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**DETAILED REVIEW PAPER ON FISH LIFE-CYCLE TESTS**

**IOMC**

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
A cooperative agreement among **UNEP, ILO, FAO, WHO, UNIDO, UNITAR and OECD**

**Environment Directorate**
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# FOREWORD

This document presents the Detailed Review Paper (DRP) on Fish Life-Cycle Tests approved at the 20th Meeting of the Working Group of National Coordinators of the Test Guidleines Programme (WNT) in April 2008.

At the Fourteenth Meeting of the WNT in May 2002, the United States submitted a standard project submission form for the development and validation of a Fish Life-Cycle Test, including the necessary literature review.

A first draft DRP was submitted by the United States in November 2002 and circulated for comments to the WNT. Comments received were numerous and at that time the draft DRP on Fish Screening Assays for Endocrine Active Substances was given a higher priority, due to the pressing need for a fish screening assay and the validation studies that were in progress. Therefore, the draft DRP on Fish Life-Cycle Tests stayed dormant for some time. In 2007, the OECD Secretariat hired a consultant to revise the draft DRP, include current literature and extend the scope to the most commonly used OECD species.

The revised DRP was circulated for comments to the experts of the Validation Management Group for ecotoxicity testing (VMG-eco) in September 2007. Comments received were addressed with responses to comments available at the last meeting of the VMG-eco and the final draft DRP was approved with minor changes by the sixth meeting of the VMG-eco in January 2008.

The DRP completes the part related to the literature review on Fish Life-Cycle Tests. At present, an analytical paper on the statistics of endpoints relevant to fish populations is under preparation, and experimental work in Germany, Japan and the United States is in progress.

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# ACKNOWLEDGEMENT

# 1.0 EXECUTIVE SUMMARY

The principal purpose of this Detailed Review Paper (DRP) is to define the basis, objectives and conduct of fish life-cycle tests for the effects of endocrine disrupting chemicals (EDCs). The main focus is on four species of fish (fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*), zebrafish (*Danio rerio*) and sheepshead minnow (*Cyprinodon variegatus*)) because relatively little life-cycle testing has been conducted to date with other species. The DRP also briefly reviews the use of fish life-cycle tests for substances other than EDCs. The paper concludes that although fish full life-cycle tests are generally more sensitive to EDCs than partial life-cycle reproduction tests, it has not yet been demonstrated that two-generation or multi-generation tests with fish offer any further advance in sensitivity (to either EDCs or non-EDCs). The early life-stage test including sexual differentiation is not included in this review as it was identified as a screening tool rather than a definitive test, and is therefore described in the DRP on Fish Screening Assays for Endocrine Active Substances (OECD, 2004). Furthermore, mechanistic endpoints diagnostic of endocrine action need only be included optionally in life-cycle test protocols, because the primary purpose of these tests is to measure definitive apical (i.e. population-relevant) endpoints for use in risk assessment.

The DRP reviews the underlying principles of existing protocols for measuring the effects of EDCs on fish, and identifies issues that might require prevalidation studies before they can be adequately addressed. This DRP critically evaluates the effect of disturbances in the endocrine system on sexual differentiation, development and reproduction in fish. A large portion of this document is focused on interpreting published data in the context of partial or full life-cycle studies for the four species of fish, which are the most likely candidate species for use in regulatory testing.

The endocrine system can be defined as any tissue or cells that release a chemical messenger (hormone) directly into the blood that signals or induces a physiological response in some target tissue. In this DRP, the focus is on sexual differentiation (growth and development), reproduction and paracrine signaling. However, the function of the endocrine system is much broader and contributes to the regulation of many physiological processes such as digestion, metabolism, growth and development. Size, sexual differentiation, sexual maturation, embryo fertilization, and maternal transfer are all examples of sensitive and critical phases in the life cycle of fish. Sensitivity to a toxicant may increase

when more of these life-stages are included in a test such as the proposed two-generation fish test, although this remains to be clearly demonstrated.

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

The exposure duration of a test needs to encompass an appropriate time of exposure necessary to elicit an effect, but not beyond a necessary time to control costs and potential exposure interruptions. The utility of the partial and full life-cycle tests, and the two-generation and multigeneration life-cycle tests has been evaluated for use in testing EDCs and non-EDCs using some designs and fish species. The partial life-cycle reproduction test is a relatively short exposure test designed to evaluate sexual reproduction in fish and the effects on the early life stages of their progeny from exposure to substances; an alternative partial life-cycle test, not including reproduction, being validated by VMG-eco, evaluates the sexual development of fish from egg to adult. The multi-generation test is designed to be a definitive test for evaluating population-level effects of substances in the environment. This long-term chronic test exposes fish through two complete life cycles, plus the early life stages of the third generation. An alternative to the multi generation life-cycle test, is the two-generation test in which the initial exposure is initiated with mature fish and the F1 generation is evaluated for embryo fertility, development, sexual maturation, reproduction and F2 viability is assessed. This test potentially incorporates maternal transfer of hormone disrupting substances from P to F1 and offers the advantage of a reduction in the time required to conduct the test. The main endpoints included in this test assess impacts upon fecundity and viability that address population level effects integral to risk assessment, while optional endpoints allow assessment of underlying mechanisms of endocrine toxicity.

Although the additional sensitivity of two-generation and multi-generation tests (in comparison with the life-cycle test) has not been conclusively demonstrated, there are some theoretical grounds for expecting that EDCs, at least, may give lower no-effect concentrations when assessed using these techniques. Pre-validation studies are therefore required to establish which, if any, life-cycle protocol is the most sensitive. In preparation for their use in risk assessment, inter-comparison of the methods employing the four species in this review must also address issues such as differences in sensitivity among the species, test reproducibility, and running costs. Alternatively, the pre-selection of one of the four species would limit the number of demonstration trials for full optimization of a method suitable for interlaboratory testing. Interlaboratory comparisons for protocol validation should be conducted with compounds that span the possible endocrine effects, including strong and weak androgen receptor agonists and antagonists, estrogen receptor agonists and antagonists, and thyroid agonists and

antagonists. Protocol validation with non-EDCs should also be conducted with a view to establishing a single fish life-cycle testing guideline for all chemicals.

## 2.0 INTRODUCTION

### 2.1 Purpose of the Review on Chronic and Long-Term Tests

1.   The DRP was initially prepared by the United States to support the development of a Fish two-generation test, as a definitive test for the assessment of endocrine disrupters in their national program. Given that the same type of test was envisaged in the OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupters, it was agreed that the draft DRP would be circulated for comments at the level of OECD expert groups, with the view to publishing a review in the Series on Testing and Assessment. The purpose of this Detailed Review Paper (DRP) is to define the basis and purpose of life-cycle tests with emphasis on four species of fish (fathead minnow, zebrafish, and Japanese medaka, sheepshead minnow) for endocrine and non-endocrine effects.  The DRP summarizes, explains, and documents decisions regarding the relevant principles, methods, and techniques recommended for life-cycle protocols, and identifies issues that might require prevalidation studies.

### 2.2 Objective of Chronic and Long-Term Tests

2.   The main driver for developing an OECD Guidance Document on fish life-cycle testing springs from the OECD Task Force on Endocrine Disrupters Testing and Assessment (EDTA), and ultimately from the Working Group of National Co-Ordinators of the Test Guidelines Programme (WNT). Through the Validation Management Group on Ecotoxicity Testing (VMG-eco), WNT and EDTA have been engaged for several years in preparing *inter alia* fish-based test methods for EDCs which are consistent with the OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals. To date (2007), VMG-eco has nearly completed the validation of a 21-day fish screening guideline which lies at Level 3 in the Conceptual Framework (*in vivo* assays providing data about single endocrine mechanisms and effects). It has also begun validation of a partial life-cycle method known as the fish sexual development test (FSDT), or non-spawning assay, which lies at Level 5 (*in vivo* assays providing data on effects from endocrine and other mechanisms) and exposes the part of the life-cycle from fertilised egg to sexually-differentiated juvenile. In the future it may also consider the validation of another partial life-cycle test (an extended fish reproduction test - also at Level 5) which exposes the part of the life cycle from adulthood through mating to fry. While all these tests are expected to play a useful role in the screening and risk assessment of EDCs, it is recognised that individual partial life-cycle tests might not necessarily be sensitive to certain mechanisms of endocrine (and non-endocrine) action, compared to tests including successive stages of the life-cycle.

3.   A fish full life-cycle, two-generation or multi-generation test (Level 5 in the EDTA Conceptual Framework) should provide an apical response to all possible modes of chemical toxicity (endocrine and non-endocrine), and as such could be considered as providing a 'gold standard' or benchmark result which provides definitive data on the long-term toxicity of a substance. The intention of such a test is not

primarily or necessarily to identify a mode of action, be it endocrine or non-endocrine. Possible modes of action are already suggested or sometimes established by other procedures before life-cycle testing commences. A guideline of this type would not, however, be required for testing all chemicals; it would allow the predictive ability of partial life-cycle tests such as the FSDT to be assessed, and it could be used in certain cases to provide definitive information on the long-term toxicity to fish of a chemical for use in environmental risk assessment.

4.   It should be noted that, in principle, fish life cycle tests will address several issues of concern about endocrine disrupters. First, because they include prolonged exposure of all life-stages, they will automatically provide scope for many or all mechanisms of action of EDCs, including those occurring in life stages not covered by present guidelines (e.g. mating adults). Secondly, the inclusion of at least two sequential generations of fish would allow the detection of substances whose effects occur a long time after exposure (e.g. abnormalities in the sex ratio of the F1 or F2 generation resulting from exposure of fry in the F0 generation). Thirdly, such tests would also permit the assessment of effects in the F1 and/or F2 generations resulting from persistent residues inherited via the egg from the F0 generation. Life-cycle tests might not, however, be able to throw much light on concerns such as unusual concentration-response curves or the possible absence of thresholds, because the detection of these effects requires the testing of a large number of concentrations which would make the test impractical. Furthermore, concerns that EDCs with identical modes of action are able to produce additive effects in mixtures could not be sensibly investigated with life-cycle tests due to the huge number of mixture combinations which such studies require. Finally, due to the apical nature of life-cycle tests, they will not necessarily reveal information about modes of action (MOA). However, such information may be available from biomarker endpoints measured in screening assays after short term exposure.

5.   It seems likely that a fish life cycle test guideline will be primarily required by regulatory authorities for investigating chemicals likely to affect development, growth and reproduction. For other chemicals of potential hazard, relatively short-term tests of non-EDCs with embryo-larval and early juvenile life stages of fish (e.g. the fish early life-stage test, OECD 1992) can be used to estimate maximum acceptable toxicant concentrations (MATCs) derived from full life-cycle tests to within a factor of 2. This has led to the widespread use of OECD Test Guideline 210 (OECD, 1992), although a USEPA fish life-cycle toxicity test method (USEPA, 1982) starting with fertilised eggs and ending with juveniles of the second generation has been employed since the early 1970s in several jurisdictions.

6.   In at least two regulatory jurisdictions (United States and the European Union), the requirement for fish-based tests of EDCs is imminent or already in existence. In the US, the Food Quality Protection Act (FQPA, 1996) and the Safe Drinking Water Act (SDWA, 1996) mandate the USEPA to screen chemicals in food and water for endocrine activity. The US Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) has recommended a two-tiered approach (screening and testing) for identifying EDCs (EDSTAC, 1998). The purpose of the testing is to determine whether a chemical or chemical mixture adversely affects the organism primarily through endocrine-mediated pathways, and to evaluate those effects with respect to the estrogen, androgen, and thyroid systems.  In addition, the tests must include exposure during the most sensitive lifestages, provide the opportunity for identification of concentration-response effects, and encompass a variety of taxa. Tier 2 will include a fish multi-generation test which, combined with other tests, will characterize the nature, likelihood, and concentration-response relationship of the endocrine disruption of estrogen, androgen, and thyroid systems in humans and wildlife.

7.   In the European Union, the new REACH (Registration, Evaluation and Authorisation of Chemicals) regulation of the European Parliament comes into force on 1 June 2007. It may require authorisation of (and hence provision of toxicity data for) EDCs if they give rise to an equivalent level of concern to substances that are carcinogenic, mutagenic or reproductive toxicants, or to those which are persistent, bioaccumulative and toxic. The draft Technical Guidance Document for REACH (TGD, 2007) specifically indicates that if a link between serious adverse effects and an endocrine mode of action can be established, a substance may be subject to authorisation. TGD (2007) goes on to state that fish life cycle test data would be appropriate for the characterisation of long-term adverse effects because they provide apical developmental and reproductive endpoints, although these are not necessarily indicative or specific to any particular mode of action (MoA). The draft TGD makes it clear that other tests (e.g. the 21 day fish screening assay) are appropriate for identifying possible endocrine MoA. Also according to the EU Directive 91/414/EEC (1991) concerning the placing on the market of plant protection products, fish full life-cycle  data are mandatory for pesticide active ingredients in particular cases.

8.   The need for regulatory testing of EDCs in Japan has not yet been enacted in legislation. However, the Japanese Ministry of the Environment has already conducted risk assessments of about 40 existing substances (including about 10 EDCs) using data from partial and full fish life-cycle tests with medaka, and further work on these lines is continuing. It is expected that once OECD has agreed a full suite of fish screening and testing guidelines for EDCs, they will be used by the Japanese government for the risk assessment of new chemicals.

9.   Finally, it should be made clear that an important objective of any toxicity test is to ensure that it is designed in such a way that it can be conducted reproducibly at an acceptable sensitivity and cost, with minimal false-positives and -negatives. This is particularly true of fish life-cycle tests which are at least of many months' duration, and which consequently may be prone to a high failure rate.

10. This DRP considers the life-history regimes associated with fathead minnow, zebrafish, Japanese medaka, and sheepshead minnow for possible application in the development of a life-cycle test guideline that will achieve the above-stated goals in the most effective and efficient manner possible. Other species will not be given detailed consideration due to the extreme shortage of life-cycle test experience with them (e.g. guppy *Poecilia reticulata*), or because their life cycles are too long for practical testing (e.g. rainbow trout *Oncorhynchus mykiss*), or due to their relatively limited use and the need to keep the DRP focused.

**2.4 List of Abbreviations**

| | |
|---|---|
| 11-KT | 11-ketotestosterone |
| 17,20-DHP | 17α, 20β-dihydroxyprogesterone |
| 17,20,21-THP | 17α, 20β, 21-trihydroxyprogesterone |
| ANOVA | analysis of variance |
| CPA | cyproterone acetate |
| CV | coefficient of variation |
| DDT | dichlorodiphenyl trichloroethane |
| DES | diethylstilbestrol |
| DRP | detailed review paper |
| E2 | 17β-estradiol |
| EAC | endocrine-active chemicals |

| | |
|---|---|
| EC50 | median effective concentration |
| ECD | electron capture detector |
| EDC | endocrine-disrupting chemical |
| EDMVS | Endocrine Disruptor Methods Validation Subcommittee |
| EDSP | Endocrine Disruptor Screening Program |
| EDSTAC | Endocrine Disruptor Screening and Testing Advisory Committee |
| EDTA | OECD Task Force on Endocrine Disrupter Testing and Assessment |
| EE2 | $17\alpha$-ethynylestradiol |
| EIA | enzyme immunoassay |
| ELISA | enzyme-linked immunosorbent assay |
| EPA | United States Environmental Protection Agency |
| ER | estrogen receptor |
| EU | European Union |
| F0: | designates the first generation of fish exposed to a test chemical, either from fertilsed eggs (in the full life-cycle tes design) or from adult life-stage (in the multi-generation test design). |
| F1: | designates the offspring of the F0. |
| F2: | designates the offspring of the F1. |
| FIFRA | Federal Insecticide, Fungicide, and Rodenticide Act |
| FQPA | Food Quality Protection Act |
| FSDT | Fish Sexual Development Test |
| FSH, GTH I | follicle stimulating hormone |
| GC | gas chromatography |
| GSI | gonadosomatic index |
| GTH | gonadotropic hormones |
| HPLC | high performance liquid chromatography |
| ICCVAM | Interagency Coordinating Committee on the Validation of Alternative Methods |
| LC | liquid chromatography |
| LH, GTH II | luteinizing hormone |
| LOEC | lowest observed effect concentration |
| M1 | 2-[(3,5-dichlorophenyl)]-carbamoyloxy-2-methyl-3-butenoic acid |
| M2 | 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide |
| MATC | maximum acceptable toxicant concentrations |
| MOA | Mode of action |
| MT | $17\ \alpha$-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 |
| Reach | Registration, Evaluation and Authorisation of Chemicals (EU regulation) |
| RIA | radioimmunoassay |
| SAB | Scientific Advisory Board |
| SAP | Scientific Advisory Panel |

| | |
|---|---|
| SDWA | Safe Drinking Water Act |
| SQRT-PCR | Semi-quantitative reverse transcriptase polymerase chain reaction |
| T | testosterone |
| T1S | Tier 1 Screening |
| T3 | triiodothyronine |
| T4 | thyroxine |
| TCDD | 2,3,7,8-tetrachlorodibenzo-p-dioxin |
| TSH | thyroid stimulating hormone |
| USEPA | United States Environmental Protection Agency |
| VMG-eco | OECD Validation Management Group for Ecotoxicity Testing |
| VTG | vitellogenin |
| WNT | OECD Working Group of National Co-Ordinators of the Test Guidelines Programme |

## 3.0 OVERVIEW AND SCIENTIFIC BASIS OF ENDOCRINE CONTROL OF FISH REPRODUCTION

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

12. In fish, as with other vertebrates, reproduction requires the coordination of a variety of physiological processes culminating in release of viable gametes and successful fertilization. In many fish species a seasonal reproductive cycle exists whereby the gonads undergo a period of recrudescence or rapid growth prior to spawning.  Fish are also similar to other vertebrate groups in that most species are gonochoristic, with separate male and female phenotypes.  However, there is great diversity in reproductive strategies among fishes, such as internal or external fertilization, oviparity (fertilized eggs mature outside of the fish) or ovoviviparity / viviparity (internal development) and synchronous (annual spawning) or

asynchronous spawning (repeated spawns during a spawning season), and synchronous and asynchronous hermaphroditism. Regardless of the reproductive strategy employed, communication between tissues involved in reproduction and the external environment is vital to ensure proper development of the gonads and ultimately, the timing of gamete production and release (Kime 1998). This latter area can be especially important for many fish species as larval / juvenile survival may depend on spawning occurring at a specific time during the year or during optimal environmental conditions (e.g. during the rainy season).

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

**3.1 Morphology & Anatomy of the Neuroendocrine System**

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

15. The most important peripheral tissue involved in neuroendocrine control of reproduction is the gonads which consist of the ovaries or testes. The thyroid system also aids in regulation of reproduction, although its specific role is less defined compared to the brain-pituitary-gonadal axis, and its involvement will not be considered to any great extent in this paper. It both cases, however, it should be clearly understood that these organs only function in the endocrine system as part of an integrated whole that includes the central nervous system and other tissues. The gonads in fishes are normally paired structures frequently lying adjacent to the air bladder or the kidney. However, numerous exceptions to this arrangement exist among fishes as in the case of the Japanese medaka, where only a single ovary develops (Redding and Patino 2000). In many fishes, the ovaries differentiate sooner in juveniles possessing a female genotype compared to the testes in genotypic males. The fish ovary has been described as essentially a hollow organ containing many lamellae that resemble the pages of a book (Scott 1987). Oogenesis, which is the process of egg development, occurs within the lamellae and can divided into several discrete phases. For example, in the zebrafish (*Danio rerio*), five stages of egg development have been described beginning with initial growth, a pre-vitellogenesis phase, vitellogenesis, maturation and then ovulation (Selman et al. 1993). Further delineation of oogenesis is possible, as oocyte maturation itself is now thought of as a two stage process (Patino et al. 2001). An important change that occurs during pre-vitellogenesis is the development of granulosa and theca cell layers around the oocyte which synthesize estradiol and the maturation inducing hormone, $17\alpha,20\beta$-dihydroxy-4-pregnen-3-one ($17\alpha,20\beta$-DP; Janz 2000). The size of mature eggs from most freshwater fishes range between 0.4 and 3 mm (Redding and Patino 2000) and during ovulation are released into the visceral cavity or lumen of the ovary where they remain until time of spawning (Scott 1987).

16. The morphology of the testes can vary considerably among the different taxa of fish. In many fishes, the testes has a characteristic whitish appearance and elongated lobular shape within which exist tubules that are surrounded by a basement membrane that divides the space into intra- and inter-tubular compartments (Nagahama 1983; Scott 1987). In teleosts, two distinctive forms of testes structure have been described and are termed tubular or lobular (Grier 1981), although technically speaking, tubules are present in both types. Most teleosts such as salmonids and cyprinids possess a lobular type testes characterized by spermatogonia being distributed along the full length of the tubules (Grier 1981). Spermatogenesis is the process of spermatozoa development and occurs through proliferation of primary spermatogonia within the tubule. Primary spermatogonia arise from germ cells typically embedded in cysts formed by Sertoli cells (Scott 1987). Another important cell type in the testes are the Leydig cells, which are found in connective tissue near Sertoli cells and are involved with synthesis of testosterone, 11-keto-testosterone and other androgens (Scott 1987; Redding and Patino 1993). Mature spermatozoa are released into the lumen of the tubule and eventually into the sperm duct which merges with a system of ducts from other tubules to form a primary duct for each testis (Redding and Patino 2000). The process of spermatozoa release into the sperm duct is called spermiation.

