 10- to 100-fold increase in VTG plasma levels after 7 to 21 days exposure (Parks et al. 1999). This latter study measured VTG using an ELISA assay based on fathead-minnow-specific anti-VTG (Parks et al. 1999). In an earlier study, significant increases in plasma VTG levels were observed after 21-day exposures to E2 water concentrations of 100 ng/L or estrone concentrations down to 31.8 ng/L (Panter et al. 1998).

The sheepshead minnow has also been demonstrated to be sensitive to VTG induction after exposure to estrogenic chemicals. Mature male sheepshead minnows exposed to E2, EE2 or DES for 16 days at various nominal concentrations ranging from 20 ng/L to 2000 ng/L (E2),

0.2 ng/L to 200 ng/L (DES), and 20 ng/L to 1000 ng/L (EE2) rapidly formed VTG with peak plasma concentrations of approximately 120 mg/mL reached in 10 to 13 days (Folmar et al. 2000). Similar results were also obtained in a later study using a nominal concentration of 200 ng/L of E2, DES, and EE2 (Denslow et al. 2001).

The measurement of VTG induction in zebrafish and medaka has been hindered until recently by a lack of antibodies developed against the specific type of VTG produced in these fish species. This lack of specificity may influence the sensitivity of the assay (Thompson et al. 2000) when compared with results from experimental studies using species-specific VTG antibodies. Therefore, unless otherwise noted, cited studies measured VTG using antibodies prepared against VTG sources from unrelated fish species.

Despite this analytical limitation, the available data indicate that estrogen-stimulated VTG induction in medaka and zebrafish is similar to observations made with fathead minnows and sheepshead minnows. For example, when adult male medaka were exposed to 20 µg/L (nominal) E2 for 4 days followed by a 5-day recovery or washout-period, significant increases in VTG immunoreactive protein were observed (Foran et al. 2000).

In another study using similarly aged male medaka (Thompson et al. 2000), fish were exposed to E2 for 21 days at nominal concentrations ranging logarithmically from 10 ng/L to 100,000 ng/L. The results of this study indicated that VTG was induced at exposure levels below 100 ng/L, with a calculated EC50 for VTG induction determined to be 200 ng/L (Thompson et al. 2000).

In a more recent study, Tabata and coworkers (Tabata et al. 2001) used extracts of non-fertilized medaka eggs to obtain polyclonal antibodies against "female-specific proteins," which probably consisted of VTG and vitelline envelope proteins (Oppen-Berntsen et al. 1999). When adult, male medaka were exposed to E2 for 3 and 5 weeks at levels down to 5 ng/L (nominal), measurable increases in female-specific proteins were detected (Tabata et al. 2001).

In adult zebrafish, a 21-day exposure to EE2 at measured concentrations down to 5 ng/L caused significant increases in VTG occurrence in blood plasma (Van den Belt et al. 2001). A similar result was also obtained in a study that exposed adult zebrafish for 14 days to a nominal EE2 concentration of 5 ng/L (Kime & Nash 1999). In this study, the level of VTG induction was roughly 3-fold greater in female than in male zebrafish (Kime & Nash 1999). Two recent studies have assessed VTG induction in zebrafish using specific VTG-detection procedures. Ota and co-workers (Ota et al. 2000)used adult male zebrafish and determined VTG induction by measurement of VTG-like mRNA in liver extracts. This study treated male zebrafish with 270 µg/L E2 (a very high concentration) for 48 hours and observed stimulated VTG gene transcription. A more thorough study of VTG induction in zebrafish was reported by (Petersen G.I. et al. 2000), in which an ELISA procedure was developed for zebrafish VTG based on antibodies produced against lipovitellin purified from zebrafish ovaries. Using this newly developed ELISA, it was later demonstrated that a 30-day exposure to nominal EE2 water

concentrations as low as 20 ng/L caused an approximately 1000-fold increase in VTG levels in whole body homogenates (Petersen et al. 2001).

7.2.1.4 Biochemical Measures: Plasma Steroid Levels. In addition to measuring VTG levels following chemical exposures, it is also desirable 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.

The most thorough study available for review 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 ng/mL 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).

A study by (Giesy et al. 2000) reported plasma E2 concentrations ranging between 1 ng/mL and 5 ng/mL and were similar in both males and females. This study also reported that a 21-day exposure to nominal nonylphenol concentrations ranging between 0.05 µg/L and 3.4 µg/L elevated E2 concentrations to a similar degree in both male and female fathead minnows (Giesy et al. 2000). 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 (Nichols et al. 1999). 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). Likewise, T concentrations were similar between sexes, varying only minimally between 6 ng/mL and 7 ng/mL (Nichols et al. 1999). In fathead 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).

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, zebrafish, and sheepshead minnows, the limited amount of data makes 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 or sheepshead minnows could be found.

7.2.1.5 Full or Partial Life-cycle Exposure. Each species under consideration has been studied as part of a multigeneration experiment with E2 or EE2. Two of the more thorough studies were performed using the sheepshead minnow and the fathead minnow. The sheepshead minnow was tested using EE2 and a partial life-cycle protocol. In this study, eight nominal EE2 concentrations were tested ranging from 0.2 ng/L to 3200 ng/L, starting when fish were approximately 6 weeks in age and continuing for 43 or 59 days (Zillioux et al. 2001). Shortly after the exposure period ended, the fish were allowed to spawn, and the embryo and larvae were monitored for 7 days. Survival was not affected up to the 200 ng/L concentration and then declined rapidly at higher concentrations (Adam et al. 2000). Fish exposed to high EE2 levels (1600 ng/L to 3200 ng/L) developed eosinophillic staining fluid in the body cavity, typical of other fish exposed to high concentrations of estrogens. At exposure rates down to 2 ng/L, effects on the testes were observed such as testis-ova and fibrosis (Adam et al. 2000). Reproductive success (defined as eggs produced per day over a 14-day spawning period) and hatching success decreased at exposure rates of 200 ng/L or higher (Adam et al. 2000).

A full life-cycle exposure was performed with fathead minnows and EE2 at nominal concentrations ranging from 0.2 ng/L to 64 ng/L (Lange et al. 2001). In this study, exposures began with fertilized eggs and continued through 28-day post-hatch of the F1 offspring. Hatching success and mortality of the P embryos and larvae were not altered by the EE2 exposures, although overall growth was diminished in P juveniles at exposure levels of 4 ng/L and higher (Lange et al. 2001). Similarly, EE2 exposures of 4 ng/L and higher prevented development of secondary sex characteristics in addition to other morphological alterations, such as anal protrusion and distended abdomens (Lange et al. 2001). Reproductive performance could only be assessed in the 0.2-ng/L and 1.0-ng/L treatment groups, in which no differences in fecundity, hatching success, and larval survival were observed with respect to control fish (Lange et al. 2001). Subsequent histological analysis of P adults indicated that over a dose range of 1.0 ng/L to 16 ng/L, a progressive increase in the number on individuals with ova-testis occurred, with 94% of all fish phenotypically female at the 64-ng/L exposure (Lange et al. 2001).

A partial life-cycle study using zebrafish with both E2 and EE2 was reported by Orn et al. (2000). In this study, newly hatched zebrafish were exposed to either E2 or EE2 at nominal exposure concentrations varying logarithmically from 0.01 µg/L to 10 µg/L E2, or 0.01 ng/L to 10 ng/L and 25 ng/L EE2 for approximately 4 months. These exposures did not significantly alter mortality or body weight; however, a shift in phenotype to predominantly female was noticed at exposure rates at or above 1 µg/L for E2 and 10 ng/L and 25 ng/L for EE2 (Orn et al. 2000). Only the 25 ng/L EE2 treatment was reported to decrease fertilization rate (Orn et al. 2000), although this was difficult to test because of the low numbers of phenotypic males.

A full life-cycle multigeneration protocol for the medaka was proposed by Patyna et al. (Patyna et al. 1999). As a pilot study, the researchers performed a partial life-cycle study by exposing juvenile medaka for 170 days at three separate E2 treatments rates (0.05, 0.5 and 5 mg/kg). A variety of histopathological effects were reported, including generalized edema in the body cavity with severe renal pathology noted as hemorrhaging, and tubular necrosis (Patyna et al. 1999). All fish appeared to be phenotypic females. These effects are all consistent with previous studies in fish using very high treatment rates of E2 (Metcalfe et al. 2001; Herman and Kincaid 1988).

7.2.2 Anti-Estrogens

7.2.2.1 Adult Exposure. There are limited data on the response of adult fish to anti-estrogens. Presumably, changes in VTG synthesis in adult fish will respond to anti-estrogens in a similar manner as that described for juvenile fish. This is supported by a recent study using primary hepatocytes isolated from adult carp liver. When the hepatocytes were incubated with 1 μM tamoxifen, a decreased synthesis of VTG occurred (Smeets et al. 1999).

Additional studies with fish models suggest certain PAHs could also have anti-estrogenic modes of action. A series of experiments using cultured rainbow trout hepatocytes treated with either α-naphthoflavone or β-naphthoflavone suggest these compounds 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 α- or β-naphthoflavone and the VTG response measured. When the hepatocytes were co-incubated with either naphthoflavone isomer, a decrease in VTG synthesis was observed (Navas & Segner2000). 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. 1996). Conversely, when the β-naphthoflavone dose was reduced to 12.5 mg/kg, a seemingly paradoxical stimulation in VTG synthesis (increase in plasma concentration) was observed (Anderson et al. 1996). This mixed stimulatory and inhibitory effect on VTG synthesis is consistent with tamoxifen like anti-estrogens, which possess agonistic activity at low concentrations (MacGregor & Jordan 1998).

Regardless of the specific mechanism of action of direct anti-estrogens, a common endpoint measured in most studies is the effect on VTG synthesis. Virtually no information is available in the literature on the specific effects of direct 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. This would be a particularly valuable area of research in the context of a multigeneration test.

7.2.2.2 Indirect Anti-estrogens. As stated previously, 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.

The importance of indirect inhibition in lowering circulating E2 levels during adult exposures is decidedly mixed, and whether this mode of action is significant in terms of reproductive performance in fish remains unclear.

Evidence supporting the hypothesis that aromatase inhibitors can lower E2 levels and alter reproductive performance comes from studies using Coho salmon (*Onchorynchus kisutch*). When pre-spawning Coho salmon were administered fadrozole by intraperitoneal injection at doses down to 0.1 mg/kg, a significant decrease in E2 plasma levels occurred 3 to 6 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). However, other established aromatase inhibitors, letrozole and clotrimazole, could not lower circulating E2 levels or suppress VTG synthesis in rainbow trout after dietary administration for 2 weeks at a dose rate of 1 mg/kg (Shilling et al. 1999).

In summary, aromatase inhibition can redirect sexual orientation 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. This latter finding needs to be evaluated in asynchronous spawning fish, such as those under consideration for a reproductive screen. Because of the generally high doses needed in fish studies to elicit a response, the environmental significance of aromatase inhibition could be questioned.

