DRAFT DETAILED REVIEW PAPER

ON

FISH SCREENING ASSAYS FOR ENDOCRINE DISRUPTION

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GREG SCHWEER
WORK ASSIGNMENT MANAGER
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, DC 20460

BATTELLE
505 King Avenue
Columbus, Ohio 43201

CONTENTS

Acknowledgements. iv

1.0 EXECUTIVE SUMMARY 1

2.0 INTRODUCTION 3
2.1 The U.S. Environmental Protection Agency Endocrine Disruptor Screening Program 3
2.2 Test-method Validation 4
2.3 Purpose of the Review 6
2.4 Rationale and Objectives of the Fish Reproductive Screening Assay 6
2.5 Methods Used in this Analysis 8
2.6 Acronyms and Definitions 9

3.0 OVERVIEW AND SCIENTIFIC BASIS OF FISH REPRODUCTIVE SCREENING ASSAY (ENDOCRINE CONTROL OF REPRODUCTION) 10

4.0 TEST SPECIES 11
4.1 Fathead Minnow (*Pimephales promelas*) Culture and Handling 12
4.1.1 Culture and Handling 12
4.2 Japanese Medaka (*Oryzias latipes*) 12
4.2.1 Culture and Handling 12
4.2.2 Application of Genetically Engineered / Inbred Strains in Toxicity Testing 14
4.3 Zebrafish (*Danio rerio*) 15
4.3.1 Culture and Handling 15
4.3.2 Application of Genetically Engineered / Inbred Strains in Toxicity Testing 15
4.4 Strengths and Weaknesses of Test Species 16

5.0 EXPOSURE PROTOCOLS FOR REPRODUCTIVE SCREENING ASSAYS 17
5.1 Route of Administration 17
5.1.1 Water 17
5.1.2 Oral (Food) 17
5.1.3 Direct Injections (Intraperitoneal / Intramuscular) 17
5.2 Dose Selection 18
5.3 Juvenile Versus Adult Exposure 18
5.4 Exposure Duration 19
5.5 Statistical Considerations 19
5.5.1 Experimental Design and Statistical Power 20
5.5.2 Decision Criteria 21

6.0 DESCRIPTION OF ASSAY ENDPOINTS 22
6.1 Growth and Morphological Alterations 22
6.1.1 Gonadosomatic Index 22
6.1.2 Sexual Differentiation 25
6.1.3 Secondary Sex Characteristics 26
6.2 Measures of Reproductive Performance 26
6.2.1 Fecundity 26
6.2.2 Gamete Viability 26
6.2.3 Changes in Spawning Behavior 27
6.3 Biochemical Measures 27
6.3.1 Estrogen Responsive Gene Products: Vitellogenin Induction 27
6.3.2 Tissue Steroid Concentrations 29

7.0 RESPONSE TO ESTROGEN AGONISTS AND ANTAGONISTS 30
7.1 Endpoint Sensitivity to 17 -estradiol or Synthetic Estrogen Exposure 30
7.1.1 Growth and Morphological Alterations 30
7.1.2 Reproductive Performance 33
7.1.3 Biochemical Measures: VTG 35
7.1.4 Biochemical Measures: Tissue Steroid Levels 37
7.2 Estrogen Antagonists 38

7.2.1 Direct-acting Anti-estrogens 38
7.2.2 Indirect-acting Anti-estrogens 40
7.3 Gender Differences 41
7.4 Strengths and Weaknesses of Test Species 42

8.0 RESPONSE TO ANDROGEN AGONISTS AND ANTAGONISTS 42
8.1 Endpoint Sensitivity to Natural and Synthetic Androgen Exposure 43
8.2 Androgen Antagonists 44
8.2.1 Direct-acting Anti-androgens 44
8.2.2 Indirect-acting Anti-androgens 45
8.3 Gender Differences 45
8.4 Strengths and Weaknesses of Test Species 45

9.0 RESPONSE TO THYROID HORMONE AGONISTS AND ANTAGONISTS 46
9.1 Endpoint Sensitivity to Thyroid Stimulation 46
9.2 Inhibition of Thyroid Function 47
9.3 Gender Differences 47
9.4 Strengths and Weaknesses of Test Species 48

10.0 RESPONSE TO OTHER HORMONAL DISTURBANCES 48
10.1 Gonadotropic Hormones 48
10.2 Progestins 49

11.0 TECHNICAL ASPECTS OF MEASUREMENT OF BIOCHEMICAL ENDPOINTS 49
11.1 Measurements of Sex Steroids in Tissues 49
11.1.1 Estrogens 50
11.1.2 Androgens 53
11.1.3 Progestins 54
11.1.4 Summary 55
11.2 Measurement of Vitellogenin 55
11.2.1 Indirect Quantification of Vitellogenin Protein: Alkaline-labile Phosphate Assay 56
11.2.2 Direct Quantification of Vitellogenin Protein: RIA 56
11.2.3 Direct Quantification of Vitellogenin Protein: Enzyme-linked Immunosorbent Assay 57
11.2.4 Quantifying Vitellogenin mRNA 61
11.2.5 MS Approaches to Quantification of Vitellogenin 62

12.0 CANDIDATE PROTOCOLS 63
12.1 Short-term (14-day) Fish Reproductive Screen 64
12.2 Short-term (21-day) Reproduction Assay with the Fathead Minnow for Identification of Endocrine Disrupting Chemicals 72
12.3 Short-term (14-day) Non-spawning Fish Screening Assay 78

13.0 RECOMMENDED PROTOCOL AND ADDITIONAL DATA NEEDS 83
13.1 Preferred Test Species 83
13.2 Exposure Protocol 84
13.3 Appropriateness of Reproductive Screen Endpoints 84
13.4 Preferred Methods for Quantification of Biochemical Endpoints 84
13.5 Significant Data Gaps 85

14.0 IMPLEMENTATION CONSIDERATIONS 85
14.1 Animal Welfare 85
14.1.1 Rationale for the Need to Use Animals 85
14.1.2 Relative Pain or Distress for Animals 85
14.2 Recommended Protocol 86
14.3 Information on Facilities and Major Fixed Equipment Needed to Conduct the Test 86

15.0 REFERENCES 86

APPENDIX A – LITERATURE SEARCH A-1

APPENDIX B – EXPERT INTERVIEWS B-1

TABLES

Table 2-1. ICCVAM Test Method Validation Process 7

Table 2-2. Acronyms 9

Table 4-1. Strengths and Weaknesses of Test Species 16

Table 12-1. Test Conditions for the 14 d or 21 d Fathead Minnow EDC Screening Assay (with Measurement of Reproductive Performance) 66

Table 12-2. Recommended Ranges of Water-quality Characteristics for Culturing and Testing the Fathead Minnow 67

Table 12-3. Short-term (14-day) Non-spawning Fish Screening Test. 79

FIGURES

Figure 2-1. EDSTAC Conceptual Framework Providing the Structure for Screening and Testing for Endocrine Disruptors 5

Figure 11-1. MALDI-MS Analysis of Rainbow Trout Vitellogenin With and Without Enzymatic Deglycosylation Step. 64

ACKNOWLEDGEMENTS

This document was prepared by:

Irvin R. Schultz[1], Ann D. Skillman[1], Michael L Blanton[1], and James J. Nagler[2]

[1]Battelle's Pacific Northwest Division
Sequim, Washington
98382
USA

[2]Department of Biological Sciences
Center for Reproductive Biology
University of Idaho
Moscow, Idaho
83844-3051
USA

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DETAILED REVIEW PAPER: FISH SCREENING ASSAYS FOR ENDOCRINE DISRUPTION

1.0 EXECUTIVE SUMMARY

The purpose of this detailed review paper (DRP) is to survey and investigate the status of various screening protocols that are currently in use or that have been proposed for use in identifying chemicals that act as endocrine disruptors to fish. The United States Environmental Protection Agency (EPA) plans to use the information contained in this DRP to design a Fish Screening Assay (standardized transferable protocol) that will be used to identify potential endocrine-disrupting chemicals.

The EPA is implementing an Endocrine Disruptor Screening Program (EDSP). In 1996, the Food Quality Protection Act was enacted by Congress to authorize the EPA to implement a screening program to evaluate whether pesticides and other chemicals found in food or water could affect endocrine systems in humans. In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed to identify and characterize the endocrine effects of environmental contaminants, industrial chemicals, and pesticides. A two-tiered approach will be utilized: Tier 1 employs a combination of *in vivo* and *in vitro* screens, and Tier 2 involves *in vivo* testing using two-generation reproductive studies. Validation of the individual screens and tests is required, and the Endocrine Disruptor Methods Validation Subcommittee (EDMVS) will provide advice and counsel on the validation assays.

The Fish Screening Assay was selected as a component of the Tier 1 screening by the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to evaluate the potential toxicity of chemicals and mixtures on the endocrine system (EDSTAC 1998). The Tier 1 screening tests were selected to obtain minimum, yet sufficient estimates of potential endocrine disrupting activity. The Committee has several stated goals for these tests. First, they should be relatively inexpensive, quick, and technically easy to perform. Second, they should be sensitive and specific, capture multiple endpoints, and be predictive across species, gender, and age. And third, they should be validated and standardized before they are used routinely by testing laboratories (EDSTAC 1998, Vol. 1, p 3-9). The purpose of using testing protocols within the EDSP is "to characterize the nature, likelihood of a dose-response relationship of endocrine disruption in humans and wildlife" (EDSTAC 1998; EPA 1997). Subsequently, the EPA has requested the development of a screening protocol that identifies compounds having the potential to affect selected endocrine processes in fish.

This DRP is a synthesis of relevant scientific peer-reviewed papers and interviews with 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 screening assay.

During the development of this DRP, a thorough review of the published and unpublished literature was conducted. The published online search was conducted and over 500 journal articles germane to the effort were reviewed. A Reference Manager Database (RMD) was created from the retrieved literature. This database and an electronic copy of the cited articles will be included in the final version of this DRP (not included in this draft) on a CD-ROM. In addition, personal interviews with leading experts in the field were conducted to gather additional information on known testing protocols and endpoints that could be used for identifying impacts from chemicals that can directly interfere with endocrine-driven development in fish.

Three species of fish that are the most promising candidates for use in reproductive screening assays were evaluated: fathead minnow (*Pimephales promelas*), Japanese medaka

(*Oryzias latipes*), and zebrafish (*Danio rerio*). The culturing and handling of all three species have been well documented for many years. All species tolerate a wide range of water-quality and water-temperature conditions, require small culture space, and produce the number of embryos needed for testing. Fathead minnows have a strong regulatory history in the United States, and the zebrafish has a strong regulatory history in Europe. The strengths and weaknesses of the three test species were evaluated for ease of culturing and handling; regulatory history; ease of determining sex characteristics; length of life cycle; and size of organisms in terms of space required for culture and testing and in terms of ease in obtaining adequate volumes of plasma.

A large number of environmental pollutants have been hypothesized to cause toxicity by binding to the estrogen receptors (estrogenic compounds) (Hoare & Usdin 2001) or to produce responses similar to estrogen without binding to estrogen receptors (estrogen-like compounds) (Gillesby & Zacharewski 1998). Consequently, experimental studies of the responses of fish exposure to natural or synthetic estrogens have received a higher degree of scrutiny than have studies on other fish hormonal systems. Fewer studies have examined the response of fish to treatment with androgenic compounds and thyroid hormone mimics. Similarly, studies of antagonistic effects of xenobiotics on fish hormone pathways are relatively recent.

Based on the literature review, three protocols were selected as potential prevalidation protocols: 1) 14-day fish reproductive assay (modified from the version described in Ankley et al. 2001); 2) 21-day reproductive test (as described in Ankley et al., 2001); and 3) 14-day fish non-reproductive screen (OECD Draft-31 December 2001). The EDSTAC had recommended a fish gonadal recrudescence assay but preliminary work indicated that a fish reproduction based assay would be more suitable. Each of the selected protocols are relatively new and have not been through validation processes. Currently there are insufficient data to make a definitive selection on which exposure protocol has the most merit and should be chosen above all others. It is recommended that a side-by-side performance evaluation of the EPA 21- and 14-day versus the OECD 14-day protocols would be beneficial to elucidating the overall performance and cost benefit of the three protocols. None of these three protocols are designed to adequately address thyroid function. Because of the overall lack of data relevant to the effects of EDCs on thyroid function in fish, it may be prudent to further investigate this aspect of endocrine disruption.

There were gaps of data found during the literature review, particularly a lack of information on the reproductive endocrinology of the test species. This paucity of information is especially true for the medaka and zebrafish. More specific data gaps include the following:

- Male-specific effects of estrogen agonists other than VTG induction.
- The effects of anti-estrogens, especially pure or Type II anti-estrogens in sexually mature test species.
- The effects of androgenic and anti-androgens in sexually mature test species, specifically, endpoints other than secondary sex characteristics that may be more sensitive to (anti-) androgens.
- Baseline data for thyroid hormone levels during reproduction in test species.
- The effects of thyroid hormone agonists (or thyroid stimulation) on reproduction.

The United States Environmental Protection Agency, Office of Science Coordination and Policy funded this DRP.

This DRP is a DRAFT and its distribution should be limited to those providing peer review.

2.0 INTRODUCTION

Note: Some of the material for this section has been taken from United States Environmental Protection Agency, Endocrine Disruptor Screening Program August 2000 Report to Congress; information included descriptions from various Work Assignments that would have been authored by the EPA Office of Science Coordination and Policy team.

2.1 The U.S. Environmental Protection Agency Endocrine Disruptor Screening Program

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 response to this attention in 1996, the passage of the two laws, the Food Quality Protection Act (FQPA) and Amendments to the Safe Drinking Water Act (SDWA), authorized the United States Environmental Protection Agency (EPA) to screen chemicals found in drinking-water sources or food to determine whether they possess estrogenic or other endocrine activity (Federal Register 2001). Pursuant to this goal, the 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..." (Federal Register 2001). 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. (42 U.S.C. § 300j-17).

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, 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). In addition to the above activities, the 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 effects similar to naturally-occurring hormones. Following the recommendations made by EDSTAC in its final report (EDSTAC 1998), the EPA established the Endocrine Disruptor Screening Program (EDSP). The aim of this program 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 chemicals, and environmental contaminants. In its final report, EDSTAC provided 71 consensus recommendations regarding the development of an EDSP (EPA 1998), which the EPA closely followed.

The EPA's EDSP is outlined in the August 11, 1998, Federal Register (63 FR 42852), and is further developed as a proposed statement of policy in the December 28, 1998, Federal Register (63 FR 71542). The EDSP 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. The Federal Register notices, SAB/SAP report, EDSP Report to Congress, 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.

As a charter member and co-chair of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the EPA will follow the interagency validation framework outlined in Validation and Regulatory Acceptance of Toxicological Test Methods (NIEHS 1997) for validating the EDSP screening and testing methods. The conceptual framework provides a number of stages and outcomes, including research, methods development, prevalidation, validation, peer review, and regulatory acceptance. Therefore, the EPA will administer the ICCVAM validation process for the EDSP with the involvement of ICCVAM. The EPA and ICCVAM have mutually agreed to this administrative arrangement to ensure that EDSP validation meets ICCVAM interagency validation principles (NIEHS 1997) and the statutorily mandated guideline development, review, and regulatory approval processes for the EPA's chemical risk management programs. For the international validation effort, validation and acceptance criteria for alternative methods have been developed by the OECD (OECD 1996).

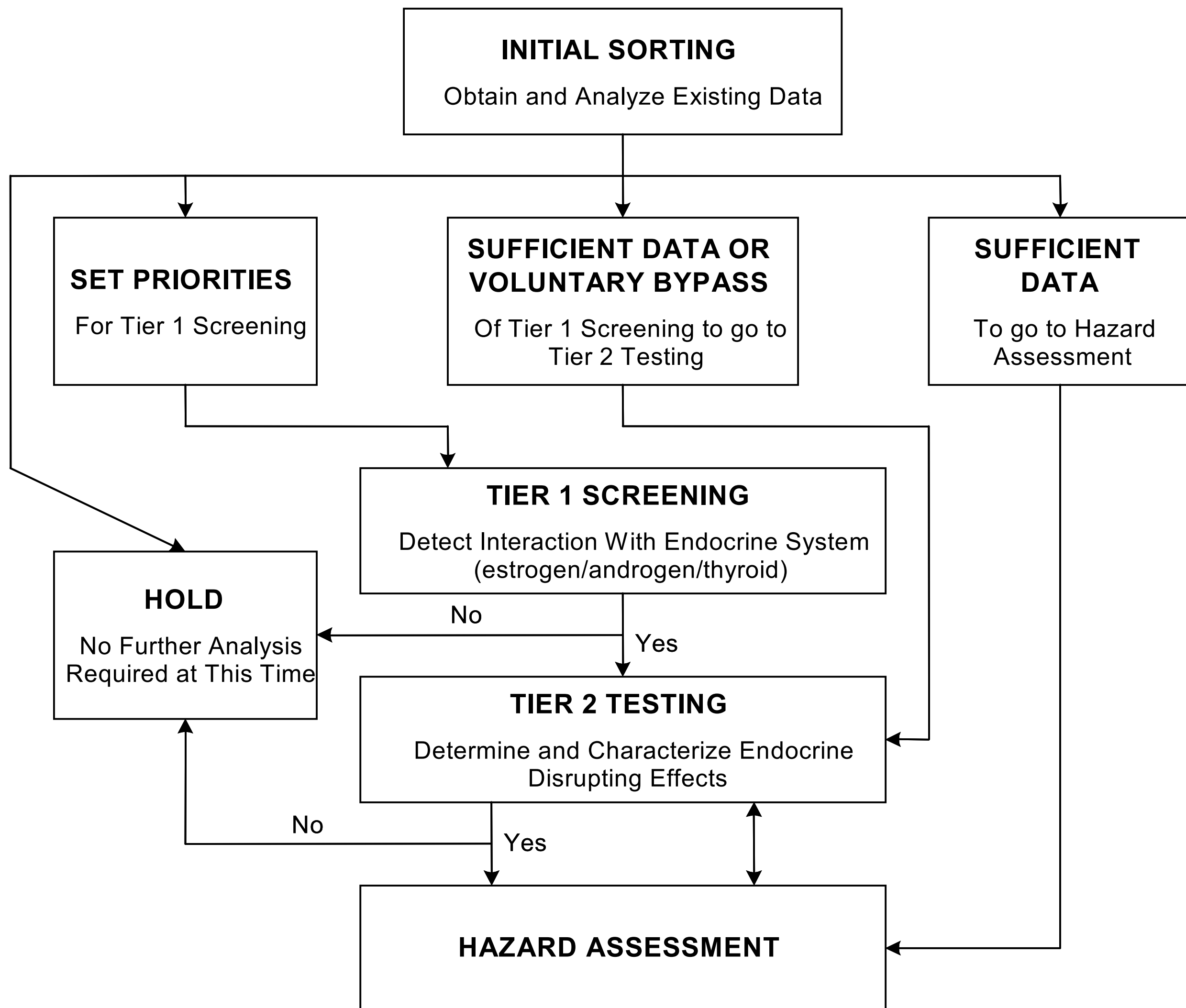
To date, the EPA has implemented the program on two fronts: 1) the development of the Endocrine Disruptor Priority Setting Database and the approach that will be used to establish priorities for screening compounds, and 2) prevalidation and validation studies on some of the Tier 1 and Tier 2 assays that 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. Provided below is a schematic for Phase 1 (Figure 2-1) of the EDSP.

2.2 Test-method Validation

The EPA EDSP is outlined in the December 28, 1998, Federal Register notice (63 FR 71542), and entails *in vitro* methods, *in vivo* screening assays, and reproduction and development toxicity tests. The different screens and tests vary in their scientific development, history of use, degree of standardization, and overall readiness for validation (EPA 1998).