**3.2 Reproductive Hormones**

*3.2.1 Hypothalamic and Pituitary Hormones*

17. A schematic representation of brain-pituitary-gonadal axis and thyroid system is depicted in Figure 3-1. Important hormones secreted by the hypothalamus are gonadotropin releasing hormone (GnRH), thyrotropin releasing hormone (TRH) and neurotransmitters such as dopamine. These hypothalamic hormones regulate release of gonadotropins and thyrotropins by the pituitary gland. An additional type of hypothalamic secretagogue that is known to influence gonadotropin release are neurotransmitters of

which the best characterized are the monoamines dopamine and serotonin (Vitale and Chiocchiol 1993; Vacher et al. 2000). GnRH is a decapeptide with at least 6 different forms described from cartilaginous and bony fish species plus an additional 2 forms isolated from lampreys (Goos et al. 1998; Dubois et al. 2002). Most GnRH's in fish are structurally conserved differing only in one or two amino acids (Goos et al. 1998). Teleost fish are similar to other vertebrate groups with the exception of placental animals in that multiple GnRH's are present in the brain (Bently 1998). Apart from the lamprey, all fish appear to possess a common GnRH identical to that found in other vertebrates, plus additional GnRH's that are specific to fish taxa (Dubois et al. 2002). Each GnRH is apparently transcribed from different genes (Dubois et al. 2002). There is recent evidence from experiments using the African catfish (*Clarias gariepinus*) that multiple GnRH's work in concert to regulate gonadotropin secretion by the pituitary (Bosma et al. 2000). There is one and possibly two distinct GnRH receptors found in fish (Goos et al. 1998). All GnRH receptors are cell surface proteins although the primary sequence of fish GnRH receptors indicate significant differences from their mammalian counterparts (Goos et al. 1998). Consistent with previous studies in the African catfish, the GnRH receptor cloned from striped bass (*Morone saxatilis*) and transfected into a Chinook salmon (*Oncorhynchus tshawytscha*) cell line exhibited reduced activation when incubated with mixtures of native GnRH's as opposed to incubation with a single GnRH (Alok et al. 2001). Interestingly, GnRH receptors are expressed in other tissues besides the pituitary including the testis (Goos et al. 1998; Alok et al. 2000). These findings may in part be related to GnRH additional roles as a neurotransmitter and autocrine functions within certain tissues (reviewed in Habibi and Huggard 1998).
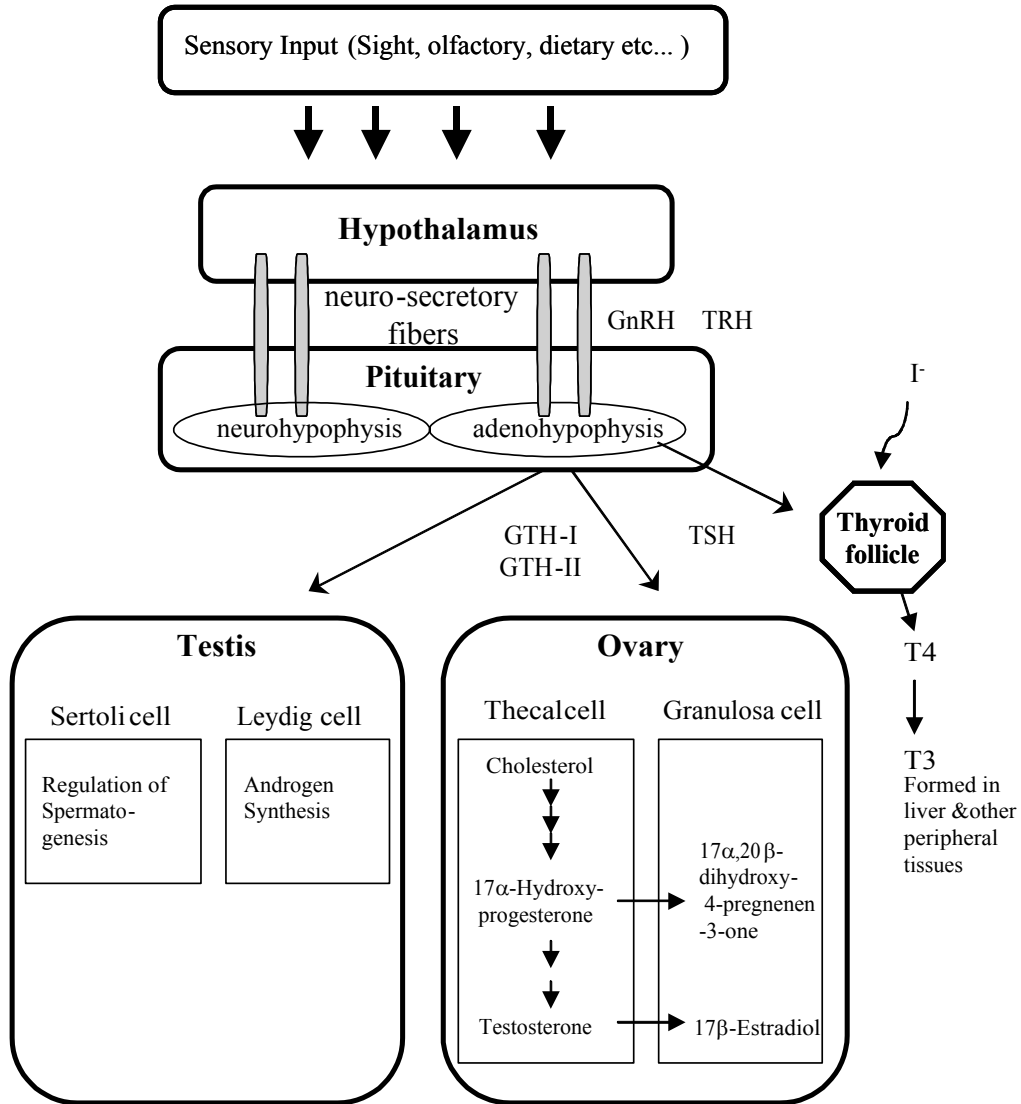
**Figure 3-1.    Outline of the Brain-Pituitary-Gonadal Axis and Thyroid System in Fish.  The Two Cell Model for Steroid Synthesis is Shown for the Ovary**

### 3.2.2  *Gonadal Hormones*

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

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

20. The synthesis and in some cases interconversion of steroids is quite complicated.  Many enzymes are involved, and their subcellular location varies between the mitochondria, endoplasmic reticulum (e.g. microsomal) and the cytoplasm.  In general, sex steroid biosynthesis can be divided into seven types of enzyme catalyzed reactions.   These are lyase, hydroxylase, hydroxysteroid dehydrogenase , isomerase, aromatase, reductase and conjugation.   Of these types of reactions, aromatase, hydroxysteroid dehydrogenase and conjugation appear to be the most important as possible targets of endocrine disruption as they are either the final step in 17β-estradiol or 11-keto-testosterone synthesis or aid in excretion of steroids.   For example, inhibition of aromatase activity can greatly diminish estrogen synthesis and produce anti-estrogenic effects in fish.  This latter topic is discussed in detail in section 7.2.2.   Steroid conjugation may be important in the production of pheromones as was mentioned previously for androgens but there is also evidence that progesterone conjugates may function as pheromones as well (Vermeirssen and Scott 2001).

21. A significant physicochemical difference between the sex steroids, thyroid hormones and the hypothalamus-pituitary hormones is that steroid and thyroid hormones are poorly soluble in water.  In

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

### 3.2.3 *Feedback Control Mechanisms*

22. As discussed in section 3.1, release of pituitary hormones is controlled by the hypothalamus and sensory input to higher brain centers. Additional regulation of hormone synthesis and secretion within the brain-pituitary-gonadal axis and thyroid system occurs from feedback control exerted by the secreted hormone(s) or hormones that are induced by hypothalamic-pituitary hormones. Feedback control mechanisms are well documented in vertebrates and can be manifested as either long or short feedback loops (Batten and Ingleton 1987). In fish, long feedback loops with the neuroendocrine system are well described with a good example being the effect of sex steroids on gonadotropin release. Gonadotropins (the tropic hormones) can stimulate the synthesis and release of sex steroids (the target hormones) by the gonads which in turn, alter the release of tropic hormones by the pituitary. A short feedback loop occurs when the secreted hormone auto-regulates its own release. Both positive and negative feedback can occur to either stimulate or inhibit release of the tropic hormone. Complicating factors that limit attempts to generalize about the type and extent of feedback loops within the brain-pituitary-gonadal axis amongst fishes are significant interspecies differences and the stage of reproductive development. In particular, there is a relative lack of studies using asynchronous spawning fishes such as those typically used in regulatory testing. The group of fish that are the best characterized with respect to feedback control mechanisms are the salmonids. For example, castration of mature Atlantic salmon (*Salmo salar*) reduced both pituitary and circulating levels of GTH-I and II suggesting a positive feedback effect of androgens (Borg et al. 1998). However, when castrated salmon were given testosterone replacement therapy during the normal time period for spawning, an initial suppression of GTH-I release occurred which was subsequently stimulated several months later near the end of the spawning season (Borg et al. 1998). In the Atlantic croaker (*Micropogonias undulatus*), gonadectomy and subsequent replacement therapy with testosterone or estrogen increased the GnRH induced GTH-II secretion from the pituitary during the early phases of gonad recrudescence (Khan et al. 1999). After maturation of the gonads, steroid treatment inhibited the responsiveness of the pituitary towards GnRH and release of GTH-II (Khan et al. 1999). Other studies involving gonadectomy have generally indicated that sex steroids have a negative feedback on gonadotropin release (Kobayashi and Stacey 1990; Larsen and Swanson 1997). In additional studies with salmonids, it would appear that the most pronounced feedback is exerted by testosterone and estrogen. In sexually maturing coho salmon (*Onchorynchus kisutch*), exposure to testosterone or estrogen but not 17α,20β-DP, has a negative feedback effect on GTH-I secretion (Dickey and Swanson 1998). In contrast, testosterone and estrogen treatments increased pituitary content of GTH-II (Dickey and Swanson 1998). Additional evidence suggests that feedback effects of testosterone are meditated in part by conversion to estrogen as aromatase inhibitors or administration of nonaromatizable androgens can block or exert a reduced effect (Trudeau et al. 1991; Khan et al. 1999).

23. An explanation for the differential feedback of steroids that is supported from research on synchronous spawning fishes such as salmonids, is based on differing effects of steroids on the pituitary gland and hypothalamus. Several studies have demonstrated that steroid treatment can increase the expression of the beta subunits for gonadotropins and pituitary content of the hormones (Querat et al. 1991; Antonopoulou et al. 1999; Mateos et al. 2002). These results imply that steroid feedback upon the pituitary is primarily mediated at the gene transcription level. In the hypothalamus of rainbow trout, steroid receptors are not expressed in GnRH neurons but instead appear to occur on neighboring fibers (Navas et al. 1995). Given this pattern of hypothalamic expression of steroid receptors, their role in feedback of GnRH release would appear to be limited to actions on neurons capable of regulating the activity of GnRH secretory neurons. In this regard, additional data from rainbow trout suggest a negative feedback of estrogen on gonadotropin secretion is linked to effects on the hypothalamus and dopaminergic activity (Linard et al. 1995; Saligaut et al. 1998). Alternatively in other fishes such as the Atlantic croaker, neuronal fibers under gamma-aminobutyric acid (GABA) neurotransmitter control may be involved (Khan and Thomas 1992). Despite the limited data in fishes, some researchers have proposed as a general mechanism that positive feedback of steroids is mediated through the pituitary and induced synthesis of gonadotropins while negative feedback is achieved by decreasing the secretion of GnRH by the hypothalamus (Mateo et al. 2002). Although this generalization is attractive for simplifying the feedback effects of steroids on gonadotropin synthesis and release, other studies examining the synthesis of the various forms of GnRH in immature male tilapia (*Oreochromis nilotica*) suggest a variety of actions including changes in biosynthesis of GnRH's occur within different regions of the hypothalamus (Parhar et al. 2000).

**3.3 Endocrine System as a Target For Chemical Toxicity**

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

25. Although sufficient observations in wildlife support the endocrine disruptor hypothesis, it is nonetheless controversial due in part to difficulties in establishing links between environmental exposure

to chemicals, changes in endocrine function and altered reproduction (Van der Kraak 1998). As discussed in previous sections, the endocrine system is complex with many potential target sites and hormonal systems may be affected. Disturbances could be directed at the brain-pituitary or at the gonadal level or a combination of both. However, there is only limited experimental data available on the vulnerability of the hypothalamus-pituitary system to chemicals. Several studies have demonstrated that gonadotropin secretion can be altered but it is unclear whether this is due to a specific endocrine disruptor type interaction or some other non-specific toxicological effect (Kime 1998). In contrast, endocrine disruptor effects on thyroid and gonadal hormones is more established. An excellent example of the latter is recent studies on wild fish living in U.K. rivers that indicate unnatural exposure to estrogen or chemicals with estrogen-like activity causes impaired reproduction (Jobling et al. 1998; 2002; van Aerle et. al. 2001).

26. In conclusion, strong evidence exists that environmental concentrations of EDCs adversely affect sexual differentiation, development and reproduction in fish. Greatest focus on potential hormone targets are the sex steroids and to a lesser extent, the thyroid hormones. The remaining sections of this detailed review paper will provide information on various aspects of life-cycle assays directed towards four species of fish (i.e., fathead minnow, zebrafish, Japanese medaka and sheepshead minnow). This paper will outline the relevant principles, methods, and techniques needed for initial protocols, and will identify issues that might require additional validation studies. The final outcome will be a standardized and readily transferable fish life-cycle protocol that can be used to definitively test potential EDCs in a regulatory arena.

# 4.0 CULTURE AND HANDLING OF TEST SPECIES

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

## 4.1 Fathead Minnow (*Pimephales promelas*)

28. The fathead minnow (*Pimephales promelas*) is a common freshwater minnow belonging to the largest family of fish, the Cyprinidae. Their original range was limited to central North America but they have since been introduced into most regions of North American (Pflieger 1975). Fathead minnows can live in

a wide range of habitats including shallow turbid waters and they are an important bait-fish species. As a result, fathead minnows are easily cultured and are readily available from commercial sources. The fathead minnow has been used extensively in aquatic toxicity testing in the U.S. and a number of testing guidelines include detailed information on their laboratory culture (EPA/600/3-87/001) (Denny 1987).

29. Fathead minnows are small (35 to 75 mm in total length) and offer ease of culture in laboratory aquaria. Adult males are territorial but are still tolerant of a number of other adult male and female fish in an aquarium. They can tolerate low dissolved oxygen and a variety of water temperatures and water quality parameters but, for optimal growth and reproduction, water temperatures should be within a range of 24ºC to 25ºC, and dissolved oxygen should remain above 60% of saturation. Adult fathead minnows are sexually dimorphic and can easily be sexed in the aquarium. The development of aggressive behavior and secondary sex characteristics are signs of sexual maturity in males, and the development of a papilla is distinct sign of sexual maturity in females. However, the time of sexual differentiation is not well documented for the fathead minnow. The generation time of fathead minnows is about 4 months under optimal conditions, although sub-optimal feeding etc. leads to longer generation times (5 months). They can be kept in breeding condition all year without manipulating light cycles and water temperature. Fathead minnows can be induced to spawn by placing spawning substrate in the aquarium, which is guarded by the male. Better spawning synchronization can be obtained by withholding the spawning substrates for several days. Spawning is usually initiated just before light and may last through the morning; therefore, it is advisable not to disturb the fish during the morning hours except to feed and collect embryos. Each spawning female will produce 30 to 250 embryos per spawn. Embryos are collected from the aquarium by removing the spawning substrate. The embryos can be incubated on the spawning substrate or they can be removed from the substrate and incubated in a container or in egg cups (glass cylinders with mesh bottoms). Fertilization can be immediately assessed with light magnification or it can be assessed 24 hours after spawning by counting the number of opaque (fertile) and white (nonfertile) embryos. Fertilization rates of 80% to nearly 100% are typical. Embryo incubation time is 4.5 to 6 days at 25ºC.

## 4.2 Japanese Medaka (*Oryzias latipes*)

30. The medaka (*Oryzias latipes*) is a freshwater fish belonging to the family of Asian rice fishes (Adrianichthyidae) indigenous to areas of Japan, Taiwan, and southeastern Asia, where ambient temperatures range from 5ºC to 35ºC (Kirchen & West 1976). The medaka has a long history as an experimental animal and a complete presentation of their biology can be found in Yamamoto (1975) http://biol1.bio.nagoya-u.ac.jp:8000/Yamabook.html, which lists over 1000 references dating from the early 1900s. The culture and handling of the medaka has been exhaustively studied and detailed guidelines are available in EPA/600/3-91/064 (Denny et al. 1991). Medaka are small (25 to 50 mm in total length) and are easy to rear and maintain in laboratory aquaria. Adults can be maintained in an aquarium with little space and can tolerate low dissolved oxygen and a wide range of temperatures and salinity) (Yamamoto 1975). Medaka are sexually dimorphic and although the sex of fully grown fish can be determined by observing the outline of the anal and dorsal fins, the observation of an anesthetized fish under a low-power microscope is recommended for confirmation and for sexing an immature fish) (Yamamoto1975).

31. Medaka have a generation interval of 2 to 3 months, but can be induced to spawn throughout the year by controlling the temperature and photoperiod, and breeding groups with a ratio of 4 males per 6 females spawn through 14 to 15 months of age. Spawning is highly predictable in time, usually within 1 hr of

first daylight, and can be controlled in the laboratory by adjusting the timing of the recommended 16-hour-light to 8-hour-dark cycle (Hirshfield 1980; Koger et al. 1999). Measures of medaka reproductive output, including gonadal morphology, fecundity, and fertility, demonstrate that through laboratory control of temperature and photoperiod, reproductive capacity of breeding groups can be calibrated before exposure, thereby establishing a baseline from which to assess effects (Koger et al. 1999). Individual females can lay an average of 25 eggs/day for up to 4 months under proper conditions of photoperiod, temperature, and food supply (Hirshfield 1980). A cluster typically contains 10 to 30 eggs that are attached to the female's vent by filaments for a number of hours until they are brushed off onto a spawning substrate simulating aquatic plants (Yamamoto1975). Even when spawning substrates such as a spawning sponge are provided, some females retain their embryos requiring manual stripping (Denny et al. 1991). The eggs should be collected as soon as possible after spawning to prevent their predation by adults. Fertilization can be easily assessed with low magnification because of the transparency of the egg chorion. The egg incubation period is approximately 1 week when kept at 28ºC (Yamamoto1975), and the embryos will tolerate a temperature range of 7ºC to 38ºC (Kirchen & West 1976).

## 4.3 Zebrafish (*Danio rerio*)

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

33. Adult zebrafish are vigorous swimmers reaching a length of 4 to 5 cm and should be fed a variety of food, including dry flake food, brine shrimp nauplia or live planktonic crustaceans. Optimal nutrition for reproduction comprises at least one feeding with live organisms and one feeding with flake food per day. Indistinct secondary sexual characteristics can make it difficult to sex a few zebrafish strains, but this is usually not a problem with most strains if maintenance conditions are adequate. Mature female zebrafish typically have a rounded, fuller body contour, and males generally have larger anal fins and areas of gold coloration. When mating, males exhibit a darker brownish colouring with anal fin stripes becoming weakly iridescent blue. Zebrafish reach sexual maturity in 9 to 12 weeks. Groups usually spawn daily, producing 25 to 45 eggs per female at sex ratios between 2:8 and 8:2. Optimal breeding in zebrafish is achieved in breeding groups with a male:female ratio of 2:1. Under such conditions, permanent spawning with 100 - 150 eggs per female per day can be achieved over periods of up to 2 weeks. Spawning takes place shortly after dawn, and the transparent, non-adhesive eggs will naturally fall to the bottom of the tank, where screens can separate the eggs from consumption by the adult fish. Eggs and sperm can be collected from individual zebrafish for controlled fertilization studies(Westerfield 2000). The embryos normally hatch after approximately 3 days of development and begin to feed 3 days post-hatch. The larvae must be reared in separate aquaria which should not be cleaned daily, but care should be taken not to over-feed (see below). Larvae can also be reared in mesh cages (mesh width: 0.4 mm).Young larvae feed on live paramecia or on proprietary breeding food (e.g. AZ 100, Tetra), and at 9 days post-hatch,

freshly hatched brine shrimp can be added to their diet, followed by adult feed with increased growth. All life stages should be fed *ad libitum* 2 to 3 times per day, and larvae should not be fed more than can be consumed in 10-15 minutes.

34. The gonadal development in the zebrafish is unique when compared with other species, including the medaka and fathead minnow (Takahashi 1977a; Andersen et al. 2003; Maack & Segner, 2003). The species is classified as an undifferentiated gonochorist and it displays juvenile hermaphroditism. The gonads of all juvenile zebrafish generally begin to differentiate into ovaries 10 to 12 days post-hatch, although there may be some variation between strains and culture conditions. After 23 to 25 days, sex differentiation begins. Some fish continue to develop ovaries, whereas in others, the ovaries will begin to degenerate and testicular development will begin. The sex reversal is completed after 40 days, and the gonads will be fully developed in 60 days post-hatch.

35. The zebrafish has now been used extensively for life cycle testing in Europe, particularly in Germany and the Nordic countries. Its behaviour in such tests is well-documented (e.g. Wenzel et al., 2001), and the important reproductive variables in control fish cultured at the Fraunhofer Institute have been helpfully summarized by Schäfers & Teigeler (2004).