7.3 Gender Differences

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 reproductive screening assay. Because individuals with a male genotype tend to undergo sexual differentiation later than females, the period of time for chemical-induced phenotypic shift is longer. This could be a concern with partial life-cycle tests initiated using older larval or juvenile life stages. In this instance, the period of vulnerability for phenotype reversal might have been passed.

In general, 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 increase) increases in plasma concentrations can be statistically significant. 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 other than female fecundity. A few studies have assessed male-specific fertility endpoints, which appear to be very sensitive to estrogenic exposure. 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.

7.4 Strength and Weaknesses of Test Species

In summary, the available data suggest that an exposure rate to E2 above 100 ng/L is needed to produce changes in the GSI and body weight, total length, and condition factors in all test species. Ethynylestradiol appears to be more potent than E2 in this respect, and exposure rates below 25 ng/L of EE2 appear to cause similar effects to those observed with E2 at higher exposure rates. All test species also appear to be comparably sensitive to early life-stage effects and reproductive performance in response to estrogen treatments. Similarly, biochemical endpoints, such as VTG, are also equally responsive among the four test species. The available data on dose-response relationships suggest that exposure of mature, male individuals to E2 concentrations of 100 ng/L and perhaps as low as 10 ng/L will produce significant increases in VTG levels in plasma and liver tissues.

Beyond these generalizations, a rigorous assessment of the strength and weaknesses of each test species is limited by the paucity of information for some species, such as the fathead and sheepshead minnows during early life stages. Also, only very limited dose-response relationships can be inferred from the available data, which are compounded by problems associated with the reporting of nominal versus measured exposure rates. A weakness common to all species is the small size at maturity, which limits the quantity of blood plasma available for analysis of VTG and sex steroids. Basic differences in life history among the proposed test species suggest some species such as the zebrafish offer advantages because of the unusual delay in time of sexual differentiation. A specific area in which the fathead minnow, medaka, and sheepshead minnow offer an advantage is the more pronounced male secondary sex characteristics.

8.0 RESPONSE TO ANDROGEN AGONISTS AND ANTAGONISTS

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 androstenedione 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 can 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 is 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).

Relatively little information is available on the effects of exposure to androgenic or anti-androgenic substances in fish, which is perhaps partly because of the complexity of the biological activity of endogenous androgens. For this reason, the discussion of (anti-) androgenic chemicals will be simplified to separate effects resulting from exposure during an immature life stage from those observed in adults resulting from short-term or continuous full life-cycle exposures.

8.1 Juvenile Life Stages

8.1.1 Sensitivity to Androgenic Steroid Exposure

Nano-injection of medaka embryos with MT has been reported. Medaka embryos of the d-rR strain were injected with graded doses of MT ranging from 0.8 ng/egg to 8000 ng/egg and then cultivated to sexual maturity (Papoulias et al. 2000b). The survival of the injected embryos at doses up to 80 ng/egg was comparable to uninjected or dosing-vehicle injected embryos (66% to 88% survival). After attaining sexual maturity, the results indicated that embryo exposure to 0.8, 800 or 8000 ng MT caused sex reversal in genetic female medaka (Papoulias et al. 2000b). In a separate study, medaka at several different stages of development (embryos, hatching day, post-hatch days 7 and 21) were immersed for 6 days in a solution of 100 µg/L T and then reared to an adult stage (Koger et al. 2000). Results of this study indicated treatment did not affect mortality, time to maturity, or sex ratios in fish, although treated fish were described as having a more pronounced intersex morphology (Koger et al. 2000). A similar lack of an effect on survival or sexual phenotype was observed in zebrafish embryos and larvae exposed to T at 2884 µg/L for up to 72 hr post-fertilization (Kishida et al. 2001). In contrast, newly hatched medaka larvae fed a diet containing 50 µg/g MT for up to 40 days were described to have numerous morphological changes during different stages of growth. A summary of these changes was reviewed in Section 6.1.1.1 but in general, MT exposure suppressed gonadogenesis (manuscript located at http://biol1.bio.nagoya-u.ac.jp:8000/KobayashiH85.html).

8.1.2 Anti-Androgens

8.1.2.1 Direct Acting. No studies of direct-acting anti-androgens in juvenile fish could be located for review. The reader is referred to Section 8.2.2 for a discussion of anti-androgens in adult fish.

8.1.2.2 Indirect Acting. There are a minimum of three mechanisms by which indirect anti-androgens can reduce intracellular steroid levels: 1) decreasing the rate of synthesis; 2) reducing the plasma-free fraction; and 3) increasing the rate of elimination. Unfortunately, only very limited information is available to assess the biological consequences of these modes of action. In contrast to E2, in which biosynthesis can be blocked using aromatase inhibitors, no equivalent androgen-specific inhibitors have been identified.

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.

The biotransformation of androgens is complex, and a variety of metabolites and conjugates can be formed and eliminated (reviewed in Borg 1994). In juvenile fathead minnows briefly exposed to T (4-hour exposure; measured concentration of 288.4 µg/L), uptake and overall elimination of T was more rapid compared with adult minnows (Parks & LeBlanc 1998). Juvenile fathead minnows also appeared to metabolize T to a greater extent than adults (Parks & LeBlanc 1998). These results imply turnover of androgens in juvenile minnows is more rapid than in adults, which could cause this life stage to be more sensitive to chemical exposures that cause a down-regulation of enzymes involved in androgen conjugation and excretion. However, it remains to be established whether changes in the rate of androgen elimination has any important biological effects in fish.

8.1.3 Strength and Weaknesses of Test Species

It is difficult to make comparisons between species. No published studies could be found using juvenile sheepshead minnows and relatively few used the zebrafish and fathead minnow. Therefore, until additional information becomes available, the medaka is clearly the preferred species for juvenile studies because of the greater emphasis placed on this species in early life-stage studies.

8.2 Sexually Mature Life Stages

8.2.1 Sensitivity to Androgenic Steroid Exposure

The effects of androgen exposure to adult or sexually differentiated individuals have received a similar level of study compared with juvenile life stages. One of the more thorough assessments of the biological effects of androgen exposure in sexually mature fish was reported for MT in fathead minnows (Ankley et al. 2001). In this study, adult fathead minnows were exposed for 12 days to measured concentrations of MT of 120 µg/L and 1700 µg/L. At these high exposure levels, some mortality was observed, and only 20% of the fish survived the high-dose exposure (Ankley et al. 2001). Nonetheless, exposure to either concentration of MT immediately caused all female minnows to stop laying eggs. A number of morphological changes in the gonads were also documented, including a reduction in the GSI in both sexes, an increase in atretic follicles in the ovaries, and only scattered spermatogenic activity in the testes (Ankley et al. 2001). Methyltestosterone exposure also reduced plasma concentrations of T and 11-KT in both sexes. Curiously, MT strongly induced VTG synthesis in both sexes. This result was attributed to the aromatization of the MT by the minnows and subsequent stimulation of the E2 receptor (Ankley et al. 2001). A particularly important observation was the formation of nuptial tubercles on female minnows. The formation of tubercles was noticeable after only 6 days exposure to MT and was suggested by the authors to be an unambiguous measure of exposure to androgenic substances (Ankley et al. 2001).

A similar observation of androgen-stimulated nuptial tubercle growth in female fathead minnows was made over 25 years ago by Smith (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 medaka was observed after oral dosing with 19-*nor*-testosterone, MT, ethisterone, T, and androstenedione (Kawamoto 1969, 1973; Uwa 1975). 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, Lambright, et al. 2001).

In summary, androgen exposure to adult fish decreases gonad growth (size) and circulating androgen levels in plasma and severely reduces reproduction in females. The available data indicate formation of male secondary sex characteristics in females is the most useful endpoint for detecting an androgenic substance. Because some androgenic substances may be aromatized, some formation of VTG may occur, which could lead to the assumption of an estrogen-like mode of action.

8.2.2 Anti-Androgens

8.2.2.1 Direct Acting. 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). When exposure occurred during gestational Day 13 thru 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 might not be anti-androgenic in fish, although further competitive binding studies are needed, particularly with 11-KT, before this conclusion is established.

In contrast to findings with male fathead minnows, oral exposure of vinclozolin at nominal levels up to 100 µg/g feed appears capable of demasculinizing adult male guppies (*Poecilia reticulata*) (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, which is a male secondary sex characteristic of this species (Baatrup & Junge 2001). 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 medaka (Hamaguchi 1978). This effect was even more pronounced in female medaka 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).

8.2.2.2 Indirect Acting. As stated in Section 8.1.2, there are a minimum of three mechanisms by which indirect anti-androgens can reduce intracellular steroid levels. Unfortunately, only very limited information is available to assess the biological consequences of these modes of action. In contrast to E2, for which biosynthesis can be blocked using aromatase inhibitors, no equivalent androgen-specific inhibitors have been identified.

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 endocrine-active compounds 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.

As stated previously, the biotransformation of androgens can be complex. In adult fathead minnows exposed to T (4-hour exposure; measured concentration of 288.4 µg/L), 11-KT was formed that appeared to be excreted directly, e.g., no conjugates could be isolated (Parks & LeBlanc 1998). This also identified significant gender differences in the rates of specific biotransformation pathways of T (Parks & LeBlanc 1998). Given the variety of androgens and their biological activity, these latter observations suggest the potential for chemicals to disturb androgen elimination and perhaps biological activity in a gender-specific manner. However, as was noted for juveniles, it remains to be established whether changes in androgen disposition has important biological effects in fish.

8.2.3 Gender Differences

During juvenile exposures, sufficiently high androgen treatments can masculinize the gonads if not cause complete sex reversal of genetic females. The available data also suggest (anti-) androgen activity is best identified in adults based on the appearance of secondary sex characteristics. In the case of androgen agonists, the development of male external characteristics in females appears to be a sensitive endpoint. As for anti-androgens, a reduced display of secondary sex characteristics in males is somewhat sensitive, although not to the degree androgen activity has on females.

8.2.4 Strength and Weaknesses of Test Species

A rigorous assessment of the strength and weaknesses of each test species with respect to (anti-) androgens is limited by the lack of information for some species, such as the fathead and sheepshead minnows during early life stages. As for estrogens, the fathead minnow, medaka, and sheepshead minnow offer an advantage because of the more pronounced male secondary sex characteristics.