The EPA and the EDMVS have chosen to follow the validation process established by the ICCVAM, of which the EPA was a charter member, for validation of the EDSP screening

and testing methods. ICCVAM was established by the NIEHS as a standing interagency committee



(taken from EDSTAC 1998; Executive Summary, page ES-5)

Figure 2-1. EDSTAC Conceptual Framework Providing the Structure for Screening and Testing for Endocrine Disruptors

to aid in the validation, acceptance, and harmonization of test methods designed to reduce animals use, refine procedures involving animals to be less stressful, and to replace animal tests wherever appropriate (Federal Register 2001). 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 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 framework for the validation process outlined by ICCVAM is shown in Table 2-1. It consists of several 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, transferable 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 DRP 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 or of questionable relevance to the biological effect of interest, or if it is not an appropriate measure of the effect, its reliability is not established. 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 validation studies of selected endocrine screens and tests 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 EPA test guidelines.

2.3 Purpose of the Review

The preparation of this DRP fulfills objective II.A.1 of the process (Table 2-1), i.e., to define the basis and purpose of the proposed reproductive screening assay(s) for three species of fish (fathead minnow, zebrafish, and Japanese medaka) 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 Rationale and Objectives of the Fish Reproductive Screening Assay

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 this apporoach 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 screening and testing program must include

exposure during the most sensitive lifestages, provide the opportunity for identification of dose-response effects, and encompass a variety of taxa.

Table 2-1. ICCVAM Test Method Validation Process

I. Test Development

II. Prevalidation/Test Optimization

A. Preliminary planning
   1. Define basis and purpose of test
   2. Develop protocol
   3. Develop control values
   4. Develop data/outcome prediction model

B. Activities
   1. Qualify and train laboratories
   2. Measure intra- and interlaboratory reproducibility
   3. Identify limitations of test

III. Determination of Readiness for Validation

A. Analysis of test development and prevalidation data

B. Standardization of protocol

IV. Test Validation

A. Formation of steering committee/management team
   1. Define purpose of validation study
   2. Design study
   3. Select participating laboratories
   4. Establish management evaluation and oversight procedures

B. Pretest procedures
   1. Implement data record-keeping procedures
   2. Select reference chemicals
   3. Code and distribute reference chemicals

C. Coded chemical testing
   1. Measure interlaboratory performance
   2. Compile and evaluate data

D. Test evaluation
   1. Analyze and summarize test results
   2. Challenge data with prediction model
   3. Peer review protocol and data
   4. Accept, revise, or reject model

V. Submission of Test for Regulatory Approval

A. Report preparation

B. Provision of supporting data

C. Preparation of results for publication

Upon completion of Tier 1 screening (T1S) 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 either the potential or no potential for having estrogen, androgen, or thyroid endocrine-disruptive effects. Through the EDSP, the most

appropriate endocrine screens and tests will be selected based upon completion of a thorough review of the scientific literature as summarized in each DRP topic. In the case of the present DRP, the recommended protocol to be used as part of a Tier 1 fish reproductive screen will be expected to complement the other screening assays such that through its completion, the following five criteria will be met.

1. The T1S battery should maximize sensitivity to minimize false negatives while permitting analysis of a yet undetermined, but acceptable, level of false positives. This criterion expresses the need to "cast the screening net widely" to not miss potential endocrine disruptors or estrogen-androgen-thyroid-active materials.

2. The T1S battery should include a range of organisms representing known or anticipated differences in metabolic activity. The battery should include assays from representative vertebrate classes to reduce the likelihood that important pathways for metabolic activation or detoxification of parent chemical substances or mixtures are not overlooked.

3. The T1S battery should be designed to detect all known modes of action for the endocrine endpoints of concern. All chemicals known to affect the action of estrogen, androgen, or thyroid hormones should be detected.

4. The T1S battery should include a sufficient range of taxonomic groups among the test organisms. Differences in endogenous ligands, receptors, and response elements among taxa can affect endocrine activity of chemical substances or mixtures.

5. The T1S battery should incorporate sufficient diversity among the endpoints and assays to reach conclusions based on "weight-of-evidence" considerations. Decisions based on the battery results will require weighing the data from several assays.

The Tier 1 screening must be relatively fast and efficient while meeting the criteria described above. The screening includes a fish reproductive assay, which fills important needs in the battery and complements the information from assays using mammals and other ecologically significant animal classes. Fish differ in steroid profiles from mammals. For example, 11-ketotestosterone (11-KT), as opposed to testosterone (T), is the most important androgen in fish, and the estrogen receptor (ER) in fish appears to differ structurally and functionally from the mammalian ER (Petit et al*.,* 1995; Gustafsson 1996). In addition, steroid receptors in eggs and for hepatic vitellogenin (VTG) have no known analogous receptors in mammals, which would suggest sites of endocrine disruption unique to oviparous animals. Therefore, this assay is essential to address these known endocrine differences.

The EDSTAC recommended that the Tier 1 Screening Battery include a fish gonadal recrudescence assay (EDSTAC 1998). In the preliminary development of this assay, it was found that this life stage paradigm would be difficult to establish as a viable test practice which could be validated and standardized in the time frame needed for implementation by the Agency (personal communication Gerald Ankley, US-EPA, ORD-MED, Duluth, MN). Given the Agency's experience with the fathead minnow, it was determined that a reproduction paradigm was practical, would address all the endpoints recommended by the EDSTAC for fish, and could meet the implementation timeline.

2.5 Methods Used in this Analysis

Provided in Appendix A is a detailed description of the methods employed for the literature search (e.g., key words, databases, and results). After the most relevant papers were identified, retrieved, and read for content, pertinent information was synthesized to create this DRP. In addition to the literature review, interviews with experts were conducted to obtain the

current views and opinions regarding assays, methods, procedures, and measurement endpoints that hold promise for developing a fish-screening assay. The results of the interviews are found in Appendix B. At the back of this report is a CD ROM that has the Reference Manager Database of all documents reviewed. This database includes the reference citation and abstract.

2.6 Acronyms and Definitions

The following are acronyms and definitions of terms used in the DRP:

Table 2-2. Acronyms

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
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
MATC	maximum acceptable toxicant concentrations
MS	mass spectrometry
MSTFA	N-methyl-N-trimethylsilyl-trifluoroacetamide
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 FISH REPRODUCTIVE SCREENING ASSAY (ENDOCRINE CONTROL OF REPRODUCTION)

Note: Some of the material for this sections has been taken directly from the EDSTAC Report Chapter 5

The endocrine system, also referred to as the hormone system, consists of glands located throughout the body, hormones that are synthesized and secreted by the glands into the bloodstream, receptors in the various target organs, and tissues that recognize and respond to the hormones. The function of the system is to regulate a wide range of biological processes, including control of blood sugar (through the hormones insulin and glucagons from the pancreas); growth and function of reproductive systems (through the hormones testosterone and estrogen and related components from the testes and ovaries); regulation of metabolism (through the hormones cortisol from the adrenal glands and thyroxin from the thyroid gland); development of the brain and the rest of the nervous system (estrogen and thyroid hormones);

and the continuum of differentiation and development of an organism that occurs throughout its life history from conception to sexual maturity and eventual senescence. Normal functioning of the endocrine system, therefore, contributes to homeostasis (the body's ability to maintain itself in the presence of external and internal changes) and to the body's ability to control and regulate reproduction, development, and/or behavior. An endocrine system is found in nearly all animals, including mammals, nonmammalian vertebrates (e.g., fish, amphibians, reptiles, and birds), and invertebrates (e.g., snails, lobsters, insects, and other species).

A large number of man-made chemicals that have been released into the environment, as well as a few natural ones, have the potential to disrupt the endocrine system of animals, including humans. Among these are the persistent, bioaccumulative, organohalogen compounds, which include some pesticides (fungicides, herbicides, and insecticides) and industrial chemicals, other synthetic products, and some metals[1]. *Our Stolen Future* (Colborn et al., 1996) was one of the first publications to propose a hypothesis that there are agents in the environment that affect, via alteration of the internal hormonal milieu, reproduction and development. The authors described effects in fish-eating birds, alligators, Great Lakes mink, frogs, invertebrates, and humans. These agents were proposed to act, even at very low 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).

Numerous studies have been published that provided evidence of compromised growth, reproduction, altered behavior and abnormal development from exposure to a variety of natural and anthropogenic chemicals in invertebrates, fish, amphibian, reptilian, avian and mammalian species (Lister & Van Der Kraak, 2001). In addition to the potential to affect a wide variety of taxa, the following four attributes further complicate the study and regulation of EDCs: 1) the chemicals of concern may have entirely different effects on the embryo, fetus, or perinatal organism than on the adult; 2) the effects are most often manifested in offspring, not in the exposed parent; 3) the timing of exposure in the developing organism is crucial in determining its character and future potential; and 4) although critical exposure occurs during embryonic development, obvious manifestations might not occur until maturity (Colborn et al., 1996).

This paper considers the effects that environmental concentrations of EDCs may have on fish reproduction and overall fitness. The following sections provide information on reproductive screening assay(s) in three species of fish (i.e., fathead minnow, zebrafish, and Japanese medaka) for endocrine effects. The document puts forward the relevant principles, methods, and techniques recommended for an initial protocol(s), and identifies issues that might require prevalidation studies to adequately address. The ultimate outcome will be a standardized transferable protocol that can be used to screen or test chemicals in a regulatory arena to determine their potential to be an EDC that could negatively affect the hormonal control of reproduction in fish.

4.0 TEST SPECIES

This review paper will focus on three species of fish that are the most likely candidates for use in reproductive screening assays: fathead minnow (*Pimephales promelas*), Japanese

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1 Many wildlife populations are already affected by these compounds. Impacts include thyroid dysfunction in birds and fish; decreased fertility in birds, fish, shellfish, and mammals; decreased hatching success in birds, fish, and turtles; gross developmental deformities in birds, fish, and turtles; metabolic abnormalities in birds, fish, and mammals; behavioral abnormalities in birds; demasculinization / feminization of male fish, birds, and mammals; defeminization / masculinization of female fish and birds; and compromised immune system in birds and mammals.

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

4.1 Fathead Minnow (*Pimephales promelas*)

4.1.1 Culture and Handling

The fathead minnow is a common freshwater minnow belonging to the largest family of fish, the Cyprinidae. Their original range was limited to central North America, but they have since been introduced into most regions of North 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 United States, and a number of testing guidelines exist, including detailed information on their laboratory culture (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 a distinct sign of sexual maturity in females. However, the time of sexual differentiation is not well documented for the fathead minnow. The generation time of fathead minnows is about 4 months. They can be kept in breeding condition all year without manipulating light cycles and water temperature. Fathead minnows can be induced to spawn by placing spawning substrate in the aquarium, which is guarded by the male. Better spawning synchronization can be obtained by withholding the spawning substrates for several days. Spawning is usually initiated just before light and can last through the morning; therefore, it is advisable not to disturb the fish during the morning hours except to feed and collect embryos. Each spawning female will produce 50 to 250 embryos per spawn. Embryos are collected from the aquarium by removing the spawning substrate. The embryos can be incubated on the spawning substrate or they can be removed by well established procedures without harm to the developing individuals from the substrate and incubated in a container or in egg cups (glass cylinders with mesh bottoms). Fertilization can be assessed immediately with light magnification or it can be assessed 24 hours after spawning by counting the number of opaque (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. The larvae are reared in separate aquaria and should be offered live brine shrimp nauplii (*Artemia*) immediately after hatch. Juvenile fish can be fed a mix of frozen brine shrimp and live nauplii, whereas adults can be fed a mixture of frozen brine shrimp and commercial flake food. All life stages should be fed *ad libitum* two to three times per day.

4.2 Japanese Medaka (*Oryzias latipes*)

4.2.1 Culture and Handling

The Japanese medaka (*Oryzias latipes*) is a freshwater killifish belonging to the family of toothcarps, Cyprinodontidae, indigenous to areas of Japan, Taiwan, and southeastern Asia, where ambient temperatures range from 5ºC to 35ºC (Kirchen & West 1976). The Japanese medaka has a long history as an experimental animal and a complete presentation of their biology can be found in Yamamoto (1975) and on the website http://biol1.bio.nagoya-u.ac.jp:8000/Yamabook.html, which lists over 1000 references dating from the early 1900s. The culture and handling of the Japanese medaka has been exhaustively studied, and detailed guidelines are available in EPA/600/3-91/064 (Denny et al. 1991). Medaka are small (25 mm to 50 mm in total length) and are easy to rear and maintain in laboratory aquaria. Adults can be maintained in an aquarium with little space and can tolerate low dissolved oxygen and a wide range of temperatures and salinity (Yamamoto 1975). Japanese medaka are sexually dimorphic, and although the sex of fully grown fish can be determined by observing the outline of the anal and dorsal fins, the observation of an anesthetized fish under a low-power microscope is recommended for confirmation and for sexing an immature fish (Yamamoto 1975). In the Japanese medaka, the sexual differentiation of the female gonad begins before hatching, whereas the male differentiates during a critical window around 13 days post-hatching (Kime 1998).

Japanese medaka have a generation interval of 2 to 3 months, and can be induced to spawn throughout the year by controlling the temperature and photoperiod. Breeding tanks are ideally set up with a ratio of 4 males per 6 females. Spawning activity normally occurs through 14 to 15 months of age. Spawning is highly predictable in time, usually within 1 hr of first daylight, and can be controlled in the laboratory by adjusting the timing of the recommended cycle of 16-hour light:8-hour dark (Hirshfield 1980; Koger et al., 1999). Measures of Japanese medaka reproductive output, including gonadal morphology, fecundity, and fertility, demonstrate that through laboratory control of temperature and photoperiod, reproductive capacity of breeding groups can be calibrated before exposure, thereby establishing a baseline from which to assess effects (Koger et al., 1999). Individual females can lay an average of 25 eggs/day for up to 4 months under proper conditions of photoperiod, temperature and food supply (Hirshfield 1980). A cluster typically contains 10 to 30 eggs that are attached to the female's vent by filaments for a number of hours until they are brushed off onto a spawning substrate that simulates aquatic plants (Yamamoto 1975). Even when spawning substrates such as a spawning sponge are provided, some females retain their embryos and require manual stripping (Denny et al., 1991). The eggs should be collected as soon as possible after spawning to prevent their predation by adults. As described in the Medaka Web site, the adhesion fibers firmly hold individual eggs as a cluster. To prevent harm by fungi, and to insure uniform potential for oxygenation, the individual eggs must be separated. Two methods exist, the first involves gently rolling the egg mass between moistened fingertips, thereby breaking attachment fibers and separating individual eggs. A slight variation of the same approach is to place the egg mass on paper towel, add 1 to 2 drops of water and place a second towel over the egg mass, and then perform gentle circular motion with the fingertips to separate eggs. The other method involves grasping the central band of adhesion fibers with tiny forceps, then with opened iridectomy scissors, separate one egg from the cluster by gently stretching attachment fibers and cutting them near the egg surface. This process is repeated until clean eggs with very short attachment fibers are separated from the original mass. Because of the transparency of the egg chorion, fertilization can be easily assessed with a dissecting microscope. The egg-incubation period is approximately 1 week when kept at 28ºC (Yamamoto 1975), and 9 to 10 days when at 25ºC to 26ºC.

There are several laboratories in the U.S.A. that have maintained research colonies of medaka for more than one decade. These research colonies are located at the Biological Survey, Columbia, Missouri; Rutgers University; University of Southern Mississippi, Gulf Coast Marine Laboratory; and at Duke University (David E. Hinton, personal communication). In general, the U.S.A. researchers have focused their attention on chronic toxicity evaluations. There is a concern with the treatment of embryonated eggs by addition of substances to the embryo-rearing medium. These are designed to afford protection from bacteria and invertebrate organisms that

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

4.2.2 Application of Genetically Engineered / Inbred Strains in Toxicity Testing

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

The see-through medaka is a recently described strain that has an almost complete absence of pigmentation in all life stages (Wakamatsu et al., 2001). The transparency of the fish allows direct visualization of the internal organs, including the reproductive organs. The introduction of a transgenic version of the STIII strain expressing the green fluorescent protein gene in primordial germ cells (Tanaka et al., 2001) permits detailed observations of the ovaries

and testes in a non-invasive manner over the life span of the individual without harming the fish and would allow repeated observations of gonad morphology during an exposure. This capability would be particularly valuable in monitoring changes in morphology of the gonads during a short-term exposure in sexually mature fish. In addition, because of the exceptional transparency of this mutant medaka, oocyte maturation can be monitored in the ovary of an individual fish (Wakamatsu et al., 2001). Application of this strain may allow the inclusion of an additional endpoint in screening assays, specifically, in-vivo examination of the effect of a chemical on normal oocyte maturation.

4.3 Zebrafish (*Danio rerio*)

4.3.1 Culture and Handling

The zebrafish, *Danio rerio,* is a member of the Cyprinidae family and is native to East India and Burma. The zebrafish has been used for aquatic toxicity regulatory purposes in Europe and worldwide as a model for studying vertebrate development and genetics. Zebrafish have been extensively studied since the 1930s, and a detailed review of their biology and laboratory use is presented by Laale (1977). Zebrafish are easy to culture and are available from commercial suppliers; detailed methods for their care in the laboratory are available in Westerfield (2000) and at http://zfin.org/zf_info/zfbook/zfbk.html. They can easily be maintained in aquaria at a temperature of 28ºC, with temperatures above 31ºC and below 25ºC resulting in abnormal breeding and development.

Adult zebrafish are vigorous swimmers, reaching a total length of 4 cm 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: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 protect the eggs from consumption by the adult fish. Eggs and sperm can be collected from individual zebrafish for controlled fertilization studies (Westerfield 2000). Viable embryos can be readily observed through the very transparent egg chorion, and the embryos normally hatch after 3 days of development.

4.3.2 Application of Genetically Engineered / Inbred Strains in Toxicity Testing

In recent years, reliable methods for the development of transgenic zebrafish has become widely available (Moav et al., 1993). The use of transgenic fish in aquatic toxicological research has been limited until recently, but offers great potential for the development of sensitive biomarkers and aid in the identification of mode of action. One approach has been to transfect zebrafish with DNA motifs that once activated will also activate a reporter gene such as luciferase that can be readily detected. Among the motifs successfully introduced into the zebrafish include dioxin, heavy metal and estrogen responsive motifs (Carvan et al., 2000a; Legler et al., 2000). This approach would be particularly valuable in the development of an androgen sensitive biomarker. With the exception of the protein spiggin, produced by the male stickleback (discussed in Section 8.0), no suitable biochemical biomarker has been developed for androgenic substances. A less explored area of research is the generation of a knock-out zebrafish. More specifically, generation of an ER null mutant zebrafish would be useful for assessing the role of E2 in sexual development in general and the impact anti-estrogens can have on reproduction.

4.4 Strengths and Weaknesses of Test Species

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

Table 4-1. Strengths and Weaknesses of Test Species

Species	Strengths	Weaknesses
Fathead minnow (*Pimephales promelas*)	- Large enough to collect individual blood plasma samples - Distinct secondary sex characteristics in both sexes - Large historical regulatory database - Many laboratories are familiar with culture and testing - Spawn on a substrate - High fertilization rate - Indigenous to North America	- Relatively long life cycle - Relatively high variability in fecundity - Relative size of the fish requires more space for culture and testing - Intersex condition is less frequently observed compared to other fishes. - Genome poorly characterized
Japanese 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	- Smaller size reduces individual blood sample volumes compared to fathead minnow - Less distinctive secondary sex characteristics - Regulatory data base less extensive compared to fathead minnow. - Limited use in short-term tests in the U.S.A.
Zebrafish (*Danio rerio*)	- Short life cycle - Small fish, making culture and testing possible in smaller spaces - Male fish go through a hermaphroditic phase as juveniles - Widely used in other medical and genetic research - Frequently used in Europe for regulatory purposes - Transgenic fish increasingly available - Anticipated that entire genome will be sequenced soon.	- Small size makes individual blood plasma samples not likely - Minimal secondary sex characteristics - Limited US regulatory data base - Limited testing experience in the US

5.0 EXPOSURE PROTOCOLS FOR REPRODUCTIVE SCREENING ASSAYS

5.1 Route of Administration

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

5.1.1 Water

Besides being ecologically relevant, water is the most common laboratory route for exposing fish to EACs. The delivery of a toxicant to water at different concentrations is well established. Laboratories around the world, using a variety of pumps, valves and gravity, have successfully conducted water exposures. A water exposure concentration is more readily correlated with water concentrations found in the aquatic environment. The biggest challenge with water exposure is preparing aqueous solutions of high concentration relative to solubility limitations of the test substances in the water. Organic solvents are often used as carriers to assist in delivering a test substance in water. The use of an organic solvent requires the maintenance of a solvent control. Organic solvents also enhance bacterial growth, which increases maintenance time during the exposure. Saturator columns have been used in some cases to eliminate the use of solvents (Ota et al., 2000).