**4.4 Sheepshead Minnow (*Cyprinodon variegatus*)**

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

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

38. The generation time of sheepshead minnows is less than 2 months. They can be kept in breeding condition all year without manipulating light cycles and water temperature. Abundant high-quality food is important in maintaining a continuous spawning population. Better spawning synchronization can be obtained by separating the sexes within an aquarium for several days. The adults can also be held at 21ºC to 25ºC and then placed into 28ºC to 30ºC water to induce spawning (Overstreet et al. 2000). Each spawning female will produce 15 to 30 embryos per spawn; therefore, a large number of females will be

needed to collect enough embryos to initiate an experiment. Spawning groups should be placed in 3- to 5-mm NITEX mesh baskets. The baskets should be placed in trays to collect the embryos that fall through the basket. Spawning should begin within 24 hours once the males and females are together. Embryos are collected from the trays holding the spawning baskets every 24 hours. The embryos can be incubated in shallow dishes or they can be incubated in egg cups (glass cylinders with mesh bottoms, or nylon mesh cylinders with Petri dish bottom). Fertilization can be immediately assessed with low magnification, or it can be assessed 24 hours after spawning by counting the number of opaque (fertile) and white (nonfertile) embryos. Fertilization rates can be low and variable for sheepshead minnows (40% to 60%). Embryo incubation time is 5 to 6 days at 28ºC.

## 4.5 Strength and Weaknesses of Test Species

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

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

| Species | Strengths | Weaknesses |
|---|---|---|
| Fathead minnow *(Pimephales promelas)* | - Large enough to collect individual blood plasma samples<br>- Distinct secondary sex characteristics in both sexes<br>- Large historical regulatory database<br>- Many laboratories are familiar with culture and testing<br>- Spawn on a substrate<br>- High fertilization rate | - Relatively long life cycle<br>- Relatively high variability in fecundity<br>- Relatively large size of the fish require more space for culture and testing<br>- No markers for genetic sex |
| Medaka *(Oryzias latipes)* | - Relatively short life cycle<br>- Relatively small fish making culture and testing possible in smaller space<br>- Female sex determined during embryo stage vs male sex determined after hatch<br>- Sex linked color strain allows determination of genetic sex<br>- High fertilization rate | - Long sticky egg strands make handling eggs difficult<br>- Small size yields small volumes of plasma<br>- Limited regulatory data base |

| Species | Strengths | Weaknesses |
|---------|-----------|------------|
| Zebrafish *(Brachydanio rerio)* | - Short life cycle<br>- Small fish making culture and testing possible in smaller space<br>- Eggs easily collected in trays<br>- Widely used in other medical and genetic research<br>- High fertilization rate<br>- Produces large numbers of eggs | - Small size yields small (but usually sufficient) volumes of plasma<br>- Minimal secondary sex characteristics<br>- No markers for genetic sex |
| Sheepshead minnow *(Cyprinodon variegatus)* | - Very short life cycle (<60days to sexual maturity), seawater costs may be offset by shorter exposure times for testing<br>- Relatively small fish making culture and testing possible in smaller space<br>- At least males may by large enough for individual blood plasma samples<br>- Distinct sexual dimorphism<br>- Relatively low variability in fecundity<br>- Relatively large historical regulatory database<br>- Many laboratories are familiar with culture and testing | - Estuarine/marine species, salinity of 15 to 30 ppt recommended, however, lower salinity may be possible (5 ppt)<br>- Culture requires a large number of females to produce enough eggs in a 24-hr period to initiate a life-cycle test<br>- Limited information on reproductive endocrinology<br>- No markers for genetic sex |

**5.0 EXPERIMENTAL DESIGN CONSIDERATIONS FOR LIFE-CYCLE FISH TESTS**

**5.1 Exposure Duration**

40. An increase in the amount of exposure time typically results in quantification of effects at lower concentrations (McKim 1977; Bresch et al. 1990; Holcombe et al. 1995; Ensenbach & Nagel 1997; Nagel et al. 1998; Parrott et al. 2000). The age of the fish during an exposure can also impact the results of a study. Sexual differentiation, sexual maturation, embryo fertilization, juvenile growth, reproduction and maternal transfer are all examples of sensitive and critical phases in the life cycle of fish. Sensitivity to a toxicant will increase when more of these endpoints are included in the experimental design. In general, early research with pesticides has shown that there is little difference in response in a variety of endpoints between the species of fish being considered (i.e., fathead minnow, medaka, sheepshead minnow, and zebrafish) (Shinomiya et al. 1997; Kawahara & Yamashita 1997. However, developmental and reproductive toxicity might occur during larval development or the juvenile or adult stage, depending upon the species examined (Arcand-Hoy & Benson 1998). Macek and Sleight (1977), Woltering (1984), Chorus (1987), and Dionne and Kiamos (1994) found that the toxicity of a significant number of the chemicals and metals tested in full life-cycle studies in fish could not be predicted from the results of only an early life-stage exposure.

41. The maintenance of partial and full life-cycle exposures is costly and can result in interruptions in exposure as a result of unforeseen test-substance behavior in water (e.g. excessive degradation by microbial flocs) 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, adds significantly to the time and effort in maintaining a long-term exposure.

42. Therefore, the exposure duration of a study needs to encompass an appropriate time of exposure necessary to elicit an effect, but not beyond a necessary time to control costs and potential exposure interruptions. As a result, the fish species with the shortest life cycle might be the preferred species for conducting partial and full life-cycle tests.

*5.1.1   Partial Life Cycle:  Exposure During F0 Phase*

43. For clarity, this section does not deal with partial life cycle tests such as the fish sexual development test, which are addressed elsewhere (OECD, 2004). This section addresses partial life-cycle exposure of fish in the scope of a fish life-cycle toxicity testing.

44. To minimize the exposure duration, partial life-cycle studies should be initiated with sexually mature fish. A partial life-cycle study exposes adult sexually mature fish (F0) for a period of time prior to and during spawning, followed by a short-term exposure of F1 embryos and juvenile fish. Fertilization of the F0 eggs should occur in the exposure solutions. The F1 exposure can be a few days to a week if the endpoints of interest are limited to embryo viability and hatching success. If F1 survival and growth endpoints are included, the exposure should continued up to 4 weeks post hatch of the F1 larvae. This is consistent with a standard fish early life-stage test, with the exception that the eggs are fertilized in the exposure solution.

45. The induction of the egg-yolk precursor protein is under estrogen control mediated by estrogen receptors (ERs) in the liver of juvenile and adult fish. Detection of vitellogenin (VTG) has become the most widely studied biomarker of exposure to endocrine-active compounds. Vitellogenin induction in adult male fish can be quantified following an exposure of less than 7 days (Kishida et al. 2001; Elonen et al. 1998). However, longer exposures are needed to measure the effects of reproductive success, changes in secondary sex characteristics, behavioral changes, and gonadal changes. The exposure of the adult F0 fish should be sustained to allow incubation of at least three or four spawns (encompassing a full spermatogenic cycle) per treatment level. For the fish species considered in this protocol, the typical exposure time for the F0 generation would be 21 days (Ankley *et al*, 2001; Harries et al. 2000; Van den Belt et al. 2001). However, sheepshead minnow and zebrafish may only need a 7-10 day exposure. In addition to this exposure period, establishing a brief spawning history with F0 fish might be desirable prior to exposure; however, this would almost double the length of time that the F0 fish would be maintained in the exposure system (Ankley *et al* 2001; Harries et al. 2000, Van den Belt et al 2001). The exposure duration will need to be lengthened if exposure begins with juvenile F0 rather than adults. The additional time and effort that this would add to the exposure protocol depends upon the starting age of the juvenile F0 fish and husbandry practices. Because raising the species of fish under consideration in this protocol to sexual maturity in laboratory cultures can be performed with ease and in a cost-effective manner, there does not appear to be an advantage to initiating the exposure with juvenile F0 fish in a partial life-cycle exposure. In summary, a partial life-cycle exposure initiated with adults would have a duration of approximately 9 weeks, consisting of 14 days of pre-exposure spawning history, 21 days of exposure of F0 fish, and up to an additional 28 days of exposure for F1 fish. For sheepshead minnow and zebrafish, partial life-cycle test duration can be reduced to 49 days.

### 5.1.2 *Full Life Cycle: Continuous Exposure During F1 Phase*

46. Full life-cycle studies have been conducted for over 25 years with sheepshead minnow, fathead minnow, and zebrafish (Carlson 1972; Nebeker, Puglisi, & DeFoe 1974; Shinomiya et al. 1997; Kawahara & Yamashita 1997; Yasuda et al. 2000; Metcalfe et al. 2000; Nakamura et al. 1998; Ensenbach & Nagel 1997; Wenzel et al., 2001), and there is also significant experience with medaka (e.g. Yokota et al., 2001; Knörr & Braunbeck, 2002; Anon. 2006) and zebrafish. Many full life-cycle studies using the fathead minnow, and to a lesser extent the sheepshead minnow in the United States, or zebrafish in Europe, have been conducted in the support of pesticide registrations. This testing is a conditional part of the registration requirements of the US Federal, Insecticide, Fungicide and Rodenticide Act (FIFRA). A full life-cycle reproductive and developmental toxicity test should begin with <24-hour-old embryos (F0), should expose all life stages of the F0 generation, and (for EDCs) ideally run on to include the period of sexual maturation in the juveniles of the F1 generation, although the test has often been stopped after the swim-up stage when testing non-EDCs. Sex determination is primarily governed by genetics; however, it can be strongly influenced by hormonal activity for critical periods of time during sexual maturation, as explained further in section 5.2. These critical exposure times are not well defined for all species, resulting in increased importance of continuous exposures. However, the critical exposure period (for estrogen exposure) has been established in the zebrafish (Andersen et al., 2003). Using good husbandry techniques, especially a balanced diet fed *ad libitum*, removing excess food, F0 fish can reach sexual maturity in 60 days (sheepshead minnow), 90 days (zebrafish), 100 days (medaka) or 120 days (fathead minnow). Allowing one to four weeks for spawning and an additional 28-54 days post hatch for the F1 generation for growth and survival endpoints, a fish full life-cycle study can be completed within 4.5 months (sheepshead minnow), 6 months (zebrafish and medaka), or 7 months (fathead minnow).

47. A full life-cycle protocol with fathead minnows was first developed in 1970, is well established (USEPA, 1982), and has been successfully conducted by a number of laboratories, but does not comprise endpoints diagnostic of ED. Furthermore, a full life-cycle protocol with zebrafish was first developed in the 1980s and changed to a minor extent in order to include endpoints for detecting endocrine disrupting effects. It has received some validation (e.g. Wenzel et al., 2001; Segner et al., 2003), and includes a 3-4 week spawning period, which produces thousands of embryos. Zebrafish, as a near-tropical species, are tested on a 12-hr-light / 12 hr-dark light cycle. There is also substantial life-cycle testing experience with medaka (e.g. Japanese government report).

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

### 5.1.3 Multiple Generation Exposures

49. Testing beyond the reproductive phase of the F1 generation would be challenging and logistically complex for many laboratories. Multiple generation exposures have been conducted; however, continuous exposure of F0, F1, and juvenile F2 generations has not been reported (Bresch et al. 1990; Patyna et al. 1999; White et al. 1999). The long-term maintenance of an exposure system requires extensive experience that is not widely available. However, studying the effects of long-term exposure on the reproductive system in fish is extremely valuable for determining population-level effects. The value of a multiple generation exposure has been recognized by advocates of zebrafish, medaka, and fathead minnow fish models (Bresch et al. 1990; Arcand-Hoy & Benson 1998; Patyna et al. 1999; White et al. 1999; Nash et al, 2004; Kidd et al, 2007; Schaefers et al, 2007). In addition to these fish models, the sheepshead minnow is a valid model for a multiple generation exposure and offers specific advantages. In considering a reasonable time frame in which to conduct a multiple generation study, it is worth noting that life-cycle tests with exposure durations of over 260 continuous days are currently conducted with fathead minnows. Therefore, it is reasonable to consider a multiple generation test that could be completed in an 8- to 10-month timeframe. The sheepshead minnow, zebrafish, and medaka reach sexual maturity within 60 to 90 days post hatch, making all three species viable candidates for a multiple generation exposure within this timeframe. The fathead minnow has an extensive testing history, can reach sexual maturity in 4 months, and should also be considered a candidate for a multiple generation exposure test.

50. The key to a feasible multiple generation exposure is early maturation and the efficient collection of spawning data. The sheepshead minnow is a continuous spawning species. Each female can produce 15 to 30 eggs per day. Assuming that a study design has 10 to 16 females per treatment level, the number of eggs collected in a week from each treatment level can approach 2000. High reproductive potential in a short period of time would result in a study with P spawning data collected within 2 months and F1 spawning data collected within 4 months.

51. The zebrafish is also a continuous spawning species. Each female can produce 25 to 45 eggs per day. Assuming that a study design has 10 females per replicate, the number of eggs collected in a week from each replicate can be 3000. This would result in a study with P spawning data collected within 2 months and F1 spawning data collected within 5 months. A multiple generation study can be completed in 6 months.

52. In contrast, by reducing the number of spawns collected from fathead minnows, a multiple generation test with this species can be completed in 10 months.

### 5.1.4    *Strength and Weaknesses of Partial versus Full Life-cycle or Multiple Generation Exposures*

53. The goal of a partial life-cycle study is to expose the part of the life-cycle which is considered (on the basis of prior information about mode of action) to be the most sensitive to a substance, and can include measurements of both biomarkers and of functionally important endpoints such as sex ratio or fecundity.

54. The strength of a partial life-cycle exposure is the reduced time it takes to conduct the test compared with a full life-cycle or multiple generation test.  A full life-cycle test would reach the beginning of P spawning in the time a partial life-cycle study would be completed.  The partial life cycle reproduction test is well-suited *inter alia* to the fathead minnow, while a partial life-cycle sexual development test is currently being validated for zebrafish and fathead minnows by VMG-eco.  The fathead minnow is relatively larger in size when compared with the sheepshead minnow, medaka, and zebrafish, which allows the collection of more blood plasma, an important consideration if protein and sex-steroid analyses are to be conducted.  The presence of VTG in male fish is a reliable biomarker of exposure to an estrogenic compound; however, the interpretation of sex-steroid levels in fish plasma is not as clear.

55. The weakness of the partial life-cycle test is its inability to expose all life stages of the fish. Therefore, the partial life-cycle study does not provide data on potential population-level effects if the most sensitive part of the life-cycle is excluded, or if there is a concern about delayed effects resulting from exposure in an earlier generation.  The full life-cycle and multiple generation exposures ensure that all life stages are exposed.  Therefore, population-level effects can be ascertained, and potentially of more importance, concentrations that do not cause population-level effects can be determined.

56. Histopathology and biomarker data might be necessary endpoints for some life-cycle studies (especially those with EDCs), but their mandatory application is probably undesirable because mechanistic information is not always required for risk assessment. However, gonad histopathology should generally be used in life cycle tests of EDCs, because it is the  key to examine a) whether a test agent effect on reproduction might result from a general toxic effect or a "physiological" endocrine effect, b) whether alterations of reproductive variables are associated with pathological changes of the gonads (for instance, the effect may be mediated at the pituitary level, or macroscopically normal gonads may show intersex phenomena at the microscopical level), and c) to confirm macroscopical sexing of the fish

57. Life-cycle testing with a smaller and faster maturing species than that used for the partial life-cycle test might reduce the study duration, and would provide useful data on the second species, but this cannot be recommended until full comparative validation studies have been conducted. However, it should be noted that a partial life-cycle study may not always be used to trigger life-cycle testing.

58. The main weakness of life-cycle studies is the difficulty in maintaining long-term exposure systems, the cost of running long-term studies, and the risk that fish may be lost to disease before the experiment is terminated. However, long-term exposures are the only reliable way to evaluate fully the chronic effects of EDCs and some other substances on fish.

**5.2 Ontogenic Period of Exposure**

59. Based on the life-history model, fish can be broadly divided into two ontogenic groupings: those with an indirect life cycle and those with direct life cycles (Balon 1975). In the case of the indirect life cycle, five distinct life-history periods are found: embryonic, larval, juvenile, adult, and senescent. Fish with an indirect life cycle tend to be oviparous. The direct life cycle is characterized by four life-history periods: embryonic, juvenile, adult, and senescent, the larval period being absent. Fish with a direct life cycle are typically viviparous, with internal fertilization and gestation – species of this type will not be considered further in this DRP, as there is only very limited experience with them in the conduct of life-cycle tests (e.g. with guppy *Poecilia reticulata*; Schäfers and Nagel, 1991). Once a fish has attained sexual maturity for the first time and entered the adult period of its life history, two patterns are observed: gonochorism or hermaphroditism (Chan & Yeung 1983). In the case of gonochoristic fish, the sex, either male or female, is fixed; whereas in hermaphroditic fish, the sex may change from male to female (protandry) or from female to male (protogyny) as adults, or both sexes can be simultaneously and functionally present. All the test species being considered in this review are gonochoristic (although the zebrafish displays juvenile hermaphroditism), and the hermaphrodite pattern of sexual development in adults will not be further discussed because experience in life-cycle testing with this group is largely absent.

60. Not surprisingly, there is considerable variability amongst different species of gonochoristic fish with respect to when during the life cycle the gonads first differentiate and sex can be determined. A generality that is emerging from fish studied to date is that ovaries differentiate in females before testes differentiate in males (Nakamura et al. 1998). Therefore, in a given species, the female gonad may begin to differentiate in a different life-history period (e.g., embryonic) from that of the male (e.g., larval or juvenile). Two pathways for gonad differentiation in fish have been documented (Yamamoto 1969). In the undifferentiated-type, the indifferent gonads pass through a female phase (i.e., initially appear as ovaries) before either taking a male path or continuing as a female. In the differentiated-type, the indifferent gonad directly develops into an ovary or testis. The zebrafish has the former, undifferentiated-type of gonadal development, in which ovaries appear around Day 10 in all juveniles and by Day 25 begin to transform into testes in males (Takahashi 1977b). Alternatively, the medaka has the differentiated-type, in which the ovaries are distinguishable during the embryonic period, and the testes in males appear later during the larval period (http://biol1.bio.nagoya-u.ac.jp:8000/). This phenomenon is probably explained by the mechanism of sex determination in these two fish in which the medaka has a clear genetic mechanism that uses sex chromosomes with male heterogamety (i.e., XY), whereas that for the zebrafish does not and likely uses a polygenic mechanism involving autosomes.

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

effects of estrogenic compounds to cause complete sex reversal or intersex conditions in males of a variety of fish, depending on when treatments are given. This shows that developmental timing of exposure is critical and important in terms of the expected effects, and reinforces the view that a partial life-cycle test runs the risk of not exposing the most sensitive life-stage.

## 5.3 Route of Administration

### 5.3.1 Water

62. Water exposure is the most common route to expose fish to EDCs, and a water-exposure concentration can be correlated with water concentrations found in the aquatic environment. The delivery of a toxicant in water at different concentrations is well established. Water exposures have been successfully conducted by laboratories around the world, using a variety of pumps, valves and gravity. The biggest challenge in a water exposure is solubilizing the test substance in the water and maintaining it in solution. Organic solvents are often used as carriers to assist in delivering a test substance in water, which requires the maintenance of a solvent control, but the use of carrier solvents in life-cycle tests is not recommended. Organic solvents enhance bacterial growth in the test system, which increases maintenance time during the exposure and encourages degradation of the test material. In some cases, saturator columns have been used to eliminate the use of solvents. Problems also arise in testing photolabile substances, when special precautions must be taken to exclude light of certain wavelengths. Further helpful guidance on testing and risk assessment of difficult substances is provided by Rufli et al. (1998) and ECETOC (2003).

63. Particular difficulties might be encountered in life-cycle tests if the water supply temporarily fails or dosing is interrupted. Loss of water could lead to stressing of fish or over-exposure, while interruption of dosing will give under-exposure. These problems can only be dealt with on a case-by-case basis, but temporary fluctuations in exposure concentration should be considered in the context of a test which continues for many months, and they may not seriously change the long-term average concentration.

### 5.3.2 Oral (Food)

64. Patyna et al. (1999) recommends that hydrophobic compounds with a $K_{ow}$ >5 should be administered via food. Dosing some commercial artificial fish food is relatively simple, but dosing food items such as live or frozen foods may not be possible. Because a balanced diet is important in the rearing of fish in an exposure system during many life stages, dosing via a variety of prepared and live foods would present a significant challenge. In addition to the need to provide a variety of chemically prepared feeds, the major problem with oral dosing is the inability to determine the dose that each fish receives. Another confounding factor of oral dosing occurs with uneaten food that remains in the tank, releasing chemical into the water, thereby combining the oral uptake with water exposure. In summary, the use of oral dosing is not recommended in life-cycle testing of chemicals for purposes of risk assessment.

### 5.3.3 Intra-Ova Injection

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

environmental concentrations is also difficult. Intra-ova injection has limited or no application in a large-scale screening and testing program because the results are hard to use for chemical risk assessment.

**5.4 Dose Selection**

66. Dose levels for both partial and full life-cycle studies should be selected with the use of range-finding data, although reliable data on doses are likely to be available from partial life-cycle experiments. Selected concentrations should be less than lethal levels and less than the level of water solubility. If the stability of the substance in the test system has been established, exposure concentrations should be measured weekly. The concentration of some test substances will decrease over time in mature life-cycle systems. If this occurs, nominal concentrations should be increased in an effort to maintain constant exposure levels in the aquaria.

67. Two or three widely spaced treatment levels would be appropriate for partial life-cycle studies if they are used purely as a screening tool for identifying EDCs. The treatment levels in that case could be separated by up to an order of magnitude, and there would be no need for a treatment level that causes no effect. However, partial life-cycle tests used for risk assessment, and in preparation for life-cycle studies, should be conducted with at least five treatment levels, and the treatment levels should be separated by approximately a factor of two. At least one of the treatment levels should be below a no-observed-effect level.