9.0 RESPONSE TO THYROID HORMONE AGONISTS AND ANTAGONISTS

The thyroid hormones, thyroxine (T4) and triiodothyronine (T3), are synthesized and released by thyroid follicles in the ventral pharyngeal region of bony fish. Secretion of thyroid hormones is under hypothalamus-pituitary control through the action of thyroid-stimulating hormone (TSH). The more biologically active thyroid hormone is T3, which is converted from T4 through deiodination via the enzyme, iodothyronine 5'-monodeiodinase. Their action at the level of the cell is mediated by specific cytosolic receptors, although they appear to interact directly with the chromatin in the nucleus. In addition to reproduction, T4 and T3 have been linked to a multitude of important functions in fish, such as growth, metabolism, and osmoregulation (Cyr & Eales 1996). 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; Legler et al. 2000; Siwik et al. 2000; Soyano, Saito, et al. 1993). 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 might 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 lake trout (*Salvelinus namaycush*) and a marine flatfish, the American plaice (*Hippoglossoides platessoides*), showed 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 gonadal secretion of thyroid hormones might be important in gametogenesis (Huang et al. 2001).

9.1 Endpoint Sensitivity to Thyroid Stimulation

A search of the scientific literature revealed only one paper that studied the effects of T4 or T3 exposure on a relevant test species. In this study, juvenile fathead minnows were exposed for 13 weeks to nominal water concentrations of 12.5, 25 and 50 µg/L T3 (Abrahams & Pratt 2000). Exposure to 50 µg/L T3 significantly decreased the growth rate of the minnows, although a clear dose-response relationship between T3 exposure and growth could not be established (Abrahams & Pratt 2000). Although data on thyroid agonists are limited, it has been proposed that certain PCB congeners or their metabolites might bind to vertebrate thyroid receptors

(Fentress et al. 2000; Brouwer et al. 1990). This potential would suggest 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 µg/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 might 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, Adams & Cyr (2000) reported that a 5 µg/kg or 25 µg/kg intra-peritoneal injection of PCB congener 77 lowered T3 levels after 1 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.

9.2 Inhibition of Thyroid Function

In contrast to the difficulties in identifying thyroid agonists, there is evidence that certain environmental contaminants can act specifically as anti-thyroidal agents. A thorough study of the effects thiocyanate on thyroid function and reproduction in fathead minnows was reported by (Lanno & Dixon 1990; Lanno & 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 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 nontoxic 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.

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, respectively) developed complex histopathology of the thyroid follicles, suggestive of overall hyperemia and hyperplasia (Sathyanesan, Joy, et al. 1978 349 /id). In the medaka, an intra-peritoneal injection of thiourea reduced the MT-stimulated growth of papillary processes of the anal fin (Fujiwara 1980). In a related study, (Wester et al. 1988) 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). A similar reduction in thyroid hormones was observed in eggs laid by exposed females. 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). Larval survivability was also unaffected by the thiourea exposure.

9.3 Gender Differences

Due to 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 might 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, might 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.

9.4 Strength and Weaknesses of Test Species

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.

10.0 CANDIDATE PROTOCOLS

The utility of the partial life-cycle two-generation test, the full life-cycle test, a two-generation test, and a multi-generational test has been evaluated for use in testing EDCs. Each test provides unique advantages, which cannot be combined into one protocol. The partial life-cycle test is a relatively short exposure test designed to evaluate sexual reproduction in fish and the effects to the early life stages of their progeny from exposure to EDCs. The partial life-cycle test can be conducted at a relatively high concentration in an effort to elicit a response and / or at low concentrations to evaluate the threshold for a reproductive response. Although a partial life-cycle test does not offer scientific advantages over the a multigenerational test, a major advantage of a partial life-cycle test is the shorter period of time necessary to conduct the in-life phase of the test, offering considerable cost savings.

The multigenerational test is designed to be a definitive test for evaluating population-level effects of EDC in the environment. The long-term chronic exposure exposes fish through two complete life cycles, plus the option to evaluate the early life stages of the third generation. This study is designed to have at least one treatment level that is statistically indistinguishable from the controls. Between 60 and 80 endpoints are collected from three generations of fish, from all of period of time necessary to conduct the significant life stages of fish, and from male and female fish. By using small fish with short life cycles, this test can be conducted in a reasonable time (as short as 4.5 months).

The partial life-cycle study allows for the use of larger and longer life-cycle species, such as the fathead minnow. The advantages of using fathead minnows in the partial life-cycle study include individual blood plasma protein and steroid analyses and greater regulatory familiarity. If the fathead minnow is used in the partial life-cycle study, then a small species with a shorter life cycle can be utilized for the multigenerational study. This will reduce the time needed for the test and will provide an evaluation of a second fish species.

An alternative to a full multigenerational full life-cycle test, is a two-generation test in which the initial exposure is initiated with mature fish and the F1 generation is evaluated for embryo fertility, development, sexual maturation, reproduction and F2 viability is assessed. With an exposure of spawning adults, the two-generation test incorporates maternal transfer from the P to F1 generation into the test. This test offers the advantage of a reduction in the time required to conduct the test when compared to the multi-generation test, allowing the use of test species with longer life cycles, including the fathead minnow. The endpoints included in this test allow inter-species comparison of the underlying mechanisms of toxicity and assess impacts upon fecundity and viability that can address population level impacts integral to risk assessment.

10.1 Partial Life-Cycle Test {Adult (P) to Juvenile (F1)}

A partial life-cycle toxicity test, which exposes P adult, sexually mature fish and the early life cycle of F1 fish, can be conducted to estimate the NOEC for the exposed fish. A pre-

exposure reproductive evaluation is conducted on the P fish. The biological endpoints evaluated include the following:

- P pre-exposure, secondary sexual characteristics and fecundity/reproduction (e.g., eggs/female)
- P post-exposure, survival, secondary sexual characteristics, fecundity/reproduction (e.g., eggs/female), GSI, histopathology, and protein and sex steroid biomarkers (e.g., VTG)
- F1 hatching success, survival, growth (length and weight).

Materials and Methods

Physical Exposure System

- Intermittent flow diluters are used in combination with mechanical or electronic syringe pumps to deliver fresh toxicant and dilution water during the exposure. If a carrier solvent is used, all treatments will have an equal solvent concentration with the exception of the dilution water control.
- Exposures will be conducted in glass aquaria, which will be impartially arranged on two separate levels (tiers). The upper level will be used for F1 embryo incubation and larval fish rearing. Larval growth chambers will be placed in each aquarium to provide the capability of simultaneously rearing two larval groups. The lower level will be used to expose P spawning groups.
- Each level will contain two to four replicates per treatment level. Sub-replicates will be established for certain endpoints – hatching success, larval exposure and spawning groups.
- Three of the four candidate species are freshwater fish. Dilution water for these species should be dechlorinated water from a noncontaminated source. Care should be used not to use surface waters, which may contain low levels of potent EDCs. Sheepshead minnow will be maintained in relatively low salinity (15 ppt; lower salinity concentrations may be possible upon further evaluation) dilution water, which can be collected from a contaminant-free source or can be prepared from a commercial mix.

Chemical System

- Two to three concentrations separated by a factor of three to ten will be selected based on a previously conducted exposure(s). The treatment levels should be lower than any concentration, which caused mortality in a standard 96-hr acute test. A dilution water control and a solvent control (if a carrier solvent is required) will be established. The solvent concentration will not exceed 100 µg/L, and every effort will be made to maintain solvent concentrations <10 µg/L.

- Analytical sampling of each exposure level will be conducted weekly to determine test substance concentration confirmation.

Test Conditions

- The water temperature for fathead minnow will be 25 ±1ºC. For the other candidate species, a water temperature of 28 ±1ºC will be maintained.
- Constant photoperiods of 16 hours light to 8 darkness will be used.
- The dissolved oxygen concentration will be maintained at =60% of saturation. Aeration may be necessary to maintain dissolved oxygen concentrations above 60% of saturation. If aeration is necessary, an experiment will be conducted to demonstrate that aeration will not alter the exposure concentrations.
- A tank volume turn over rate of 6 per 24-hour period will be established, or 90% replacement in 9 hours.

Biological Methods

A general description of the biological methods is provided below.

- Larval fish will be fed *ad libitum* several times daily and adults twice daily. Combinations of live and frozen brine shrimp and dry foods will be used.

- Reproductive P Adults, Pre-Exposure Phase

The P adult fish should be of the same age and cultured together under identical conditions. Sexual maturity fish, as evidenced by secondary sexual characteristics and behavior, will be collected from the culture and randomly assigned to spawning groups appropriate for the species. Additional spawning groups should be established as replacement fish. A pre-exposure phase will be conducted under identical exposure conditions (i.e., water quality and flow, space, light, temperature, feeding, spawning structure, etc). The pre-exposure phase will last until spawning is established in all spawning groups to be used in the study. This period could last up to14 days, depending on the species. During the pre-exposure, the reproductive potential of all spawning groups will be evaluated. Any fish that dies during the pre-exposure phase will be replaced with a fish from the original culture. Any spawning group that does not produce viable embryos during the pre-exposure phase will not be used. The number of eggs/female/day should be calculated for each spawning group.

- Reproductive P Adults, Exposure Phase

The spawning groups selected for the exposure will be placed to the lower unit of the exposure system. The exposure system should be functioning properly for several days prior to adding the spawning groups. Those fish not selected for the exposure can be

sacrificed and used to collect pre-spawning endpoints, if desired. The reproductive period will last from 7 to 28 days, depending on the species being exposed.

- F1 Embryo Exposure (hatching success)

All embryos will be counted daily. Representative spawns will be incubated and hatching success determined. The embryos used to determine hatching success will be contained in small incubation containers (sub-replicates) and observed daily until hatch.

- F1 Larval Exposure

Upon completion of the hatch, impartially selected larval fish will be added to larval growth chambers (sub-replicates). The larval fish exposure will be concluded 4 weeks (28 days) post hatch.

Statistical Analysis

If a solvent control is used in the exposure, a t-test will be used to determine whether there are differences between the two control groups. If there are no differences between the control groups, the controls will be pooled for the determination of treatment-related effects. If there is a difference between the control groups, the solvent control data will be used for the determination of treatment-related effects. Continuous data will be analyzed using ANOVA and pair-wise comparison tests (e.g., Dunnett's Test) to determine differences between treatments and controls. Discrete data will be analyzed by a contingency table test (e.g., Fisher's Exact Test).

Reporting Requirements

- Identification of the laboratory and testing site(s), dates of testing and key personnel involved in the study
- Identification of the test substance, which may include chemical name, additional designations (e.g., trade name), chemical designation (CAS number), empirical formula, molecular structure, manufacturer, lot or batch number, water solubility, vapor pressure, purity of test substance
- Characterization and origin of the dilution water
- Scientific name of the test organism, source, and culturing information
- Exposure system description, dilution water volume, construction materials used, depth and volume of test containers
- Description of the test substance delivery system and stock solution preparation
- Information regarding test temperatures, dissolved oxygen concentration, pH, salinity, photoperiod, and light intensity used
- Tabular presentation of all measured and calculated endpoints

- Description of reference (or inclusion as an appendix) to chemical and statistical procedures applied
- Analytical results of test concentration measurements and QC samples
- The NOEC and LOEC values will be provided, as well as the endpoints and statistical procedures used to establish these values
- Deviations from the protocol not addressed in protocol amendments, together with a discussion of the impact on the study
- Good Laboratory Practice (GLP) compliance statement signed by the Study Director
- Date(s) of Quality Assurance reviews, and dates reported to the Study Director and management, signed by the Quality Assurance Unit
- Location of the raw data and report.