5.1.2 Oral (Food)

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

5.1.3 Direct Injections (Intraperitoneal / Intramuscular)

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

Direct injection procedures impose greater stress on the fish because of the extra handling, anesthetic exposure, and trauma inflicted by the injection (Heming 1989). The tolerance of the injections may vary across species. For example, a recent study in fathead minnows indicated repetitive intraperitoneal injections did not alter plasma sex steroid levels or reproductive performance in this species (Kahl et al., 2001). Regardless of the actual level of stress imposed on a fish, direct injection methods should be considered as a final option after water and oral exposures. An occasional problem with direct injection procedures is loss of delivered dose through leakage of the injectate through the entry wound (Horsberg 1994). Depending on the toxicokinetic behavior of the test compound, multiple injections would likely be needed to maintain some type of consistency in tissue concentrations during the assay and the required frequency could be challenging to estimate. These problems would make it difficult to reproduce the effective administered dose (e.g., internally absorbed dose x test duration) across individuals, which can add to the variability in the measurement of toxicological endpoints.

5.2 Dose Selection

The basic considerations for dose selections are that a sub-lethal exposure is used and in the case of water exposures, water solubility limits are not exceeded. Ideally, the dose level used should not alter the growth of the test fish. Because the purpose of the reproductive screening assay is not to obtain extensive dose-response data, the number of test concentrations can be limited to one or two exposure rates or injection dosages.

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

5.3 Juvenile Versus Adult Exposure

The utility of juvenile fish in short term screening assays has recently been demonstrated for (anti)-estrogens (Panter et al., 2002). The primary advantage of using juvenile fish in an endocrine screen is the reduction in costs associated with maintenance and performance of the assay. The use of juvenile fish also allows for a corresponding reduction in the quantity of water and test chemical required for the test. This factor could be especially important when using test chemicals that are expensive to produce or difficult to obtain in sufficient quantities and with high chemical purity. The use of juvenile life stages would also allow the inclusion of additional fish species that are too large at sexual maturity for practical use in a screening assay (e.g., salmonids). The application of a juvenile salmonid species as a test organism has been explored with estrogen mimics and shown to be responsive to induction of VTG and changes in other biomarkers indicative of steroid mimic exposure (Thorpe et al., 2001). Changes in blood plasma steroid concentrations as a result of chemical treatment can also be detected using juvenile fish.

The main disadvantage of using juvenile fish lies in the inability to directly assess reproductive performance. Other features of juvenile assays that are limiting include the lack of sexual differentiation, maturation, and the absence of secondary sex characteristics. Lack of this latter attribute may prevent detection of (anti)-androgens (see review in section 8.0). Because information regarding spawning performance cannot be obtained, the potential exists for a test chemical to cause decreased reproductive performance without observable changes in biomarker activity (VTG, E2, or 11-KT changes). A specific example of this problem could occur with EACs that act as aromatase inhibitors. Aromatase inhibitors reduce the rate of synthesis of estrogen and are capable of causing a reduction in estrogen tissue concentrations. This effect is best detected in fish by the decreased E2 levels and expression of VTG in female individuals during spawning (Afonso et al., 1999a; Ankley 2002). Because juvenile fish may not as readily exhibit a decrease in VTG, the use of immature fish would not be effective as a reproductive screen.

5.4 Exposure Duration

A variety of concerns need to be considered in the determination of the duration of the toxicant exposure. An underlying principle of toxicology is that an increase in the amount of exposure time typically results in identification of effects at lower concentrations (Ensenbach & Nagel 1997; Holcombe et al., 1995; McKim 1977; Nagel et al., 1998; Parrott et al., 2000). Thus, the longer the duration of the exposure, the more likely an effect will be observed. With respect to a reproductive screening assay, the use of a high toxicant concentration might allow for a shorter exposure period to be used. The exposure duration should be sufficiently long to allow for extensive absorption of the toxicant and potential biotransformation events (e.g., disposition) to occur to a significant extent. As a useful approximation, an exposure duration lasting sufficiently long to achieve a pseudo-equilibrium between toxicant concentrations in the exposure media and those in fish tissues would be acceptable. For most chemicals, this equilibrium would be expected to occur within 30 days of exposure (Veith et al., 1979). However, for lipophilic chemicals, a pseudo-equilibrium might be difficult to achieve in this time span. Aspects of the basic reproductive biology of the test species also need to be considered in the exposure duration. The three fish being considered are all fractional spawners with an approximately 4-day spawning interval (Jensen et al., 2001). Therefore, the exposure should continue for a sufficient time to allow for several spawning cycles or intervals to pass to ensure any toxicant effects on gametogenesis and fertilization are captured. This minimal time period would also be needed to record daily fecundity data, which can be quite variable in fractional spawners (Lange et al., 2001; Harries et al., 2000).

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

5.5 Statistical Considerations

The purpose of the reproductive screening assay is to detect potential EDCs and their likely mode of action in fish. Thus, a screening assay must be biologically sensitive while providing a mechanistic link between changes in endpoints and reproductive dysfunction, statistically powerful, and cost effective (Munkittrick et al., 1998). The screening assay is meant to detect potential EDCs and not to produce a precise estimate of toxicity. The statistical

considerations here are restricted to the demands of the screening assay. The amount of information obtained from the screening assay can be limited to detecting a series of specific differences in reproductive traits when both genders are exposed or can be used to determine whether or not gender-specific differences can be detected when gender-selective exposure is used.

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

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

5.5.1 Experimental Design and Statistical Power

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

Independence of treatment application, including the creation of the treatment, incorporates the variability associated with someone else, somewhere else making and applying the same treatment. Thus, the inference made associated with the treatments under test is extended to someone else repeating the experiment. The random sample of organisms from a given population actually limits the inference to that population. However, one can evaluate the stability of the inherent variability of the population over time. An experimental unit is defined as the group of material to which a treatment is applied independently in a single trial of the experiment (Cochran & Cox 1957). Replication of experimental units for each treatment provides a measure of all the necessary sources of variability needed to extend the inference across time and space. A reduction in the sources of variability that are truly independent constrains the inference (Hurlbert 1984). Thus, if only one solution of each treatment is made and then divided between replicates, the source of variation associated with making the treatment

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

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 (Figure 2). 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 coefficients of variation (CVs) (standard deviation/mean x 100%). In terms of power, high CVs have low power for detecting small-scale differences (Figure 2). Experiments with the standard number of 5 replicates per treatment and CVs for the measured response greater than 50% will be unlikely to detect differences smaller than 70% between the test and reference treatment response at a type I error rate of $\alpha = 0.05$. For a given CV, one can increase power by increasing the number of replicates. The choice of the test species and endpoints with the least inherent variability, by default, require the least replication for a given level of power and thus are more cost effective.

5.5.2 Decision Criteria

In some cases, individual responses would be diagnostic of a specific endocrine mode of action (e.g., induction of vitellogenin in males caused by estrogen receptor agonists), but in most cases the pattern of responses of the various endpoints would have to be considered to assess which (if any) endocrine pathway has been affected. In general, each endpoint will be evaluated to determine if at least one concentration of a test material produces a significantly different mean response (greater or lesser response depending on the endpoint) from the control mean response using Dunnett's test when there are at least two doses greater than zero being tested (Dunnett 1955; ARS 1977; Chapman et al., 1996). This procedure uses an experiment-wise error rate. The degrees of freedom for testing are the number of experimental units (not sub-replicates) minus one times the number of doses plus the no-dose controls. When the variance for testing is based on the variance among fish in a treatment chamber (i.e., sub-replicates) instead of between true replicates, the inference of repeatability through time and space as described above is invalid (Hurlbert 1984). If only one dose and one control are tested, then a t-test or Fisher's Exact test can be used (Chapman et al., 1996).

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

Appropriate data transformations will be applied to maintain homogeneity of the within-class variances (i.e., data expressed as a percentage will be arcsine-square root transformed,

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

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.

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

Finally, statistical significance and biological significance should not be confused. Unfortunately, statistical significance is often used in place of biological significance when little biological knowledge is available. If the sample size of a test is very large, the probability of detecting a statistically significant difference between means becomes large. Thus, we must balance statistical power with the need to detect biologically meaningful differences.

6.0 DESCRIPTION OF ASSAY ENDPOINTS

6.1 Growth and Morphological Alterations

6.1.1 Gonadosomatic Index

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

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

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 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 (Devlaming et al., 1982). However, this latter approach may not be practical in some species such as the fathead minnow as egg dimater can vary substantially between batches of eggs laid by individual females (Harries 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 data set 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 Histology Techniques. Several authors have described the histological preparation of reproductive and other tissues of model fish species to assess toxicant effects on the

reproductive organs (Orn et al., 2000; Wester & Canton 1986; Lange et al., 2001). Histological methods are widely practiced and relatively routine but there are variations in methodology that need to be standardized. A protocol for both paraffin-embedded and glycol-methacrylate-embedded histology was described by Jensen et al. (2001). Paraffin embedment is a longer established and more commonly practiced method of embedding, adapted to processing high volumes of tissue samples. Glycol-methacrylate embedment is a more specialized technique that results in the ability to more finely differentiate cellular and tissue structure. Because less heat in applied that in paraffin embedment, particulary if a non-exothermic accelerator is used, less shrinkage of tissues occurs a higher density matrix is formed to support tissue structures, and thinner tissue sections can be cut than with paraffin. Both methods are sufficiently routine that various references are available to the methodological detail. Paraffin is recommended embedment medium when immunohistochemical methods are applied and use of small fish species will likely benefit from localization of specific proteins within gonads or other endocrine related organs/tissues.

A standard chemical fixative should be used for evaluations, since choice of fixative can affect degree of tissue shrinkage, alter tinctorial or staining properties of the tissues, and prevent certain follow-up definitive procedures should they be desired. The use of routine fixatives such as neutral buffered formalin, Bouin's fixative and buffered glutaraldehyde-formaldehyde fixatives in regard to fixation of fish reproductive tissues has been reported (Jensen et al., 2001; Schwaiger et al., 2000; Miles-Richardson et al., 1999b; Wester & Canton 1986). With small aquarium fishes and certainly with early life stages (larvae and juveniles) it is possible to fix entire individuals and then process these. For juveniles where penetration of fixative may become an issue (i.e., lead to inadequate fixation) surgically opening the abdominal wall in the ventral midline prior to placement in fixative will result in improved penetration. To minimize autolysis and ensure satisfactory results, tissues should be placed in the fixative immediately after excision from specimens and adequate fixation time allowed prior to initiation of the histological matrix infiltration and embedment procedures. Typically, sections are stained with hematoxylin and eosin to observe tissue and organ structures. However, an array of alternative staining procedures may also be used to permit differentiation of various cell types.

To evaluate the gonads of model fish used in these studies, transverse sections should be taken along the long axis of the gonad at 4-µm to 5-µm intervals in a serial-step fashion. Multiple sections are desirable and standardized procedures for producing such multiple sections need to be established for any protocol.

6.1.1.2 Interpretation of Histopathological Results. Histology is a qualitative to semi-quantitative tool widely used to describe alterations, detect and localize specific changes and is essential for developmental biology and pathology. The method provides a permanent record of alterations in the microscopic anatomy of tissues. Histology has been recognized as the most accurate for staging reproductive development in fish (West 1990), and although it can be a sensitive method, it is not as sensitive as measurement of some biochemical biomarkers such as VTG (Miles-Richardson et al., 1999b). The primary difficulty in applying histological analysis is that interpretation of changes in tissues may vary from one investigator to another. Therefore, to maximize the repeatability of interpretation, individual histological slides are blind coded to decrease bias. Additionally, multiple pathologists may independently investigate and describe alterations in a randomized subset of the animals, meet and confer to reach consensus on specific changes, and then individually and separately examine all slides and record incidences of alteration. Where desired, scoring methods may be used to provide a semi-quantitative result.

Histological analysis can be used to compare test or unknown cases with standardized stages of development and to compare normal with abnormal structures. For example, the effects on male reproductive follicles have been 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., 1999b). 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. In females, normal or arrested development of oocytes can be determined in comparison with the normal or control developmental sequence; abnormal formation of degenerative oocytes can be observed histologically. West (West 1990) recommends histology as the most appropriate method for staging ovarian development and abnormalities. This author described the staging of oocytes based on their microscopic appearance and the occurrence of atretic oocytes, which may result either from normal follicular resorption in, for example, seasonal cyclic spawners, or a toxicopathological process affecting the ovaries. Ovarian development is generally described as the numerical proportion of oocytes in stages of development from primary to early and late vitellogenic oocytes, and appearance of the corpus luteum in the post-spawning condition.

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 due to plane of section variations 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.

Histopathology continues to be widely applied in assays for effects of xeno-estrogens and other xeno-biotic agents (Legler et al., 2000; Wester & Canton 1986; Wester & Canton 1990). Although histopathological analysis provides a direct assessment of the microscopic condition of tissues, the need for careful control is generally recognized where the method is applied to studies requiring critical evaluation and repeatability.

6.1.2 Sexual Differentiation

After the gonads have differentiated into either ovaries or testes in gonochoristic fishes, there may be a considerable period of time during which these tissues develop before gametes are produced for the first time. In oviparous female fishes, oogenesis must be completed, which involves vitellogenesis, before oocytes are fully formed and ready for ovulation. The testes of the male must complete the sequential processes of spermatogenesis and spermiation to produce viable sperm. The development of the gonads is regulated by endocrine signals originating in the hypothalamus and pituitary, and sex steroids, particularly 11-KT in the male 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. Therefore, EACs that affect any aspect of the reproductive endocrine system causing reduced synthesis or release of gonadotropins, or that interfere with gonadal steroidogenesis, will delay or prevent reproduction from occurring (Arcand-Hoy & Benson 1998). This can be observed by examining the state of development of gonads of exposed fish relative to a suitable control, via the gonadosomatic index or histological staging (Le Gac et al., 2001). A delay in spawning (i.e., time to first spawning/sexual maturity) and spawning frequency are commonly assessed endpoints in toxicological lifecycle studies of fish that presumably result from this mode of action. Fecundity might also be impacted in both sexes when fewer germ cells develop or

mature. This can be detected by quantifying the number of eggs spawned in females or assessing sperm number in males.

The effects of alterations on sexual development are technically easy to measure and any of the three 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). However, it should be pointed out that in reproductive screening assays using spawning fish, any specific effects on sexual development are likely to be missed. In this respect, assays that incorporate gonadal recrudescence as part of the protocol are more likely to be sensitive to effects on sexual development.

6.1.3 Secondary Sex Characteristics

Secondary sex characteristics in fish are hormonally controlled, making them viable endpoints for the evaluation of endocrine disruption. All of the species considered have some secondary sex characteristics, such as females having distinct genital papilla. The male fathead minnow has distinct breeding tubercles on the snout and dorsally located fatpad. The male zebrafish and medaka have larger or longer anal fins than the females, and the male medaka dorsal fin has a cleft. The mature male fathead minnow and medaka have distinct coloration.

It is important to identify how the observation and measurement of secondary sex characteristics will be used. General observation or qualitative results can be made for the three species under consideration. Qualitative results would be supporting evidence of endocrine disruption and would be useful in a screening-type test. Actual measurements or quantitative results could reduce the candidate species to the Japanese medaka and fathead minnow, as both sexes in these species have strong secondary sex characteristics. Quantitative analysis can be performed on semi-empirical observations of secondary sex attributes in chemically exposed fish. An excellent example is provided by Papoulias et al. (2000), who used a readily available statistical software package (SPSS, Chicago IL) to perform stepwise discriminant analysis to assess the predictability of secondary sex characteristics in identifying genotypic sex in the medaka.

6.2 Measures of Reproductive Performance

6.2.1 Fecundity

Female fecundity is the more common endpoint measured as part of an assessment of reproductive performance. Because the general spawning strategy for fish is to produce many eggs with limited or no parental protection, a large number of eggs can be produced by a female in a relatively short time.

More variability is seen in spawning data compared with data endpoints, such as fertilization success, hatching success, length, weight, and survival. Because of the limitations of replication, other endpoints might routinely be more sensitive than fecundity when measured based on the number of eggs deposited over time. Because all three species of fish being considered are fractional spawners, the time to deposit eggs might be the most critical endpoint. For example, the number of eggs-per-female-per-day over a 4-week period might be similar, but higher treatment levels might induce spawning over a shorter time. However, if eggs are released from the ovary before they are mature, fertilization success might also be affected.

6.2.2 Gamete Viability

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 would be useful additions to a reproductive screen protocol. Egg viability can be affected in a number of ways, including reduced VTG incorporation into the oocyte causing smaller sized eggs or disturbances in the levels of sex and thyroid hormones (reviewed in Kime & Nash 1999). Sperm viability is frequently assessed by motility, and a strong correlation can exist between sperm motility and fertilization success (Lahnsteiner et al., 1998). Accurate measurement of sperm motility has become easier in recent years with the advent of computer-assisted motion analysis. However, it should be acknowledged that the specialized equipment for this procedure is not routinely available in most laboratories.

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

6.2.3 Changes in Spawning Behavior

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

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

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

6.3 Biochemical Measures

6.3.1 Estrogen Responsive Gene Products: 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 ERs in the liver. Both male and female fish can be induced to synthesize VTG after estrogen exposure or after exposure to estrogen-mimics. Vitellogenin can also be induced in both juvenile and adult individuals, and detection of VTG synthesis has become the most widely studied biomarker of exposure to EACs. In many fish, induction of VTG has been shown to be extremely sensitive to estrogen exposure. For example, in sexually mature male rainbow trout, a 28-day exposure to 0.3 ng/L EE2 caused a statistically significant increase in plasma VTG levels compared with those of control trout, with a predicted no observable effects level (NOEL) of less than 0.1 ng/L (Sheahan et al., 1994). For the test species under consideration as a reproductive screen, VTG synthesis has also been demonstrated to be sensitive to estrogen exposures, although somewhat less sensitive than that observed in rainbow trout. The reduced sensitivity appears to be due in part to difficulties in obtaining sufficient quantities of purified VTG protein and species-specific antibodies for use in enzyme-linked immunosorbent assay- (ELISA) or radioimmunoassay (RIA)-based measurement techniques (Section 6.3.1) (Parks et al., 1999; Van den Belt et al., 2001).

Although there are a variety of methods to detect VTG in plasma, the most widely applied methods are the immunoassays, ELISA and RIA. These methods exploit the highly specific interaction of antibodies and the antigen VTG to detect the protein in a variety of samples, including plasma, tissue samples, and culture medium. Radioimmunoassay and ELISA typically use homologous polyclonal anti-VTG bodies to quantify VTG, although antibody cross-reactivity allows for VTG detection among species. The methods offer similar sensitivities in the samples analyzed; however, ELISA typically requires the dilution of samples to reduce interferences in the assay, effectively reducing the sensitivity compared to RIA. The small volumes of plasma available from individual fish of the test species can be a limiting sensitivity factor, requiring dilution of the sample to perform ELISA. The ELISAs are used more frequently to measure VTG because, unlike RIA, it does not require radioactive isotopes, uses stable reagents, and is relatively easy to set up and use. Although these immunologically based methods have been developed and validated for a wide range of teleosts, more modern direct methods, such as mass spectrometry, may be applied in the future. The specifics of ELISA and RIA are discussed in Sections 11.2.2 and 11.2.3.