**5.5 Statistical Considerations**

68. The objective of the life cycle fish test is to provide the most ecologically-relevant, economic and statistically efficient estimate of toxicity associated with endocrine disruption and reproductive fitness for a given chemical. Thus, the assay must be biologically sensitive, have minimal variability associated with exposure throughout the duration of the test, and have a statistically powerful inference. Biological sensitivity is a function of the choice of species tested, the relevance of the endpoints measured to species survival, and the route and duration of the chemical exposure. Design-associated variability in exposure is a function of exposure route and duration, chemical stability and purity within the testing environment, and the testing protocol. The power of a statistical inference is a function of the inherent variability in response; design-associated variability; the degrees of freedom and the source of variability for testing; and the estimation process and decision criteria. Other areas in this section have discussed biological sensitivity; this subsection will focus on design-associated variability and statistical power. Extensive discussion of the statistical analysis of ecotoxicity data from OECD tests can be found in the recent (2006) OECD Series on Testing and Assessment Report Number 54 (ENV/JM/MONO(2006)18): *Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application* (posted at http://appli1.oecd.org/olis/2006doc.nsf/linkto/env-jm-mono(2006)18, with Annexes at http://appli1.oecd.org/olis/2006doc.nsf/linkto/env-jm-mono(2006)18-ann).

*Controlling variability*

69. It is important to recognize that there is naturally very high variability in fish reproduction, both between species and between individuals of some species. This particularly applies to the numbers of eggs produced (i.e. to the important endpoint of fecundity). It is a natural feature of fish which should not be ignored by only testing species with low inherent variability, and the statistical design of a life-cycle test must therefore be capable of taking high variability in fecundity into account. In particular, it is

considered important to measure spawning in several randomly selected breeding groups in order to minimize the effects of this variability on statistical power.

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

71. Ideally, an experimental design incorporates randomness, independence, and replication (Cochran & Cox 1957). Randomness and independence are used to remove bias, and replication provides a measure of variability across similar test units within a treatment (Chapman et al. 1996). Randomization of 1) experimental containers within a testing environment, 2) treatment application to experimental containers, and 3) application of organisms to experimental containers allows one to incorporate the variability associated with the environmental conditions, the containers, and the organism equally across all treatments. Thus, when one evaluates the difference between treatment means, the variability associated with experimental environment, experimental containers, and organisms being treated can be used to determine whether any differences between means are likely to be as a result of chance, or indicate that the treatments truly had different effects.

*Defining the experimental unit*

72. 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. Pseudoreplication is defined as the use of inferential statistics to test for treatment effects with data from experiments in which either treatments are not replicated (though samples may be) or replicates are not statistically independent. For either type of pseudoreplication, one would obtain an error term that is invalid for testing the hypothesis associated with the mean responses from the population of fish over time and space. Without true treatment replication, the effect of the treatment is confounded with the variability of the response. It is possible that the response is mainly due to a poor random sample of fish exposed to the treatment rather than the treatment itself. Thus, the strength of the inference of causality is diminished. A reduction in the sources of variability that are truly independent constrains the inference (Hurlbert 1984). Thus, if only one solution of each treatment is made and then divided between replicates, the source of variation associated with making the treatment is not included in the variability for testing, and the inference is limited. Some would say that this variability is nuisance noise, too small to be of concern, and costly to include. Therefore, if this source of variability is not included, it should at least be acknowledged. The variability between replicate experimental units may also include noise that was not randomized out due to a poor randomization or variable measurement error. These sources of variability can be reduced without loss to inference.

73. For some endpoints (e.g., percent males/sex ratio, survival and testis-ova induction) the experimental unit must be the tank, and relatively large numbers of replicate tanks are required for high power to detect significant differences between treatments and the control. However, for endpoints based on individual measurements (fecundity and fertilisation success per female, time to hatching, hatching success, and

growth) it is possible to argue that the individual fish is the experimental unit. Some statisticians will argue that this ignores pseudoreplication (Hurlbert 1984), but others point out that it may not be practically possible to run ecotoxicity tests for some endpoints without treating individual animals as individual replicates, even if they are housed together. Information on within-tank and between-tank variability may be a useful guide to whether individual fish across different tanks can be treated as if they are replicates (probably within a nested ANOVA design). In such case, either the mean response of the fish in a tank should be analyzed (taking survival into account if there is differential mortality) or a nested variance structure should be used (i.e., fish(tank(conc))).

### *Importance of statistical power*

74. 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 (i.e., the significance level that is chosen, which is usually 5% ($p=0.05$), and the percentage of difference one wishes to detect. One can control the latter three components; however, the variability in response is inherent in the organism being tested. Thus, the choice of which species should be tested and the relevant endpoints which should be 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. For example, control data from experiments with fathead and sheepshead minnows with only 2 replicates per treatment provide estimates of CVs for a variety of endpoints that can be used to evaluate power. The measured response with a CV greater than or equal to 15% will be unlikely to detect differences smaller than 50% between the test and reference treatment response at a type I error rate of $\alpha= 0.05$ (Figure 5-1). For a given CV, one can increase power by increasing the number of replicates. The choice of the test species and endpoints with the least inherent variability, by default, requires the least replication for a given level of power and, thus, cost less to run.
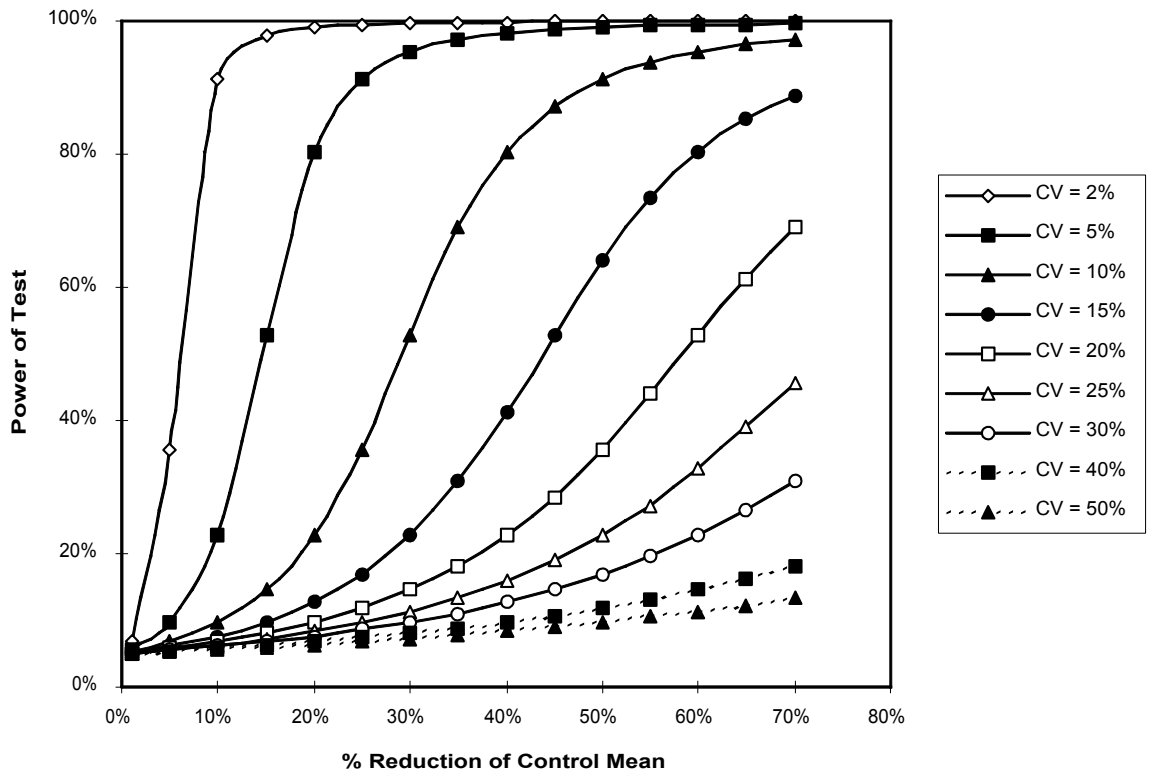
**Figure 5-1.    Power of a one-sided independent-samples t-test as a function of the percent reduction detected between the test and reference means with 2 replicates per treatment, a = 0.05**

75.     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 treatments.

*Data treatment*

76. Appropriate data transformations should be applied to maintain homogeneity of the within treatment variances (i.e., data for quantal responses expressed as a percentage will be arcsine-square root transformed, counts should be square root transformed, and continuous data should be transformed to the natural logarithm) (Snedecor & Cochran 1980).  A rank transformation or nonparametric statistic should be used when the commonly used data transformations are not successful in controlling heterogeneity (Daniel 1978).  Steel's rank sum test (Steel 1959) is a nonparametric alternative for comparing a control to at least two doses greater than zero.  This procedure uses an experiment-wise error rate.  Fisher's Exact Test for binomial data (e.g., the number hatched divided by the number fertile) or the Wilcoxon test can be used to compare two test vessels and check whether there is any statistically significant difference (e.g., a water control and a solvent control, when a solvent carrier was used in the test) before deciding to pool them for further statistical analysis. This operation increases the statistical power (Steel & Torrie 1980; Chapman et al. 1996).

77. 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). Tukey (J. W. Tukey, Exploratory Data Analysis, Wiley, 1977) suggest defining an outlier as an observation more than 1.5 IQR above the third quartile or 1.5 IQR below the first quartile.  This rule, implemented in standard software packages such as SAS, would designate a higher percentage of observations as outliers that that in the cited paragraph, though still a very small percentage arising purely by chance.  If an explanation cannot be found 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.

*Statistical tests for the NOEC/LOEC determination*

78. Green and Springer (2005) proposed the following decision tree for an appropriate statistical analysis.

Both solvent control and non-solvent control are present.

**Yes** → | **No** →

Compare controls using Wilcoxon.
Do controls differ?

**Yes** → | **No**

Drop Non-solvent control

Combine controls*, retaining subgroups

Dose Response Experiment?

**Yes** | **No**

Expect monotone dose response & there are >2 doses** in test?

Compare treatments to a common control?

**Yes** | **No**

Use step-down trend test (e.g. based on Jonkheere, or alternatively, if data are normal and homogeneous, Williams test )

Assess data for normality and variance homogeneity. Data normal *and* homogeneous?

Assess data for normality and variance homogeneity. Data normal and homogeneous?

Non-standard design. Not discussed here.

**Yes** | **No**

Use parametric pairwise comparison (e.g. Dunnett's test)

Go to
(A)
On next page

**Yes** | **No**

Use parametric pairwise comparison (e.g. Dunnett's test)

Use non-parametric pairwise comparison (e.g. Mann-Whitney with Bonferroni correction)

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* Both scientific judgment and regulatory guidance must be considered in deciding whether to pool non-solvent and solvent controls. The flow chart depicts appropriate actions if pooling is permissible given these constraints.

** Doses include 0-dose control

Note: If there are <5 experimental units per treatment, or there are massive ties (see text) then exact trend or pairwise tests should be used if possible.

A

Data normally distributed?

Yes

No

Use Tamhane-Dunnett test or perform pairwise
comparisons (eg. using
-Dunn's Test with Bonferroni-Holm correction or
-Mann-Whitney with Bonferroni-Holm Correction or
-Unequal variance t-test with Bonferroni-Holm
 Correction )

Use non-parametric pairwise comparison (e.g.
Dunn's test or Mann-Whitney with Bonferroni-Holm
correction)

Note: If there are <5 experimental units per treatment, or there are massive ties (see text)  then exact trend or pairwise tests
should be used if possible.

**Figure 5.2. Statistical flow chart describing the recommended statistical protocol for Phase-2 studies**

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

79. Typical considerations for sample size are based on the number of endpoints to be collected and whether a fish has to be sacrificed to collect the data. With chronic dosing protocols, a dose response is expected (i.e., over some specified range of doses there will be varying intensity of endpoint response that is significantly different from un-dosed or control fish). Additionally, it is assumed that at some dose there will be no difference between the dosed and un-dosed fish. A specific concern related to multigeneration tests is the need for adequate F1 offspring to be produced to allow for sufficient replication of adult F1 responses. If at h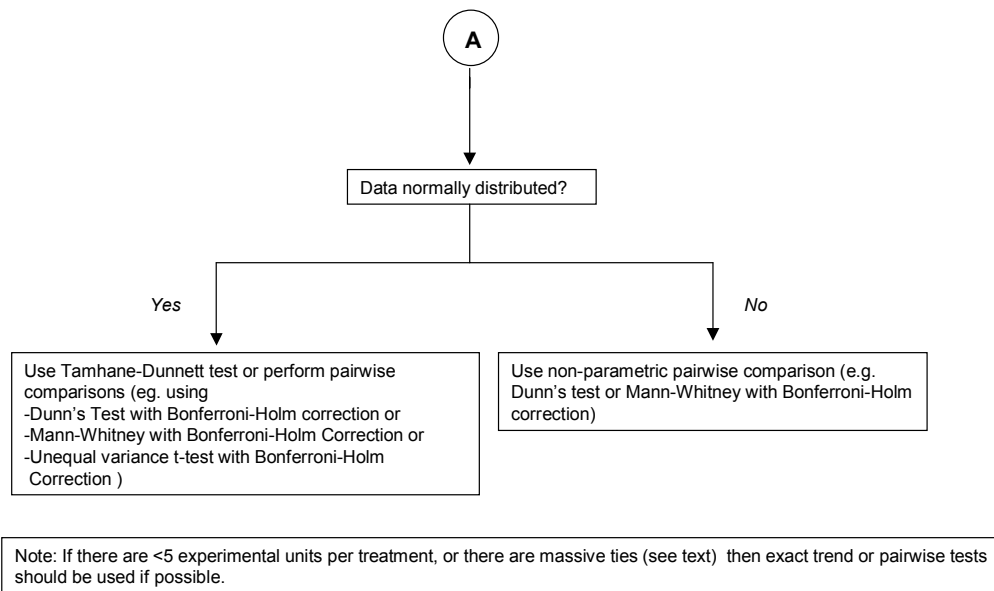igh doses complete or substantial embryo-larval lethality occurs, the assay, in effect, approximates a screening test, as only F0 reproductive effects can be assessed. To guard against this possibility, prior results on fertilization and hatching success obtained as part of a screening test should be carefully scrutinized to avoid excessively high exposure rates. Furthermore, it may be necessary to increase the sample size of F0 spawners at high exposure rates to ensure adequate collection of surviving F1 larvae. However, due to the size of the system needed to culture and expose fish through a (partial) life cycle and the need for multiple treatment levels, the number of replicates that can be maintained will be limited. Regulatory testing and studies from the literature routinely use a number of treatment levels with two true replicates per treatment level. A dilution water control and a carrier control (if used) are always required. Groupings of fish within a replicate are advisable if uncertainty regarding F1 larval toxicity exists.

80. As a useful guide, 100 embryos per replicate has been a standard sample size for starting a life-cycle exposure used for regulatory purposes. This number is twice the number previously recommended by regulatory agencies (Rexrode & Armitage 1986), but ensures adequate numbers of larvae can be procured for continuation of the exposure to maturity. The literature generally has smaller starting sample sizes ranging from approximately 20 embryos or larval fish per replicate (Carlson1972; Nebeker et al. 1974; White et al. 1999) to 240 larval fish (Nimrod & Benson 1998). One hundred embryos allows for a good examination of hatchability, fry survival, and growth of the F0 generation. It also allows for adequate numbers of fish to set up spawning groups and collect sexually mature but never spawned fish for GSI, blood biomarkers, and histopathology. Fewer F0 post-spawn fish will be available, with more females than males for most species. Collecting 50 to 100 F1 embryos per F0 spawning group would also be adequate for the F1 exposure. Additional embryos from the F0 spawning groups can be collected for other endpoints, e.g., eggs per female and fertilization or hatching success. Finally, 50 to 100 F2 embryos per F1 spawning group will be sufficient for evaluating growth and survival of the third generation. Two generation and multi-generation designs have relatively few parental fish contributing to subsequent generations where sex ratio is assessed. Therefore, sex ratio skews resulting from clutch to clutch bias will adversely affect the power of this endpoint in these generations. This is not an issue with the one generation design where the F0 generation can be initiated with mixed eggs from as many clutches as possible.

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

81. The no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) have been used to evaluate data from a multiple generation study typically when the differences between exposure levels are no greater that a factor of approximately 2 to 2.5. A multiple generation study will

generate up to approximately 20 to 30 NOEC/LOEC pairs of endpoints for both the F0 and F1 generations, plus an additional three or four endpoints for the F2 generation. This would provide a total of up to 60+ NOEC/LOEC pairs for evaluation of a NOEC for fish chronic toxicity. These endpoints are life-stage specific, sex specific, and generation specific.

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

83. Even though the NOEC is widely used, it should not be relied on as the sole indicator of low toxicity. The largest test concentration for which statistical differences have failed to be detected is a direct function of the power of the test. For certain endpoints, such as F0 eggs/female/day, it is unlikely that any effects will be detected with only two replicates per treatment, given the variability between replicates. It is also conceivable that short-term range finding experiments will have difficulty in predicting the location of an NOEC in a multigeneration test.

*Regression analysis for ECx determination*

84. An alternative to the NOEC determination is the analysis of regression to determine a given effect concentration ECx, if a regression model fit to the data can be found. However, care must be taken not to estimate an $EC_x$ value that lies outside the range of the data (i.e. interpolation) because this introduces large error limits. The difficulty in interpreting the results from a multigeneration study might lie in whether a statistically significant or non-significant result will have true population-level effects i.e. what size of ECx is considered biologically significant? Precision and accuracy of the $EC_x$ is a function of the spread between treatment concentrations, the number of concentrations tested (Chapman et al. 1996), the appropriateness and goodness-of-fit of the model to the data. This issue of population significance may be addressed by using the data from fish reproduction tests to estimate the effects of EDCs on demographic endpoints, such as the the intrinsic rate of population increase or risk of extinction (Grist et al., 2003).

85. The design and analysis requirements for estimating the NOEC are different from those for fitting a concentration-response model (Chapman et al. 1996); (Stephan & Rogers 1985). ANOVA methods require experimental unit replication and achieve greater power in testing as a function of the number of replicates. As shown in Figure 5-1 and Table 5-1, the different endpoints would require different amounts of replication to achieve the same level of power. Transformation of the data to satisfy homogeneity of variance is required for the parametric test and the regression approach. Estimation of the NOEC does not require the assumption of a specific model, such as a lognormal, and ANOVA methods such as the t-test and Dunnett's test are robust against mild violation of the normality assumptions to non-normal errors (Sheefé, 1959) on which they are, in fact based, they are not impervious to non-normality. Second the t-test and Dunnett tests are not the most appropriate tests to use, as argued elsewhere in these comments.

One of the drawbacks of these two tests is their complete ignorance of the dose-response nature of the experiment. One of the drawbacks of the regression approach is the use of specific regression models to capture this dose-response, when there is no agreed biological basis for any routinely used model. Tests such as Williams and Jonckheere assume a monotone dose-response but no specific parametric form. A non-parametric regression model could provide a similar flexibility for ECx estimation.

86. Individual responses should not be assumed random responses from a normal population. Either the mean response of the fish in a tank should be analyzed (taking survival into account if there is differential mortality) or a nested variance structure should be used (i.e., fish(tank(conc))). In the context of tests for sexratio, the replicate is clearly identified as the tank. Presumably that applies here as well. Further, due to the unpredictable nature of survival and fertility in the multi-generation test and the large variability in specific endpoints, it is desirable to have some level of treatment replication in order to provide a more accurate estimate of the mean population response for a given dose. The number of replicates would depend on the maximum expected variability in response for each dose. The variability in response may be a function of the dose. In this case, either a weighted analysis should be conducted or a data transformation applied that satisfies the assumption of homogeneity of variance.

87. Benefits of the regression approach include 1) estimation of the pattern (e.g., slope) of toxicity as a function of dose; 2) estimation of the distance between effect concentrations and environmental concentrations; 3) estimation of $EC_x$ and their associated confidence intervals for x equal to a low to medium effect; 4) the $EC_x$ estimates are not limited to doses on test; 5) both measured and nominal concentrations can be used; and 6) the ability to compare concentration-response curves across endpoints (Chapman et al. 1996). The size of the resulting confidence intervals (i.e., precision of the estimated $EC_x$) is a function of the inherent variability in the response and the number and spacing of the concentrations tested. But, the result of providing a confidence interval that does not include a measure of uncertainty can be a false positive sense of precision from a small confidence interval arising from a model that does not fit to the data at the concentration being estimated.

### Model(s) selection

88. Hoeting *et al*, 1999, state that "Basing inferences on [a single model] alone is risky; presumably, ambiguity about model selection should dilute information about effect sizes and predictions since 'part of the evidence is spent to specify the model.' Leamer (1978), Draper (1995) and Hodges (1978), make essentially the same observation."

89. As implied in Raftery (1996), confidence intervals for ECx, which are often cited as a way of measuring estimate quality, are model dependent and underestimate, sometimes considerably, the error in the estimate.

90. Among the possible ways of remedying this lack, Raftery (1996), Hoeting *et al* (1999), Raftery, Madigan and Hoeting (1997) among others, advocate what they term Bayesian model averaging as a way of capturing and quantifying model uncertainty.

### Concentration spacing for an analysis of regression approach

91. Guidelines often require five concentrations that are geometrically spaced and sub lethal plus a no-dose control. Thus, a range-finding test would be required to determine appropriate concentrations.

Regression modeling is flexible enough to handle a wide range of concentration-response patterns including non-monotonic. If there are only one or two responses that are neither 0 nor 100% affected and at least one response is greater than 50% affected, the Spearman-Karber non-parametric method can be used to estimate an $EC_{50}$. Finally, the regression approach can handle a wide range of responses including continuous responses, counts, and quantal data by re-expressing or transforming the data (e.g., log (y+c); $(y+c)^{1/2}$, and probit respectively).