10.2 Full Life-Cycle Test {Egg (P) to Juvenile (F1)}

A full life cycle test has been developed for use with fathead minnows (Benoit 1981) and for the sheepshead minnow (Hansen et al. 1978). Tables 10-1 and 10-2 present a summary of the full life cycle test with the fathead minnow and the sheepshead minnow. The full life-cycle test is initiated with fertilized eggs (P) and the fish are continuously exposed through reproductive maturity, followed assessment of the early development of the F1 generation.

The biological endpoints evaluated include the following:

- P embryo time-to-hatch, hatching success, larval survival and length, weight of thinned fish, survival, secondary sexual characteristics, fecundity/reproduction (e.g., eggs/female), growth.
- F1 hatching success, survival, growth (length and weight)

Table 10-1. Full Life-cycle Test with Fathead Minnow

Timeline	Test Day	# Organisms	Endpoints
Month 1	Embryo Incubation Day 0 - 5	200 per concentration 50 per replicate\	P Embryo time-to-hatch Hatching success
	Larval Exposure Day 6 -35	100 per concentration 25 per replicate	P Larval survival and length
Month 2	Day 65	Reduced to 50 per concentration 25 per duplicate	P Larval survival and length Weight (thinned out fish)
Months 3 through 5	P Juvenile to Adult Day 65 - 150	Reduced to 8 pairs per concentration 4 pairs per duplicate	P Survival 2^o sex characteristics and internal examination of gonads Lengths and weights (male and female thinned fish)

Timeline	Test Day	# Organisms	Endpoints
Months 6 through 9	P Spawning Period Day 150 - 270	8 pairs per concentration 4 pairs per duplicate	Eggs/female Spawns/female Eggs/spawn
	F1 Hatching Success Day 150 - 275	50 embryos per number of spawns >50 eggs	F1 Embryo time-to-hatch Embryo hatching success
Months 6 through 11	F1 4 - 8 Week Larval Exposure Day 150 - 330	25 larvae per group 2 groups per duplicate 4 groups per concentration	F1 4 and 8 week survival and length and weight
Month 10 - 11	P Termination Day 300 - 330	Survivors	P Survival 2^o sex characteristics and internal examination of gonads Lengths and weights (male and female thinned fish)

Table 10-2. Full Life-cycle Test with Sheepshead Minnow

Timeline	Test Day	# Organisms	Endpoints
Month 1	Embryo Incubation Day 0 - 5	200 per concentration 50 per replicate	P Embryo time-to-hatch Hatching success
	Larval Exposure Day 6 -35	Reduced to 50 larvae per concentration 25 per replicate (Day 35)	P Larval survival and length Weight (thinned fish)
Month 2	Juvenile to Adult Day 62	50 per concentration 25 per replicate	P Survival and length Time to maturity 2^o sex characteristics
Month 3	P Spawning Period Day 62 - 76 (possibly 2^{nd} round of spawn groups= Day 90)	Minimum : 4 groups of 3 females/2 males per concentration 2 groups per replicate	eggs/female/day
	F1 Hatching Success Day 62 - 95	50 embryos per number of spawns >50 eggs	F1 Embryo time-to-hatch Embryo hatching success

Month 4	F1 4-Week Larval Exposure Day 95 - 123	25 larvae per group 2 groups per duplicate 4 groups per concentration	F1 Survival Length and weight
	P Termination Day 95	Survivors	P Survival 2^o sex characteristics and internal examination of gonads Lengths and weights (male and female thinned fish)

Materials and Methods

Physical Exposure System

- Intermittent flow diluters are used in combination with mechanical or electronic syringe pumps to deliver fresh toxicant and dilution water during the exposure. If a carrier solvent is used, all treatments will have an equal solvent concentration with the exception of the dilution water control.
- Exposures will be conducted in glass aquaria, which will be impartially arranged on two separate levels (tiers). The upper level will be used for P, and F1, and F2 embryo incubation and larval and juvenile fish rearing. Larval growth chambers will be placed in each aquarium to provide the capability of rearing two larval groups, simultaneously. The lower level will be used to expose P and F1 spawning groups.
- Each level will contain two replicates per treatment level. Sub-replicates will be established for certain endpoints – hatching success, larval exposure and spawning groups.
- Three of the four candidate species are freshwater fish. Dilution water for these species should be dechlorinated water from a non-contaminated source. Care should be used not to use surface waters, which may contain low levels of potent EDCs. Sheepshead minnow will be maintained in relatively low salinity (15 ppt, lower salinity concentrations may be possible upon further evaluation) dilution water, which can be collected from a contaminant free source or can be prepared from a commercial mix.

Chemical System

- A range of five concentrations separated by a factor of at least two, that will cover the full dose response curve, will be selected based on a previously conducted exposure. A dilution water control will be established and a solvent control, if a carrier solvent is required. The solvent concentration will not exceed 100 µg/L and every effort will be made to maintain solvent concentrations <10 µg/L.
- Analytical sampling of each exposure level will be conducted weekly to determine test substance concentration confirmation.

Test Conditions

- The water temperature for fathead minnow will be 25 ±1ºC and for sheepshead minnow a water temperature of 28 ±1ºC will be maintained.
- Constant photoperiods of 16 hours light to 8 hours darkness will be used.
- The dissolved oxygen concentration will be maintained at =60% of saturation. Aeration may be necessary to maintain dissolved oxygen concentrations above 60% of saturation. If aeration is necessary, an experiment will be conducted to demonstrate that aeration will not alter the exposure concentrations.
- A tank volume turn over rate of 6 per 24-hour period will be established, or 90% replacement in 9 hours.

Biological Methods

Tables 10-1 and 10-2 provide species-specific time lines and data endpoints to be collected in each significant life stage for each generation of the fish. A general description of the biological methods is provided below.

Larval fish will be fed *ad libitum* several times daily and adults twice daily. Combinations of live and frozen brine shrimp and dry foods will be used.

- P Embryo Exposure (hatching success)

The exposure will begin with embryos as soon after fertilization as practical = 48 hours, ideally = 24 hours. Eggs obtained to initiate the study will be collected from numerous spawns. For some species, subsamples can be microscopically examined to estimate fertilization success before incubation. The embryos will be contained in small incubation containers (sub-replicates) and observed daily until hatch.

- P Larval Exposure

Upon completion of the hatch, impartially selected larval fish will be added to larval growth chambers (subreplicates). The larval fish exposure will be concluded 4 weeks (28 days) post hatch.

- P Juvenile Exposure

At this time, subreplicates will be combined and the juvenile fish will be cultured until they reach sexual maturity. The time to sexual maturity varies based on husbandry (diet is the most critical) and species.

- Reproductive P Adults

When fish reach sexual maturity, as evidenced by secondary sexual characteristics and behavior, spawning groups appropriate for the species will be formed. The spawning groups will be established in the lower unit of the exposure system. Those fish not selected for the spawning groups will be sacrificed and prespawning endpoints will be collected from these fish. The reproductive period will last for 14 to 28 days, depending on the species exposed.

- F1 Embryo Exposure (hatching success)

All embryos will be counted daily. Representative spawns will be incubated and hatching success determined as described for the P embryo exposure.

F1 Larval Exposure

The F1 larval exposure will be conducted following the same procedures presented for the P larval exposure.

- F1 Juvenile Exposure

The F1 juvenile exposure will be conducted following the same procedures presented for the P juvenile exposure.

Statistical Analysis

If a solvent control is used in the exposure, a t Test will be used to determine if there are differences between the two control groups. If there are no differences between the control groups, the controls will be pooled for the determination of treatment related effects. If there is a difference between the control groups, the solvent control data will be used for the determination of treatment related effects. Continuous data will be analyzed using ANOVA and pair-wise comparison tests (e.g., Dunnett's test) to determine differences between treatments and controls. Discrete data will be analyzed by a contingency table test (e.g., Fisher's Exact Test).

Reporting Requirements

- Identification of the laboratory and testing site(s), dates of testing, and key personnel involved in the study.
- Identification of the test substance which may include chemical name, additional designations (e.g., trade name), chemical designation (CAS number), empirical formula, molecular structure, manufacturer, lot or batch number, water solubility, vapor pressure, purity of test substance.
- Characterization and origin of the dilution water.

- Scientific name of the test organism, source and culturing information.
- Exposure system description, dilution water volume, construction materials used, depth and volume of test containers
- Description of the test substance delivery system and stock solution preparation.
- Information regarding test temperatures, dissolved oxygen concentration, pH, salinity, photoperiod and light intensity used.
- Tabular presentation of all measured and calculated endpoints.
- Description of reference (or inclusion as an appendix) to chemical and statistical procedures applied.
- Analytical results of test concentration measurements and QC samples.
- The NOEC and LOEC values will be provided, as well as the endpoints and statistical procedures used to establish these values.
- Deviations from the protocol not addressed in protocol amendments, together with a discussion of the impact on the study.
- Good Laboratory Practice (GLP) compliance statement signed by the Study Director.
- Date(s) of Quality Assurance reviews, and dates reported to the Study Director and management, signed by the Quality Assurance Unit.
- Location of the raw data and report.

10.3 Multi-Generation Test {Egg (P) to Juvenile (F2)}

The multi-generation toxicity test, which exposes all life-stages of two generations of fish, is presented for the species under consideration in this review in Tables 10-3 through 10-6. The test is initiated with eggs and two full generations of fish are exposed during the test. This test can be conducted to estimate the NOEC for the exposed fish and the biological endpoints evaluated include the following:

- P and F1 hatching success, survival, growth (length and weight), time-to-maturity, sex ratio, secondary sexual characteristics, fecundity/reproduction (e.g., eggs/female), and protein and sex steroid biomarkers (e.g., VTG).

- F2 hatching success, survival and growth.