In addition to the yolk-forming VTG, several eggshell envelope proteins, collectively called zona radiata proteins, are also induced upon exposure to estrogen. Zona radiata proteins are synthesized in the liver and ovaries and incorporated into the developing oocytes in a manner analogous to VTG. Although induction of both zona radiata proteins and VTG is estrogen dependent, stimulation of zona radiata synthesis appears to require lower levels of estrogen compared with VTG (Sohoni et al., 2001). Furthermore, evidence suggests that zona radiata proteins are preferentially induced by weak estrogenic compounds, such as bisphenol-A and *o,p*'-DDT (Arukwe et al., 2000). This latter finding might be significant for a 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.

A third estrogen responsive gene product is the ER itself. Estrogen receptors are autoregulated in rainbow trout (Riehl et al., 1999), meaning that E2 is involved in a positive feedback loop to upregulate its own receptor. Therefore, ERs are estrogen responsive genes with regulatory estrogen responsive elements. Numerous studies have demonstrated in rainbow trout that liver ER mRNA and protein is upregulated by E2-17â treatment (Arcand-Hoy & Benson 1998; Gray et al., 1999; Riehl et al., 1999), and after *in vivo* exposure to an endocrine disruptor, nonylphenol (Cheek et al., 2000). The transcription rate of ER mRNA (or the lack thereof) could be a useful and sensitive measure of estrogenic endocrine disruptor activity in other fish species too. A number of xenobiotics have been shown to induce comparable effects on the synthesis of ER and VTG mRNA when added to rainbow trout liver cells *in vitro* (Skinner et al., 1999; Winn et al., 2000). Although the use of VTG as a biomarker in fish dominates the literature, future

research could warrant the substitution of ER or zona radiata protein induction for VTG as a useful endpoint in reproductive screening assays.

6.3.2 Tissue Steroid Concentrations

The measurement of plasma levels of E2, 11-KT, and T can be used as endpoints to assess sex-steroid status in male and female fish, provided there is some knowledge about normal population levels when the samples are taken. In the context of a laboratory toxicology experiment, a control group of sufficient size is an absolute requirement. Plasma levels of E2 have limited utility in males, because levels of this steroid are usually low or non-detectable. The measurement of plasma E2 is most useful in sexually maturing females because of the gradual rise in this hormone during the period of vitellogenesis. A good example is the well-documented case for the rainbow trout, an iteroparous, seasonally breeding fish with group-synchronous ovarian development in which maximum levels are attained well before spawning (Scott et al., 1980a; Vanbohemen & Lambert 1981). In many fish, E2 begins to decline by the time of final maturation, probably as a consequence of the completion of yolk synthesis by the ovary, and E2 levels drop significantly after that. A distinct switch is seen in fish ovarian steroidogenic pathways from estrogens to progestins at this time (Nagahama 1999).

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

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

7.0 RESPONSE TO ESTROGEN AGONISTS AND ANTAGONISTS

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

A comparatively large number of environmental pollutants have been hypothesized to cause toxicity via binding to the ER (estrogenic compounds) (Hoare & Usdin 2001) or to produce responses similar to estrogen without binding to ERs (estrogen-like) (Gillesby & Zacharewski 1998). As a result, experimental studies of the responses of fish exposure to natural or synthetic estrogens have received a higher degree of scrutiny than have studies on other fish hormonal systems. In this respect, relatively few studies have examined the response to treatment with androgenic compounds and thyroid hormone mimics. Similarly, studies of antagonistic effects of xenobiotics on hormone pathways have only recently been studied in fish.

A large number of experimental studies on EACs have used salmonid fish species or other synchronous annual spawning fishes. With respect to fish species used in reproductive screening tests, the preferred test species in the majority of studies are the fathead minnow and the Japanese medaka. Experimental studies using zebrafish typically focus on full-life-cycle or multi-generation studies with a noticeable lack of studies using short-term exposures to sexually mature zebrafish.

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

A brief overview of the molecular biology of the ER has been placed in section 7.2.1 to provide a context for descriptions of anti-estrogen effects in fish. The reader may find it helpful to refer to that section for the ensuing discussion on estrogen agonists.

7.1 Endpoint Sensitivity to 17β-estradiol or Synthetic Estrogen Exposure

7.1.1 Growth and Morphological Alterations

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

An additional confounding factor that is problematic with asynchronous fractional spawning fishes such as those under consideration in this review is the daily fluctuation in gonad weight during the spawning cycle. This fluctuation is especially pronounced in females, where the GSI can vary as much as 45% over a 2-day period, depending on the sampling day during the

spawning interval (Jensen et al., 2001). In contrast, the daily fluctuation in the GSI in males is usually less variable, and may be as little as 1% during the spawning cycle (Jensen et al., 2001). This latter observation tends to make GSI measurements in males a more sensitive and commonly reported parameter in reproductive studies of estrogenic substances. Another important male attribute in this regard is that the microscopic morphology of the testis is much more consistent (as compared with ovarian morphology) during the spawning cycle and is less influenced by environmental changes (Koger et al., 1999).

7.1.1.1 Weak Estrogen Agonists. Perhaps the best characterized class of contaminants considered to be weakly estrogenic are the alkylphenol ethoxylates and their environmental degradation products, alkylphenols. The basis for the presumption of an estrogenic mode of action for these and other contaminants stems from both in-vitro and in-vivo studies measuring induction of VTG, typically in salmonid species (Jobling & Sumpter 1993; White et al., 1994; Lech et al., 1996). With respect to growth and morphological endpoints in proposed test species, the effects of these compounds can be ambiguous. For example, in adult fathead minnows, a 42 day exposure to nonylethoxylate or 4-nonylphenol at measured concentrations ranging from 0.15 to 5.5 and 0.05 to 3.4 µg/L respectively, did not alter the appearance of secondary sex characteristics in males (Miles-Richardson et al., 1999). Gonad histology was only altered in males after the 1.1 µg/L or 3.4 µg/L 4-nonylphenol exposures, which caused increased Sertoli cell numbers and necrotic spermatozoa (Miles-Richardson et al., 1999). In breeding pairs of fathead minnows exposed for 21 days to either 4-nonylphenol or butyl benzyl phthalate at measured concentrations of 0.62 to 58.6 and 69 to 82 µg/L respectively, effects on GSI and secondary sex characteristics were reported only from the 4-nonylphenol treatments (Harries et al., 2000). Specifically, 4-nonylphenol exposure rates of 48.1 and 58.6 µg/L decreased the GSI and appearance of nuptial tubercles in males. A more sensitive male secondary sex characteristic was the thickness of the dorsal fat pad, which was significantly decreased at 4-nonylphenol exposure rates at or above 3.1 µg/L (Harries et al., 2000). This study also reported that 4-nonylphenol treatment rates of 8.1 µg/L and higher reduced the GSI in females, although not to a statistically significant extent. An additional weak estrogen that has been studied in fathead minnows is the organochlorine pesticide methoxychlor. A 21-day exposure of fathead minnows to measured methoxychlor concentrations of 0.55 and 3.56 µg/L did not alter GSI in males and females, although the higher treatment rate caused an increase in the numbers of atretic follicles in the ovaries (Ankley et al., 2001).

In the medaka, effects on growth and morphological effects of weak estrogens have only been studied using extended exposure periods initiated during juvenile life stages. For example, a study reported by Metcalfe et al. (2001) exposed newly hatched larvae to either bisphenol A, mixtures of nonylphenol mono- and di-ethoxylates and di-2-ethylhexylphthlate for 85 to110 days at concentrations ranging from 10 to 200, 25 to 100, and 500 to 5,000 µg/L respectively. At these exposure rates for the nonylphenol ethoxylates and di-2-ethylhexylphthlate, no effects on growth or morphology were observed. However, bisphenol A treatments at 50 µg/L and above caused testicular fibrosis and a decrease in the numbers of spermatozoa in testicular lobules (Metcalfe et al., 2001). In an earlier study, medaka larvae exposed for three months to 4-nonylphenol at concentrations of 50 (measured range 15.4 to 51.8) and 100 (measured range 55.9 to 104.2) µg/L were shown to undergo testis-ova formation in males (Gray & Metcalfe 1997). In a separate study, medaka larvae were exposed to nonylphenol and methoxychlor at measured levels of 0.44 to 1.93 µg/L and 0.18 to 2.31 µg/L for 28 days followed by a post-dosing recover period of 28 days (Nimrod & Benson 1998). The results indicated that neither nonylphenol nor methoxychlor altered growth or gonad weight after any of the treatment rates (Nimrod & Benson 1998).

7.1.1.2 Estradiol and Synthetic Estrogen Agonists. Several studies have been conducted on the effects of short-term exposure to estrogenic chemicals on the growth of fathead minnows. In juveniles, a 21-day exposure 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 did not significantly alter body weight or total length compared with control fish (Panter et al., 2000b). 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). In adult 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., 1999b). In adult 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 these 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., 2000). Thus, a reduction in testis mass (or GSI) and lethality caused by short-term exposure to E2 in adult fathead minnows occurs between a relatively narrow exposure window of 60 to 100 ng/L and 800 to 1000 ng/L. These exposure rates to E2 have also been documented to cause severe testicular lesions described as a loss of germinal cells and presence of degenerate spermatozoa (Miles-Richardson et al., 1999b). Additional testicular histopathology noted was proliferation of Sertoli cells that in extreme cases led to complete occlusion of seminiferous tubules (Miles-Richardson et al., 1999b). Estradiol-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 exposure rates down to nominal concentrations of 17 ng/L (Miles-Richardson et al., 1999b).

Several of the aforementioned studies using fathead minnows also reported changes in growth and development of secondary sex characteristics. An 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., 1999b). 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., 1999b).

Experimental results for the Japanese medaka and zebrafish are generally consistent with findings in fathead minnows that changes in the GSI or overall body mass are insignificant at E2 or EE2 / DES exposure rates below 100 ng/L, whereas histopathological changes are noticeable below exposure rates of 100 ng/L. For example, Scholz and Gutzeit (2000) exposed freshly hatched medaka for 2 months to nominal EE2 concentrations up to 100 ng/L, followed by a 6-week recovery period, and observed similar growth (total body weight and length) in both male and female individuals compared with control fish. In this study, the GSI of male fish was unchanged but was decreased in females at EE2 exposure levels of 10 and 100 ng/L (Scholz & Gutzeit 2000). A shift in phenotype from male to female also was observed in all fish exposed to 100 ng/L of EE2 (Scholz & Gutzeit 2000). A similar study was described in (Metcalfe et al., 2001), in which newly hatched medaka fry were exposed to E2 and EE2 concentrations ranging logarithmaticaly from 0.0001 µg/L to 1.0 µg/L for 90 days. The results indicated 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). This study also reported a number of histopathological changes in exposed medaka, the most prominent being an 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).

An additional study that exposed newly hatched medaka fry to E2 was reported by Nimrod & Benson (1998). In this study, measured E2 exposure levels from 0.01 µg/L to 1.66

µg/L were continued for 28 days followed by a post-dosing recover period of 28 days. 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). These studies are similar in that all exposures were initiated using juvenile medaka.

There is a notable lack of studies that include histopathological examination of the gonads in adult, sexually mature medakas after short-term exposure to E2 or synthetic analogues. This general observation can also be made for zebrafish, with a single notable exception. Adult zebrafish were exposed to EE2 for 21 days, using nominal concentrations of 10 ng/L and 25 ng/L (Van den Belt et al., 2001). At these exposure rates, EE2 decreased the GSI in both males and females (Van den Belt et al., 2001). In addition, 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).

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 factor. Ethynylestradiol appears to be more potent than E2 in this respect, and exposure rates below 50 ng/L of EE2 have been noted to cause similar effects to those observed with E2 at higher exposure rates. A surprisingly limited amount of information is available on the histopathological changes caused by short-term exposure to E2 or synthetic analogues in sexually mature fish. This paucity of information hinders the interpretation and significance of reported findings, as effects observed in adults that have been exposed to estrogenic compounds as juveniles may reflect exposure prior to sexual differentiation, rather than at maturity, and therefore, may have little relevance in the context of a reproductive screen using sexually mature fish. Weakly estrogenic chemicals appear to require exposure rates 10 –100 times higher than for E2 to cause changes in GSI and gonad histology.

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. The buildup of eosinophilic staining material in male fish can be pronounced when exposure rates to E2 are high (e.g., exceeding 1 mg/kg or mg/L doses) and has been suggested to be a toxic consequence of VTG induction (Herman & Kincaid 1988). A more recent study of male flounders (*Paralichthys dentatus*) exposed to E2 suggested excessive VTG induction overwhelms the kidney by damaging the glomeruli, which allows fluid to drain directly into the nephron (Zaroogian et al. 2001). More specific descriptions of the histopathology caused by estrogenic substances require additional study using exposure protocols more closely aligned to those under consideration in reproductive screening assays. It is also important to mention here that caution should be exercised when making dose-response interpretations from studies that do not measure exposure concentrations (e.g., only nominal concentrations are reported). In virtually all reviewed studies in which measured concentrations are reported, actual concentrations of E2 or EE2 are less than nominal, sometimes by as much as a factor of 10 (e.g., 10% of nominal). Thus, the absence of an observed effect at a nominal exposure concentration when compared with a positive response at a similar, but measured exposure rate may in fact be due to actual test concentrations significantly lower than the reported nominal exposure rate.

7.1.2 Reproductive Performance

The ultimate measure of reproductive performance is successful spawning, or more specifically, the successful fertilization of sufficient numbers of eggs such that an acceptable number of larvae are produced that become young of the year fish and ultimately recruited into the population. With regard to development of a reproductive screen, the experimental evaluation of estrogenic or estrogen-like compounds has primarily focused on measuring fecundity, fertilization rate, spawning frequency (duration between spawns), and hatching success as measures of reproductive performance. Also, several studies of reproduction in

appropriate test species used exposure protocols significantly different from that suitable for a screening assay. For this reason, the ensuing discussion is restricted to those studies that initiated exposures to adult individuals.

7.1.2.1 Weak Estrogen Agonists. There have been several studies in fathead minnows that measured various endpoints of reproductive performance after exposure to weak estrogens. For example, in adult female fathead minnows exposed to methoxychlor for 21 days at measured concentrations of 0.55 µg/L and 3.56 µg/L, the mean fecundity rate decreased from 20.5 to 8.3 eggs/female/day (control versus 3.56 µg/L treatment) (Ankley et al., 2001). The decrease in fecundity was attributed to both a decrease in egg numbers per spawn and less frequent spawning (increased spawning interval) (Ankley et al., 2001). Similar results were obtained in a study that exposed pair breeding fathead minnows to 4-nonylphenol, at measured concentrations of 0.65 µg/L, 8.1 µg/L and 57.7 µg/L for 21 days. At these water concentrations, 4-nonylphenol exposure reduced fecundity (total eggs spawned / 21 days) to 60%, 45% and < 5% of control values respectively, while also decreasing the spawning frequency at exposure rates of 8.1 µg/L and higher (Harries et al., 2000). This same study also tested butyl benzyl phthalate, which had no effect on fecundity at a measured exposure rate of 75.5 µg/L. In another study of 4-nonylphenol using a 42-day exposure protocol to measured concentrations ranging from 0.05 – 3.4 µg/L, fecundity was observed to decrease at exposure levels of 0.16 µg/L and higher, although only the 3.4 µg/L treatment was statistically significant (Giesy 2000). Finally, a recent study of nonylphenol ethoxylates (a mixture of 17 oligomers) in fathead minnows at measured concentrations between 0.21 µg/L and 7.9 µg/L observed no significant effects on fecundity (Nichols et al., 2001). This latter study also noted that the variance relative to the sample size was high, which reduced the statistical power to discriminate between treatments (Nichols et al., 2001).

In the medaka, the effect of 4-nonnylphenol exposures on reproductive performance in females was studied using a protocol that exposed each gender separately before mating to control medaka of the opposite sex (Shioda & Wakabayashi 1999). The results indicated that nominal exposures to 4-nonnylphenol at 6.45 µg/L or higher significantly decreased fecundity and hatching success of eggs produced from exposed females (Shioda & Wakabayashi 2000). In contrast, hatching success of eggs fertilized by exposed males was not affected although high variability limited the sensitivity of this endpoint (Shioda & Wakabayashi 2000). In a similarly designed study, the reproductive performance of pair breeding zebrafish was evaluated after 21-day exposures to nominal octylphenol concentrations of 12.5, 25, 50 and 100 µg/L (Van den Belt et al., 2001). Reproductive success was quantified from the percentage of breeding pairs that produced viable offspring and was unaffected by all octylphenol treatments (Van den Belt et al., 2001).

7.1.2.2 Estradiol and Synthetic Estrogen Agonists. The effect of E2 exposure on reproductive performance in the medaka was reported by Shioda and Wakabayashi (1999) in their previously cited study using gender specific exposures. The results of the study indicated that nominal E2 exposures of 817 ng/L or higher significantly decreased fertilization success of eggs fertilized by exposed males. In females, E2 treatments at or above 27.2 ng/L decreased both fecundity and fertilization success (Shioda & Wakabayashi 2000). Similarly results were obtained with pair breeding zebrafish exposed for 21-days 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 reproductive success of spawning females 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 control reference value reported by the authors (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 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). In adult fathead minnows exposed to various E2

concentrations for 19 days, a decrease in fecundity was reported with a calculated E2 exposure median effective concentration (EC_{50}) of 120 ng/L (Kramer et al., 1998).

In summary, short-term exposure of sexually mature fish to estrogenic compounds reduces fertilization success in both males and females. Both E2 and particularly EE2 appear to decrease fertilization at exposure rates below that needed to alter growth of the gonads, and thus appears to be a more sensitive indicator of reproductive disturbances. In some species, such as the fathead minnow, cumulative egg production measured before and during an exposure appears to be a sensitive measure of fecundity and overall reproductive performance of females. However, in other species, such as zebrafish, problems associated with extreme inter-individual variation in fecundity of control females prevent the determination of cumulative fecundity as a useful endpoint (Van den Belt et al., 2001). Although reproductive performance of male fish appears to be equally sensitive to estrogenic compounds, little information is available on the type of reproductive disturbances estrogenic compounds induce on male gamete quality.

7.1.3 Biochemical Measures: VTG

7.1.3.1 Weak Estrogen Agonists. Many of the aforementioned weak estrogen agonists also cause induction of VTG in the test species of interest and several of the more pertinent studies are reviewed below. For example, male fathead minnows exposed to methoxychlor at 3.56 µg/L for 21 days had significantly elevated plasma VTG concentrations (>4000 fold; Ankley et al., 2001). However, VTG was not significantly elevated in actively spawning female minnows undergoing the same treatment. Male fathead minnows exposed to 48.8 – 58.6 µg/L 4-nonylphenol had plasma levels of VTG that were 4,000 to 45,000 times greater than control males (Harries et al., 2000). In this study, 4-nonylphenol exposure also elevated VTG in spawning females to an extent that was 2 to 10 times greater than control females. In the male medaka, seven day exposure to nominal 4-nonylphenol concentrations of 20 and 50µg/L increased VTG mRNA expression in the liver as measured by RT-PCR (Islinger et al., 2002). Also in the male medaka, exposure to 4-tert-octylphenol has been reported to induce VTG synthesis at exposure rates between 20 and 230 µg/L (Gronen et al., 1999).

7.1.3.2 Estradiol and Synthetic Estrogen Agonists. Recent studies with fathead minnows indicate the pattern of VTG synthesis and overall elimination of the protein from blood plasma of male individuals is roughly comparable to that of salmonid species. For example, when adult male fathead minnows were given an intra-peritoneal injection of E2 at 0.5 mg/kg or 5.0 mg/kg, the plasma concentration of VTG peaked after 48 to 144 hours post injection and declined slowly, with plasma levels still within 50% of maximal values after 18 days (Korte et al., 2000). These findings are similar to those obtained in rainbow trout given intra-arterial injections of EE2. When trout were injected with EE2 at doses ranging from 0.001 to 1 mg/kg, VTG concentrations in blood plasma peaked after 8 days and then declined with an apparent biological half-life of 44 hrs (Schultz et al., 2001).