92. In summary, ANOVA designs for fish testing appear inferior to regression designs, and the latter are considered to show more promise for fish life cycle tests given the generally large inherent variability in egg production (fecundity) between individuals, which inevitably reduces the power of the ANOVA approach. Final decisions on which design strategy to use should be made on a case-by-case basis, taking into account factors such as the known variability in reproductive output of the species in question.

# 6.0 DESCRIPTION OF ASSAY ENDPOINTS REFLECTIVE OF REPRODUCTIVE DYSFUNCTION

## 6.1 Growth and Morphological Alterations

### 6.1.1 Gonadal Development

93. 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).

94. The GSI can be potentially useful as part of a life cycle study, because reduction in relative gonad mass can occur as a response to certain types of endocrine-active compounds. Note, however, that GSI did not provide useful results during the validation by VMG-eco of the 3 week fish screen, maybe because exposure time was insufficient for gonad mass to change significantly. Although frequently reported, the appropriateness for comparison of GSIs between control and treatment groups is based on several specific assumptions, many of which may be difficult to validate in fish such as those considered in this review. Specifically, one key assumption is that linear relationships between gonad weight and body weight are constant throughout varying stages of gonadal development (Wall et al. 2000). This criterion can be challenging to meet with females of fish species that are asynchronous, fractional spawners, as inter-individual variation in ovarian weight can be high during the spawning cycle. Two approaches researchers have used to overcome this limitation are synchronization of spawning cycles through environmental manipulations (Soyano et al. 1993; Van den Belt et al. 2001), and more commonly, simply including sufficient numbers of replicates in an exposure tank to "average" out the variation across individuals.

95. 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 $= 100 \times$ gonad weight / body weight. Typical values for reproductively active fathead minnows, medaka, and zebrafish are as follows: fathead minnows: females 8% to 12%; males ~1% (Jensen et al. 2001); zebrafish: females 6.7% ±1.6%; males 0.98% ±0.2 % (Van den Belt et al. 2001); medaka: females 8.5%; males 1.5% (Scholz & Gutzeit 2000).

96. Although measurement of the GSI is a simple procedure, its determination and application as a useful indicator of reproductive status has been questioned. It has been frequently noted in fish that differences

in mass of the reproductive organs are not always attributable to changes in overall gamete production or gamete size. For example, a reproductive study of Gizzard shad (*Dorosoma cepedianum*) indicated that only 50% of the variability in ovarian weight could be explained by differences in fecundity or mean egg volume (Jons & Miranda 1997). Other authors have suggested that a better predictor of gonad maturation in females is measuring oocyte diameter (Wall et al. 2000).

97. 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.

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

*6.1.1.1 Histopathology in Juveniles.*

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

100.    Detailed descriptions of histological changes caused by hormone active compounds were described in the 1980s in juvenile guppy and medaka (Wester et al., 1985; Wester and Canton, 1986). Zebrafish histopathological data are also presented in Van der Ven et al. (2003), and http://www.rivm.nl/fishtoxpat

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

102.    Although inhibition of gonad development is often reported, in some cases enhanced development is also found, such as with sodium bromide where stimulation of gonad development and thyroid (as observed histologically) has been reported (Wester et al., 1988). Moreover, histopathological investigations may also produce (unexpected) mechanistic data. See also http://www.rivm.nl/fishtoxpat.

*6.1.1.2 Histopathology in Sexually Mature Individuals.*

103.    During the validation studies for the 21-day fish endocrine screening assay, a guidance document was developed for the preparation and evaluation of gonad histopathology. This document contains guidance for the staging of gonads and for the evaluation of most commonly found gonad alterations. The guidance document is not yet publicly available and it is currently considered as a working paper**.** The primary structural effect of EDCs on mature fish is observed in reproductive follicles.  In female fish, ovarian development can be staged by enumerating the proportion of a fixed number of ova in either normal or degenerative stages of development.  In regard to the normal development of oocytes West (1990) reviewed histological staging in fish and notes the following developmental sequence: 1) chromatin nucleolar stage, 2) perinucleolar stage, 3) yolk vesicle (cortical alveoli) formation, 4) vitellogenic (yolk) stage, and 5) ripe (mature) stage.  Although West (1990) concludes that histology is the most sensitive and appropriate method for staging ovaries, he also notes that the final stages of oocyte maturation are often difficult to follow in histological material because of the shrinkage and distortion of the cells during processing and the loss of ovulated cells during histological processing.  However, shrinkage and distortion of ovaries is not a general observation in routine paraffin processing (for results see http://www.rivm.nl/fishtoxpat).

104.    Detailed descriptions of histological changes caused by hormone active compounds in adult guppy and medaka have been published (Wester et al., 1985; Wester and Canton, 1986). Zebrafish histopathological data are also presented in Van der Ven et al. (2003), and http://www.rivm.nl/fishtoxpat. In fathead minnows exposed to E2, Miles-Richardson et al. (1999) reported an increased number of atretic follicles and fewer secondary and Graffian follicles in comparison to unexposed female fish.  Other authors have also reported that the ovaries in female fish exposed to estrogenic chemicals show fewer mature follicles and a greater number of atretic follicles (Ankley et al. 2001; Länge et al. 2001).  There

have been many other studies of reproductive histopathology of females on exposure to EDCs, including exposures of medaka to lindane (Wester & Canton, 1986).

105. Effects on male reproductive follicles are reported to include degeneration of spermatocytes, generalized atrophy, the development of ova-testes and a proliferation of Sertoli cells related to the concentration of exposure (Miles-Richardson et al. 1999). These authors evaluated testicular lesions based on the degree or severity of relative or absolute Sertoli cell proliferation and the percentage of seminiferous tubules affected. The semi-quantitative method of Sertoli cell proliferation rated the proportion of affected Sertoli cells from 0 to 4, corresponding to the quartile percentage of affected cells. Degenerative changes included germ cell syncytia, mineralization of spermatozoa and necrotic spermatozoa. Studies of testicular ultrastructure following *in vivo* treatment with EE2 have been published by Islinger et al. (2003).

106. It is worth noting that, apart from well established EDCs, other compounds may also exert specific adverse effects on gonads that may jeopardise reproductive capacity, such as spermatogenetic degeneration and necrosis after exposure to methyl mercury (Wester and Canton, 1992).

### 6.1.2 *Histopathology of Non-Reproductive Tissues*

107. Direct effects of estrogen or androgen (anti-) agonists may occur in tissues that express receptors for these compounds leading to histologically detectable changes, particularly when exposure causes excessive or unnatural stimulation of these pathways. In addition, indirect effects may occur in peripheral tissues resulting from excessive stimulation of receptor-gene products such as VTG. In this latter example, unnaturally high VTG levels are responsible for the accumulation of excess protein in various organs and the frequently noted accumulation of eosinophilic fluid in tissues during prolonged exposure to high E2 levels (Herman & Kincaid 1988; Metcalfe et al. 2001). More specific histopathological effects reported from exposure to estrogenic compounds include changes in liver ultrastructure (e.g. Islinger et al., 2003) such as vacuolization of hepatocytes, and degeneration of renal glomeruli (Metcalfe et al. 2001). A very significant and prominent feature of estrogen stimulation is the altered morphology of the hepatocytes as presented for the first time in the case of endocrine disruption by Wester et al., (1985), plus effects on various other organs (heart, gonads, kidney, pituitary). Indeed this altered hepatocyte morphology as a hallmark for vitellogenesis has been shown to be rather sensitive in comparison with VTG ELISA (van der Ven et al., 2003b).

108. Effects of chemicals acting on the thyroid gland are also detectable by histological analysis. Histological changes in the thyroid gland have been found to be the most sensitive parameter observed in fathead minnows exposed to a anti-thyroidal chemical (Lanno & Dixon 1994). This study showed that a sufficiently high dose results in hyperplasia of the thyroid causing a visible goiter. Clearly, at lower doses, physiologically significant thyroid hyperplasia can occur but will only be detected histologically. Other thyroid active compounds can produce complex effects such as thyroid hypertrophy and hyperplasia of follicular epithelium that are only detectable through histological analysis (Sathyanesan et al. 1978). For further information on thyroid changes in laboratory fish exposed to EDCs, see Wester et al. (1986) for effects in medaka expose to the pseudoestrogen βHCH, in guppies and medaka by sodium bromide (Wester et al., 1988) and van der Ven et al. for thyroid hyperplasia in zebrafish exposed to the antithyroidal drug propylthiouracyl (van der Ven et al, in preparation; see for details http://www.rivm.nl/fishtoxpat).

109. Other possible toxicant-induced effects detected by histological means in the medaka include lipomatosis (development of adipose tissue) in the liver and kidney, and buildup of amorphous eosinophilic precipitate in renal glomeruli, liver sinusoids and around the splenic capsule (Wester & Canton 1986).

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

### 6.1.3 Sexual Development

111. Once the gonads have differentiated into either ovaries or testes in gonochoristic fish there may be a considerable period of time during which these tissues develop before gametes are produced for the first time. In oviparous female fish, oogenesis must be completed, which involves vitellogenesis, before oocytes are fully formed and ready for ovulation. The testes of the male must complete the sequential processes of spermatogenesis and spermiation to produce viable sperm. The development of the gonads is regulated by endocrine signals originating in the hypothalamus and pituitary, and sex steroids, particularly 11-ketotestosterone (11-KT) in the male is known to be important for male germ cell production (Borg 1994). The intraovarian role for estrogens in the female is not known, but reduced E2 titers will affect VTG production by the liver and zonagenesis or production of egg membrane. Therefore, endocrine-active compounds that affect any aspect of the reproductive endocrine system causing reduced synthesis or release of gonadotropins, or interfere with gonadal steroidogenesis will delay or prevent reproduction from occurring (Arcand-Hoy & Benson 1998). This can be observed by examining the state of development of gonads of exposed fish relative to a suitable control, via the GSI or histological staging (Le Gac et al. 2001). A delay in spawning (i.e., time to first spawning/sexual maturity) and spawning frequency are commonly assessed endpoints in toxicological life-cycle studies of fish that presumably result from this mode of action. Fertility may also be affected in both sexes when fewer germ cells develop and mature. This can be detected by quantifying the number of eggs produced in females or assessing sperm number in males.

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

### 6.1.4 Secondary Sex Characteristics

113. The development of secondary sex characteristics in fish is hormonally controlled, making them viable endpoints for the evaluation of endocrine disruption. All of the species considered have some secondary sex characteristics. The female zebrafish and fathead have a distinct genital papilla. The male fathead minnow has distinct breeding tubercles on the snout and a 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. Adult male zebrafish, especially mating individuals, are coloured darkly and intensively compared to females, although it should be noted that colouration in this species is not under endocrine control. The mature male fathead minnow, medaka and sheepshead minnow also have distinct coloration. The male sheepshead minnow has a distinct vertical band along the posterior edge of the caudal fin. The female

sheepshead minnow has a distinct spot located on the anterior portion of the dorsal fin. Other species specific secondary sex characteristics are discussed in Section 4.1 to 4.4.

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

## 6.2 Measures of Reproductive Performance

### 6.2.1    Fecundity

115.    Fecundity can be measured as GSI by sacrificing fish or by counting eggs and spawns from spawning groups of fish.  Since the general spawning strategy for fish is to produce many eggs with limited or no parental protection, a large number of eggs can be produced by a female in a relatively short time.  The collection of eggs is a very time consuming phase in the study especially if the eggs are to be evaluated for fertilization or hatching success.

116.    There is more variability in spawning data when compared with data endpoints like fertilization success, hatching success, length, weight and survival.  GSI is also less variable, but requires sacrificing females.  Due to the limitations of replication, other endpoints may routinely be more sensitive than fecundity, when measured based on the number of eggs deposited over time.  Since all four species of fish being considered are continuous spawners, the time to deposit eggs may be the most critical endpoint. For example, the number of eggs per female per day  over a four-week period may be similar, but higher treatment levels may induce spawning over a shorter period of time.  However, if eggs are released from the ovary before they are mature, fertilization success may also be affected.

### 6.2.2    Gamete Viability and Fertilization Success

117.    In addition to the formation of adequate numbers of gametes for reproduction, it is also important that the gametes possess high viability for successful fertilization and larval survivability.  Screening assays that expose both genders simultaneously and then determine fertilization success will effectively measure gamete viability.  However, it is also helpful to measure gender-specific effects on gamete viability as this may provide important clues to the mode of action.  Identifying a gender-specific mode of action would be particularly valuable for interpreting the significance of laboratory results using controlled fertilization trials and extending this to natural reproductive conditions.  In this regard, specific measures of the viability of oocytes for fertilization and the ability of spermatozoa to fertilize eggs could be useful additions to a multigeneration protocol.  The subsequent two sections briefly discuss possible mechanisms for impairment of gamete viability and approaches to assess viability.

*6.2.2.1 Sperm Viability.*

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

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

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

*6.2.2.2 Egg Viability*

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

### 6.2.3    Fertility and Embryonic Development

122.    The formation of the zygote heralds the beginning of the embryonic period in animal life history. The embryonic period in the life history model for fish ends with "swim-up", the transition from the endogenous yolk supply to exogenous feeding (Balon 1975).  In oviparous fish the embryonic period will encompass all development within the egg and can extend after hatching until the yolk supply is exhausted (sac-fry).  All four test species proposed in this review are oviparous, which is advantageous

for the study of EDCs and early development as the eggs are released by the female and develop external to the parents. Therefore, they can be easily studied after spawning and fertilization without invasive procedures on the adults.

123.    The early development is rapid, in all four test species proposed, as a consequence of their small size and short life cycle. All are typically raised at warm water temperatures, which aids in accelerating their development. The embryonic development of the zebrafish (Kimmel et al. 1995) and medaka (Iwamatsu 1994) are very well characterized and the web sites (zebrafish- http://zfin.org/; medaka-http://biol1.bio.nagoya-u.ac.jp) for each provide detailed explanations and diagrams of all distinguishable stages (e.g., 128-cell, 256-cell, etc.). The utility of measuring embryonic development in the zebrafish or medaka is the transparent characteristics of the vitelline envelope (i.e., eggshell) such that every stage during development can be visually assessed in the living animal. This allows periodic data collection over time from the same individual. In addition, these fish embryos develop at water temperatures (25ºC to 28ºC) near that of normal room temperature and the eggs are small and easily studied using conventional light microscopy. These features, among others, have made the study of zebrafish embryos an important model for toxicant effects on development (Tanaka et al. 2001). For example, a study by Blader and Strahle (1998) described the effects of ethanol on prechordal plate formation in the zebrafish embryo using conventional microscopic techniques. Unfortunately, there are no similar information sources on stages of embryonic development for the fathead minnow (USEPA 1996) or sheepshead minnow. This is a major negative in the use of these species in a multiple generation test, especially when many effects of endocrine disruptors occur during early development. However, a number of studies have been performed with these latter two species in which endpoints such as hatching success or growth during embryological development have been reported (Hansen et al. 1983) (Creech et al. 1998; Lange et al. 2001). While these endpoints are useful, the greater resolution and detail afforded by the zebrafish or medaka is superior for assessing changes during early development.

### 6.2.4    Changes in Spawning Behavior

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

125.    Quantitative spawning behavior is not routinely collected in life-cycle studies and is not widely published. In one study male medaka spawning behavior was significantly altered by exposure to octylphenol, a known estrogen agonist (Gray et al. 1999). However, other endpoints were as sensitive or more sensitive than the male spawning behavior. In zebrafish, male mating behavior was shown to be clearly retarded by estrogen agonists like ethinylestradiol and octylphenol. When mating started, spawning occurred but the fertilization rate was significantly reduced. When testing estrogen agonists, fertilization capacity was shown to be the most sensitive endpoint, and retardation of spawning behavior was nearly as sensitive (Wenzel and Schäfers, unpublished 2001). A full review of this field has not been

undertaken here, because there is no evidence that reproductive behaviour is more sensitive than other variables, and such behaviour is very difficult to quantify in a reproducible way.

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

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

### 6.3 Biochemical Measures

127.    Biochemical measures can be used to uncover the mechanism of action of both EDCs and non-EDCs. This section focuses on the specific biochemical biomarkers that have been found sensitive to some EDCs, but it should be noted that they give little if any useful information for environmental risk assessment i.e. it is difficult or impossible to relate them to potential impacts at the population level. It is expected that biomarkers of an EDC's action will have been measured before a life-cycle test is triggered (e.g. in the 3-week fish screening test, and/or during a partial life-cycle test), and therefore it may not be necessary to repeat such work. It is consequently recommended that biochemical markers of MoA should only be included optionally, or not at all, in life-cycle tests.

### 6.3.1 Vitellogenin Induction

128.    Vitellogenin is an egg-yolk precursor protein that is synthesized in the liver of fish prior to its transport to the ovaries and incorporation into developing oocytes. Synthesis of VTG is under estrogen control mediated by estrogen receptors in the liver. Juvenile and adult fish, both male and female, can be induced to synthesize VTG after estrogen exposure or after exposure to estrogen-mimics. Detection of VTG synthesis has become the most widely studied biomarker of exposure to endocrine-active compounds. Although there are a variety of methods to detect VTG in fish, the most widely applied methods are the immunoassays, enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA). However, mRNA-based methods (using semi-quantitative reverse-transcriptase polymerase chain reaction, or SQRT-PCR) are likely to be useful for small species or individuals from which little tissue can be taken for analysis. The immunoassays 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, while SQRT-PCR detects induction of the mRNA coding for VTG. ELISA is used more frequently to measure VTG because, unlike RIA, it does not require radioactive isotopes, uses stable reagents, and offers ease in set up and use.

*6.3.1.1. Induction During Juvenile Life Stages*

129.     The induction of VTG can be measured through whole-body sampling of juvenile fish, as early as 2-4 days post-hatch in some fishes from which samples of plasma are not available (Todorov et al. 2002). This measure of induction during juvenile life stages has been shown to be a sensitive measure of exposure to estrogens. For example, in a study of mixed-sex juvenile fathead minnows aged  45 days-post-hatch, VTG levels showed a statistically significant increase after a 4-day exposure to 2 ng/L of the synthetic steroid EE2 (Panter et al. 2002).  When juvenile fathead minnows were exposed to 2.9 μg/L of the synthetic estrogen diethylstilbestrol (DES), VTG was induced to a level 100 times greater than control fish after 4 days (Panter et al. 2002).  In addition to rapid detection of induction, the VTG levels in the EE2 and DES exposed fish remained elevated during the 21-day exposure period and reached maximal levels reported for cyprinids (Panter et al. 2002).   In addition to synthetic estrogens, the natural phytoestrogen, genistein, and the weak estrogen mimic, 4-tert-pentylphenol (TPP), induced VTG in juvenile fish (Panter et al. 2002).  Although potent estrogens can elicit a measurable response in several days, a minimum of a 14-day exposure was necessary to measure induction in the majority of the chemicals tested in this study.  In addition to measuring estrogenic activity in juvenile fathead minnows through VTG induction, anti-estrogenic activity of some substances has been demonstrated through a measured reduction in VTG synthesis relative to control fish.  For example, in the Panter et al. (2002) study, the pharmaceutical anti-estrogen ZM 189,154 caused a statistically significant reduction in VTG levels in juvenile fathead minnows relative to control fish. In juvenile zebrafish, significant vitellogenin induction has been measured in whole-body homogenate after exposure to 1.5 ng/l EE2 from day 20 post hatch to day 38 post hatch under flow-through conditions. Significant vitellogenin reduction was obtained in whole-body homogenate after exposure to 50 ng/l methyltestosterone from day 20 post hatch to day 38 post hatch (Örn et al., 2003).

130.     Detecting VTG by immunoassay may be difficult or impossible in the juveniles of small species, or in certain very small adults. QRT-PCR can be used in these cases because it is exquisitely sensitive to the mRNA coding for VTG. However, one drawback of this approach should be noted – whereas VTG itself generally persists for a period of up to several weeks in the tissues and blood plasma of male fish, its mRNA is rather transient, and so has to be measured soon after EDC-exposure. For example, Kirby et al. (2003) were able to measure VTG mRNA in male sand gobies (*Pomatoschistus* spp.) that had been recently lab-exposed to EE2, but were unable to detect it in wild males which were known to have been exposed to high but fluctuating estrogen concentrations in sewage-contaminated estuaries. Other studies (not exhaustive) which have employed QRT-PCR to measure VTG mRNA include Islinger et al. (1999), Celius et al. (2000), Pawlowski et al. (2000), Denslow et al. (2001), Ackermann et al. (2002), Bowman et al. (2002), Islinger et al. (2002), Lattier et al. (2002), Rasmussen et al. (2002), Zerulla et al. (2002), and Islinger et al. (2003).

*6.3.1.2 Induction During Adult Life Stages*

131.     In adult fish, induction of VTG has been shown to be extremely sensitive to estrogen exposure. For example, in sexually mature male rainbow trout, a 28-day exposure to 0.3 ng/L EE2 caused a statistically significant increase in plasma VTG levels compared with those of control trout, with a predicted no observable effects level (NOEL) of less than 0.1 ng/L (Sheahan et al. 1994).  For the test species under consideration in multiple generation tests, VTG synthesis has also been demonstrated to be sensitive to estrogen exposures, although somewhat less sensitive than that observed in rainbow trout (discussion of VTG induction in candidate species is presented in section 7.2.1.3).   The reduced

sensitivity appears to be due in part to difficulties in obtaining sufficient quantities of purified VTG protein and species-specific antibodies for use in ELISA- or RIA-based measurement techniques (Section 6.3.1) (Parks et al. 1999; Van den Belt et al. 2001). Rose et al. (2002) found vitellogenin induction in whole-body homogenate of adult male zebrafish on exposure to 3.0 ng/l EE2 (measured concentration) after 8 days of exposure in a flow-through system and corresponding induction after exposure to E2 at 21 ng/l. By logistic regression analysis they calculated $EC_{50}$-values of 2.51 and 41.2 ng/l for EE2 and E2 respectively.