Table 10-3. Multigeneration Test with Sheepshead Minnow

Timeline	Test Day	# Organisms	Endpoints
Month 1	P Hatching Success Day 0-5	200 per concentration 100 per replicate 50 per incubation cup	P hatching success Embryo time-to-hatch
	P Larval Exposure (Early Life-Stage) Day 6-33	100 per concentration 50 per replicate 25 per growth chamber	P survival Lengths

Timeline	Test Day	# Organisms	Endpoints
Month 2	P Juvenile Exposure Day 33-60	50 per concentration 25 per replicate	P survival Time to maturity Sex ratio Male, female length and weight GSI 2º sex characteristics Blood plasma biomarkers Histopathology
Month 3	P Reproduction Phase Day 60-74	8 males/20 females per concentration 4 males/10 females per replicate (2 groups of 2 males/5 females)	Time to maturity Sex ratio 2º sex characteristics Pre-spawn condition Fecundity (eggs/female) Fertilization success Spawning behavior
	P Adult Termination Day 74	Survivors	Male, female length and weight GSI Blood plasma biomarkers Histopathology
	F1 Hatching Success Day 60-79	200 embryos per concentration 100 per replicate 50 per incubation cup	F1 hatching success Embryo time-to-hatch
	F1 Larval Exposure (Early Life-Stage) Day 66-107	100 larval fish per concentration 50 per replicate 25 per growth chamber	F1 survival Length
Month 4	F1 Juvenile Exposure Day 93-135	50 per concentration 25 per replicate	F1 survival Time to maturity Sex ratio Male, female length and weight GSI 2º sex characteristics Blood plasma biomarkers Histopathology
Month 5	F1 Reproduction Phase Day 135-149	8 males/20 females per concentration 4 males/10 females per replicate (2 groups of 2 males /5 females)	Time to maturity Sex ratio 2º sex characteristics Pre-spawn condition Fecundity (eggs/female) Fertilization success Spawning behavior
	F1 Adult Termination Day 149	Survivors	Male, female length and weight GSI Blood plasma biomarkers Histopathology
Month 5	F2 Hatching Success Day 135-154	200 embryos per concentration 100 per replicate 50 per incubation cup	F2 hatching success Embryo time-to-hatch

Timeline	Test Day	# Organisms	Endpoints
Month 6	F2 Larval Exposure (Early Life-Stage) Day 140-182	100 larval fish per concentration 50 per replicate 25 per growth chamber	F2 survival Length Weight

Table 10-4. Multigeneration Test with Zebrafish

Timeline	Test Day	# Organisms	Endpoints
Month 1	P Hatching Success Day 0-7	200 per concentration 100 per replicate 50 per incubation cup	P hatching success Embryo time-to-hatch
	P Larval Exposure (Early Life-Stage) Day 7-35	100 per concentration 50 per replicate 25 per growth chamber	P survival Lengths
Month 2, 3	P Juvenile Exposure Day 35-90	100 per concentration 50 per replicate	P survival Time to maturity Sex ratio Male, female length and weight GSI 2^{o} sex characteristics Whole body homogenate biomarkers Histopathology
Month 3	P Reproduction Phase Day 90-104	32 males/20 females per concentration 16 males/10females per replicate	Time to maturity Sex ratio 2^{o} sex characteristics Pre-spawn condition Fecundity (eggs/female) Fertilization success Spawning behavior
	P Adult Termination Day 104	Survivors	Male, female length and weight GSI Whole body homogenate biomarkers Histopathology
Month 3	F1 Hatching Success Day 90-111	200 embryos per concentration 100 per replicate 50 per incubation cup	F1 hatching success Embryo time-to-hatch
Month 4	F1 Larval Exposure (Early Life-Stage) Day 97-139	100 larval fish per concentration 50 per replicate 25 per growth chamber	F1 survival Length

Timeline	Test Day	# Organisms	Endpoints
Month 5	F1 Juvenile Exposure Day 125-178	100 per concentration 50 per replicate	F1 survival Time to maturity Sex ratio Male, female length and weight GSI 2^{o} sex characteristics Whole body homogenate biomarkers Histopathology
Month 6	F1 Reproduction Phase Day 178-192	32 males/20 females per concentration 16 males/10females per replicate	Time to maturity Sex ratio 2^{o} sex characteristics Pre-spawn condition Fecundity (eggs/female) Fertilization success Spawning behavior
	F1 Adult Termination Day 192	Survivors	Male, female length and weight GSI Whole body homogenate biomarkers Histopathology
Month 7	F2 Hatching Success Day 178-199	200 embryos per concentration 100 per replicate 50 per incubation cup	F2 hatching success Embryo time-to-hatch
	F2 Larval Exposure (Early Life-Stage) Day 206-227	100 larval fish per concentration 50 per replicate 25 per growth chamber	F2 survival Length Weight

Table 10-5. Multigeneration Test with Fathead Minnow

Timeline	Test Day	# Organisms	Endpoints
Month 1	P Hatching Success Day 0-5	200 per concentration 100 per replicate 50 per incubation cup	P hatching success Embryo time-to-hatch
	P Larval Exposure (Early Life-Stage) Day 6-33	100 per concentration 50 per replicate 25 per growth chamber	P survival Lengths
Month 2, 3, 4	P Juvenile Exposure Day 33-120	50 per concentration 25 per replicate	P survival Time to maturity Sex ratio Male, female length and weight GSI 2^{o} sex characteristics Blood plasma biomarkers Histopathology

Timeline	Test Day	# Organisms	Endpoints
Month 5	P Reproduction Phase Day 120-150	6 males/12 females per concentration 3 males/6 females per replicate (3 groups of 1 males /2 females)	Time to maturity Sex ratio 2^{o} sex characteristics Pre-spawn condition Fecundity (eggs/female) Fertilization success Spawning behavior
	P Adult Termination Day 150	Survivors	Male, female length and weight GSI Blood plasma biomarkers Histopathology
	F1 Hatching Success Day 120-155	200 embryos per concentration 100 per replicate 50 per incubation cup	F1 hatching success Embryo time-to-hatch
Month 5, 6	F1 Larval Exposure (Early Life-Stage) Day 125-183	100 larval fish per concentration 50 per replicate 25 per growth chamber	F1 survival Length
Month 6, 7, 8	F1 Juvenile Exposure Day 153-275	50 per concentration 25 per replicate	F1 survival Time to maturity Sex ratio Male, female length and weight GSI 2^{o} sex characteristics Blood plasma biomarkers Histopathology
Month 8, 9	F1 Reproduction Phase Day 241-305	6 males/12 females per concentration 3 males/6 females per replicate (3 groups of 1 males /2 females)	Time to maturity Sex ratio 2^{o} sex characteristics Pre-spawn condition Fecundity (eggs/female) Fertilization success Spawning behavior
Month 8, 9, 10	F2 Hatching Success Day 241-310	200 embryos per concentration 100 per replicate 50 per incubation cup	Male, female length and weight GSI Blood plasma biomarkers Histopathology
Month 10, 11	F2 Larval Exposure (Early Life-Stage) Day 268-338	100 larval fish per concentration 50 per replicate 25 per growth chamber	F2 hatching success Embryo time-to-hatch
			F2 survival Length Weight

Table 10-6. Multigeneration Study with Medaka

Timeline	Test Day	# Organisms	Endpoints
Month 1	P Hatching Success Day 0-7	200 per concentration 100 per replicate 50 per incubation cup	P hatching success Embryo time-to-hatch
	P Larval Exposure (Early Life-Stage) Day 7-35	100 per concentration 50 per replicate 25 per growth chamber	P survival Lengths
Month 2	P Juvenile Exposure Day 35-63	100 per concentration 50 per replicate	P survival Time to maturity Sex ratio Male, female length and weight GSI 2^{o} sex characteristics Whole body homogenate biomarkers Histopathology
Month 3	P Reproduction Phase Day 63-77	24 males/40 females per concentration 12 males/20 females per replicate	Time to maturity Sex ratio 2^{o} sex characteristics Pre-spawn condition Fecundity (eggs/female) Fertilization success Spawning behavior
	P Adult Termination Day 77	Survivors	Male, female length and weight GSI Whole body homogenate biomarkers Histopathology
	F1 Hatching Success Day 64-84	200 embryos per concentration 100 per replicate 50 per incubation cup	F1 hatching success Embryo time-to-hatch
Month 4	F1 Larval Exposure (Early Life-Stage) Day 71-112	100 larval fish per concentration 50 per replicate 25 per growth chamber	F1 survival Length
	F1 Juvenile Exposure Day 126-140	100 per concentration 50 per replicate	F1 survival Time to maturity Sex ratio Male, female length and weight GSI 2^{o} sex characteristics Whole body homogenate biomarkers Histopathology

Timeline	Test Day	# Organisms	Endpoints
Month 5	F1 Reproduction Phase Day 140-154	24 males/40 females per concentration 12 males/20 females per replicate	Time to maturity Sex ratio 2° sex characteristics Pre-spawn condition Fecundity (eggs/female) Fertilization success Spawning behavior
	F1 Adult Termination Day 154	Survivors	Male, female length and weight GSI Whole body homogenate biomarkers Histopathology
	F2 Hatching Success Day 141-161	200 embryos per concentration 100 per replicate 50 per incubation cup	F2 hatching success Embryo time-to-hatch
Month 6	F2 Larval Exposure (Early Life-Stage) Day 148-189	100 larval fish per concentration 50 per replicate 25 per growth chamber	F2 survival Length Weight

Materials and Methods

Physical Exposure System

- Intermittent flow diluters are used in combination with mechanical or electronic syringe pumps to deliver fresh toxicant and dilution water during the exposure. If a carrier solvent is used, all treatments will have an equal solvent concentration with the exception of the dilution water control.

- Exposures will be conducted in glass aquaria, which will be impartially arranged on two separate levels (tiers). The upper level will be used for P, F1, and F2 embryo incubation and larval and juvenile fish rearing. Larval growth chambers will be placed in each aquarium to provide the capability of rearing two larval groups, simultaneously. The lower level will be used to expose P and F1 spawning groups.

- Each level will contain two replicates per treatment level. Sub-replicates will be established for certain endpoints – hatching success, larval exposure and spawning groups.

- Three of the four candidate species are freshwater fish. Dilution water for these species should be dechlorinated water from a non-contaminated source. Care should be used not to use surface waters, which may contain low levels of potent EDCs. Sheepshead minnow will be maintained in relatively low salinity (15 ppt, lower

salinity concentrations may be possible upon further evaluation) dilution water, which can be collected from a contaminant free source or can be prepared from a commercial mix.

Chemical System

- A range of five concentrations separated by a factor of at least two, that will cover the full dose response curve, will be selected based on a previously conducted exposure. A dilution water control will be established and a solvent control, if a carrier solvent is required. The solvent concentration will not exceed 100 µg/L and every effort will be made to maintain solvent concentrations <10 µg/L.

- Analytical sampling of each exposure level will be conducted weekly to determine test substance concentration confirmation.

Test Conditions

- The water temperature for fathead minnow will be 25 ±1ºC. For the other candidate species a water temperature of 28 ±1ºC will be maintained.

- Constant photoperiods of 16 hours light to 8 hours darkness will be used.

- The dissolved oxygen concentration will be maintained at =60% of saturation. Aeration may be necessary to maintain dissolved oxygen concentrations above 60% of saturation. If aeration is necessary, an experiment will be conducted to demonstrate that aeration will not alter the exposure concentrations.

- A tank volume turn over rate of 6 per 24-hour period will be established, or 90% replacement in 9 hours.

Biological Methods

Tables 10-1 through 10-4 provide species-specific time lines and data endpoints to be collected in each significant life stage for all generations of the fish. A general description of the biological methods is provided below.