Although intra-peritoneal and intra-arterial exposures are useful routes of administration for understanding the kinetics of VTG induction and elimination, 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). Juvenile fathead minnows have also been demonstrated to undergo VTG synthesis after a short-term estrogen exposure. Exposure to EE2 concentrations as low as 4 ng/L for 21 days caused an approximately 5- to 10-fold increase in VTG levels compared with control fish, which weighed 150 mg at the beginning of the exposure (Panter et al., 2000a). A similar 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).

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, which could be used in ELISA or RIA procedures. This 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. 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 EC_{50} 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 medakas 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 formation in blood plasma (Van den Belt et al., 2001). A similar result was also obtained in a study which exposed adult zebrafish for 14 days to a nominal EE2 concentration of 5 ng/L (Kime & Nash1999). In this study, the level of VTG induction was roughly 3-fold greater in female than in male zebrafish (Kime & Nash1999). 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 of E2) for 48 hours and observed stimulated VTG gene transcription. A more thorough study of VTG induction in zebrafish was reported by Petersen 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).

In summary, all three test species of interest respond to a challenge dose of estrogen or an estrogenic substance (e.g., EE2) by the rapid synthesis of VTG. 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.

7.1.4 Biochemical Measures: Tissue Steroid Levels

In addition to measuring VTG levels following chemical exposures, it may also be helpful to measure plasma concentrations of endogenous E2 and the androgens, 11-KT and T. In contrast to salmonids, for which an extensive database is available on seasonal cycles in circulating plasma concentrations of reproductive hormones, relatively little information is available for the proposed test species. Furthermore, the effect of chemical exposure on circulating hormone levels in these species is largely untested, and the ensuing discussion is largely limited to a review of reported values in control fish.

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

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

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

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

7.2 Estrogen Antagonists

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

7.2.1 Direct-acting Anti-estrogens

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

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

Based on studies using mammalian animal models, direct anti-estrogens can be classified into two broad categories: 1) analogs of tamoxifen or its metabolites (Type I) which are actually weak ER agonists at low concentrations and antagonists at high exposure concentrations, and 2) pure anti-estrogens (Type II), which have no agonist-like properties. Both types of anti-

estrogens are competitive inhibitors of E2 binding to the ER, but differ in their effectiveness at blocking ER mediated transcription. Tamoxifen analogs form a receptor complex that is only partially active in initiating gene activation and may cause a single round of gene transcription, although high concentrations are completely inhibitory (MacGregor & Jordan 1998). Pure anti-estrogens bind to the ER immediately after synthesis in the cytoplasm prior to nuclear localization (Dauvois et al., 1992). The inactivated receptor complex appears to be rapidly degraded, preventing estrogen-mediated gene transcription from occurring (Dauvois et al., 1992).

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

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

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

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

Regardless of the specific mechanism of action of direct anti-estrogens, a common endpoint measured in most studies is the effect on VTG synthesis. Only limited information is available in the literature on the specific effects of anti-estrogens on reproduction in fish. Thus, it is difficult to state what effect, if any, anti-estrogens may have on reproductive success. Based

on the results indicating partial suppression of VTG synthesis, some effect on oogenesis would be expected but this remains to be demonstrated. The recent study of fadrozole in fathead minnows suggest low reproduction can occur (Ankley et al., 2002).

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

7.2.2 Indirect-acting 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. 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.

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

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).

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 decreased expression of the CYP19 gene (Kitano et al., 2000).

At lower treatment rates, fadrozole and other aromatase inhibitors can transiently lower circulating levels of E2. For example, pre-spawning Coho salmon administered fadrozole by

intraperitoneal injection at doses down to 0.1 mg/kg had significantly lower E2 plasma levels 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). Interestingly, other established aromatase inhibitors, letrozole and clotrimazole, have been shown to be sensitive inhibitors of aromatase activity *in vitro* using trout ovarian microsomes, but could not lower circulating E2 levels or suppress VTG synthesis in juvenile rainbow trout after dietary administration for 2 weeks at a dose rate of 1 mg/kg (Shilling et al., 1999).

With respect to fish species under consideration as reproductive screen, only two studies were found that examined aromatase inhibition. Most recently, sexually mature fathead minnows were exposed for 21 days to measured concentrations of fadrozole of 1.4, 7.3 and 57 µg/L (Ankley et al., 2002). This study measured brain aromatase activity after the 57 µg/L treatment and observed a significant reduction in activity in both sexes. Not surprisingly, both E2 and VTG levels in female minnows was decreased in a dose dependent manner with the fadrozole treatments (Ankley et al., 2002). Interestingly, both 11-KT and T were significantly elevated in male fish after the 7.3 and 57 µg/L treatments. With respect to reproduction, female fecundity decreased from mean control values of 20.5 eggs / female / day to 8.9, 1.5 and 1.2 eggs / female / day after the respective fadrozole treatments of 1.4, 7.3 and 57 µg/L (Ankley et al., 2002). The decreased fecundity at the two higher treatment rates was attributed to cessation of spawning after two days of treatment. In a previous study of fadrozole using juvenile fathead minnows, nominal concentrations of 25, 50, and 100 µg/L did not alter growth or reduce VTG synthesis (Panter et al., 2000a).

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. However, a recent study of perch collected from a Swedish lake receiving drainage from a nearby landfill reported that many fish had a lowered ovarian somatic index and reduced levels of circulating E2 that were associated with decreased aromatase activity (Noaksson et al., 2001). This pattern of effects is consistent with those observed with aromatase inhibitors and suggests inhibition of E2 synthesis could be an environmentally relevant mode of action for EACs.

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. 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. Similarly, VTG synthesis after an E2 challenge has been shown to be sensitive to anti-estrogen exposure in males. In regards to reproduction, most endpoints either directly assess female effects (fecundity) or combined gender effects (fertilization success). This makes it difficult to assess specific gender

sensitivities. A few studies have assessed male-specific fertility endpoints, which appear to be very sensitive to estrogenic exposure (Schultz et al., 2002). However, more research is needed to determine whether male fertility is more sensitive to estrogenic exposures. A specific endpoint for which females appear to be more sensitive is changes in circulating steroid levels, which tend to be more dynamic during the spawning cycle. In addition, morphological and reproductive responses to aromatase inhibitors and to a lesser extent, direct acting anti-estrogens appear to be more sensitive endpoints in female fish.

7.4 Strengths and Weaknesses of Test Species

A rigorous assessment of the strength and weaknesses of each test species with respect to endpoint sensitivity is limited by the paucity of information for some species, particularly the zebrafish. Also, only very limited dose-response relationships can be inferred from the available data, which are comfounded 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.

Despite these limitations, several endpoints appear to be equally sensitive in all test species. Similar sensitivities were observed for growth and gonad morphology and VTG induction. All three species appear capable of VTG induction in response to E2 or EE2 exposures as low as 25 ng/L. In addition, all three species appear capable of induced spawning in a short-term assay, and effects on fecundity have been documented after similar exposures to E2 or EE2 (e.g., 25 ng/L to 100 ng/L). A specific area in which the fathead minnow and medaka offer an advantage is the more pronounced male secondary sex characteristics. Beyond these comparisons, the greater application of fathead minnows in toxicity evaluations combined with recent studies on reproductive endocrinology provides a larger database for comparison of endpoints in control versus treated fish.

8.0 RESPONSE TO ANDROGEN AGONISTS AND ANTAGONISTS

In contrast to the predominate role E2 plays in the development of feminine characteristics, several androgenic steroids appear to be involved with male differentiation and reproduction. In male teleost fishes, 11-oxygenated androgens, especially 11-KT, appear to be the most important endogenous androgenic steroids, although T and androstendione have also been shown to be important in male sexual development (Borg 1994; Fostier et al., 1983). In females, 11-KT plasma concentrations are very low, often below 1 ng/mL, whereas T levels may approach E2 concentrations during portions of the spawning cycle in some fractional spawners, such as the fathead minnow (Jensen et al., 2001). The elevated T levels in females 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).

The biosynthesis of androgens primarily occurs in the testes and ovaries, although extra-gonadal synthesis has been demonstrated in several tissues including the liver and brain (Fostier et al., 1983). In the testis, synthesis of 11-KT and T primarily occurs in the Leydig and Sertoli cells (Redding & Patino 1993). Testosterone may serve as a precursor to the synthesis of the 11-oxygenated androgens (Shibata et al., 2000), and administration of T to both male and female fathead minnows results in the formation and excretion of 11-KT (Parks & LeBlanc 1998).

Although 11-KT is a more biologically potent androgen than T, early studies of androgen binding in cytosolic and nuclear extracts of fish tissues (reviewed in Borg 1994) suggested T is preferentially bound to tissue extracts compared with 11-KT. This discrepancy between biological activity and tissue binding might in part be explained by the recent discoveries of multiple androgen receptors, each with different tissue distributions, binding affinities, and

binding capacities for various androgens (Sperry & Thomas 1999a; Sperry & Thomas 1999b). Importantly, multiple androgen receptor subtypes appear to be expressed in Leydig and Sertoli cells and also in developing spermatocytes (Takeo & Yamashita 2001).

The variety of androgenic substances naturally occurring in fish, combined with the potential for cell- and androgen-receptor-specific responses, makes it difficult to generalize about the biological effects of xeno-androgens to an extent comparable for estrogenic substances. Relatively little information is available on the effects of exposure to androgenic or anti-androgenic substances in fish which is perhaps partly due to the complexity of the biological activity of androgens. An additional limitation for the characterization of androgenic substances is the lack of a suitable biochemical biomarker that is as broadly applicable as VTG is for estrogenic substances. An encouraging development in this area is the recent molecular characterization of the adhesive protein spiggin, which is produced by the kidney of male three-spined sticklebacks (*Gasterosteus aculeatus*) for use in nest building during the breeding season (Jakobsson et al., 1999). Most importantly from a reproductive screening standpoint, spiggin was shown to be only induced by administration of 11-keto-androgens as neither T nor dihydrotestosterone was effective at stimulating its synthesis (Korte et al., 2000).

8.1 Endpoint Sensitivity to Natural and Synthetic Androgen Exposure

It is well established that exposure of juvenile fish to potent androgenic substances can lead to formation of testis-ova in females or a complete shift in phenotype from female to male (Nakamura et al., 1998); (Koger et al., 2000). However, the effects of androgen exposure to adult or sexually differentiated individuals has received less study. An assessment of the biological effects of androgen exposure in sexually mature fish was reported for methyltestosterone (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, high 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 (1974). Additional studies in other fish species support the conclusion that formation of secondary male characteristics in females during androgenic exposure can be diagnostic for this mode of action. For example, in the adult medaka, a 15-day dietary exposure to 11-KT at dose rates ranging from 25 µg/g to 500 µg/g-food increased the number of papillary processes (growths) on anal fin rays in females in a dose-responsive manner (Hishida & Kawamoto 1970). In a series of related studies, appearance of papillary processes on the anal or dorsal fin ray in female medakas was observed after oral dosing with 19-*nor*-testosterone, MT, ethisterone, T, and androstenedione (Kawamoto 1973; Kawamoto 1969; Uwa 1975). 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 a intromissive organ. This development is under androgen control and female mosquitofish administered 11-KT in their diet at dose rates as low as 20 µg/g-food will develop a gonopodium or exhibit a modified anal

fin suggestive of gonopodium formation within 20 days of exposure (Angus et al., 2001). This phenomenon appears to be environmentally relevant, as masculinization of mosquitofish has been observed in the wild populations (Howell et al., 1980) and appears to be the result of androgenic substances, such as androstenedione found in pulp-mill effluents released into surface waters (Jenkins et al., 2001; Parks et al., 2001).

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

In summary, androgen exposure to juvenile fish that are sexually undifferentiated can lead to phenotypic reversal. In adult fish, androgen exposure decreases gonad growth (size) and circulating androgen levels in plasma and severely reduces reproduction in females. The available data indicate 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 Androgen Antagonists

8.2.1 Direct-acting Anti-androgens

A well-characterized environmental contaminant known to antagonize androgenic steroid action is vinclozolin, a chlorinated fungicide widely used as a pesticide for over 20 years. The anti-androgenic activity of vinclozolin was first described in developing rats exposed in-utero or lactationally via oral dosing of vinclozolin to the pregnant mother (Gray et al., 1994; Kelce et al., 1994). When exposure occurred during gestational day 13 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 may 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 (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 & Junge2001).

In an earlier study using the Japanese medaka, the potent anti-androgen cyproterone acetate (CPA) was also shown to retard development of male secondary sex characteristics. A 21 day exposure to nominal CPA concentrations ranging from 100 µg/L to 2000 µg/L reduced the number of papillary processes on anal fin rays in male medakas (Hamaguchi 1978). This effect was even more pronounced in female medakas co-exposed to 20 µg/L MT. In this latter experiment, CPA suppressed the MT stimulated formation of papillary processes in a dose-dependent manner (Hamaguchi 1978).

8.2.2 Indirect-acting Anti-androgens

As discussed previously for indirect anti-estrogens (Section 7.2.2), there would conceivably be a minimum of three mechanisms indirect anti-androgens could reduce intracellular steroid levels: 1) decreasing the rate of synthesis; 2) reducing the plasma free fraction; and 3) increasing the rate of elimination. Unfortunately, there is only very limited information available to assess the biological consequences of these modes of action. In contrast to the biosynthesis of E2, which can be blocked using aromatase inhibitors, no equivalent androgen-specific inhibitors have been identified. However, it is known that E2 administration to juvenile or post-differentiating fish decreases androgen secretion by the testes, and this effect is due to diminished expression of enzymes involved in their biosynthesis (Fitzpatrick et al., 1993; Govoroun et al., 2001). These results imply that xenobiotics capable of inhibiting androgen steroidogenesis could be effective at lowering circulating T or 11-KT levels. However, demonstration of this mode of action has not yet been made.

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

The biotransformation of androgens is complex, and a variety of metabolites and conjugates may be formed and eliminated (reviewed in Shibata et al., 2000). Both T and 11-KT excretion have been well studied in trout, where it appears conjugation to glucuronide is the most important elimination pathway (Yeoh et al., 1996). However, adult fathead minnows briefly exposed to T (4-hour exposure; measured concentration of 288.4 µg/L) formed 11-KT, which appeared to be excreted directly, i.e., no conjugates could be isolated (Parks & LeBlanc1998). Regardless of the potential for species differences in conjugation, it remains to be established whether changes in the rate of androgen elimination have any important biological effects in fish.

8.3 Gender Differences

The available data suggest (anti-) androgen activity is best identified in the test species 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 diagnostic for this mode of action. As for anti-androgens, a reduced display of secondary sex characteristics in males is suggestive of this mode of action, although it may not be as diagnostic as with agonistic androgen activity on females.

8.4 Strengths and Weaknesses of Test Species

The complexity of androgen activity and the similarity of many endpoints to other modes of action (for example, see discussion of thyroid hormones and secondary sex characteristics below) indicate a single test species may not provide sufficient diagnostic power for adequately identifying chemicals that disturb androgen activity. An obvious weakness of the proposed test species is the lack of a suitable biochemical biomarker sensitive to androgen agonists. In this respect, inclusion of the three spine stickleback in a testing program would be beneficial, as the

11-KT-induced synthesis of spiggin appears to be a very sensitive indicator of androgen exposure in this species. In addition, fish species possessing a gonopodium, such as the guppy and mosquitofish, appear to be superior in identifying both androgen agonists and anti-androgens through changes in the overall extent of masculinization of males. Although both the fathead minnow and medaka appear capable of responding to (anti-)androgens by changes in secondary sex characteristics, the lack of both sensitivity and specificity for these modes of action in the test species is a concern.

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 pharynx region of bony fishes. Secretion of thyroid hormones is under hypothalamus-pituitary control through the action of thyroid-stimulating hormone (TSH). Of the two, 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; Soyano et al., 1993; Legler et al., 2000; Siwik et al., 2000). Conversely, E2 administration has been recently reported to lower circulating T3 levels in immature trout (Alestrom et al., 1994). In the medaka, MT exposure was reported to stimulate thyroid activity (Nishikawa 1976). These studies would suggest the possibility that sex-steroid agonists may alter thyroid function. Despite these intriguing results, relatively few studies in fish have investigated whether environmental contaminants alter thyroid hormone status or directly interact with thyroid receptors to impair reproduction. However, one group of environmental contaminants, the polychlorinated biphenyls (PCBs), is of particular concern relative to thyroid hormones because of their structural similarity. Studies in 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 (Brown et al., 1997; 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 may be important in gametogenesis (Huang et al., 2001).

9.1 Endpoint Sensitivity to Thyroid Stimulation

As alluded to in the introductory comments, it has been proposed that certain PCB congeners or their metabolites may bind to vertebrate thyroid receptors (Fentress et al., 2000; Kovriznych & Urbancikova 2001). This potential would suggest 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 may not be a good indicator

of mode of action, as a complicating factor is the strong feedback control of T4 and T3 levels. For example, (Iwamatsu et al., 2000) reported that a 5 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 study of the effects of thiocyanate on thyroid function and reproduction in fathead minnows was reported by (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 time spawning activity was monitored. The results indicated that fathead minnows exposed to 16.6 mg/L and 32.6 mg/L thiocyanate completely lacked or underwent incomplete development of secondary sex characteristics. These fish also made no attempt to reproduce (Lanno & Dixon 1994). However, toxicity was quite high at these exposure levels, with reported mortalities during the exposure to be 30% and 63% at the 16.6-mg/L and 32.6-mg/L exposure levels, respectively (Lanno & Dixon 1994). A lower exposure rate of 7.3 mg/L was non-toxic but still impaired reproduction, as measured by the delay in time of first spawning and decreased fecundity (Lanno & Dixon 1994). Antagonism of thyroid function was evidenced at 7.3 mg/L to 32.6 mg/L thiocyanate exposure rates by the development of overt goitrous nodules along the branchial region of the lower jaw. Histopathological examination of these fish indicated a clear dose-response relationship between thiocyanate exposure rate and the extent of the hyperplastic and colloidal goitrous follicles (Lanno & Dixon 1994). The time to first appearance of the goiters or gender-specific differences of goiter formation were not discussed in this study.

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

9.3 Gender Differences

Because of the paucity of studies available for review, little discussion of gender differences to thyroid stimulation or inhibition can be made. Certainly, more research is needed focusing on thyroid agonists or direct stimulation by T3 and T4 and subsequent effects on reproduction. The limited data on anti-thyroid compounds suggest both male medaka and

fathead minnows may be a more sensitive model. In these species, impairment of the appearance of secondary sex characteristics, such as papillary processes on the anal fin in medaka or nuptial tubercles in minnows, may become apparent after short-term exposures. However, it is unclear whether significant goiter formation can occur in these species over the relatively short exposure periods (14 to 21 days) used in screening assays. In this respect, histopathological analysis of the thyroid follicles would be particularly helpful in identifying anti-thyroidal chemicals.

9.4 Strengths 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 RESPONSE TO OTHER HORMONAL DISTURBANCES

The reproductive endocrine system of fish is complex and is not restricted to the actions of estrogens, androgens, and thyroid hormones. Indeed, endocrine tissues "upstream" of the gonads and thyroid gland, namely the pituitary and hypothalamus, are the central regulators of reproductive activities (Peter & Crim 1979). The hypothalamus stimulates the release of two gonadotropic hormones (GTHs), follicle-stimulating hormone (FSH, GTH I) and luteinizing hormone (LH, GTH II), from the anterior pituitary gland in fish. These GTHs regulate steroidogenesis and gametogenesis (Schulz et al., 2001; Vanderkraak et al., 1998). In turn, estrogens and androgens, via receptors in the pituitary and hypothalamus, modify the release of GTHs through a system of elaborate feedback pathways (Goos 1987; Iwamatsu et al., 1999). Endocrine disruptors that mimic natural estrogens or androgens could interfere with GTH regulation through the hypothalmo-pituitary axis and affect reproductive development (Arcand-Hoy & Benson 1998).