132.    This section has focused on VTG as this protein is the best studied and most commonly measured estrogen responsive gene product in fish. However, other proteins such as the eggshell envelope proteins, collectively called zona radiata proteins, are also induced upon exposure to estrogen. Zona radiata proteins are synthesized in the liver and ovaries and incorporated into the developing oocytes in a manner analogous to VTG. Stimulation of zona radiata synthesis appears to require lower levels of estrogen compared with VTG (Sohoni et al., 2001). Furthermore, evidence suggests that zona radiata proteins are preferentially induced by weak estrogenic compounds, such as bisphenol-A and *o,p'*-DDT (Arukwe et al., 2000). This latter finding might be significant for a reproductive screening program, as chemicals with a weak estrogenic mode of action can be difficult to identify if VTG is not significantly induced. Thus, zona radiata proteins might be a more sensitive endpoint in this respect.

### 6.3.2    Tissue Steroid Concentrations

#### 6.3.2.1  17β-estradiol Concentrations During Juvenile and Adult Life Stages

133.    The measurement of plasma levels of E2 can be used as an endpoint to assess estrogen sex steroid status in female fish, provided there is some knowledge about normal population levels when the samples are taken. In the context of a laboratory toxicology experiment a control group of sufficient size is an absolute requirement. Plasma levels of E2 have limited utility in males because levels of this steroid are usually low or non-detectable. An exception may be the male fathead minnow, which is discussed below. The measurement of plasma E2 is most useful in sexually maturing females due to the gradual rise in this hormone during the period of vitellogenesis. A good example is the well-documented case for the rainbow trout, an iteroparous (reproducing over more than one reproductive or spawning period), seasonally breeding fish with group-synchronous ovarian development in which maximum levels are attained well before spawning (Scott et al. 1980a; Vanbohemen & Lambert 1981). In many fish, E2 begins to decline by the time of final maturation, probably as a consequence of the completion of yolk synthesis by the ovary, and E2 levels drop significantly after that. A distinct switch is seen in fish ovarian steroidogenic pathways from estrogens to progestins at this time (Nagahama 1999). Therefore, in juvenile fish that have immature gonads and have not begun active vitellogenesis ovarian synthesis of E2 will be negligible and plasma levels are consequently very low. Since many toxicants or environmental contamination scenarios typically affect sex steroids by depressing plasma titers it may be difficult to discern an effect in juvenile fish that already have very low levels of these hormones.

134.    In the four test species proposed, there are no data on plasma E2 levels in juveniles. There are limited data for adult fathead minnow and medaka; none exist for sheepshead minnow or zebrafish. One study on female fathead minnow reported mean plasma levels peaking at 10 ng E2/mL one day after spawning before decreasing to ~4 ng E2/mL (Jensen et al. 2001). Another reported mean plasma levels in the range of 3 ng E2/mL to 11 ng E2/mL for adult female fathead minnows, that were unaffected by three-week exposure to sewage treatment effluent (Sternberg & Moav 1999). There are three reports of

the detection of E2 in the plasma of male fathead minnows; these values fall in the range of 0.5 ng E2/mL to 5 ng E2/mL (Jensen et al. 2001; Makynen et al. 2000; Sternberg & Moav 1999). In a study on the effect of vinclozolin (an anti-androgen) in male fathead minnows plasma E2 levels were significantly elevated in a group treated for three weeks with 700 µg/L, compared with the control (Makynen et al. 2000). Measuring E2 plasma levels in male fathead minnow may be a useful endpoint dependent on the toxicant used. One study reports plasma E2 levels in adult female medaka during the daily period of vitellogenesis and spawning (Soyano et al. 1993). These data show low levels of 4 ng E2/mL plasma at 16 hours before spawning, with a peak of 16 ng E2/mL plasma at 8 hours prior to spawning before levels decrease again. At least in fathead minnow and medaka it can be concluded that it will be important to have appropriate controls (and adequate sample sizes) to compare to treated fish because plasma E2 levels fluctuate considerably over narrow temporal periods due to their short reproductive cycle.

*6.3.2.2 11-ketotestosterone Concentrations During Juvenile and Adult Life Stages*

135.    Similar to E2 in female fish, 11-KT is the sex steroid characteristic of the sexually maturing male. Very low levels (e.g., pg/mL range) are sometimes reported in adult female fish (Jensen et al. 2001; Simpson & Wright 1977) although the physiological significance, if any, is not understood. In toxicology studies 11-KT is routinely measured in the blood of male fish to assess androgen status, with the expectation being that reduced levels of this hormone are synonymous with reproductive dysfunction. This correlation has not been conclusively established, although the necessity of 11-KT for fish spermatogenesis has emerged (Miura et al. 1991; Schulz et al. 2001). Plasma levels of 11-KT are very low in juvenile fish that are not sexually maturing (Simpson & Wright 1977). Once spermatogenesis is under way in the testes, and later spermiation, considerable amounts of 11-KT appear in the blood (Fostier et al. 1982; Scott et al. 1980b; Simpson & Wright 1977). Therefore, provided an appropriate control group is available point measures of 11-KT could be used in sexually maturing males to assess the effects of EDs.

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

## 7.0 RESPONSE TO ESTROGEN AGONISTS AND ANTAGONISTS

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

### 7.1 Juvenile Life Stages

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

### *7.1.1    Sensitivity to E2 or Synthetic Estrogen Exposure*

#### *7.1.1.1    Embryonic Exposure*

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

1. direct uptake from the water after fertilization and during subsequent embryogenesis; and

2. maternal transfer of the chemical to the developing oocyte prior to ovulation. An example of the first route of exposure is provided by Andersen et al. (2003) who exposed zebrafish to 15.4 ng EE2/l in different critical life stages and observed 100% females after exposure from hatch to 60 days post hatch and from 20 to 60 days post hatch. Örn et al. (2003) found a sex ratio significantly skewing toward females after exposure of zebrafish from 20 to 60 days post hatch to only 0.6 ng EE2/l (lowest measured concentration).

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

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

142.    An interesting study of maternal transfer of E2 in zebrafish was described by Olsson et al. (1999), in which sexually mature female zebrafish were injected i.p. with E2 at 0.27 µg/kg or 272 µg/kg and mated to unexposed males.  Mortality of embryos produced from the pairings was high at both treatment doses.  The surviving larvae were reared to maturity and observed to be 90% female at the high E2 exposure.  Additional histopathological effects noted were indications of bile stasis in the livers of both treatment groups (Olsson et al. 1999). However, caution should be exercised with these results because it is possible that the 'female' gonads were really protogynous gonads of delayed males, or an artifact cause by differential mortality of males.

*7.1.1.2. Larval and Juvenile Exposure*

143.    This section treats larval and juveniles together because the relevant studies sometimes expose fish for the larval and juvenile periods combined.

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

145. A thorough study was described by Metcalfe et al. (2001), in which newly hatched medaka fry were exposed to E2 and EE2 concentrations ranging logarithmically from 0.0001 µg/L to 1.0 µg/L for 90 days. The results indicated both total length and body weight increased at the highest concentration of E2, whereas body weight decreased at similar concentrations of EE2 (Metcalfe et al. 2001). A number of histopathological changes were observed in exposed medaka, including accumulation of an eosinophilic fluid in the body cavity in general, and in the kidney and liver specifically, after E2 or EE2 exposures at or above 0.1 µg/L (Metcalfe et al. 2001). The sex ratio of exposed fish was also shifted toward females at exposure rates above 0.1 µg/L, whereas formation of testis-ova in male individuals became noticeable in E2 treatments as low as 0.01 µg/L (Metcalfe et al. 2001). The authors also noted that when testis-ova occurred, a gradient appeared to exist with oocytes in the posterior region and testes in the anterior region of the gonads (Metcalfe et al. 2001).

146. Another study in the medaka exposed freshly hatched larvae for 2 months to nominal EE2 concentrations up to 0.1 µg/L, followed by a 6-week grow-out period (Scholz & Gutzeit 2000). Similar growth rates (total body weight and length) in both male and female individuals were observed compared with control fish, although the GSI of female fish was decreased at EE2 exposure levels of 0.01 µg/L and 0.1 µg/L (Scholz & Gutzeit 2000). This study also observed a shift in phenotype from male to female in all fish exposed to 0.1 µg/L of EE2 (Scholz & Gutzeit 2000). Interestingly, EE2 exposure rates of 0.001 and 0.01 µg/L did not cause formation of testis-ova in male medaka (Scholz & Gutzeit 2000). In a similarly designed study, newly hatched medaka fry were exposed to measured E2 concentrations ranging from 0.01 µg/L to 1.66 µg/L for 28 days followed by a post-dosing recovery period of 28 days. At the end of the recovery period, both the total length and body weight appeared to decrease with increased E2 exposure levels, although the authors concluded that these differences were not significant (Nimrod & Benson 1998). Wester et al. (1986) described effects of the pseudoestrogen βHCH in medaka exposed during early life stages (from egg onwards) where testis-ova was later induced, together with other histopathological changes.

147. The effects of EE2 on zebrafish development following exposure at several life stages, especially the larval stage, were extensively studied during the EU-funded IDEA project (Segner et al., 2003a). This work showed that while exposure from fertilization to 21 days post fertilization (dpf) had no subsequent effects, exposure from 42 to 75 dph caused a delay in the time to spawning, altered mating behaviour, reduced the number of eggs produced per female, and reduced fertilization success, these effects all occurring at ≥3 ng EE2/l. Holbech et al. (2006) studied exposure of zebrafish larvae to 17β-estradiol from fertilised eggs to 60 days post hatch. At 54 ng/L, significant vitellogenin induction was measured in whole body homogenates, and sex ratio was significant altered towards female. Maack and Segner (2004) found that feminising effects in zebrafish following juvenile exposure to estrogen were reversible at the adult stage as both fish with ovaries and fish with testes were found. However, reproductive capabilities (fertilisation) were altered following exposure to estrogen at the gonad transition stage around 20 to 35 days post hatch.

148. Fenske *et al.* (2005) and Schafers *et al* (2007) examined effects of larval /juvenile exposure to ethinyl estradiol on the reproductive capabilities of zebrafish, and compared them with effects following life-cycle exposure. Both authors found that early life stage exposure resulted in reversible effects, while life-cycle exposure resulted in irriversible effects (9.3 ng/L)or partially reversible effects (3ng/L) on reproduction (fecundity and fertility).

149.    Brion *et al* (2004) also studied the effects of exposure of zebrafish (group spawner) to 17β-estradiol during various life-stages on reproductive capacity. Exposure during early life-stage (between 21 and 42 days post hatch) impacted the fecundity, but not fertility or hatching rate. The authors recognized that variability in fecundity was due to spawning frequency, rather than batch size.

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

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

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

### 7.1.2    *Anti-estrogens*

153.    The biological effects of anti-estrogens in fish development and reproduction have only recently been studied, and relatively few studies of anti-estrogens are reported.  For purposes of discussion, a direct anti-estrogen is a chemical known to interfere with signaling through competitive inhibition of binding established to the ER(s).  Chemicals that alter the normal turnover of estrogen in the fish (e.g., alter synthesis or elimination rates of estrogen) are considered indirect acting anti-estrogens.  This latter category includes aromatase inhibitors.

*7.1.2.1.Embryo-Larval Exposure*

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

*7.1.2.2.Juvenile Exposure*

155.    As with earlier life stages, only a single study could be found that exposed anti-estrogens to a juvenile life stage.  In this study, juvenile fathead minnows were exposed to the pure Type II anti-estrogen, ZM189,154, for 21 days at measured concentrations of 7.9, 33.7, and 95.3 μg/L.  A significant decrease in VTG formation was observed at all exposure levels after 14 days of exposure without causing significant changes in body weight or total length of the fish (Panter et al. 2000a).

156.    Andersen et al. (2004) studied effects of the anti-estrogen ZM 189, 156 on zebrafish exposed from 20 to 60 days post hatch. Vitellogenin concentration was significantly increased at 100 and 200μg/L. The percentage of females declined and the number of undifferentiated fish increased. No information is available though on potential effects on reproduction.

### 7.1.3.    Indirect Anti-estrogens

157.    For purposes of this review, an indirect-acting anti-estrogen is considered to be a xenobiotic that significantly lowers the unbound or free E2 concentration in blood plasma.  The best-characterized, indirect-acting anti-estrogens are the aromatase inhibitors.  In fish, aromatase activity is due to at least two separate enzymes encoded by multiple CYP19 genes (Tchoudakova & Callard 1998) and is the critical enzyme responsible for the final, irreversible step in estrogen synthesis from androgen precursors (Simpson et al. 1994).  The existence of multiple P450-aromatase isoenzymes suggests tissue-specific, differential expression, which could explain the exceptionally high activity found in the teleost brain (Tchoudakova & Callard 1998).  In any case, inhibition of this enzyme has been demonstrated to adversely affect sexual differentiation and reproduction.

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

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

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

160.    Andersen et al. (2004) studied effects of the aromatase inhibitor fadrozole on zebrafish exposed from 20 to 60 days post hatch. Vitellogenin concentration was significantly increased at 10 and 100µg/L. The percentage of female declined and the number of undifferentiated fish increased. No information is available though on potential effects on reproduction. Kinnberg et al. (2007) studied the effects of the aromatase inhibitor prochloraz onzebrafish exposed from 0 to 60 days post hatch. An increased proportion of males and intersex fish was noted at 202 µg/L; vitellogenin concentration was decreased in females at 202 µg/L, while an increase in vitellogenin was noted at intermediate concentrations (16, 64 µg/L). However, there was no investigation of longer term effects on reproduction.

### 7.1.4    Strength and Weaknesses of Test Species

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

### 7.2 Sexually Mature Life Stages

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

*7.2.1   Sensitivity to E2 or Synthetic Estrogen Exposure*

*7.2.1.1 Adult Exposure*

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

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

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

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

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

also noted that besides reducing the overall size of the ovaries, exposure to 10 ng/L and 25 ng/L EE2, greatly reduced the number of mature, yolk-filled oocytes (Van den Belt et al. 2001).

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

*7.2.1.2 Reproductive Performance*

169.     A thorough study of reproductive performance was reported by Ankley et al. (2001), in which adult fathead minnows were exposed to methoxychlor, a weak estrogenic chemical, for 21 days at measured concentrations of 0.55 μg/L and 3.56 μg/L.  The results from this study indicated the mean fecundity rate decreased from 20.5 to 8.3 eggs/female/day (control versus 3.56 μg/L treatment), and was attributed to both a decrease in egg numbers per spawn and less frequent spawning (increased spawning interval) (Ankley et al. 2001).  An earlier study exposed adult fathead minnows to various E2 exposure rates for 19 days and observed a decrease in fecundity with a calculated E2 median effective concentration (EC50) of 120 ng/L for decreased egg production (Kramer et al. 1998).  An additional study of note exposed pair breeding fathead minnows to the weak estrogenic pollutant, 4-nonylphenol, at measured concentrations between 60 μg/L and 80 μg/L for 21 days.  At this water concentration, 4-nonylphenol caused a significant reduction in both fecundity and spawning frequency (Harries et al. 2000).

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

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

*7.2.1.3 Biochemical Measures: VTG*

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

173.    The sheepshead minnow has also been demonstrated to be sensitive to VTG induction after exposure to estrogenic chemicals.  Mature male sheepshead minnows exposed to E2, EE2 or DES for 16 days at various nominal concentrations ranging from 20 ng/L to 2000 ng/L (E2), 0.2 ng/L to 200 ng/L (DES), and 20 ng/L to 1000 ng/L (EE2) rapidly formed VTG with peak plasma concentrations of approximately 120 mg/mL reached in 10 to 13 days (Folmar et al. 2000).  Similar results were also obtained in a later study using a nominal concentration of 200 ng/L of E2, DES, and EE2 (Denslow et al. 2001).

174.    The measurement of VTG induction in zebrafish and medaka has also been broadly reported Several specific and sensitive ELISAs for zebrafish vitellogenin have been developed and validated (Fenske et al., 2001; Holbech et al., 2001; Brion et al., 2002). Also, specific ELISAs have been developed for VTG in medaka (liver and blood) (Nishi et al., 2002; Nozaka et al, 2004). These homologous ELISAs have successfully been used to measure VTG concentration, either from whole body homogenate for zebrafish, or from blood or liver for medaka (Andersen et al. 2003;Brion et al. 2002;Brion et al. 2004;Fenske et al. 2001;Rose et al. 2002;van der Ven et al. 2003a;Seki et al. 2006)

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

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

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

178.    In adult zebrafish, a 21-day exposure to EE2 at measured concentrations down to 5 ng/L caused significant increases in VTG occurrence in blood plasma (Van den Belt et al. 2001).  A similar result was also obtained in a study that exposed adult zebrafish for 14 days to a nominal EE2 concentration of 5 ng/L (Kime & Nash 1999).  In this study, the level of VTG induction was roughly 3-fold greater in female than in male zebrafish (Kime & Nash 1999).  Two recent studies have assessed VTG induction in zebrafish using specific VTG-detection procedures.  Ota and co-workers (Ota et al. 2000) used adult male zebrafish and determined VTG induction by measurement of VTG-like mRNA in liver extracts.  This study treated male zebrafish with 270 μg/L E2 (a very high concentration) for 48 hours and observed stimulated VTG gene transcription.  A more thorough study of VTG induction in zebrafish was reported by (Petersen et al. 2000), in which an ELISA procedure was developed for zebrafish VTG based on antibodies produced against lipovitellin purified from zebrafish ovaries.  Using this newly developed ELISA, it was later demonstrated that a 30-day exposure to nominal EE2 water concentrations as low as 20 ng/L caused an approximately 1000-fold increase in VTG levels in whole body homogenates (Petersen et al. 2001).

*7.2.1.4 Biochemical Measures: Plasma Steroid Levels*

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

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

181.    A study by (Giesy et al. 2000) reported plasma E2 concentrations ranging between 1 ng/mL and 5 ng/mL and were similar in both males and females.  This study also reported that a 21-day exposure to nominal nonylphenol concentrations ranging between 0.05 μg/L and 3.4 μg/L elevated E2 concentrations to a similar degree in both male and female fathead minnows (Giesy et al. 2000).  In a prior study, the plasma concentrations of E2 and T were measured in mature fathead minnows that were caged in either a reference site or in wastewater treatment effluents (Nichols et al. 1999).  Estradiol concentrations in both male and female minnows from the reference site were considered similar and varied between 1.4 ng/mL and 11.1 ng/mL (Nichols et al. 1999).  Likewise, T concentrations were similar between sexes, varying only minimally between 6 ng/mL and 7 ng/mL (Nichols et al. 1999).  In fathead minnows that were exposed to various wastewater treatment effluents, a general decrease in both E2 and T was observed in both male and female individuals (Nichols et al. 1999).

182.    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 female fathead minnows while causing a significant decrease in T and 11-KT in male individuals (Ankley et al. 2001).

183.    With regard to medaka, zebrafish, and sheepshead minnows, the limited amount of data makes it difficult to make comparisons with fathead minnows, other than evidence for cyclic levels of E2 in females.  For example, in the medaka, E2 plasma concentrations appear to oscillate during the spawning cycle, with peak concentrations occurring approximately 8 hours before spawning (Soyano et al. 1993).  The only published ecotoxicological data on E2, T, or 11-KT levels in zebrafish or sheepshead minnows that could be found concern the response of female zebrafish to ethinylestradiol (Hoffman et al., 2006).  Plasma E2 and T titres were significantly reduced following 48 and 168 h exposures to EE2 concentrations in the range 15-100 ng/L.

*7.2.1.5 Full or Partial Life-cycle Exposure*

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

185.    A full life-cycle exposure was performed with fathead minnows and EE2 at nominal concentrations ranging from 0.2 ng/L to 64 ng/L (Länge et al. 2001).  In this study, exposures began with fertilized eggs and continued through 28-day post-hatch of the F1 offspring.  Hatching success and mortality of the F0 embryos and larvae were not altered by the EE2 exposures, although overall growth was diminished in F0 juveniles at exposure levels of 4 ng/L and higher.  Similarly, EE2 exposures of 4 ng/L and higher prevented development of secondary sex characteristics in addition to other morphological alterations, such as anal protrusion and distended abdomens.  Reproductive performance could only be assessed in the 0.2-ng/L and 1.0-ng/L treatment groups, in which no differences in fecundity, hatching success, and larval survival were observed with respect to control fish.  Subsequent histological analysis of F0 adults indicated that over a dose range of 1.0 ng/L to 16 ng/L, a progressive increase in the number on individuals with ova-testis occurred, with 94% of all fish phenotypically female at the 64-ng/L exposure (Länge et al. 2001).

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

reported to decrease fertilization success (Örn et al. 2000), although this was difficult to test because of the low numbers of phenotypic males.

187.    More recently, Nash et al. (2004) conducted a life cycle experiment with zebrafish exposed to EE2, starting with reproductively active adults (F0) and continuing exposure for the entire lifetime of the F1 generation. 5 ng EE2/L caused complete infertility in the F1 generation by reducing fecundity by 56% and disturbing male sexual differentiation – the males either had no functional testes, or undifferentiated or intersex gonads, although their sexual behaviour and spawning act appeared normal. On the other hand, 5 ng EE2/L had no impact on the reproductive success of the parental F0 generation. Segner et al. (2003) exposed one generation of zebrafish to EE2 from the fertilized egg to reproductive maturity, and obtained a LOEC of 1.7 ng/L for several reproductive endpoints, including time to spawning, mating behaviour, eggs per female, and fertilization success. These data suggest that zebrafish are of similar sensitivity to fathead minnows when exposed to EE2 for a whole life cycle.