Larval fish will be fed *ad libitum* several times daily and adults twice daily. Combinations of live and frozen brine shrimp and dry foods will be used.

- P Embryo Exposure (hatching success)

The exposure will begin with embryos as soon after fertilization as practical = 48 hours, ideally = 24 hours. Eggs obtained to initiate the study will be collected from numerous

spawns. For some species, subsamples can be microscopically examined to estimate fertilization success before incubation. The embryos will be contained in small incubation containers (sub-replicates) and observed daily until hatch.

- P Larval Exposure

Upon completion of the hatch, impartially selected larval fish will be added to larval growth chambers (subreplicates). The larval fish exposure will be concluded 4 weeks (28 days) post hatch.

- P Juvenile Exposure

At this time, subreplicates will be combined and the juvenile fish will be cultured until they reach sexual maturity. The time to sexual maturity varies based on husbandry (diet is the most critical) and species.

- Reproductive P Adults

When fish reach sexual maturity, as evidenced by secondary sexual characteristics and behavior, spawning groups appropriate for the species will be formed. The spawning groups will be established in the lower unit of the exposure system. Those fish not selected for the spawning groups will be sacrificed and prespawning endpoints will be collected from these fish. The reproductive period will last for 7 to 28 days, depending on the species exposed.

- F1 Embryo Exposure (hatching success)

All embryos will be counted daily. Representative spawns will be incubated and hatching success determined as described for the P embryo exposure.

- F1 Larval Exposure

The F1 larval exposure will be conducted following the same procedures presented for the P larval exposure.

- F1 Juvenile Exposure

The F1 juvenile exposure will be conducted following the same procedures presented for the P juvenile exposure.

- Reproductive F1 Adults

The F1 reproductive exposure will be conducted following the same procedures presented for the P reproductive exposure.

- F2 Embryo Exposure (hatching success)

The F2 embryo exposure will be conducted following the same procedures presented for the F1 embryo exposure.

- F2 Larval Exposure

The F2 larval exposure will be conducted following the same procedures presented for the P and F1 larval exposures.

Statistical Analysis

If a solvent control is used in the exposure, a t Test will be used to determine if there are differences between the two control groups. If there are no differences between the control groups, the controls will be pooled for the determination of treatment related effects. If there is a difference between the control groups, the solvent control data will be used for the determination of treatment related effects. Continuous data will be analyzed using ANOVA and pair-wise comparison tests (e.g., Dunnett's test) to determine differences between treatments and controls. Discrete data will be analyzed by a contingency table test (e.g., Fisher's Exact Test).

Reporting Requirements

- Identification of the laboratory and testing site(s), dates of testing, and key personnel involved in the study.
- Identification of the test substance which may include chemical name, additional designations (e.g., trade name), chemical designation (CAS number), empirical formula, molecular structure, manufacturer, lot or batch number, water solubility, vapor pressure, purity of test substance.
- Characterization and origin of the dilution water.
- Scientific name of the test organism, source and culturing information.
- Exposure system description, dilution water volume, construction materials used, depth and volume of test containers
- Description of the test substance delivery system and stock solution preparation.
- Information regarding test temperatures, dissolved oxygen concentration, pH, salinity, photoperiod and light intensity used.
- Tabular presentation of all measured and calculated endpoints.
- Description of reference (or inclusion as an appendix) to chemical and statistical procedures applied.
- Analytical results of test concentration measurements and QC samples.
- The NOEC and LOEC values will be provided, as well as the endpoints and statistical procedures used to establish these values.

- Deviations from the protocol not addressed in protocol amendments, together with a discussion of the impact on the study.
- Good Laboratory Practice (GLP) compliance statement signed by the Study Director.
- Date(s) of Quality Assurance reviews, and dates reported to the Study Director and management, signed by the Quality Assurance Unit.
- Location of the raw data and report.

10.4 Two Generation Test {Adult (P) to Juvenile (F2)}

Objective

A two generation life-cycle toxicity test, which exposes the adult P, full F1generation,and measures F2 viability, can be conducted to estimate the NOEC for the exposed fish. Table 10-7 summarizes this test utilizing the fathead minnow. The test can also be applied to zebrafish, medaka and sheepshead minnow (Tables 10-8 through 10-10). Variables including the time-line of the test, the number of fish required in the test, and obtaining endpoints such as Vtg plasma levels will be associated with the different species. For example, zebrafish are difficult to sex and will require more fish for testing and plasma is difficult to obtain from the smaller species limiting the biochemical measures in plasma. The biological endpoints evaluated will include the following:

- P Survival, secondary sex characteristics, reproductive behavior, spawning activity, fecundity, fertilization success
- F1 hatching success, survival, growth (length and weight), time-to-maturity, sex ratio, secondary sexual characteristics, fecundity/reproduction (e.g., eggs/female), and protein and sex steroid biomarkers (e.g., VTG).
- F2 hatching success, survival and growth.

Materials and Methods

Physical Exposure System

- Intermittent flow diluters will be used in combination with mechanical or electronic syringe pumps to deliver fresh toxicant and dilution water during the exposure. If a carrier solvent is used, all treatments will have an equal solvent concentration with the exception of the dilution water control.

- Exposures will be conducted in glass aquaria, which will be impartially arranged on two separate levels (tiers). The upper level will be used for F1, and F2 embryo incubation and larval and juvenile fish rearing. Larval growth chambers will be placed in each aquarium to provide the capability of rearing two larval groups, simultaneously. The lower level will be used to expose P and F1 spawning groups.

Table 10-7. Two-Generation Test with Fathead Minnow

Timeline	Test Day	# Organisms	Endpoints
Pre-test	7 to 21 days Pre-exposure	4 females/2 males per replicate	2^o sex characteristics Reproductive Behavior Spawning activity Fecundity Fertilization success
P	Initiate Exposure 0 to 21 days	4 females/2 males per replicate	2^o sex characteristics Reproductive Behavior Spawning activity Fecundity Fertilization success
F1	Day 14 to 21	50 embryos per replicate	Hatching success Time to hatch Normal/ abnormal
P	P Adult Termination	Survivors	Weight and length Sex GSI Gonad Histopathology Vtg and Sex steroids
F1	Day 26 to 16-18 weeks (post -hatch)	25 larvae per replicate	Survival Length at 4 and 8 weeks Time to maturity Sex ratio 2^o sex characteristics Pre-spawn condition
F1	18 to 21 weeks	4 females/2 males per replicate	Survival 2^o sex characteristics Reproductive Behavior Spawning activity Fecundity Fertilization success
F2	18 to 21 weeks	50 embryos per replicate	Hatching success Time to hatch Normal/ abnormal
F1	F1 Adult Termination	Survivors	Weight and length Sex GSI Gonad Histopathology Vtg and Sex steroids
F2	21 to 25 weeks	25 larvae per replicate	Survival Weight and length at 4 weeks

Table 10-8. Two-Generation Test with Sheepshead Minnow

Timeline	Test Day	# Organisms	Endpoints
Pre-test	4 to 7 days Pre-exposure	5 females/2 males per replicate	2^o sex characteristics Reproductive Behavior Spawning activity Fecundity Fertilization success
P	Initiate Exposure 0 to 14 days	5 females/2 males per replicate	2^o sex characteristics Reproductive Behavior Spawning activity Fecundity Fertilization success
F1	Day 10 - 14	50 embryos per replicate	Hatching success Time to hatch Normal/ abnormal
P	P Adult termination	Survivors	Weight and length Sex GSI Gonad Histopathology Vtg and Sex steroids
F1	Day 19 to 7 - 8 weeks (post -hatch)	25 larvae per replicate	Survival Length at 4 and 8 weeks Time to maturity Sex ratio 2^o sex characteristics Pre-spawn condition
F1	8 -10 weeks	5 females/2 males per replicate	Survival 2^o sex characteristics Reproductive Behavior Spawning activity Fecundity Fertilization success
F2	8 - 10 weeks	50 embryos per replicate	Hatching success Time to hatch Normal/ abnormal
F1	F1 Adult Termination	Survivors	Weight and length Sex GSI Gonad Histopathology Vtg and Sex steroids
F2	10 - 14 weeks	25 larvae per replicate	Survival Weight and length at 4 weeks

Table 10-9. Two-Generation Test with Zebrafish

Timeline	Test Day	# Organisms	Endpoints
Pre-test	4 to 7 days Pre-exposure	10 females/16 males per replicate	2^0 sex characteristics Reproductive Behavior Spawning activity Fecundity Fertilization success
P	Initiate Exposure 0 to 14 days	10 females/16 males per replicate	2^0 sex characteristics Reproductive Behavior Spawning activity Fecundity Fertilization success
F1	Day 10 to 14	50 embryos per replicate	Hatching success Time to hatch Normal/abnormal
P	P Adult termination	Survivors	Weight and length Sex GSI Gonad Histophathology Vtg and Sex steroids
F1	Day 21 to 12 to 14 weeks (post-hatch)	25 larvae per replicate	Survival Length at 4 and 8 weeks Time to maturity Sex ratio 2^o sex characteristics Pre-spawn condition
F1	14 to 16 weeks	10 females/16 males per replicate	Survival 2^0 sex characteristics Reproductive Behavior Spawning activity Fecundity Fertilization success
F2	14 to 16 weeks	50 embryos per replicate	Hatching success Time to hatch Normal/abnormal
F1	F1 Adult Termination	Survivors	Weight and length Sex GSI Gonad Histopathology Vtg and Sex steroids
F2	16 to 20 weeks	25 larvae per replicate	Survival Weight and length at 4 weeks

Table 10-10. Two-Generation Test with Medaka

Timeline	Test Day	# Organisms	Endpoints
Pre-test	7 to 21 days Pre-exposure	20 females/12 males per replicate	2° sex characteristics Reproductive behavior Spawining activity Fecundity Fertilization success
P	Initiate exposure 0 to 21 days	20 females/12 males per replicate	2° sex characteristics Reproductive behavior Spawining activity Fecundity Fertilization success
F1	Day 14 to 21	50 embryos per replicate	Hatching success Time to hatch Normal/abnormal
P	F0 adult termination	Survivors	Weight and length Sex GSI Gonad Histopathology Vtg and Sex steroids
F1	Day 21 to 16 - 18 weeks (post-hatch)	25 larvae per replicate	Survival Length at 4 and 8 weeks Time to maturity Sex ratio 2° sex characteristics Pre-spawn condition
F1	18 to 21 weeks	20 females/12 males per replicate	Survival 2° sex characteristics Reproductive behavior Spawining activity Fecundity Fertilization success
F2	18 to 21 weeks	50 embryos per replicate	Hatching success Time to hatch Normal/abnormal
F1	F1 adult termination	Survivors	Weight and length Sex GSI Gonad Histopathology Vtg and Sex steroids
F2	21 to 25 weeks	25 larvae per replicate	Survival Weight and length at 4 weeks

- Each level will contain two replicates per treatment level. Sub-replicates will be established for certain endpoints – hatching success, larval exposure and spawning groups.