Environmental factors that affect GTH function could affect other endocrine systems in the gonads of fish that have direct consequences on reproduction. One of the most important and well-studied in fish is that of the progestin sex steroids. Progestins are critical for final gamete maturation (Nagahama 1999) and have been shown, particularly in cyprinids, to act as pheromones involved with reproductive behavior (Stacey et al., 1994). Progestins are discussed in this review primarily for their potential to be a useful endpoint, since they play key reproductive roles.

10.1 Gonadotropic Hormones

Few studies have investigated GTH response directly as a consequence of exposure to endocrine disruptors in fish. One study in the platyfish indicates effects of endocrine disruptors on the brain and pituitary that affect the gonads (Hamazaki et al., 1987). Other research provides indirect evidence. An extragonadal mode of action could explain the findings of Jobling et al. (Jobling et al., 1996) and Ashfield et al. (1998), in which *in vivo* exposure to nonylphenol was shown to reduce testicular and ovarian growth in sexually maturing rainbow trout. The mechanism of action of this endocrine disruptor could be on the gonad directly, or indirectly via effects on GTH synthesis or release from the pituitary. Valuable endpoints for detecting the effects of endocrine disruptors would be circulating plasma GTH levels in exposed fish versus a suitable control group. In salmonids, FSH (GTH I) and LH (GTH II) are released at different points during the reproductive cycle (Prat et al., 1996; Swanson et al., 1989); FSH appears first in the blood and controls gametogenesis, whereas LH is released later and regulates final gamete maturation and spawning. There is scope to measure these GTHs independently, and it is possible that one may serve as a more sensitive or suitable endpoint over the other at key points during the reproductive cycle.

10.2 Progestins

The progestins are a unique class of sex-steroid hormones produced by the gonads and are viable candidates to be affected by endocrine disruptors. Progestins usually appear suddenly in the blood of fish and in high concentration (i.e., ng/ml plasma) during the final period of reproductive development in the female and male (Scott et al., 1983; Truscott et al., 1986). They have been shown to be critical for the final maturation of the gametes from both sexes, and are often referred to as the maturation-inducing steroid. Substantial evidence suggests that in some fish, such as the goldfish, progestins also serve as sex pheromones (Stacey et al., 1994). A number of different progestins (e.g., 17α, 20β-dihydroxyprogesterone [17,20-DHP], 17α, 20β, 21-trihydroxyprogesterone [17,20,21-THP]) have been detected in bony fish, and the major maturation-inducing steroid is not the same in all fish species that have been studied to date. Although in test species such as the zebrafish, 17,20-DHP is the most potent sex steroid at initiating oocyte maturation (Selman et al., 1994). The regulation of progestin synthesis in the gonads is through LH; therefore, environmental agents that affect GTH synthesis or release during the final phase of reproductive development could be expected to influence progestin production.

No known environmental agents that mimic natural progestins in fish or antagonize the receptors for these steroids have been described. One study, however, has shown that exposure to an environmental estrogen, EE2, can significantly affect progestin status (Schultz et al., 2002). Plasma 17,20-DHP levels were significantly elevated in sexually maturing male rainbow trout exposed sublethally to EE2 for 2 months leading up to the time of spawning. A variety of factors could have affected plasma levels, including endocrine perturbations in extra-testicular tissues, altered binding of 17,20-DHP to plasma proteins, and/or decreased elimination of 17,20-DHP. Interestingly, the quality of the sperm from the exposed fish was compromised, as determined from fertilization trials.

11.0 TECHNICAL ASPECTS OF MEASUREMENT OF BIOCHEMICAL ENDPOINTS

11.1 Measurements of Sex Steroids in Tissues

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

In the intact animal, the blood vascular system is the most convenient and popular to sample for sex-steroid measurements in fish. Provided the animal is large enough (i.e., >50 g), a blood sample can be easily obtained in a non-lethal manner via heart puncture or caudal vessels with a needle and syringe. It should be noted that toxicant exposure can alter red-blood-cell volume, resulting in a change in concentration of plasma constituents; therefore, hematocrit analysis is recommended as a preliminary step before quantification of steroids in plasma is initiated. A 100-µL blood sample will yield sufficient plasma (~40 µL) to permit detection of most of the common sex steroids with currently available assays, provided the fish has begun to sexually mature. In the case of small fish, lethal blood sampling, in which the tail is excised, might be required. In addition, when blood volumes are limited from small fish, pooling from a number of individuals might be necessary. Sex steroids have been measured in homogenates of small, whole fish with mixed results, depending on the assay used (e.g., fish embryos) (Feist et al., 1990; Okimoto et al., 1991). For sex-steroid measurement, considerations of body size, the

number of individuals in each treatment group, and state of sexual maturity need to be incorporated into experimental and statistical designs.

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

11.1.1 Estrogens

Estrogens belong to the estrane (i.e., C_{18}) series of steroid hormones, with a phenolic group at Position 3. The ovaries of sexually maturing bony fish have a high synthetic capacity for estrogen production (Vanbohemen & Lambert1981). The principal estrogen in bony fish is 17β-E2, although estrone and estriol have also been detected (Ozon 1972a). In the female, estrogens are significant for their involvement with oocyte development and female sexual behavior (Fostier et al., 1983). The highest concentrations of E2 are found in body fluids, particularly blood plasma, of sexually maturing females. In bony fish, plasma concentrations of E2 range up to and exceed 100 ng E2/mL plasma, depending on the species and state of sexual maturity (SCOTT et al., 1980). Much lower E2 levels, with a maximum of usually not more than 1.0 ng E2/mL plasma, have been reported in the males of some species (Truscott et al., 1986; Makynen et al., 2000; Nichols et al., 1999). The adrenal/interrenal tissues or testes in the male might be responsible for this biosynthesis.

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

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

Quantification of E2 by RIA has been done primarily to determine the sex of the fish when this cannot be easily deduced from external morphology, and/or to assess the state of sexual maturity in known females. Schulz (1984) showed that increasing concentrations of plasma E2 in female rainbow trout correlates well with a histological staging scheme based on increasing oocyte size, histological appearance of the ovaries, and GSI. The available data on

plasma E2 measurements by RIA in fathead minnow in relation to a reproductive cycle are limited. Jensen et al. (2001) reported mean plasma levels peaking at 10 ng E2/mL one day after spawning before decreasing to ~4 ng E2/mL. Average plasma E2 concentrations are in the range of 6 ng E2/mL and 0.5 ng E2/mL in mature females and males, respectively. One study reports plasma E2 levels measured by RIA in mature female medaka during the daily period of vitellogenesis and spawning (Soyano et al., 1993). These data show low levels of 4 ng E2/mL plasma at 16 hours before spawning, with a peak of 16 ng E2/mL plasma at 8 hours prior to spawning before levels decrease again. There are no available plasma E2 data measured by RIA for zebrafish.

11.1.1.2 Enzyme-linked Immunosorbent Assay. The principle of ELISA is also based on the reaction between an antigen (the steroid of interest) and a specific antibody, one of which is passively adsorbed to a solid surface (e.g., the wells of a microtiter plate (Crowther 1995). There are a variety of schemes for ELISA, but the one used most widely for sex-steroid measurements is a modification of the direct-labeled antigen method (Varadaraj 1990). In this ELISA, the antibody is attached to the solid phase via a secondary antibody, and the antigen, both non-isotopically labeled and unlabeled steroid, is added to compete for the antibody. The labeled steroid is modified by addition of an enzyme moiety (e.g., acetylcholinesterase). After a suitable incubation period, the steroids are washed out of the well, and color development reagents are added to react with the bound labeled steroid remaining. Provided the amount of labeled steroid is held constant, increasing amounts of unlabeled steroid will reduce the amount of labeled steroid that is bound, resulting in a decrease in the amount of color development detected. Therefore, the degree of color development will be inversely proportional to the amount of unlabeled steroid added. The specific wavelength of the color produced is detected by a spectrophotometer. A standard curve can be developed with known amounts of unlabeled steroid, with which unknown samples can be compared.

Commercially developed ELISA kits for E2 (e.g., Cayman Chemical) are available, but have not been applied extensively to measurement of E2 levels in fishes. A limited number of individual research laboratories have reported E2 ELISA methods used on fish plasma with in-house developed reagents (Asahina et al., 1989; Okimoto et al., 1991; Varadaraj1990). These assays are sensitive (detection limit = 10 pg E2/mL plasma) and specific, with minimum cross-reactivity with other related steroids. It appears that ELISAs are not suitable for quantifying E2 in whole-body homogenates of fish because of protein or lipid interference in the assay (Okimoto et al., 1991). Similar to RIA, the principal consideration in the application of ELISA is the volume of blood necessary in order to obtain sufficient plasma (i.e., 5 µL to 10 µL) with which to work, in conjunction with the reproductive status of the test fish.

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

11.1.1.3 Liquid/Gas Chromatography with Mass Selective Detection (LC/GC-MS). Analysis of sex steroids using gas-liquid chromatographic techniques and mass selective detection has been performed since the mid 1960s (see review of early studies by Sjovall & Axelson 1982). Routine analysis of sex steroids using mass spectrometry is a more recent occurrence with the

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

As with any quantitative analytical method utilizing chromatography, an internal standard(s) is added to account for sample loss during subsequent steps in the analysis and fluctuations in detector response. Preferred internal standards for MS analysis are isotopically labeled steroid analogues, especially those synthesized with carbon-13, which typically contain minimal quantities of unlabeled steroid (Wolthers & Kraan 1999). The internal standard is added directly to the sample prior to extraction and later during the MS detection, a minimum of two fragment ions are simultaneously measured that originate from the endogenous steroid and labeled analogue. The ratio of the respective peak heights (or peak areas) is then used for quantitation. For some sex steroids such as E2 and T, ^{13}C-labeled analogues are available at a reasonable cost. However, for other reproductive hormones such as 11-KT, this may not be the case and structural analogues as opposed to isotopic analogues are used as internal standards. In the case of 11-KT, non-endogenous anabolic steroids such as 19-*nor*-testosterone can be used as an internal standard (Schultz et al., 2001).

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

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

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

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

11.1.2 Androgens

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

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

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

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

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

11.1.3 Progestins

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

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

11.1.4 Summary

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

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

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

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

11.2 Measurement of Vitellogenin

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

development of a standard procedure for VTG measurement, as batch differences in binding affinity for VTG could be excessive.

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

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

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

11.2.2 Direct Quantification of Vitellogenin Protein: RIA

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

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

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

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

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

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

Antibody-capture. In an antibody-capture competitive ELISA, a known amount of antigen is coated onto the surface of the microwell plate, and the binding sites that remain free of antigen are blocked. Known amounts of the primary antibody are pre-incubated with samples, or standards, containing variable amounts of antigen. The antigen binds to the antibody such that if a sample has a high level of antigen, only a small amount of antibody will be free to bind to the antigen coating the plate. Following this pre-incubation, the samples are added to the wells of the

plate, and the antibodies that did not bind to the antigen in the sample bind to the test plate. In the next step, a known amount of the enzyme-linked secondary antibody specific to the primary antibody is added to the wells. An enzyme substrate is added and the color change is measured by a microwell plate reader. Because of the competitive nature of this ELISA, the color change is inversely proportional to the antigen present in the sample.

In the late 1980s, Nunez Rodriguez et al. (1989) developed and validated an antibody-capture competitive ELISA for sole (*Solea vulgaris*) VTG. The assay used a polyclonal antibody to circulating VTG and was based upon the competition between soluble VTG in the samples and standards and the VTG adsorbed on the microwell plates. The time-course of the assay was relatively brief, the reagents were deemed stable and inexpensive when compared with other methods (RIA), the intra- and inter-assay reproducibility provided comparable values, parallelism of the competition curves demonstrated the integrity of the assay, and the sensitivity was 2.5 ng/mL VTG in the sample assayed (125 ng/mL plasma). Following this protocol, antibody-capture competitive ELISAs were developed and validated for a wide range of teleosts, including fathead minnow (*Pimephales promelas*) (Nakamura 2000), and carp (*Cyprinus carpio*) (Edmunds et al., 2000). The reported sensitivity of these homologous antibody-capture competitive ELISAs expressed as the lowest detectable standard in the working range of the assay (approximately 80% to 90% of binding) range from 1 ng/mL to 33 ng/mL in the sample analyzed, which is comparable to the sensitivity achieved with RIA and sandwich ELISA (Blader & Strahle1998; Edmunds et al., 2000; Giesy et al., 2000; Hatakeyama et al., 1999; Maack et al., 1999; Nakamura 2000; Rodina & Horvath 1999; Scholz & Gutzeit 2000; Shepherd et al., 2001; Tanaka et al., 2001; Tyler et al., 1999). It should be noted that although ELISA detection limits in the low ng/mL range are reported, the practical detection limit for accurate quantification of low levels of VTG in samples is significantly higher. This is because of the required dilution of the sample resulting from small plasma volumes available from individual test fish, or because of the need to eliminate interferences in the ELISA.

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

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

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

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

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

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

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

Aspects of the materials and methods for the teleost sandwich ELISA are similar to the competitive antibody-capture ELISA with the following exceptions. For the secondary antibody, Fab' fragment is prepared from the rabbit IgG and conjugated with enzyme (e.g., horseradish peroxidase). The plates are coated with primary antibody, typically 200 μL of 40 μg/mL,

overnight at 4ºC. The plates are blocked (e.g., BSA), and the samples are diluted and are added directly to the antibody wells. The remaining steps are similar to the competitive antibody-capture ELISA.

In the late 1980s, with the recent application of ELISA to measure VTG in fish-blood plasma with a competitive antibody-capture ELISA (Kiparissis & Metcalfe 1997; Kwon et al., 2000; Shinomiya et al., 1997) developed a homologous sandwich ELISA to detect VTG in the plasma of whitespotted charr (*Salvelinus leucomaenis*). Subsequently, this method was applied to developing and validating similar ELISAs (Bauer & Goetz 1998; Grant 1995; Miles-Richardson et al., 1999a; Wester & Van 2000). In developing the sandwich ELISA for charr, Kwon et al. (2000) prepared antibodies in this ELISA against lipovitellin. Similarly, Okumura et al. (1995) developed a sandwich ELISA for the Japanese eel with anti-lipovitellin antibodies, and the secondary antibody was biotinylated (with steptavidin-HRP steps) to magnify the signal with a detection limit of 0.8 ng/mL. Korsgaard and Pedersen (1998) developed a sandwich ELISA and achieved a detection limit of 1 ng/mL VTG with affinity purification applied to increase the specificity of the anti-VTG antibodies and careful blocking procedures.

A homologous zebrafish sandwich ELISA using anti-lipovitellin antibodies and lipovitellin to generate a standard curve with a detection limit of 0.2 ng/mL sample analyzed has recently been developed and applied to whole body homogenates (Andersen et al., 2000). Purified carp VTG and anti-VTG carp antibodies, or a complete carp VTG sandwich ELISA kit, are commercially available (Biosense). This carp-based sandwich ELISA can be used to measure fathead minnow VTG, and sandwich-based kits to detect zebrafish and Japanese medaka VTG are under development. The carp VTG sandwich ELISA kit has a detection limit of 0.2 ng/mL sample analyzed, with intra- and inter-assay variation of 3% to 8%, and this ELISA can be applied to the fathead minnow to measure VTG induction.

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

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

achieved with the sensitive carp sandwich ELISA through the use of homologous VTG to prepare the standard curve in the assay.

11.2.4 Quantifying Vitellogenin mRNA

An alternative to measuring the VTG protein is to quantify the messenger ribonucleic acid (mRNA) for VTG that codes for the protein. In oviparous fishes, the liver of both sexes contain VTG gene(s) that are responsive to E2 (Carvan et al., 2000b; Mallett 1997; Riehl et al., 1999). Estrogen mimics will upregulate VTG mRNA transcription, which has been shown to be a sensitive indicator of exposure to these compounds (Skinner et al., 1999). There is also evidence that physiological levels of androgen alone can increase VTG mRNA expression by male rainbow trout hepatocytes *in vitro* (Kawahara & Yamashita 2000). Vitellogenin mRNA expression might not, however, result in translation of protein, because plasma VTG levels in male trout are typically very low (Copeland et al., 1986).

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

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

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

In response to E2 injection, fathead minnow VTG mRNA has been measured in liver samples by RPA and shown to be very sensitive, 1000-fold more so than VTG protein detected by ELISA (Kishida & Callard 2001). The measurement of medaka or zebrafish VTG mRNA has

not been attempted with RPA, although zebrafish VTG sequence information is available (Kuwahara et al., 2000) such that probes could be designed.

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

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

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

11.2.5 MS Approaches to Quantification of Vitellogenin

The characterization of protein-based biomarkers such as VTG has become an integral part of a reproductive-screening program. As reviewed in the preceding sections, immunoassays are the most commonly used approach in VTG analysis. Although not without considerable merit, immunoassays can suffer from problems related to antibody specificity and the limitation that only a single specific protein can be assayed during each RIA or ELISA. Thus, development of a secondary detection procedure for VTG suitable for use as a reference method in external quality assessments would be desirable. Additionally, if at some point in the future other biomarker proteins were to be added to the measured endpoints, additional immunoassays need to be developed. In these respects, mass spectrometry (MS) offers the potential for becoming a reference method for VTG (analogous to its use in steroid quantification) and for

combining multiple protein analysis from a single tissue sample. However, the application of MS techniques towards protein quantification in general is a relatively new field of study (Leonil et al., 2000) and has been applied to VTG or structurally related proteins in only a few instances (Chen & Kuo 1998; Reith et al., 2001).

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

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

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

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

12.0 CANDIDATE PROTOCOLS

In the following sections, three protocols have been chosen to be discussed in detail: 1) 14-day fish reproductive assay (modified from the version described in Ankley et al., 2001); 2) 21-day reproductive assay (as described in Ankley et al., 2001); and 3) 14-day fish non-reproductive screen (OECD Draft-31 December 2001). Each of the three protocols is relatively new and has not been through a validation processes. The strengths and weaknesses of the candidate protocols will be discussed in Section 13. Three additional assays were considered but ultimately were not selected for detailed description here. These addtional assays are a 21-day reproductive screen using pair breeding fathead minnows (Harries et al., 2000), a 14 day juvenile

fish protocol that is primarily designed to detect (anti)-estrogens (Panter et al., 2002) and an assay that uses monosex carp that are genetic males. This latter assay has been used with both juvenile and adult carp in tests with weak estrogenic chemicals (Gimeno et al., 1998a; Gimeno et al., 1998b). It also should be pointed out that although the ensuing descriptions are discussed specficially for fathead minnows, they are also adaptable for the Japanese medaka and zebrafish.