188.    Schafers et al. (2007) studied the concentration- and time-dependent effects of EE2 exposure on reproductive capabilities of the zebrafish. Exposures from 0 to 75 days post hatch, and from 0 to 177 days post hatch (F1 life-long) impaired adult fecundity and fertilization success, with complete and irreversible inhibition at 9.3 ng/L after life-long exposure.

189.    A full life-cycle multigeneration protocol for the medaka was proposed by Patyna et al. (Patyna et al. 1999).  As a pilot study, the researchers performed a partial life-cycle study by exposing juvenile medaka for 170 days at three separate E2 treatments rates (0.05, 0.5 and 5 mg/kg).  A variety of histopathological effects were reported, including generalized edema in the body cavity with severe renal pathology noted as hemorrhaging, and tubular necrosis (Patyna et al. 1999).  All fish appeared to be phenotypic females.  These effects are all consistent with previous studies in fish using very high treatment rates of E2 (Metcalfe et al. 2001; Herman and Kincaid 1988). Life cycle experiments with medaka have since been conducted with estrogen mimics, including bisphenol A (Kang et al., 2002) and nonylphenol (Yokota et al., 2001). Furthermore, a life cycle test starting with fertilized medaka eggs exposed to EE2 has been conducted (Japanese government report, unpublished) which gave a NOEC for fertility reduction of 3 ng/L in the F0 generation, but few clear effects in the F1 generation apart from the induction of VTG (NOEC = <1 ng/L). Many but not all of the life-cycle studies with medaka suggest that biomarkers or histopathology provide more sensitive endpoints than reproductive endpoints such as fecundity or fertility.

### 7.2.2    *Anti-Estrogens*

#### 7.2.2.1 Adult Exposure

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

Additional studies with fish models suggest certain PAHs could also have anti-estrogenic modes of action. A series of experiments using cultured rainbow trout hepatocytes treated with either β-naphthoflavone or 3-methylcholanthrene suggest these compounds can interfere with ER activation by E2

(Navas & Segner, 2000). In this study, hepatocytes were co-incubated with 1 μM E2 and several concentrations of β-naphthoflavone or of 3-methylcholanthrene and the VTG response measured. When the hepatocytes were coincubated with ß-naphthoflavone 0.78 μM or higher or with 3-methylcholanthrene 6.25 μM, a decrease in VTG synthesis was observed (Navas & Segner, 2000). The reduction in VTG production caused by ß-naphthoflavone disappeared when the hepatocytes were simultaneously incubated with the aryl hydrocarbon receptor antagonist a-naphthoflavone 12.5 μM. Authors suggested that the inhibition in the production of VTG caused by PAHs was mediated through the activation of aryl hydrocarbon receptor.  Similar findings have been reported by in-vivo studies that administered E2 and various doses of β-naphthoflavone.  When rainbow trout were administered 0.5 mg/kg E2 and 25 mg/kg or 50 mg/kg β-naphthoflavone, a decrease in VTG synthesis was observed (Anderson et al. 1996). Conversely, when the β-naphthoflavone dose was reduced to 12.5 mg/kg, a seemingly paradoxical stimulation in VTG synthesis (increase in plasma concentration) was observed (Anderson et al. 1996). This mixed stimulatory and inhibitory effect on VTG synthesis is consistent with tamoxifen like anti-estrogens, which possess agonistic activity at low concentrations (MacGregor & Jordan 1998).

191.    Regardless of the specific mechanism of action of direct anti-estrogens, a common endpoint measured in most studies is the effect on VTG synthesis.  Virtually no information is available in the literature on the specific effects of direct anti-estrogens on reproduction in fish.  Thus, it is difficult to state what effect, if any, anti-estrogens may have on reproductive success.  Based on the results indicating partial suppression of VTG synthesis, some effect on oogenesis would be expected but this remains to be demonstrated.  This would be a particularly valuable area of research in the context of a multigeneration test.

### 7.2.2.2 Indirect Anti-estrogens

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

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

194.    Evidence supporting the hypothesis that aromatase inhibitors can lower E2 levels and alter reproductive performance comes from studies using Coho salmon (*Onchorynchus kisutch*).  When pre-spawning Coho salmon were administered the aromatase inhibitor fadrozole by intraperitoneal injection at doses down to 0.1 mg/kg, a significant decrease in E2 plasma levels occurred 3 to 6 hours after dosing (Afonso et al. 1999b).  Additional studies in Coho salmon demonstrated that fadrozole treatment inhibited oocyte development and overall ovarian growth (Afonso et al. 1999a). Fadrozole has also been shown to decrease plasma E2 in female fathead minnows as well as vitellogenin production, and increase plasma T

and 11-KT in males (Ankley et al., 2002). In that study, fecundity was decreased after exposure for 21 days to concentrations ranging from 2 to 50 µg/L; histological assessment of ovaries confirmed a decrease in mature oocytes and an increase in preovulatory atretic follicles.

195.    More recent studies exposed adult fathead minnows for about three weeks to fenarimol and prochloraz,  which are hypothesized to reduce endogenous estrogen activity though either acting as an inhibitor of aromatase activity or through directly binding to the estrogen receptor as an antagonist. In fenarimol experiments, VTG levels in females and fecundity decreased after exposure to 497 µg/L (Thorpe et al, 2007) and 1mg/L (Ankley et al, 2005), mainly due to the spacing of spawning events. Embryo viability was investigated in the Thorpe experiment and did not appear to be affected. In the prochloraz experiment, VTG levels in females and fecundity decreased after exposure to 100 µg/L (Ankley et al, 2005), thus showing more potency than fenarimol.

196.    However, other established aromatase inhibitors, letrozole and clotrimazole, could not lower circulating E2 levels or suppress VTG synthesis in rainbow trout after dietary administration for 2 weeks at a dose rate of 1 mg/kg (Shilling et al. 1999).

**7.3 Gender Differences**

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

198.    In general, male individuals appear to be more sensitive to estrogenic compounds based on gonad morphology and assessment of testis-ova formation.  The GSI is likely to be more sensitive to estrogen exposure in males, as the relative testis mass is a less variable parameter in asynchronous spawners, making this parameter more sensitive as a result of improved statistical power.  Estrogen-induced changes in secondary sex characteristics are also a more sensitive parameter in male fish (particularly fathead minnows).  An additional endpoint in which male fish appear to be more sensitive is the induction of VTG.  Because of the naturally lower background levels of VTG in males, even slight (<10-fold increase) increases in plasma concentrations can be statistically significant.

199.    In regards to reproduction, most endpoints either directly assess female effects (fecundity) or combined gender effects (fertilization success).  This makes it difficult to assess specific gender sensitivities other than female fecundity.  A few studies have assessed male-specific fertility endpoints, which appear to be very sensitive to estrogenic exposure.  However, more research is needed to determine whether male fertility is more sensitive to estrogenic exposures, although this may be the case for zebrafish in some experimental designs.  A specific endpoint for which females appear to be more sensitive is changes in circulating steroid levels, which tend to be more dynamic during the spawning cycle.  In addition, morphological and reproductive responses to aromatase inhibitors and to a lesser extent, direct acting anti-estrogens appear to be more sensitive endpoints in female fish.

## 7.4 Strength and Weaknesses of Test Species

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

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

## 8.0 RESPONSE TO ANDROGEN AGONISTS AND ANTAGONISTS

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

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

### 8.1 Juvenile Life Stages

#### 8.1.1   Sensitivity to Androgenic Steroid Exposure

204.     Nano-injection of medaka embryos with MT has been reported.   Medaka embryos of the d-rR strain were injected with graded doses of MT ranging from 0.8 ng/egg to 8000 ng/egg and then cultivated to sexual maturity (Papoulias et al. 2000b).  The survival of the injected embryos at doses up to 80 ng/egg was comparable to uninjected or dosing-vehicle injected embryos (66% to 88% survival).  After attaining sexual maturity, the results indicated that embryo exposure to 0.8, 800 or 8000 ng MT caused sex reversal in genetic female medaka (Papoulias et al. 2000b).  In a separate study, medaka at several different stages of development (embryos, hatching day, post-hatch days 7 and 21) were immersed for 6 days in a solution of 100 µg/L T and then reared to an adult stage (Koger et al. 2000).  Results of this study indicated treatment did not affect mortality, time to maturity, or sex ratios in fish, although treated fish were described as having a more pronounced intersex morphology (Koger et al. 2000).

205.     A similar lack of an effect on survival or sexual phenotype  was observed in zebrafish embryos and larvae exposed to T at 2884 µg/L for up to 72 hr post-fertilization (Kishida et al. 2001).  In contrast, newly hatched medaka larvae fed a diet containing 50 µg/g MT for up to 40 days were described to have numerous morphological changes during different stages of growth.  A summary of these changes was reviewed in Section 6.1.1.1 but in general, MT exposure suppressed gonadogenesis (manuscript located at http://biol1.bio.nagoya-u.ac.jp:8000/KobayashiH85.html).  Örn et al. (2003) used a juvenile zebrafish partial life-cycle assay in which the endpoints were vitellogenin induction and and sex ratio. Juvenile zebrafish were exposed from day 20 to 60 post-hatch under flow-through conditions and subgroups of fish were collected at day 38 post-hatch for vitellogenin measurement in whole-body homogenate and at day 60 post hatch for sex-ratio determination. Exposure with the androgen methyltestosterone (MT)

resulted in an all-male population at the lowest exposure concentration of 26 ng MT/l and significant vitellogenin reduction at 50 ng MT/l.

206. There does not appear to be any information in the literature on the effects of androgens on early life stages of fathead minnows.

207. While effects of androgenic exposure during early life-stage have been studied in zebrafish, mainly leading to male biased sex ratio (Orn et al. (2006); Holbech et al. (2006)), relatively little information exists on the long-tern effects on reproduction of androgenic exposure in juvenile fish. Seki et al. (2006) conducted a life-cycle experiment with methylt testosterone on medaka. Further to the masculination of secondary sex characteristics in fish, at higher concentrations (27 ng/L), no ovary could be discerned; at lower concentrations (9 ng/L), fish had swollen abdomens, due to enlarged ovaries, and fecundity and fertility of the F0 generation declined.

### 8.1.2 Anti-Androgens

#### 8.1.2.1 Direct Acting

208. The reader is referred to Section 8.2.2 for a discussion of anti-androgens in adult fish. The only study of anti-androgenic effects in young fish that could be found in the literature involved exposure of newly-fertilized fathead minnows to the anti-androgenic pesticide vinclozolin for 34 days, followed by grow-out for 4-6 months in clean water (Makynen et al., 2000). However, no adverse effects were observed except for slightly reduced growth at the top concentration (1200 µg/L).

#### 8.1.2.2 Indirect Acting

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

210. 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.

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

### 8.1.3    Strength and Weaknesses of Test Species

212.    It is difficult to make comparisons between species.  No published studies could be found using juvenile sheepshead minnows although more are now available for the zebrafish and fathead minnow. However, on the basis of available information, there seems to be little to choose between fatheads, zebrafish and medaka.

## 8.2 Sexually Mature Life Stages

### 8.2.1    Sensitivity to Androgenic Steroid Exposure

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

214.    A similar observation of androgen-stimulated nuptial tubercle growth in female fathead minnows was made over 25 years ago by Smith (Smith, 1974).  Additional studies in other fish species support the conclusion that formation of secondary male characteristics in females during androgenic exposure can be diagnostic for this mode of action.  For example, in the adult medaka, a 15-day dietary exposure to 11-KT at dose rates ranging from 25 µg/g to 500 µg/g-food increased the number of papillary processes (growths) on anal fin rays in females in a dose-responsive manner (Hishida & Kawamoto 1970).  In a series of related studies, appearance of papillary processes on the anal or dorsal fin ray in female medaka was observed after oral dosing with 19-*nor*-testosterone, MT, ethisterone, T, and androstenedione (Kawamoto 1969, 1973; Uwa 1975).  Of these androgenic substances, 19-*nor*-testosterone was the most potent at stimulating formation of papillary processes with a dose rate as low as 0.6 µg/g-food producing a measurable effect (Kawamoto 1969).  In the male mosquitofish (*Gambusia affinis*), the anal fin develops into a gonopodium, which serves as an intromissive organ.  This development is under androgen control, and female mosquitofish administered 11-KT in their diet at dose rates as low as 20 µg/g-food will develop a gonopodium or exhibit a modified anal fin suggestive of gonopodium formation within 20 days of exposure (Angus et al. 2001).  This phenomenon appears to be environmentally relevant, as masculinization of mosquitofish has been observed in wild populations (Howell et al. 1980) and appears to be the result of androgenic substances, such as androstenedione found in pulp-mill effluents released into surface waters (Jenkins et al. 2001; Parks, Lambright, et al. 2001).

215. More recently, Ankley et al. (2003) and Jensen et al. (2006) have studied the effects of beta- and alpha trenbolone, respectively, on reproduction of fathead minnows in partial life cycle tests. The results were rather similar, both qualitatively and quantitatively, with significantly reduced fecundity, induction of nuptial tubercles in females, and reduced VTG in females in concentrations of 0.01-0.03 µg/L.

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

### 8.2.2 Anti-Androgens

#### 8.2.2.1 Direct Acting

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

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

In this latter experiment, CPA suppressed the MT stimulated formation of papillary processes in a dose-dependent manner (Hamaguchi 1978).

219.     Recently, Jensen et al. (2004) have studied the effects of another anti-androgen, flutamide, on fathead minnows in a partial life-cycle 3 week reproduction study. They showed that a flutamide concentration of 651 µg/L caused increases of T titres in females and E2 titres in males, changes which were accompanied by increased VTG in both sexes. This somewhat unusual endocrinological picture was associated with reduced fecundity and embryo hatch, and a variety of histopathological changes in the gonads.

*8.2.2.2 Indirect Acting*

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

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

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

**8.2.3     Gender Differences**

223.     The available data suggest (anti-) androgen activity is best identified in adults based on the appearance of secondary sex characteristics, a non-invasive observation after relatively short time exposure (i.e. 3 weeks).  In the case of androgen agonists, the development of male external characteristics in females appears to be a sensitive endpoint.  As for anti-androgens, a reduced display of secondary sex characteristics in males is somewhat sensitive, although not to the degree androgen activity has on females.  However, it is clear that both androgens and anti-androgens can also have significant effects on fecundity and hatching success.

**8.2.4     Strength and Weaknesses of Test Species**

224.     A rigorous assessment of the strength and weaknesses of each test species with respect to (anti-) androgens is limited by the lack of information for some species, such as the fathead and sheepshead

minnows during early life stages.  As for estrogens, the fathead minnow, medaka, and sheepshead minnow offer an advantage because of the more pronounced male secondary sex characteristics, but fecundity and hatching success appear to be generally applicable endpoints.

## 9.0 CANDIDATE PROTOCOLS

225.    The utility of the Partial Life-Cycle Reproduction Test, the Full Life-Cycle test, a Two-Generation Test, and a Multi-Generation Test has been evaluated for use in testing EDCs and non-EDCs. Each test provides unique advantages, which cannot be combined into one protocol.  The Partial Life-Cycle Test is a relatively short exposure test designed to evaluate sexual reproduction in fish and the effects to the early life stages of their progeny from exposure to chemicals.  The Partial Life-Cycle Test can be conducted at a relatively high concentration in an effort to elicit a response and / or at low concentrations to evaluate the threshold for a reproductive response. Although a Partial Life-Cycle Test has scientific disadvantages compared with a multiple generation test (e.g. it omits a full evaluation of sexual development, or of effects of maternally-transferred test material), a major advantage of the former is the shorter period of time necessary to conduct the in-life phase of the test, offering considerable cost savings.

226.    By comparison, the Full Life-Cycle Test exposes fish from the fertilized egg stage of the F0 generation to the juvenile stage of the F1 generation, and is hence somewhat longer than the Partial Life-Cycle Test. It has been widely used for evaluating non-EDCs. However, it may not always be able to evaluate EDCs fully because it may miss delayed effects of maternally transferred test material on reproduction.

227.    The Multi-Generation Test is designed to be a definitive test for evaluating population-level effects of EDC and non-EDCs in the environment.  This long-term chronic test exposes fish through two complete life cycles, starting with fertilized eggs of the F0 generation, and continuing to the early life stages of the F2 generation.  Furthermore, it evaluates the effects of maternal transfer on two generations, and between 60 and 80 endpoints could be collected from three generations of both male and female fish. However, the number of endpoints can be reduced by omitting optional mechanistic biomarkers, especially when testing non-EDCs. By using small fish with short life cycles, this test can be conducted in a reasonable time (as short as 4.5 months).

228.    A possible alternative to a full Multi-Generation Test, is a Two-Generation Test in which the exposure is initiated with mature F0 fish and the F1 generation is evaluated for embryo fertility, development, sexual maturation, reproduction. F2 viability is then assessed up to the juvenile stage. With an exposure of spawning adults, the Two-Generation Test incorporates limited maternal transfer from the F0 to F1 generation into the test, but also evaluates some effects of maternal transfer from the F1 to F2 generations. This test offers the advantage of a reduction in the time required to conduct the test when compared to the Multi-Generation Test, allowing the use of test species with longer life cycles, including the fathead minnow.  As with the Multi-Generation Test, the optional endpoints included in the Two-Generation Test allow inter-species comparison of the underlying mechanisms of toxicity, and the main endpoints assess impacts upon fecundity and viability that can address population level impacts integral to risk assessment. A possible disadvantage of starting the test with adults is that reproductive output is likely to vary considerably between individuals, thus implying the need for considerably more replicates than in the Full Life-Cycle or Multi-Generation Tests.

229.    The various candidate test schemes are described in detail below for each species, and their various advantages and disadvantages are summarized in Table 9-11.

**9.1 Partial Life-Cycle Reproduction Test {Adult (F0) to Juvenile (F1)}**

230.    A Partial Life-Cycle Toxicity Test, which exposes F0 adult, sexually mature fish and the early life cycle of F1 fish, can be conducted to estimate the ECx or NOEC for the exposed fish (always bearing in mind that an ECx or NOEC derived from a partial life-cycle test may be less sensitive than equivalent statistics from a life-cycle test).  A pre-exposure reproductive evaluation is conducted on the F0 fish.  It should be noted that the Fish Sexual Development Test (FSDT) whose validation is already in progress by VMG-eco, which exposes fish from the fertilized egg stage through to sexual maturity, can also be considered a partial life-cycle test, but it omits the stage of sexual reproduction and will not be considered further here. The biological endpoints evaluated in the adult-to-juvenile Partial Life-Cycle Test include the following:

- F0 pre-exposure,  fecundity/reproduction (e.g., eggs/female)
- F0 post-exposure, survival, secondary sexual characteristics, fecundity/reproduction (e.g., eggs/female), GSI, histopathology, and protein and sex steroid biomarkers (e.g., VTG)
- F1 hatching success, survival, growth (length and weight).
- Mechanistic endpoints (e.g. VTG, E2, T, 11-KT) can be added optionally, as can gonad histopathology

**Materials and Methods**

Physical Exposure System

- Intermittent flow diluters are used in combination with mechanical or electronic syringe pumps to deliver fresh toxicant and dilution water during the exposure.  If a carrier solvent is used, all treatments will have an equal solvent concentration with the exception of the dilution water control.
- Exposures will be conducted in glass aquaria, which will be impartially arranged on two separate levels (tiers).  The upper level will be used for F1 embryo incubation and larval fish rearing.  Larval growth chambers will be placed in each aquarium to provide the capability of simultaneously rearing two larval groups.  The lower level will be used to expose F0 spawning groups.
- Each level will contain two to four replicates per treatment level.  Fewer or no replicates may be appropriate for regression designs, although some level of true replication (e.g., duplicates) is usually preferable even in regression designs. Groups within replicates will be established for certain endpoints – hatching success, larval exposure and spawning groups.
- Three of the four candidate species are freshwater fish.  Dilution water for these species should be dechlorinated water from a noncontaminated source.  Care should be used not to use surface waters, which may contain low levels of potent EDCs.  Sheepshead minnow will be maintained in relatively low salinity (15 ppt; lower salinity concentrations may be possible upon further evaluation) dilution water, which can be collected from a contaminant-free source or can be prepared from a commercial mix.

Chemical System

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

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

Test Conditions

- The water temperature for fathead minnow will be 25 ±1°C and for the zebrafish will be 26 ±1°C. For the other candidate species, a water temperature of 28 ±1°C will be maintained.
- Constant photoperiods of 16 hours light to 8 darkness will be used except for zebrafish where the photoperiod will be 12 h light / 12 h dark.
- The dissolved oxygen concentration will be maintained at =60% of saturation. Aeration may be necessary to maintain dissolved oxygen concentrations above 60% of saturation. If aeration is necessary, an experiment will be conducted to demonstrate that aeration will not alter the exposure concentrations.
- A tank volume turn over rate of 6 per 24-hour period will be established, or 90% replacement in 9 hours.

Biological Methods

A general description of the biological methods is provided below.