- Three of the four candidate species are freshwater fish. Dilution water for these species should be dechlorinated water from a non-contaminated source. Care should be used not to use surface waters, which may contain low levels of potent EDCs. Sheepshead minnow will be maintained in relatively low salinity (15 ppt, lower salinity concentrations may be possible upon further evaluation) dilution water, which can be collected from a contaminant free source or can be prepared from a commercial mix.

Chemical System

- A range of five concentrations separated by a factor of at least two, that will cover the full dose response curve, will be selected based on a previously conducted exposure. A dilution water control will be established and a solvent control, if a carrier solvent is required. The solvent concentration will not exceed 100 µg/L and every effort will be made to maintain solvent concentrations <10 µg/L.

- Analytical sampling of each exposure level will be conducted weekly to determine test substance concentration confirmation.

Test Conditions

- The water temperature for fathead minnow will be 25 ±1ºC. For the other candidate species a water temperature of 28 ±1ºC will be maintained.
- Constant photoperiods of 16 hours light to 8 hours darkness will be used.
- The dissolved oxygen concentration will be maintained at = 60% of saturation. Aeration may be necessary to maintain dissolved oxygen concentrations above 60% of saturation. If aeration is necessary, an experiment will be conducted to demonstrate that aeration will not alter the exposure concentrations.
- A tank volume turn over rate of 6 per 24-hour period will be established, or 90% replacement in 9 hours.

Biological Methods

Table 10-7 provides a time line of the test utilizing fathead minnows, with shorter time lines resulting with the use of sheepshead minnow zebrafish and medaka, (Tables 10-8 through 10-10). The data endpoints to be collected in each significant life stage for all generations of the fish are presented in these tables (10-7 through 10-10). A general description of the biological methods is provided below.

Larval fish will be fed *ad libitum* several times daily and adults twice daily. Combinations of live and frozen brine shrimp and dry foods will be used.

- Mature P Adults Pre-exposure

Sexually maturity fish, as evidenced by secondary sexual characteristics and behavior, will be formed into spawning groups appropriate for the species. Zebrafish do not display clear secondary sexual characteristics and a number of fish will be selected to allow for this characteristic. Typically, the spawning groups will be established in the lower unit of the exposure system. The reproductive period will last for 4 to 28 days, depending on the species, and during this time survival, secondary sex characteristics, reproductive behavior, spawning activity, fecundity, fertilization success data will be collected.

- Mature P Adults Exposure

Following the pre-exposure period, the exposure will be initiated and for 14 to 21 days the survival, secondary sex characteristics, reproductive behavior, spawning activity, fecundity, fertilization success data will be collected.

- F1 Embryo Exposure (hatching success)

The exposure will begin with embryos as soon after fertilization as practical = 48 hours, ideally = 24 hours. Eggs will be collected from the P adult spawning groups near the end of the adult exposure period. For some species, subsamples can be microscopically examined to estimate fertilization success before incubation. The embryos will be contained in small incubation containers (sub-replicates) and observed daily until hatch.

- F1 Larval Exposure

Upon completion of the hatch, impartially selected larval fish will be added to larval growth chambers (subreplicates). The larval fish exposure will be concluded 3 to 4 weeks post hatch.

- F1 Juvenile Exposure

At this time, subreplicates will be combined and the juvenile fish will be cultured until they reach sexual maturity. The time to sexual maturity varies based on husbandry (diet is the most critical) and species. All embryos will be counted daily. Representative spawns will be incubated and hatching success determined.

- Reproductive F1 Adults

The F1 reproductive exposure will be conducted following the same procedures presented for the P reproductive exposure.

- F2 Embryo Exposure (hatching success)

The F2 embryo exposure will be conducted following the same procedures presented for the F1 embryo exposure.

- F2 Larval Exposure

The F2 larval exposure will be conducted following the same procedures presented for the F1 larval exposures.

Statistical Analysis

If a solvent control is used in the exposure, a t Test will be used to determine if there are differences between the two control groups. If there are no differences between the control groups, the controls will be pooled for the determination of treatment related effects. If there is a difference between the control groups, the solvent control data will be used for the determination of treatment related effects. Continuous data will be analyzed using ANOVA and pair-wise comparison tests (e.g., Dunnett's test) to determine differences between treatments and controls. Discrete data will be analyzed by a contingency table test (e.g., Fisher's Exact Test).

Reporting Requirements

- Identification of the laboratory and testing site(s), dates of testing, and key personnel involved in the study.
- Identification of the test substance which may include chemical name, additional designations (e.g., trade name), chemical designation (CAS number), empirical formula, molecular structure, manufacturer, lot or batch number, water solubility, vapor pressure, purity of test substance.
- Characterization and origin of the dilution water.
- Scientific name of the test organism, source and culturing information.
- Exposure system description, dilution water volume, construction materials used, depth and volume of test containers
- Description of the test substance delivery system and stock solution preparation.
- Information regarding test temperatures, dissolved oxygen concentration, pH, salinity, photoperiod and light intensity used.
- Tabular presentation of all measured and calculated endpoints.
- Description of reference (or inclusion as an appendix) to chemical and statistical procedures applied.
- Analytical results of test concentration measurements and QC samples.
- The NOEC and LOEC values will be provided, as well as the endpoints and statistical procedures used to establish these values.
- Deviations from the protocol not addressed in protocol amendments, together with a discussion of the impact on the study.
- Good Laboratory Practice (GLP) compliance statement signed by the Study Director.

- Date(s) of Quality Assurance reviews, and dates reported to the Study Director and management, signed by the Quality Assurance Unit.
- Location of the raw data and report.

11.0 RECOMMENDED PROTOCOL AND ADDITIONAL DATA NEEDS

After considering the strengths and weaknesses of the various candidate approaches, the two-generation test is the recommended protocol. At this time, this protocol is not adequately developed to a point that a standardized transferrable protocol can be written without additional prevalidation studies. Developing a two-generation protocol that can be conducted with multiple species offers distinct advantages. However, if the two-generation test is conducted with multiple species, the reliability and reproducibility of the test must be demonstrated. Issues such as differences in sensitivity among the species must addressed for use of the test in risk assessment. Demonstration trials with multiple species will be required and the two-generation protocol requires significant cost and time commitments. The number of demonstration trials for full optimization of a method suitable for interlaboratory testing could be limited with the pre-selection of a species, such as the fathead minnow. Interlaboratory comparisons should be conducted with compounds that span the possible endocrine effects (strong and weak androgen receptor, estrogen receptor, and thyroid agonists and antagonists).

Each of the test species under consideration in this review has specific strengths and weaknesses. Important to the development of the protocol is the consideration that the selected test species provide definitive data endpoints necessary to identify relevant population effects covering a wide range of EDC modes of action. It is also important that the standardized transferable protocol can be readily followed and executed by participating test laboratories.

The length of time required to conduct a test protocol is an important consideration. The sheepshead minnow, zebrafish, and medaka all have significantly shorter life cycles than the fathead minnow, and of these three species, the sheepshead minnow likely provides the shortest life cycle.

Another important consideration for a fish life cycle test is the ease of handling eggs, which is the most labor-intensive aspect of the in-life phase of the test. In addition to time considerations is the potential for damaging eggs in the process of handling, which could jeopardize fertilization and hatching success endpoints. All fish eggs tend to be sticky because of the filaments surrounding the egg. Fathead minnow eggs are the easiest eggs to handle, as they are laid on a substrate and, as a result, are clean and easy to retrieve. The remaining three species have similar spawning patterns; eggs can be collected from a chamber isolated below the spawning population. Medaka eggs are more difficult to handle compared with sheepshead minnow and zebrafish eggs because of the long strands that extend from each egg. Sheepshead minnow eggs are slightly larger than the zebrafish eggs, making them somewhat easier to handle.

The preparation of spawning groups is another critical phase in the test. Sex identification must be done quickly and accurately, without causing undue stress to the fish during handling. The sheepshead minnow and fathead minnow have the strongest natural sexual dimorphism of the four species being considered and, therefore, offer ease of sex determination and establishing spawning groups. However, the d-rR orange-red strain medaka is superior, because the coloration of the males is a gender-specific genetic trait.

The period during which sex is determined in a species offers a critical endpoint of endocrine disruption, particularly in a partial life-cycle exposure. The time of sex determination is well documented in the literature for the zebrafish and medaka; however, this is not the case for sheepshead minnow and fathead minnow, which limits their consideration as a test species.

Regulatory and historical recognition should be considered in the selection of a test species. Fathead minnow and sheepshead minnow have wide EPA acceptance as test species, whereas the zebrafish and medaka do not have a significant regulatory history in the U.S. It should be noted however, that a number of North American research scientists are using the zebrafish and medaka, which offer advantages, including smaller size and shorter life cycles compared to fathead minnows. Numerous early life-stage and full life-cycle studies have been conducted with both the sheepshead minnow and the fathead minnow, with studies using the fathead minnow being significantly more common. Both of these species are widely available in the U.S. Because fathead minnow and sheepshead minnow have been used extensively in the U.S. for regulatory purposes, sufficient data are available to compare the statistical sensitivity of endpoints for both species. Based on a limited but fair comparison, most endpoints have the same sensitivity, except for the reproductive endpoints, which are less variable and thus more sensitive for sheepshead minnow.

Testing and culture space is a final consideration for choosing a test species. Zebrafish are the smallest fish and should provide the most efficient use of culture and testing space in a laboratory. Medaka are also relatively small fish, whereas the sheepshead minnow and fathead minnow are larger fish and require more floor space. The main advantage of using the larger fish is the potential to collect individual blood plasma samples for steroid and protein analyses.

11.1 Exposure Protocol

The fish two-generation life cycle test is initiated with mature fish in which reproductive fitness has been established. The exposure of the adult P fish incorporates the maternal transfer of EDCs in the initiation of the test. The fish two generation test offers the advantage of a reduced test time line when compared to the multigenerational test. The reduced time line will aid the use of fish species with longer life cycles, including the fathead minnow. The significant database that exists for the fathead minnow and characteristics discussed in previous sections, such as ease of egg handling and large plasma volumes, coupled with the reduced time line of the two generation test suggest that this combination may offer a test that is robust and can be conducted in a cost and time effective manner.

It should be noted that the multigenerational protocol for the sheepshead minnow can be successfully conducted in a time frame (approximately 4.5 months) similar to two-generation test with the fathead minnow. Although this time frame is several weeks longer than the traditional EPA sheepshead minnow full life-cycle study, it provides reproductive data endpoints for the P and F1 generations and also assesses growth and survival endpoints for the P, F1, and F2 generations. The exposure is continuous throughout all life stages and generations. Although this exposure may not reflect real-world exposure conditions, it will establish a level at which a continuous exposure will be a minimal risk. Additional testing might be needed on individual EDCs if real-world exposure levels are intermittent but at levels greater than a continuous-exposure level that caused significant effects. The partial life-cycle and standard, full life-cycle protocols collect a more limited data set, thus necessitating extrapolation of potential effects of long-term chronic exposure.