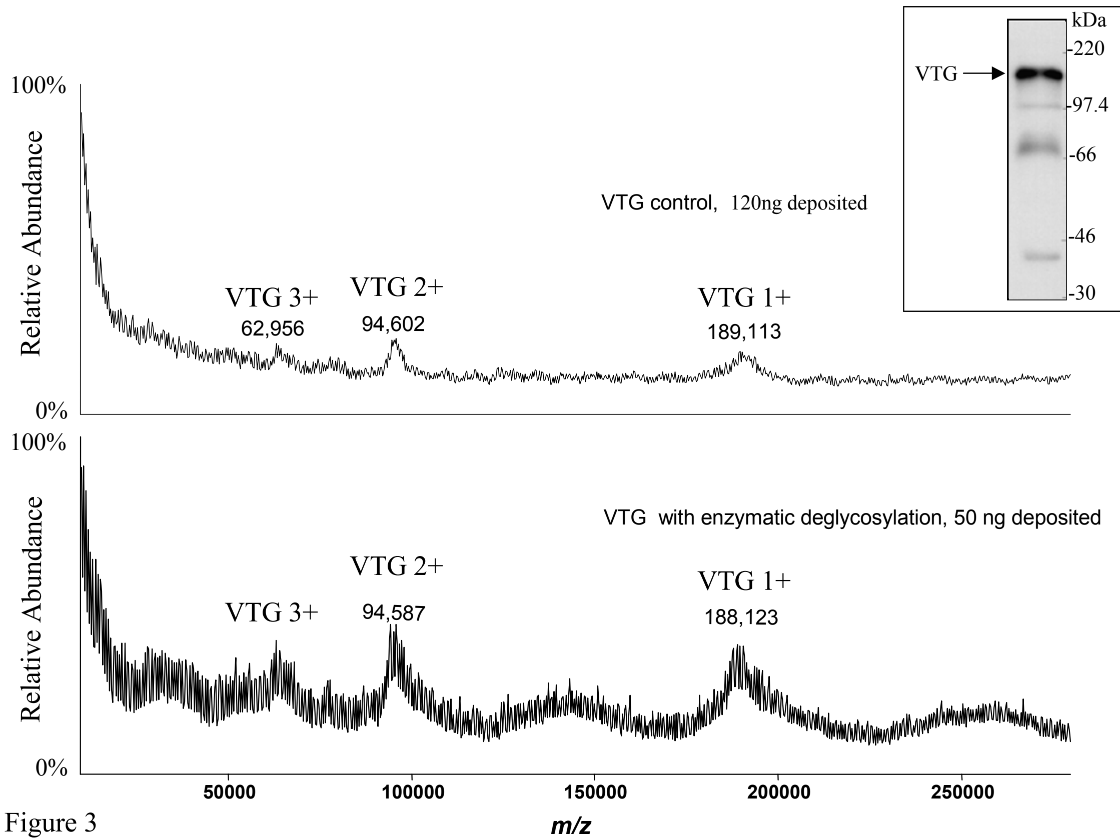

Figure 11-1. MALDI-MS Analysis of Rainbow Trout Vitellogenin With and Without Enzymatic Deglycosylation Step. 120 mg VTG Combined With 500 nL of 10 M Ferrulic Acid/0.1% TFA/25% Formic Acid (data from Wunschel & Wahl 2002). Inset figure is a Western Blot of same VTG sample using a polyclonal antibody against trout VTG (from Schultz et al., 2001)

12.1 Short-term (14-day) Fish Reproductive Screen

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

Note: The protocol described below is modified from the full description of a short-term (21-day) assay method described in EPA (2001).

INTRODUCTION

This protocol describes a 14-day reproduction assay using the fathead minnow (Pimephales promelas). This test is a14-day short-term assay that measures the reproductive performance of groups of fathead minnows as the primary indicator for endocrine disruption.

Additional measurements of morphology, histopathology, and biochemical endpoints are performed to aid identification of the specific toxicological mode of action of the test chemical.

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

PRINCIPLE OF THE ASSAY

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

DESCRIPTION OF THE METHOD

Test Animals and Assay System

Test Animals: The procedure described here is initiated with adults, as opposed to embryos or larvae. The test should be started with newly-mature fish (typically 4-6 months old), as opposed to older animals that have been actively reproducing for some period of time, for example, in a culture setting. Thus, to maintain a ready supply of known-quality animals at the desired age for routine testing, it is preferable to maintain a fathead minnow culture, as opposed to purchasing the animals prior to testing. Field collected fathead minnows generally should not be used to initiate cultures or for testing. Information on general culture and testing requirements for the fathead minnow are provided in Table 12-2.

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

Assay System: Glass, stainless steel, or other chemically inert material should be used for construction of the test system. The dimensions of the test chambers must be such that the animals can interact in a fashion conducive with successful spawning. The test chamber contains 10 L of test solution, which is renewed at least once every 4 h. This particular animal loading/water renewal rate is within recommended guidelines and in studies conducted according to this method, has maintained acceptable water quality (Tables 12-1, 12-2), while not utilizing an excessive amount of test material.

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

Test type	Flow-through
Water temperature	25 ±1ºC
Illumination	Quality Fluorescent bulbs (wide spectrum)
Light intensity	10-20 ìE/M2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)
Photoperiod	16 h light, 8 h dark
Test chamber size	18 L (40 x 20 x 20 cm) (minimum)
Test solution vol.	10 L
Volume exchanges of test solutions	Minimum of six daily
Flow rate	Approx 3.5L/g fish/day
Age of test organisms	Reproducing adults (120 day minimum)
No. of fish per test chamber	4 females and 2 males
No. of treatments	2 (plus appropriate controls)
No. replicate test chambers per	4
No. of fish per test	16 females and 8 males
Feeding regime	Frozen adult brine shrimp twice daily
Aeration	None unless DO concentration falls below 4.9 mg/L
Dilution water	Clean surface, well, or reconstituted water
Dilution factor	37385
Chemical exposure duration	21-day test; 14-day test
Endpoints	Adult survival, reproductive behavior, secondary sexual characteristics, GSI and gonadal histology, plasma VTG and sex steroids (E2, T, 11-KT) concentrations, fecundity, fertility, embryo hatch and larval survival.
Test acceptability	Dissolved oxygen >60% of saturation; mean temperature of 25 ±1ºC; 90% survival of in the controls; successful egg production in controls

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

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

Experimental Design

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

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

Exposure via Water

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

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

Analytical Determination

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

PERFORMANCE OF THE ASSAY

Assay Initiation and Conduct

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

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

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

Observations

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

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

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

Fertilization Success: After the spawning substrate has been removed from the tank, the embryos should be carefully rolled off it with a gentle circular motion of an index finger and visually inspected under appropriate magnification. If spawning occurred that morning, embryos typically will be undergoing late cleavage, and determination of the fertility rate (number embryos/number of eggs x 100) is easily achieved. Infertile eggs are opaque or clear with a

white dot where the yolk has precipitated; viable embryos remain clear for 36 to 48 h until reaching the eyed stage. An alternative to the microscopic approach to determining fertilization success is to enumerate eyed embryos at this time. If the latter approach is used for fertility determination, the embryos should be placed in "incubation" chambers and held in a system apart from the adults to avoid possible predation.

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

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

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

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

Blood Sampling: At the conclusion of the exposure, the fish will be anesthetized by transfer to an oxygenated solution of MS-222 (100 mg/L buffered with 200 mg NaHCO3/L), and weighed. Blood will be collected from the caudal artery/vein with a heparinized microhematocrit capillary tubule. Depending upon the size of the fathead minnow (which usually is sex-dependent), blood volumes generally range from 30 to 80 µl. Plasma is separated from the blood via centrifugation (3 minutes at 15,000 x g) and stored with protease inhibitors at -80ºC, until analyzed for VTG and sex steroids.

Gonad Size and Morphology

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

Routine histological procedures can be used to assess condition of testes and ovaries from the fish. Gonads should be placed in an appropriate fixative, such as 4% formaldehyde/1% glutaraldehyde, and embedded in paraffin or plastic. Serial sections 4 µm to 5 µm thick should

be cut along the long axis of the gonad. At a minimum, two serial sections should be collected from at least three steps equally spaced between the leading edge of the tissue and the midline of the gonad, for a total of six-tissue sections/sample. Sections can be stained with hematoxylin and eosin, and should be evaluated by an experienced histologist without prior knowledge of the treatment regime associated with specific samples. In general, evaluation of the testis is based on the amount of germinal epithelium present, and the degree of spermatogenic activity. The ovary is evaluated based upon relative numbers of perinucleolar, cortical alveolar, and vitellogenic oocytes.

Vitellogenin

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

Sex Steroids

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

PERFORMANCE CRITERIA

- Water quality characteristics should remain within the limits of tolerance depicted in Tables 1 and 2
- There should be documentation (via appropriate analytical chemistry) of purity of the test material, as well as delivery of chemical to the fish (e.g., concentrations of the chemical in test water)
- There should be more than 90% survival of control animals over the duration of the chemical exposure, and the control fish in each replicate should spawn, at a minimum, every 3 to 4 days
- There should be greater than 95% fertility and hatchability of eggs and embryos, respectively, from the control animals.

DATA REPORTING

Treatment and Interpretation of Results

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

Test Report

The test report must include the following:

Test Substance:

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

- Additional information should be provided, when available, such as its solubility in water, octanolwater partition coefficient, vapor pressure, toxicity to fathead minnow.

Test Species:

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

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

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

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

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

- The photoperiod and light intensity used during the exposure.

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

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

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

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

Results:

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

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

12.2 Short-term (21-day) Reproduction Assay with the Fathead Minnow for Identification of Endocrine Disrupting Chemicals

Note: The protocol description below is a summary of the full description detailed in EPA (2001).

INTRODUCTION

This guideline describes a 21-day reproduction assay with the fathead minnow *(Pimephales promelas)* that considers reproductive fitness as an integrated measure of toxicant effects. It also enables measurement of a suite of histological and biochemical endpoints that potentially are directly reflective of effects associated with the classes of EDCs of concern. The test is a short-term assay that assesses reproduction and also could incorporate aspects of early development.

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

PRINCIPLE OF THE TEST

An overview of the test and relevant test conditions are provided in Table 12-2. The test is initiated with mature adults that have a record of reproductive success as measured both by fecundity (number of eggs) and embryo viability (e.g., hatchability). This is established during a "pre-exposure" period of 14 days to 21 days in the same system/tanks as will be utilized for the chemical test. The test is conducted at a minimum of two chemical concentrations, as well as appropriate controls, with a minimum of four experimental units (replicates) per treatment. Each replicate tank contains four female and two male fish. Chemical delivery can be via an aqueous route (with or without carrier solvents). The exposure is conducted for up to 21 days, during which appearance of the fish, behavior, and fecundity are assessed daily. Viability of resultant embryos (e.g., hatching success, developmental rate, occurrence of malformations, etc.) can be assessed in animals held either in clean water, or the same treatment regime to which the adults were exposed. At conclusion of the test, blood samples are collected from the adults for determination of sex steroids and VTG, and the gonads sampled for measurement of the GSI and histological analyses.

DESCRIPTION OF THE METHOD

Test animals and assay system

Test animals: The procedure described herein is unique to most fathead minnow tests to date in that it is initiated with adults, as opposed to embryos or larvae. The test should be started with newly-mature fish (typically 4 to 6 months old), as opposed to older animals that have been actively reproducing for some period of time, for example, in a culture setting. Thus, to maintain a ready supply of known-quality animals at the desired age for routine testing, it is preferable to maintain a fathead minnow culture, as opposed to purchasing the animals prior to testing. Field collected fathead minnows generally should not be used to initiate cultures or for testing. Information on general culture and testing requirements for the fathead minnow are provided in Table 12-2.

Water: The fathead minnow can reproduce successfully over a wide range of water quality. Therefore, no specific water type is required for this test. Any uncontaminated surface,

well, or reconstituted water in which the fish can be cultured successfully should be acceptable. Minimal recommended water quality characteristics are listed in Table 12-2. The animals should be tested using a flow-through water renewal system that enables maintenance of adequate water quality (temperature, dissolved oxygen, low ammonia, etc.), as well as ensuring a consistent exposure to the parent chemical (for those tests where water is the route of exposure).

Assay System: Glass, stainless steel, or other chemically inert material should be used for construction of the test system. The dimensions of the test chambers must be such that the animals can interact in a fashion conducive with successful spawning. The test chamber contains 10 L of test solution, which is renewed at least once every 4 h. This particular animal loading/water renewal rate is within recommended guidelines and in studies conducted according to this method and has maintained acceptable water quality (Tables 12-1 and 12-2), while not utilizing an excessive amount of test material.

Experimental Design

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

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

Exposure via Water

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

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

Analytical Determination

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

PERFORMANCE OF THE ASSAY

Assay Initiation and Conduct

Pre-exposure: The pre-exposure phase of the assay should be started with animals that have achieved reproductive maturity, as evidenced by initial development of secondary sex characteristics, but have not been held in a culture/test situation conducive to routine spawning. These animals, which typically are 4 to 6 months old, are held in a "mass" culture tank that corresponds to the date the animals were hatched. They typically represent the pooled offspring of 10 to 20 pairs of adult fish from the culture facility. Four females and two males should be randomly assigned to the replicate exposure chambers at each anticipated treatment concentration. In addition to the number of replicates actually exposed at each concentration (e.g., four), a number of additional tanks also should be started; these can serve as "replacement" units for tanks in which pre-exposure spawning is not observed, and/or mortality occurs before initiation of the chemical exposure. Identification of gender may be difficult to resolve for some fish; these animals should not be used for the assay. At this stage in development, males will exhibit nuptial tubercles, while females possess an ovipositor; in addition, males tend to be larger and darker than females from the same cohort.

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

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

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

Observations

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

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

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

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

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

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

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

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

Appearance of Adults: Observations of physical appearance of the adults should be made over the course of the test, and at conclusion of the study. From the perspective of screening EDCs, characteristics of particular importance include: body color (light or dark), coloration

patterns (presence of vertical bands), body shape (head and pectoral region), and specialized secondary sex characteristics (size of dorsal nape pad, number of nuptial tubercles in males; ovipositor size in females). Notably, chemicals with certain MOA cause abnormal occurrence of secondary sex characteristic in animals of the opposite sex.

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

GONAD SIZE AND MORPHOLOGY

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

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

VITELLOGENIN

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

SEX STEROIDS

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

PERFORMANCE CRITERIA

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

- There should be documentation (via appropriate analytical chemistry) of purity of the test material, as well as delivery of chemical to the fish (e.g., concentrations of the chemical in test water)
- There should be more than 90% survival of control animals over the duration of the chemical exposure, and the control fish in each replicate should spawn, at a minimum, every 3 to 4 days
- There should be greater than 95% fertility and hatchability of eggs and embryos, respectively, from the control animals.

DATA REPORTING

Treatment and Interpretation of Results

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

Test report

The test report must include the following:

Test substance:

- The report must include a detailed description of the test substance, including information on its CAS number, source, lot number and purity.
- Additional information should be provided, when available, such as its solubility in water, octanolwater partition coefficient, vapor pressure, toxicity to fathead minnow.

Test species:

- Information must be provided on the fathead minnows used in the test. This information must include the source of the fish, age and condition of the fish at the initiation of the test, and the pre-exposure reproductive performance.
- Any observed abnormalities in reproductive behavior or performance of control fish must also be reported.

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

- Information on the source, treatment of, and basic chemical characteristics of the dilution water.
- Means and ranges for water temperature, dissolved oxygen, pH, hardness, alkalinity and conductivity.
- The photoperiod and light intensity used during the exposure.
- The chamber size, number of females and males per replicate, and number and composition of spawning substrates.

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

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

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

Results:

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

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

12.3 Short-term (14-day) Non-spawning Fish Screening Assay

Note The following protocol is adapted from a draft guideline of an OECD workgroup (Draft proposal–31 December 2001) prepared by Tom Hutchinson based on procedures employed at the Brixham Laboratories of AstraZeneca.

INTRODUCTION

This test protocol describes a 14-day bioassay for identifying EDCs in adult fathead minnows based on the protocol described by the OECD Draft proposal–31 December 2001. The test is intended to detect the EDCs with a mode of action that is (anti)- androgenic or (anti)-estrogenic. This 14-day test is intended to identify changes in biochemical, morphological, and histological endpoints considered to be endocrine specific in fathead minnows. Reproductive performance parameters are not assessed in this test protocol. The concept for this protocol is derived from work in Europe on the fathead minnow (*Pimephales promelas*) (Panter et al., 1998a and 1998b) and in North America (Ankley et al., 2001).

This test guideline addresses a 14-day chemical exposure period that does not require a pre-exposure validation period. The test is started with mature male and female fish. During the subsequent 14-day chemical exposure, survival and secondary sexual characteristics are observed. At termination of the test, measurements are made of external morphology, GSI, gonadal histology, and plasma concentrations of VTG and sex steroids (E2, T, 11-KT). This 14-day period may be reduced if the exposed fish exhibit a clear response to the test chemical in terms of altered secondary sexual characteristics (most likely to occur during methyl-testosterone exposures [Ankley et al, 2001]).

PRINCIPLE OF THE ASSAY

An overview of the assay and relevant test conditions is provided in Table 12-3. The assay is initiated with healthy, sexually dimorphic adult fish (males and females contained in separate test chambers to prevent induction of spawning). To minimize the confounding effect of natural spawning cycles, these fish are selected from pre-spawning populations. The assay is conducted using three chemical concentrations, as well as controls, with two individual vessels per treatment (one vessel containing 10 males, the other 10 females). Chemical delivery is preferably by a flow-through water system or orally by adding the test chemical to food.

Table 12-3. Short-term (14-day) Non-spawning Fish Screening Assay

Test type	Flow-through
Water temperature	25 ±2ºC
Illumination	Fluorescent bulbs (wide spectrum)
Light intensity	10-20 µE/M2/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)
Photoperiod	16 h light, 8 h dark
Test chamber size	18 L (40 x 20 x 20 cm) (minimum)
Test solution volume	10 L
Volume exchanges of test solutions	Minimum of five daily
Flow rate	Approx 3.5L/g fish/day
Age of test organisms	Pre-breeding Adults (5-6 months)
Weight of Fish	Females: 1.5 +/- 10%; Males 2.5 +/- 10%
No. of fish per test chamber	10
No. of treatments	3 minimum (plus appropriate controls)
No test chambers per treatment	2
No. of fish per test concentration	10 females and 10 males
Feeding regime	Frozen adult brine shrimp twice daily
Aeration	None unless DO concentration falls below 4.9 mg/L
Dilution water	Clean surface, well, or reconstituted water
Dilution factor	37324
Pre-Exposure period	None
Chemical exposure duration	14-d
Endpoints	Adult survival & behavior; secondary sexual characteristics, GSI, and gonadal histology, plasma VTG and sex steroids (E2, T, 11-KT) concentrations.
Test acceptability	Dissolved oxygen >60% of saturation; mean temperature of 25 ±2ºC; 90% survival of in the controls

The test duration is 14 days, during which the behavior and appearance of the fish are observed daily. At the end of the test fish are terminated in MS-222 solution and blood samples are collected for determination of VTG and sex steroids as described for the previous 14 / 21 day reproductive performance assays. The gonads are removed for measurement of the GSI and histological analyses. Early signs of a treatment induced effect, such as male fish appearing feminized, may indicate that completing other endpoint analyses (VTG and gonad histology) is not necessary. In all cases, however, blood and gonads must be collected to support the gross observations with biochemical (VTG) or histological endpoints, if necessary.

DESCRIPTION OF THE METHOD

Test Animals and Assay System

Test animals: The test should be started with mature fish (at least five to six months old). Test fish should be selected from a population of a single stock, preferably from the same spawning, acclimated for at least two weeks prior to the test under conditions of water quality and illumination similar to those used in the test. Fish will be fed a ration of 1% body weight per day throughout the holding period and during the 14-day bioassay.

Water: No specific water type is required for this test. Any uncontaminated surface, well, or reconstituted water in which the fish can be cultured successfully should be acceptable. Minimal recommended water quality characteristics are listed in Table 2. The animals will be tested using a flow-through water renewal system that maintains adequate water quality (temperature, DO, low ammonia, etc.), and ensures a consistent exposure to the parentchemical (for those tests where water is the route of exposure).

Assay system: Five-gallon glass exposure vessels will be used for the test system. As recommended by Ankley et al. (2001), the dimensions of the test chambers must be such that the animals can interact in a fashion conducive to successful spawning. Each test chamber contains 10 L of test solution, which is renewed at least once every four hours. This particular animal loading/water renewal rate is within recommended guidelines and has maintained acceptable water quality in studies conducted according to this method (Tables 12-1 and 12-2), while not using an excessive amount of test material.

Experimental Design

For the 14-day assay, the highest concentration should not have caused significant mortality in previous range-finding tests (note, this may also be determined by water solubility limitations) and the lower concentration/dose should be a factor of 5 to 10 times lower than the highest test concentration. The third exposure concentration will be intermediate between the high and low concentrations in the 14/21-day reproductive performance assays. Two exposure tanks are used for each test concentration. One replicate will contain 10 males and the other replicate will contain 10 females. Two control tanks also are used containing 10 males and 10 females. Based on this design, a minimum of 80 fish is required per assay (20 fish in each of three treatments, plus one control).