11.2 Appropriateness of Reproductive Screen Endpoints

Endpoints such as the number of eggs/female/day, fertilization, hatching success, and GSI should be collected at every opportunity. These endpoints are clear and direct measurements of potentially significant population-level effects. Endpoints such as histopathology and biochemical analyses can demonstrate exposure to EDCs and provide information on modes of action. The interpretation of histological material requires experience in identifying normal and abnormal structures in organs and tissues. It is recommended that histology interpretation be conducted by a certified fish pathologist with training and experience in veterinary and fish pathology. For histopathological analysis to be especially useful in reproductive screening tests, it is important to ensure maximum control of the subjective nature of the interpretations, and to limit the interpretation to repeatable results that can be verified by different investigators. As an aid toward to this end, the following steps should be implemented:

- Staging and abnormalities are best defined by incremental changes (i.e., presence or absence of particular structures) and in all cases, need to be codified in a standardized definition that can be used by multiple investigators.

- Measurement of the size of structures is subject to a high degree of measurement error as a result of variations in planes of sections and must, therefore, be compared using appropriate statistical tests.

- Methods for fixation, embedding, sectioning, and staining need to be standardized.

- Unknown samples should be read and interpreted in a blind fashion.

Impacts of EDCs on the sex determination of larval fish could be an endpoint that provides important information with the potential to detect endocrine disruption in the early life stages of fish. Raising fish to sexual maturity could be a cost-effective analysis of this endpoint. At this time, relatively little information is available on the impact of EDCs on sex-determination effects of embryonic or larval stages of fish.

11.3 Preferred Methods for Quantification of Biochemical Endpoints

The most widely used assays for individual sex steroids based on competitive binding with a specific antibody include RIA and ELISA. Both are equally suitable, sensitive, and specific for detecting the common sex steroids of interest (E2, T, 11-KT) in fish blood plasma. 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. In comparing the two antibody-based methods, RIA and ELISA, a major disadvantage of the RIA is the use of radioisotopes. The handling of radioisotopes in RIA, the equipment required for radioactive detection, and problems with radioisotope disposal are less desirable compared with ELISA. Therefore, the preferred method to quantify steroids in the fathead minnow is the ELISA.

For quantification of VTG in fathead minnows, ELISA offers ease of use, sensitivity, and specificity for measuring VTG and, unlike RIA, does not require the use of radioactive isotopes. ELISA methods to quantify VTG in plasma and tissue homogenates of fathead minnows include the competitive antibody-capture and sandwich ELISA, with slightly greater sensitivity achieved with sandwich ELISAs. Antibody-capture ELISAs require larger amounts of purified VTG to perform the assay, whereas sandwich ELISAs require larger amounts of multiple VTG antibodies. If accurate quantification and a high degree of specificity are desired, the use of purified VTG homologous to the test species to produce antibodies and as standards is required. Due to the cross reactivity of antibodies, carp-based ELISA systems are available to measure the induction of VTG. Purified carp vitellogenin and anti-VTG carp antibodies are available commercially for use in ELISA. If desired, the loss of specificity and sensitivity with the use of carp-based systems can be addressed though the use of purified fathead minnow VTG as a standard; however, fathead minnow VTG and antibodies are not currently commercially available. Therefore, ELISA is the recommended method to measure VTG levels in the fathead minnow. Factors including cost, supply, and sensitivity and specificity of homologous versus carp-based ELISA to measure VTG in fathead minnows will determine the specifics of the recommended ELISA protocol. VTG induction can be measured with a carp-based ELISA, the most sensitive of which is the sandwich ELISA. However, new mass spectrometry methods offer exciting possibilities for VTG analysis and in the future might prove to be the preferred method.

11.4 Significant Data Gaps

The general techniques for the long-term exposure and the evaluation of the reproductive potential of fish are fairly well established. For the sheepshead minnow, additional assessment of growth and reproductive performance in different salinities could be conducted. Testing and culture of sheepshead minnow at lower salinities will offer distinct cost-controlling advantages and will assist the solubilization of some potential EDCs during testing. There is a need for basic endocrinology studies in this species, as little is known on sex-steroid levels. Further testing using model steroids is also needed, particularly with androgenic and anti-androgenic substances, for which no data are currently available for this species. In addition, the

determination of sexual differentiation for the preferred fish species might be important information in evaluating the results of a life-cycle test. Methods of sexual differentiation are established for zebrafish and medaka, but are not published for fathead minnow or sheepshead minnow.

There are distinct advantages to developing a two-generation protocol as opposed to the alternative protocols. The two-generation protocol allows evaluation of the effect of maternal transfer of a chemical during oogenesis and subsequent reproductive performance of the F0 offspring (not covered in partial life cycle protocols). Both the two-generation and multi-generation protocols are better suited to evaluate population level effects. When cost is an overriding consideration, then a two- as opposed to a multi-generation test is less expensive to perform. However, if the two-generation test is to be conducted with the species examined in this review, it will be necessary to demonstrate the reliability and reproducibility of the test with the candidate species. Specific protocol variables, such as the size of the spawning groups for a particular species, must be determined for the candidate species under consideration for the two-generation test. The individual elements of the protocol must be optimized and the required number of interlaboratory comparisons must be determined.

12.0 IMPLEMENTATION CONSIDERATIONS

12.1 Animal Welfare

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

12.1.1 Rationale for the Need to Use Animals

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.

12.1.2 Relative Pain or Distress for Animals

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 to induce spawning. 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.

12.2 Recommended Protocol

There are advantages to developing a two-generation protocol that can be conducted with multiple species. However, if the two-generation test is conducted with the species examined in this review, it will be necessary to demonstrate the reliability and reproducibility of the test with these species. Additionally, inter-comparison of results of the method using the different species must address issues such as differences in sensitivity among the species for use in a risk assessment. The two-generation protocol requires significant cost and time commitments and demonstration trials with multiple species will required. The pre-selection of a species, such as the fathead minnow, would limit the number of demonstration trials for full optimization of a method suitable for interlaboratory testing. Validation of the protocol through interlaboratory comparisons should be conducted with compounds that span the possible endocrine effects, including strong and weak androgen receptor agonists and antagonists, estrogen receptor agonists and antagonists, and thyroid agonists and antagonists.

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

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

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APPENDIX A

LITERATURE SEARCH

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A bibliographic summary of all citations listed in the reference list and other relevant documents used in the review of literature for the development of this DRP will be provided in the final version of this document. Because of the extensive nature of this literature search, the information, including abstracts where available, will be placed on a CD-ROM for ease of use. In addition, the individual papers cited in this study will be available electronically on this CD. The database format for this literature review is through Reference Manager, which permits multiple sort capabilities and search queries through key words, authors, periodicals, and other parameters.

APPENDIX B

EXPERT INTERVIEWS

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Questionnaires were sent to four experts to obtain current views and opinions regarding promising assays, methods, procedures, and measurement endpoints that could be used in developing a standard transferable protocol for conducting a fish two-generation test. At the time of this writing only one of those people had responded and the questions and his responses are shown below.

The one responder at the time of this writing is:

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1 Are you aware of any gray literature that you would recommend that addresses reproductive and developmental effects of chemicals in fish?

(A) I do not understand the meaning of the gray literature.

2 The primary advantage of using a partial life cycle test instead of multi-generational tests is the reduction in cost. With respect to assessing reproductive and developmental effects, do you believe the disadvantages in using a partial life cycle (e.g. chemical exposure during juvenile → adult → embryo →larval stages) protocol outweigh the cost savings?

(A) I do not understand the partial life cycle test you are talking about. In Japan, we did a partial life cycle test using medaka (chemical exposure from fertilized egg to adult). We only can check the effects of chemicals on the first generation in the partial life cycle test. The partial life cycle test is not designed to study effects of chemicals on next generation and reproduction, which are important to do risk assessment of chemicals. The partial life cycle test is less expensive, however, I believe that we need a test which can be studied reproduction and next generation.

3) Based upon your expertise and experience, what reproductive and developmental endpoints are the most sensitive to toxicants during partial or full life cycle exposures? For example, are changes in the concentration of circulating sex hormones sensitive endpoints? Are morphological / histopathological changes (ovo-testes or ?) more reliable endpoints?

(A) In our own experience using medaka, reduction of reproduction (less number of eggs and low fertilization) was observed in lower concentrations of stronger estrogenic chemicals. In weaker estrogenic chemicals, the concentration which induced secondary sex

characteristics and ovotestis, was almost the same as that induced reduction of reproduction. Medaka spawns daily, therefore,sex hormone levels will change even in a day. I do not think sex hormone levels are good endpoints. Histological changes seem to be good endpoints.

4) Is there significant benefit in measuring biochemical (sex steroids, vitellogenin) and morphological / histological endpoints during larval - juvenile life stages in addition to measurements at sexual maturity? Or, to phrase the question another way, other than growth and mortality, is there any benefit in measuring additional endpoints in larval / juvenile life stages during partial / full life cycle exposures?

(A) In Japan, we have a FLF medaka which can be identify sex by body color and abnormalities of gonadal differentiation even in juvenile period. In general, vitellogenin assay can be used to check estrogenic activity, however, it is difficult to check whether you are using female or male. I believe, growth and mortality are good endpoints.

5) Based on your experience, can you recommend any specific chemicals, exposure durations (partial or multi-generational) and doses that would be appropriate for validating partial and multi-generational assay/protocols that are designed to test for reproductive and developmental effects in fish?

(A) We are making a protocol for medaka, which will be opened in the near future.

6) Currently, four fish species are under consideration for use in partial or full life cycle tests: fathead minnow, medaka, zebrafish and sheepshead minnow. All of these species have the same basic attributes that make them attractive for testing: inexpensive to keep; short or relatively short generation time and previous use in some capacity for toxicity testing. Based on your experience or familiarity, are there other characteristics that make one of these species significantly better for reproductive and developmental studies?

(A) In medaka, sex is stable and we have genetic sex markers such as dr-R and FLF which can be used to detect abnormality of sex differentiation. Quite recently , a gene which determine sex of medka has been discovered in our Insitute (Prof. Nagahama's group). This article will appear in Nature soon. I believe that medaka has several advantages. I am personally studying sex differentiation of zebrafish, which has by 23 days after hatching, then a half fish will differentiate testis. We have a method to produce all male fish and study sex differentiation. But this is tricky way. I do not have experience in the use of fathead minnows.

7) Can you provide any other comments? For example, from your experiences in testing alkylphenolic compounds in the medaka, can you provide additional comments related to question # 2; e.g. are there additional endpoints that could be measured in embryo – larval life stages that would be useful in assessments of reproductive or developmental toxicity?

(A) No, we do not.