Exposure via Water

Water exposures are preferably conducted using a proportional diluter without a carrier solvent. When necessary, concentrated aqueous stock solutions of the test chemical will be prepared using a saturator method described by Kahl et al. (1999).

Analytical Determination

Water concentrations of the test chemical in the exposure chambers should be measured prior to adding fish to verify target concentrations. Additionally, water samples should be analyzed weekly for the test chemicals.

PERFORMANCE OF THE ASSAY

Assay Initiation and Conduct

Chemical exposure: After a 48-hr acclimatization period, chemical exposure will be initiated and continued for 14 days during which time behavior and appearance of the fish will be observed on a daily basis.

Observations

Fish will be examined daily during the test period, and any external abnormalities (such as hemorrhage or discoloration) will be noted. Any mortality is recorded and the dead fish removed as soon as possible. Dead fish will not be replaced.

Behavior of Adults: Abnormal behavior (relative to controls) such as hyperventilation, loss of equilibrium, or feeding abstinence is noted during the daily observations. Alterations in reproductive behavior, particularly loss of territorial aggressiveness by males, also should be noted.

Appearance of Adults: The external appearance of the adults should be assessed as part of the daily observations, and any unusual changes noted. External features of particular importance include body color (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and specialized secondary sex characteristics (size of dorsal nape pad, number of nuptial tubercles in males; ovipositor size in females). These observations are especially important for assessing endocrine active agents that are (anti)-androgenic.

Termination At 14-Days: Gonad Size snd Morphology and Biochemical Endpoints (VTG; optional measurement of sex steroids)

Blood collection: At conclusion of the exposure, fish are anesthetized by transfer to an oxygenated solution of MS-222 (100 mg/L buffered with 200 mg $NaHCO_3$/L) and weighed, and blood collected from the caudal artery/vein with a heparinized microhematocrit capillary tubule. Depending upon fathead minnow size (which usually is sex dependent), blood volumes generally range from 30 to 80 µl. Plasma is separated from the blood via centrifugation (3 min at 15,000 x g) and stored with protease inhibitors at -80ºC until analyzed for VTG and sex steroids.

Gonad size snd morphology: After blood sampling, fish should be weighed, and the gonads removed and weighed (to the nearest 0.1 mg) for determination of the GSI (GSI = 100 x gonad wt/body wt). Typical GSI values for reproductively active fathead minnows range from 8% to 13% for females and from 1% to 2% for males. Many chemicals that reduce fecundity also will reduce the GSI in one or both sexes. After removal of the gonads, the remainder of the fish carcass will be discarded.

Routine histological procedures should be used to assess the condition of the testes and ovaries from the fish. Gonads will be placed in fixative, (4% formaldehyde/1% glutaraldehyde) and embedded in paraffin. Serial sections 4 µm to 5 µm thick will be cut along the long axis of the gonad. At least two serial sections should be collected from at least three areas equally

spaced between the leading edge of the tissue and the midline of the gonad, for a total of six tissue sections per sample. Sections are stained with hematoxylin and eosin and evaluated by an experienced histologist without prior knowledge of the treatment regime associated with specific samples. Evaluation of the testis is based on the amount of germinal epithelium present and the degree of spermatogenic activity. The ovary is evaluated based upon relative numbers of perinucleolar, cortical alveolar, and vitellogenic oocytes.

Vitellogenin: VTG in plasma samples can be measured using ELISA. For the ELISA, either fathead minnow or polyclonal Carp (*Cyprinus carpio*) VTG antibody and purified VTG protein can be used.

Sex Steroids

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

Performance Criteria

- Water quality characteristics should remain within the limits of tolerance depicted in Tables 1 and 2.
- Purity of the test material must be documented by the vendor (for test compounds purchased commercially). Documentation also will be maintained for chemical analysis of exposure water (e.g., verification of exposure concentrations of the chemical in test water).

DATA REPORTING

Treatment and Interpretation of Results

Any endpoints that are significantly affected by the test chemical will be reported as such.

Test Report

The test report will include the following:

Test Substance:

- The report will include a detailed description of the test substance, including information on its CAS number, source, lot number, and purity.
- Additional information should be provided, when available, such as solubility in water, octanol:water partition coefficient, vapor pressure, and toxicity to fathead minnow.

Test Species:

- Information that must be provided on the fathead minnows used in the test includes the source of the fish, age and condition of the fish at the initiation of the test, and the pre-exposure reproductive performance.
- Any observed abnormalities in reproductive behavior or performance of control fish must also be reported.

Test conditions: The report must specify the following conditions under which the test was performed:

- Source, treatment, and basic chemical characteristics of the dilution water
- Means and ranges for water temperature, dissolved oxygen, pH, hardness, alkalinity, and conductivity
- Photoperiod and light intensity used during the exposure
- Chamber size, number of females and males per replicate, and number and composition of spawning substrates
- Food used to feed the fish during the exposure, including supplier and lot number
- Basic nature of exposure (i.e., flow-through) in addition to specific information related to the exposure type (e.g., the number of volume exchanges of dilution water daily)
- Use of solvent or dispersant if any, the specific solvent or dispersant and the concentrations to which the fish were exposed.

Results:

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

13.0 RECOMMENDED PROTOCOL AND ADDITIONAL DATA NEEDS

13.1 Preferred Test Species

The Fathead minnow (*Pimephales promelas*) was selected as the preferred test species based upon a number of factors including:

- An extensive chemical toxicological database including a large database addressing Endocrine Active Compounds (EACs)
- Recent studies on the circulating levels of sex hormones provide reference values for interpreting the impact of potential EAC's. Comparatively less information has been reported for the medaka and zebrafish.
- Ready availability throughout the U.S.

In addition to these factors, fathead minnows are similar to medaka and zebrafish with respect to the following:

- Rapid development and sexual maturation
- Ease of culturing and performance in laboratory setting

13.2 Exposure Protocol

Currently there are insufficient data to make a definitive selection on which exposure protocol has the most merit and should be chosen above all others. It is recommended that a side-by-side performance evaluation of the 21 versus the 14 day protocols would be beneficial to elucidating the overall performance and cost benefit of the two protocols. With out having such a study comparison the 21-day is recommended due to the greater amount of data that is generated. In addition, another point of consideration is an extension to the 21-day protocol described in Section 12.2 to have a gender specific exposure regime to help to further describe the mode of action of EAC. This additional exposure regime would enable a determination of gender specific effects to be established, however, it would add additional cost to the test and the benefits would need to be established to justify the additional cost. A comparative evaluation of the described fathead minnow assays has been commissioned by the Agency to ascertain the most appropriate exposure protocol.

13.3 Appropriateness of Reproductive Screen Endpoints

The measurement endpoints as described in each candidate protocol provide meaningful data. There are two endpoints with limited value. Single estimates of the concentrations of steroids in tissues (e.g. blood or plasma) are difficult to interpret because of limited baseline data available for comparison and the natural fluctuation that occurs during the spawning cycle (e.g., E2). Additionally, it is unclear how information on spawning behavior is to be used or how it specifically adds to the determination of mode of action. Because this parameter coincides with daily fecundity measurements, it does add complexity and cost in the performance of the assays. A comparative evaluation of the described fathead minnow assays has been commissioned by the Agency to assess the value of the various endpoints recommended.

13.4 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 (or EIA). 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, ELISA 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, while 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 *VTG* 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 vs. carp-based ELISA to measure VTG in fathead minnows will determine the specifics of the recommended ELISA protocol. In a fish reproductive screen assay, induction may be measured with carp-based ELISA, the most sensitive of which is the sandwich ELISA. A comparative evaluation of VTG methods has been commissioned by the Agency to ascertain the most appropriate method(s) to recommend in the optimized protocol.

13.5 Significant Data Gaps

An overall data gap is the lack of information on the reproductive endocrinology of the test species. This is especially true for the medaka and zebrafish. More specific data gaps include the following:

- Male specific effects of estrogen agonists other than VTG induction.
- The effects of anti-estrogens, especially pure or Type II anti-estrogens in sexually mature test species.
- The effects of androgenic and anti-androgens in sexually mature test species, specifically, endpoints other than secondary sex characteristics that may be more sensitive to (anti-) androgens.
- Baseline data for thyroid hormones levels during reproduction in test species.
- The effects of thyroid hormone agonists (or thyroid stimulation) on reproduction.

14.0 IMPLEMENTATION CONSIDERATIONS

14.1 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.

14.1.1 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.

14.1.2 Relative Pain or Distress for Animals

It is not anticipated that the animals would suffer prolonged pain or distress. The study protocols necessitate 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.

14.2 Recommended Protocol

Pre-validation studies following the ICCVAM validation process should be initiated. None of the three candidate protocols have been through a validation processes and each of the protocols have not been routinely used by laboratories outside of the protocol development stages. Based on available information, selection of one candidate protocol would be difficult. It is therefore recommended that a pre-validation study be performed that would evaluate the three candidate protocols "side by side" using the same chemicals.

Validation of the study design through interlaboratory comparisons should be conducted once a preferred protocol has been identified using 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

Although the goal of this DRP is the development of a fish reproduction screening test, it should be noted that currently there is a lack of information on thyroid function and the potential impairment from EDCs. If thyroid function and impairment is of interest, then additional effort should be directed to further review this topic. An alternative screening test that is specific to thyroid function may be beneficial. However, the fish species considered for the fish screening test (fathead minnow, medaka, and zebrafish) may not be the species of choice for elucidating thyroid impairment. A potential alternative could be the use of an anadromous species, such as salmon or steelhead, so that smoltification performance (the ability of the fish to transition from freshwater to salt water) could be measured. The thyroid system plays an important role in this processes.

14.3 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 reproductive screening test. However, it should be noted that those facilities that have in-house fish culture would be advantageous but not a prerequisite to running the test.

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APPENDIX A

LITERATURE SEARCH

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 a later version of this document. Because of the extensive nature of this literature search, the information, including abstracts where available, will be printed 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

On the following pages are the questionnaire and responses of key experts contacted 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 screening assay. Below is a list of those contacted. At the time of this writing, three have replied to the questionnaire, and their responses follow. Acknowledging the busy schedules of the participants, we are currently conducting follow-up calls to gather additional information and will be providing extended responses in a subsequent version of this DRP.

Questionnaires were sent to the following identified experts:

Tom Hutchinson
Zeneca - Brixham Environmental Laboratory
Freshwater Quarry
Brixham
Devon TQ5 8BA
ROYAUME-UNI / UNITED KINGDOM
+44 1803 882 882
+44 1803 882 974 (fax)
E-mail: tom.hutchinson@brixham.zeneca.com

Pieter Wester
RIVM
Laboratory for Pathology and Immunobiology
Antonie van Leeuwenhoeklaan 1
PO Box 1
NL- 3720 BA Bilthoven
PAYS-BAS / THE NETHERLANDS
+31 30 274 2242
+31 30 274 4437 (fax)
E-mail: p.wester@rivm.nl

Prof. Taisen Iguchi
Okazaki National Research Institutes
Center for Integrative Bioscience
Bioenvironmental Science
38 Nishigonaka, Myodaiji, Okazaki 444-8585, Japan
TEL: +81-564-55-7525
FAX: +81-564-55-7527
E-mail: taisen@nibb.ac.jp

Dr. Grace H. Panter
AstraZeneca Global Safety Heallth & Environment
Brixham Environmental Lab, Freshwater Quarry,
Brixham TQ5 8BA, Devon, England
E-mail: grace.panter@brixham.astrazeneca.com

Dr. Dan G. Cyr
Fisheries & Oceans Canada
Maurice Lamontagne Institute
Mont Joli, PQ,
Canada
Telephone 1-514-630-8833
Email: Daniel.cyr@INRS-SANTE.uquebec.ca

Dr. Nancy Denslow
Univ Florida, Dept Biochem & Mol Biol,
POB 100156 HC, Gainesville, FL 32610
USA
Tel.: _1-352-392-9665;
E-mail address: denslow@biotech.ufl.edu

General Questionnaire for Development of Fish Screening Assay for Endocrine Disruption

1) Do you know of any gray literature that you would recommend that investigate the status of various *testing* methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) endocrine systems in fish.

2) What is your preferred fish screen method for elucidating endocrine disruption in fish? For example, the EPA Ankley's 21 day fish screen or the 14-day OCED method or an alternative method that you would recommend, please consider the following questions?

    a. What are the limitations of your preferred method?

    b. What are the strengths?

    c. What would you recommend to further enhance this method or what changes would you recommend (if any)?

    d. In running this method/procedure are their any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share…or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay.

    e. Do you have any unpublished data relevant to these assays that you would be willing to share? If so, are there any restrictions?

    f. Are their variations of the assay that should be considered that you did not have time to validate or access. Anything you would change about the assay to increase it's sensitivity, efficiency, relevancy, or robustness?

    g. Quality control

        1. What are the quality control measures to be included in these assays that you favor?

        2. Are there specific or special circumstances when additional quality control measures need to be added?

3) Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on fish endocrine systems? For example, are sex steroids good endpoints? Is measuring thyroid function a viable option? Etc.

4) Based on your experience, what chemicals, duration, and doses would you recommend to be used to validate an assay/protocol?

5) Is there anyone else you can think of that we should contact? If so, whom? Can we mention your name when we contact him/her?

6) Other comments?

From Professor Taisen Iguchi, Okazaki National Research Institutes, Center for Integrative Bioscience, Bioenvironmental Science, Okazaki, Japan

1) Do you know of any gray literature that you would recommend that investigate the status of various *testing* methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) endocrine systems in fish.

I do not know any gray literatures.

2) What is your preferred fish screen method for elucidating endocrine disruption in fish? For example, the EPA Ankley's 21 day fish screen or the 14-day OCED method or an alternative method that you would recommend, please consider the following questions?

If we study only estrogenic chemicals, 14-day OECD method is preferred.

a. What are the limitations of your preferred method?

The 14-day OECD method has power to detect chemicals affecitng by receptor mediated mechanism. However, it will weak to detect chemicals which act receptor non-mediated ways, and the data will not be used for estimation of hazard levels of chemicals.

b. What are the strengths?

c. What would you recommend to further enhance this method or what changes would you recommend (if any)?

We need to standardize the method of vitellogenin measurement including quality of assay kit.

d. In running this method/procedure are their any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share…or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay.

VTG is a fragile protein, therfore, we need to shorten the sampling time.

e. Do you have any unpublished data relevant to these assays that you would be willing to share? If so, are there any restrictions?

We are standardizing VTG antibody and assay method for Medaka.

f. Are their variations of the assay that should be considered that you did not have time to validate or access. Anything you would change about the assay to increase it's sensitivity, efficiency, relevancy, or robustness?

I will ask researchers about this question and reflect it to our VTG protocol.

g. Quality control

1. What are the quality control measures to be included in these assays that you favor?

We need pure VTG, which is quite hard to prepare.

2. Are there specific or special circumstances when additional quality control measures need to be added?

3) Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on fish endocrine systems? For example, are sex steroids good endpoints? Is measuring thyroid function a viable option? Etc.

In Medaka, we thought occurrence of ovotestis is sensitive, however, sometimes we could not see dose-response in apperarance of ovotestis in recent studies.

This seems that ovotesis is not a good endpoint. We are still discussing the endpoint. VTG is a good candidate of endpoint.

4) Based on your experience, what chemicals, duration, and doses would you recommend to be used to validate an assay/protocol?

I personally recommend OECD reference chemicals. In Medaka, partial life cycle test is useful. We are preparing a test guideline.

5) Is there anyone else you can think of that we should contact? If so, whom? Can we mention your name when we contact him/her?

6) Other comments?

From Dr. Dan G. Cyr, Fisheries & Oceans Canada, Maurice Lamontagne Institute, Mont Joli, PQ, Canada

1) Do you know of any gray literature that you would recommend that investigate the status of various *testing* methods that have been used to identify chemicals that affect (i.e., inhibit or enhance) endocrine systems in fish?

No. All the literature on thyroid function is published in peer review journals and I am unaware of publications in the gray literature on EDCs and thyroid

2) What is your preferred fish screen method for elucidating endocrine disruption in fish? For example, the EPA Ankley's 21 day fish screen or the 14-day OCED method or an alternative method that you would recommend, please consider the following questions?

Combination of measuring thyroid hormone by RIA and deiodinase activity or mRNA levels in liver.

a. What are the limitations of your preferred method?

Deiodinase mRNA seqyuences vary between species and therefore new cDNA probes or primersequences (for RT-PCR) need to be generated for different species.

b. What are the strengths?

The strength of using both hormone levels and deiodinase is that this gives information of circulating hormone levels, activation or deactivation of thyroxine, and since deiodiase is itself regulated by thyroid hormones then it is an indicator of hormone action at the level of the cell.

c. What would you recommend to further enhance this method or what changes would you recommend (if any)?

More tools are necessary, particularly at the molecular level, to assess hormone action at the level of the cell since this is the most important function of the hormone.

d. In running this method/procedure are their any steps that are especially difficult that require special attention i.e., lessons learned that come after numerous runs that you would like to share…or is there any special set up strategy you would recommend that would save time or resources that come from experience in running the assay?

Thyroid hormone levels in fish plasma, for example, are low and the majority of commercial kits do not work. Other basic approaches for RNA isolation and rapid freezing are standard.

e. Do you have any unpublished data relevant to these assays that you would be willing to share? If so, are there any restrictions?

No.

f. Are their variations of the assay that should be considered that you did not have time to validate or access. Anything you would change about the assay to increase it's sensitivity, efficiency, relevancy, or robustness?

We have not seen major effects of EDCs on thyroid hormones in wild fish species. Ideally it would be nice to show effects.

g. Quality control

1. What are the quality control measures to be included in these assays that you favor?

In this case we used livers from either non-exposed or vehicle exposed fish,

2. Are there specific or special circumstances when additional quality control measures need to be added?

Not really, although since estrogens alter thyroid hormone levels and their metabolism it is important to use immature fish.

3) Based upon your expertise and experience, what endpoints would be most appropriate for elucidating the effects of chemicals on fish endocrine systems? For example, are sex steroids good endpoints? Is measuring thyroid function a viable option? Etc.

See above for the tests we use. It would also be a good idea to look at thyroid hormone receptor binding in vitro, but a good tests would need to be developped.

4) Based on your experience, what chemicals, duration, and doses would you recommend to be used to validate an assay/protocol?

The best thyroid inhibitor is probably sodium ipodate/iopanoic acid. Although needs to be injected. In mammals, PCB congeners can also be good inhibiotors although they appear to be less effective in fish.

5) Is there anyone else you can think of that we should contact? If so, whom? Can we mention your name when we contact him/her?

Scott Brown at Environment Canada and Geoff Eales at the Univ. Manitoba

6) Other comments?

Correspondence from Dr. Tom Hutchinson, CBiol FIBiol, Head of Research & Environmental Effects, AstraZeneca Global Safety, Health and Environment, Brixham Environmental Laboratory, Freshwater Quarry, Brixham, Devon, UK

Apologies for my delay but I have not been able to open the word attachment.

Basically, I think the approach being taken by EPA on the fathead minnow short-term reproduction ('FishSTR') test is scientifically sound. The protocol described by Ankley et al in ET&C has a range of ED-specific endpoints (e.g., VTG) and non-specific endpoints (e.g., fecundity). For this reason, I believe that the full range of endpoints is appropriate for testing compounds shown to have the intrinsic capability to interact with hormonal axes (e.g., from in vitro data) and provides valuable reproductive toxicity data for risk assessment purposes. Further work needs to be undertaken to try to reduce the scale of the method if it is to be a screen of comparable utility to the rodent uterotrophic assay. One way to do this is to exclude fecundity measurements and focus on ED-specific endpoints (e.g., VTG; molecular markers such as mRNA for CYP19 may also be useful in the future).

I am back at my desk next week so please telephone me for further information.

Kind regards,

Tom