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

- Reproductive F0 Adults, Pre-Exposure Phase

The F0 adult fish should be of the same age and cultured together under identical conditions. Sexually mature fish, as evidenced by secondary sexual characteristics and behavior, will be collected from the culture and randomly assigned to spawning groups appropriate for the species. Additional spawning groups should be established as replacement fish. A pre-exposure phase will be conducted under identical exposure conditions (i.e., water quality and flow, space, light, temperature, feeding, spawning structure, etc). The pre-exposure phase will last until spawning is established in all spawning groups to be used in the study. This period could last up to14 days, depending on the species. During the pre-exposure, the reproductive potential of all spawning groups will be evaluated. Any fish that dies during the pre-exposure phase will be replaced with a fish from the original culture. Any spawning group that does not produce viable embryos during the pre-exposure phase will not be used. The number of eggs/female/day should be calculated for each spawning group.

- Reproductive F0 Adults, Exposure Phase

The spawning groups selected for the exposure will be placed in the lower level of the exposure system. The exposure system should be functioning properly for several days prior to adding the

spawning groups. Those fish not selected for the exposure can be sacrificed and used to collect pre-spawning endpoints, if desired. The reproductive period will last from 7 to 28 days, depending on the species being exposed.

- F1 Embryo Exposure (hatching success)

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

- F1 Larval Exposure

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

Statistical Analysis

If a solvent control is used in the exposure, a two-tailed t-test will be used to determine whether there are differences between the two control groups. If there are no differences between the control groups, the controls will be pooled for the determination of treatment-related effects. In some cases, it may be better to analyse results separately for dilution controls, solvent controls, and pooled controls, and report the power of all analyses as an aid to interpretation. If there is a difference between the control groups, the solvent control data will be used for the determination of treatment-related effects. In ANOVA designs, continuous data will be analyzed, after checking for homogeneity of variances, using ANOVA and pair-wise comparison tests (e.g., Dunnett's Test) to determine differences between treatments and controls. Quantal data will be analyzed by a contingency table test (e.g., Fisher's Exact Test with Bonferroni-Holm adjusted significance level). In regression designs, an appropriate model will be fitted to the data and should be checked to ensure that it is valid. The nature of these checks will depend on the type of data being analyzed (Chapman et al., 1996). Concentration-response models based on the normal distribution will require a check for homogeneity of variance and model fit (Draper and Smith 1981). When a concentration-response model based on a distribution other than the normal is fitted, both over-dispersion and model adequacy should be checked (Collett 1991).

**Reporting Requirements**

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

- Information regarding test temperatures, dissolved oxygen concentration, pH, salinity, photoperiod, and light intensity used
- Tabular presentation of all measured and calculated endpoints
- Description of reference (or inclusion as an appendix) to chemical and statistical procedures applied
- Analytical results of test concentration measurements and QC samples
- The ECx, or the NOEC and LOEC values will be provided, as well as the endpoints and statistical procedures used to establish these values. When an ECx is reported, the slope of the dose response and the confidence limits around the slope and the ECx should all be reported. When a NOEC is reported, the treatment means, their standard errors, the error degrees of freedom and the least significant difference, or standard error of the difference, should all be reported, along with the power of any inferential tests.
- If a ECx is reported, an assessment of goodness-of-fit should be reported, along with an assessment of the appropriateness of the model if such exists. If a NOEC is reported, the tests used and its justification (including an assessment of the assumptions on which it is based) should be reported. The handling of the multiple control issue should also be reported, where multiple controls were used (e.g., where a solvent was used).
- Deviations from the protocol not addressed in protocol amendments, together with a discussion of the impact on the study
- Good Laboratory Practice (GLP) compliance statement signed by the Study Director
- Date(s) of Quality Assurance reviews, and dates reported to the Study Director and management, signed by the Quality Assurance Unit
- Location of the raw data and report.

## 9.2 Full Life-Cycle Test {Egg (F0) to Juvenile (F1)}

231.	A Full Life Cycle Test has been developed for use with fathead minnows (Benoit 1981) and for the sheepshead minnow (Hansen et al. 1978), although there is no reason why it cannot also be operated with medaka or zebrafish. Tables 9-1 and 9-2 present a summary of the Full Life Cycle Test with the fathead minnow and the sheepshead minnow, and these summaries can easily be adapted for use with medaka and zebrafish. The Full Life-Cycle Test is initiated with fertilized eggs (F0) and the fish are continuously exposed through reproductive maturity, followed by assessment of the early development of the F1 generation.

232.	The biological endpoints evaluated include the following:

- F0  embryo time-to-hatch, hatching success, larval survival and length, weight of thinned fish, survival, secondary sexual characteristics, fecundity/reproduction (e.g., eggs/female), growth.
- F1  hatching success, survival, growth (length and weight)
- Mechanistic endpoints (e.g. VTG, E2, T, 11-KT) can be added optionally, as can gonad histopathology

**Table 9-1.  Full Life-cycle Test with Fathead Minnow**

| Timeline | Test Day | # Organisms | Endpoints |
|---|---|---|---|
| Month 1 | Embryo Incubation Day 0 - 5 | 200 per concentration 50 per replicate | F0 Embryo time-to-hatch  Hatching success |
| | Larval Exposure Day 6 -35 | 100 per concentration 25 per replicate | F0   Larval survival and length |
| Month 2 | Day 65 | Reduced to 50 per concentration 25 per duplicate | F0 Larval survival and length  Weight ( thinned out fish) |
| Months 3 through 5 | F0 Juvenile to Adult Day 65 - 150 | Reduced to 8 pairs per concentration 4 pairs per duplicate | F0 Survival  $2^{o}$ sex characteristics and internal examination of gonads  Lengths and weights  (male and female thinned fish) |
| Months 6 through 9 | F0 Spawning Period Day 150 - 270 | 8 pairs per concentration 4 pairs per duplicate | Eggs/female Spawns/female Eggs/spawn |
| | F1 Hatching Success Day 150 - 275 | 50 embryos per number of spawns >50 eggs | F1 Embryo time-to-hatch  Embryo hatching success |
| Months 6 through 11 | F1 4 - 8 Week Larval Exposure Day 150 - 330 | 25 larvae per group 2 groups per duplicate 4 groups per concentration | F1 4 and 8 week survival and length and weight |
| Month 10 -11 | F0 Termination Day 300 - 330 | Survivors | F0 Survival  $2^{o}$ sex characteristics and internal examination of gonads  Lengths and weights  (male and female thinned fish) |

**Table 9-2. Full Life-cycle Test with Sheepshead Minnow**

| Timeline | Test Day | # Organisms | Endpoints |
|---|---|---|---|
| Month 1 | Embryo Incubation Day 0 - 5 | 200 per concentration 50 per replicate | F0 Embryo time-to-hatch   Hatching success |
| | Larval Exposure Day 6 -35 | Reduced to 50 larvae per concentration 25 per replicate ( Day 35) | F0 Larval survival and length   Weight (thinned fish) |
| Month 2 | Juvenile to Adult Day 62 | 50 per concentration 25 per replicate | F0 Survival and length   Time to maturity   $2^o$ sex characteristics |
| Month 3 | F0 Spawning Period Day 62 - 76 (possibly $2^{nd}$ round of spawn groups= Day 90) | Minimum : 4 groups of 3 females/2 males per concentration 2 groups per replicate | eggs/female/day |
| | F1 Hatching Success Day 62 - 95 | 50 embryos per number of spawns >50 eggs | F1 Embryo time-to-hatch   Embryo hatching success |
| Month 4 | F1 4-Week Larval Exposure Day 95 - 123 | 25 larvae per group 2 groups per duplicate 4 groups per concentration | F1 Survival   Length and weight |
| | F0 Termination Day 95 | Survivors | F0 Survival   $2^o$ sex characteristics and internal examination of gonads   Lengths and weights   (male and female thinned fish) |

**Materials and Methods**

These are the same as for a partial life-cycle reproduction test (see Section 9.1). Biological methods differ and are described below.

Biological Methods

Tables 9-1 and 9-2 provide species-specific time lines and data endpoints to be collected in each significant life stage for each generation of the fish. A general description of the biological methods is provided below.

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

- F0 Embryo Exposure (hatching success)

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

- F0 Larval Exposure

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

- F0 Juvenile Exposure

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

- Reproductive F0 Adults

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

- F1 Embryo Exposure (hatching success)

All embryos will be counted daily. Representative spawns will be incubated and hatching success determined as described for the F0 embryo exposure.

- F1 Larval Exposure

The F1 larval exposure will be conducted following the same procedures presented for the F0 larval exposure.

- F1 Juvenile Exposure

The F1 juvenile exposure will be conducted following the same procedures presented for the F0 juvenile exposure.

Statistical Analysis

The same procedures as for the partial life-cycle reproduction test described in section 9.1 apply.

**Reporting Requirements**
The same requirements as for the partial life-cycle reproduction test described in section 9.1 apply.


**9.3 Multi-Generation Test {Egg (F0) to Juvenile (F2)}**

233.    The Multi-Generation Toxicity Test, which exposes all life-stages of two generations of fish, is presented for the species under consideration in this review in Tables 9-3 through 9-6. The test is initiated with eggs and two full generations of fish are exposed during the test. This test can be conducted to estimate the ECx or NOEC for the exposed fish and the biological endpoints evaluated include the following:

- F0 and F1 hatching success, survival, growth (length and weight), time-to-maturity, sex ratio, secondary sexual characteristics, fecundity/reproduction (e.g., eggs/female).
- F2 hatching success, survival and growth.
- Mechanistic endpoints (e.g. VTG, E2, T, 11-KT) can be added optionally, as can gonad histopathology

**Table 9-3. Multigeneration Test with Sheepshead Minnow**

| Timeline | Test Day | # Organisms | Endpoints |
|---|---|---|---|
| Month 1 | F0 Hatching Success Day 0-5 | 200 per concentration 100 per replicate 50 per incubation cup | F0 hatching success Embryo time-to-hatch |
| | F0 Larval Exposure (Early Life-Stage) Day 6-33 | 100 per concentration 50 per replicate 25 per growth chamber | F0 survival Lengths |
| Month 2 | F0 Juvenile Exposure Day 33-60 | 50 per concentration 25 per replicate | F0 survival Time to maturity Sex ratio Male, female length and weight GSI $2^o$ sex characteristics Blood plasma biomarkers (optional) Histopathology (optional) |
| Month 3 | F0 Reproduction Phase Day 60-74 | 8 males/20 females per concentration 4 males/10 females per replicate (2 groups of 2 males/5 females) | Time to maturity Sex ratio $2^o$ sex characteristics Pre-spawn condition Fecundity (eggs/female) Fertilization success Spawning behavior (optional) |
| | F0 Adult Termination Day 74 | Survivors | Male, female length and weight GSI Blood plasma biomarkers (optional) Histopathology (optional) |
| | F1 Hatching Success Day 60-79 | 200 embryos per concentration 100 per replicate 50 per incubation cup | F1 hatching success Embryo time-to-hatch |
| | F1 Larval Exposure (Early Life-Stage) Day 66-107 | 100 larval fish per concentration 50 per replicate 25 per growth chamber | F1 survival Length |
| Month 4 | F1 Juvenile Exposure Day 93-135 | 50 per concentration 25 per replicate | F1 survival Time to maturity Sex ratio Male, female length and weight GSI $2^o$ sex characteristics Blood plasma biomarkers (optional) Histopathology (optional) |
| Month 5 | F1 Reproduction Phase Day 135-149 | 8 males/20 females per concentration 4 males/10 females per replicate (2 groups of 2 males /5 females) | Time to maturity Sex ratio $2^o$ sex characteristics Pre-spawn condition Fecundity (eggs/female) Fertilization success Spawning behavior (optional) |

| Timeline | Test Day | # Organisms | Endpoints |
|---|---|---|---|
| | F1 Adult Termination Day 149 | Survivors | Male, female length and weight GSI Blood plasma biomarkers (optional) Histopathology (optional) |
| Month 5 | F2 Hatching Success Day 135-154 | 200 embryos per concentration 100 per replicate 50 per incubation cup | F2 hatching success Embryo time-to-hatch |
| Month 6 | F2 Larval Exposure (Early Life-Stage) Day 140-182 | 100 larval fish per concentration 50 per replicate 25 per growth chamber | F2 survival Length Weight |

**Table 9-4. Multigeneration Test with Zebrafish**

| Timeline | Test Day | # Organisms | Endpoints |
|---|---|---|---|
| Month 1 | F0 embryo-larval exposure<br>Day 10-31 | 100 embryos per replicate (encaged)<br><br>2 replicates per treatment | Hatching success<br>Time to hatch<br>Normal/abnormal<br>Weekly survival rate |
| Month 2, 3 | F0 Juvenile Exposure<br>Day 31-70 | 100 per concentration<br>50 per replicate | F0 survival<br><br>Length at 3 and 10 weeks |
| Month 3 | F0 Reproduction Phase<br>Day 70-105 | 30 fish per replicate | Survival<br>Time to maturity<br>Sex ratio<br>$2^o$ sex characteristics, preliminary<br>Time to first spawning<br>Fecundity (eggs/female)<br>Fertilization success<br>Spawning behavior (optional) |
| | F0 Adult Termination<br>Day 105 | Survivors | Male, female length and weight<br>Sex<br>GSI<br>Biomarkers (optional)<br>Gonad histopathology (optional) |
| Month 3, 4 | F1 Embryo-larval exposure<br>Day 98-126 | 100 embryos per replicate (encaged)<br><br>2 replicates per treatment | Hatching success<br>Time to hatch<br>Normal/abnormal<br>Weekly survival rate<br>Weight and length after 4 weeks |
| Month 5, 6 | F1 juvenile exposure<br>Day 126-165 | 100 per concentration<br>50 per replicate | F1 survival<br><br>Length at 3 and 10 weeks |
| Month 5, 6 | F1 reproduction phase<br>Day 165-200 | 30 fish per replicate | Survival<br>Time to maturity<br>Sex ratio, preliminary<br>$2^o$ sex characteristics<br>Time to first spawning<br>Fecundity (eggs/female)<br>Fertilization success<br>Spawning behavior (optional) |

| Timeline | Test Day | # Organisms | Endpoints |
|---|---|---|---|
| Month 6 | F1 Adult termination Day 200 | Survivors | Male, female length and weight Sex, definite sex ratio* GSI Biomarkers (optional) Gonad histopathology (optional) |
| Month 6, 7 | F2 Embryo-larval exposure Day 193-221 | 100 embryos per replicate (encaged) 2 replicates per treatment | Hatching success Time to hatch Normal/abnormal Weekly survival rate Weight and length after 4 weeks |
| Month 8, 9 | F2 juvenile exposure Day 221-260 | 100 per concentration 50 per replicate | F2 survival Length at 3 and 10 weeks |
| * eggs per female and day recalculated based on the data at group termination | | | |

**Table 9-5. Multigeneration Test with Fathead Minnow**

| Timeline | Test Day | # Organisms | Endpoints |
|---|---|---|---|
| Month 1 | F0 Hatching Success Day 0-5 | 200 per concentration 100 per replicate 50 per incubation cup | F0 hatching success Embryo time-to-hatch |
| | F0 Larval Exposure (Early Life-Stage) Day 6-33 | 100 per concentration 50 per replicate 25 per growth chamber | F0 survival Lengths |
| Month 2, 3, 4 | F0 Juvenile Exposure Day 33-120 | 50 per concentration 25 per replicate | F0 survival Time to maturity Sex ratio Male, female length and weight GSI $2^o$ sex characteristics Blood plasma biomarkers (optional) Histopathology (optional) |
| Month 5 | F0 Reproduction Phase Day 120-150 | 6 males/12 females per concentration 3 males/6 females per replicate (3 groups of 1 males /2 females) | Time to maturity Sex ratio $2^o$ sex characteristics Pre-spawn condition Fecundity (eggs/female) Fertilization success Spawning behavior (optional) |
| | F0 Adult Termination Day 150 | Survivors | Male, female length and weight GSI Blood plasma biomarkers (optional) Histopathology (optional) |
| | F1 Hatching Success Day 120-155 | 200 embryos per concentration 100 per replicate 50 per incubation cup | F1 hatching success Embryo time-to-hatch |
| Month 5, 6 | F1 Larval Exposure (Early Life-Stage) Day 125-183 | 100 larval fish per concentration 50 per replicate 25 per growth chamber | F1 survival Length |

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

**Table 9-6. Multigeneration Study with Medaka**

| Timeline | Test Day | # Organisms | Endpoints |
|---|---|---|---|
| Month 1 | F0 Hatching Success Day 0-7 | 200 per concentration 100 per replicate 50 per incubation cup | F0 hatching success Embryo time-to-hatch |
| | F0 Larval Exposure (Early Life-Stage) Day 7-35 | 100 per concentration 50 per replicate 25 per growth chamber | F0 survival Lengths |
| Month 2 | F0 Juvenile Exposure Day 35-63 | 100 per concentration 50 per replicate | F0 survival Time to maturity Sex ratio Male, female length and weight GSI $2^{o}$ sex characteristics Whole body homogenate biomarkers (optional) Histopathology (optional) |
| Month 3 | F0 Reproduction Phase Day 63-77 | 24 males/40 females per concentration 12 males/20 females per replicate | Time to maturity Sex ratio $2^{o}$ sex characteristics Pre-spawn condition Fecundity (eggs/female) Fertilization success Spawning behavior (optional) |
| | F0 Adult Termination Day 77 | Survivors | Male, female length and weight GSI Whole body homogenate biomarkers (optional) Histopathology (optional) |
| | F1 Hatching Success Day 64-84 | 200 embryos per concentration 100 per replicate 50 per incubation cup | F1 hatching success Embryo time-to-hatch |
| Month 4 | F1 Larval Exposure (Early Life-Stage) Day 71-112 | 100 larval fish per concentration 50 per replicate 25 per growth chamber | F1 survival Length |
| | F1 Juvenile Exposure Day 126-140 | 100 per concentration 50 per replicate | F1 survival Time to maturity Sex ratio Male, female length and weight GSI $2^{o}$ sex characteristics Whole body homogenate biomarkers (optional) Histopathology (optional) |

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

**Materials and Methods**

These are the same as for a partial life-cycle reproduction test (see Section 9.1). Biological methods differ and are described below.

Biological Methods

Tables 9-1 through 9-4 provide species-specific time lines and data endpoints to be collected in each significant life stage for all generations of the fish. A general description of the biological methods is provided below.

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

- F0 Embryo Exposure (hatching success)

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

- F0 Larval Exposure

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

- F0 Juvenile Exposure

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

- Reproductive F0 Adults

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

- F1 Embryo Exposure (hatching success)

All embryos will be counted daily. Randomly selected spawns will be incubated and hatching success determined as described for the F0 embryo exposure.

- F1 Larval Exposure

The F1 larval exposure will be conducted following the same procedures presented for the F0 larval exposure.

- F1 Juvenile Exposure

The F1 juvenile exposure will be conducted following the same procedures presented for the F0 juvenile exposure.

- Reproductive F1 Adults

The F1 reproductive exposure will be conducted following the same procedures presented for the F0 reproductive exposure.

- F2 Embryo Exposure (hatching success)

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

- F2 Larval Exposure

The F2 larval exposure will be conducted following the same procedures presented for the F0 and F1 larval exposures.

Statistical Analysis

The same procedures as for the partial life-cycle reproduction test described in Section 9.1. apply.

Reporting Requirements

The same requirements as for the partial life-cycle reproduction test described in section 9.1 apply.

**9.4 Two Generation Test {Adult (F0) to Juvenile (F2)}**

**Objective**

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234.    A Two Generation Life-Cycle Toxicity Test, which exposes the adult F0, full F1 generation,and measures F2 viability, can be conducted to estimate the ECx or NOEC for the exposed fish. Tables 9-7 through 9-10 summarize this test utilizing the fathead minnow, zebrafish, medaka and sheepshead minnow. Variables including the time-line of the test, the number of fish required in the test, and obtaining endpoints such as VTG plasma levels will be associated with the different species. For example, zebrafish are sometimes difficult to sex and will require more fish for testing and plasma is difficult to obtain from the smaller species limiting the biochemical measures in plasma. The biological endpoints evaluated will include the following:

- F0   Survival, secondary sex characteristics, reproductive behavior, spawning activity, fecundity, fertilization success
- F1 hatching success, survival, growth (length and weight), time-to-maturity, sex ratio, secondary sexual characteristics, fecundity/reproduction (e.g., eggs/female)
- F2 hatching success, survival and growth.
- Mechanistic endpoints (e.g. VTG, E2, T, 11-KT) can be added optionally, as can gonad histopathology

**Materials and Methods**

These are the same as for a partial life-cycle reproduction test (see Section 9.1). Biological methods differ and are described below.

**Table 9-7. Two-Generation Test with Fathead Minnow**

| Timeline | Test Day | # Organisms | Endpoints |
|---|---|---|---|
| Pre-test | 7 to 21 days Pre-exposure | 4 females/2 males per replicate | $2^o$ sex characteristics<br>Reproductive Behavior (optional)<br>Spawning activity<br>Fecundity<br>Fertilization success |
| F0 | Initiate Exposure 0 to 21 days | 4 females/2 males per replicate | $2^o$ sex characteristics<br>Reproductive Behavior (optional)<br>Spawning activity<br>Fecundity<br>Fertilization success |
| F1 | Day 14 to 21 | 50 embryos per replicate | Hatching success<br>Time to hatch<br>Normal/ abnormal |
| F0 | F0 Adult Termination | Survivors | Weight and length<br>Sex<br>GSI<br>Gonad Histopathology (optional)<br>Vtg and Sex steroids (optional) |
| F1 | Day 26 to 16-18 weeks (post -hatch) | 25 larvae per replicate | Survival<br>Length at 4 and 8 weeks<br>Time to maturity<br>Sex ratio<br>$2^o$ sex characteristics<br>Pre-spawn condition |
| F1 | 18 to 21 weeks | 4 females/2 males per replicate | Survival<br>$2^o$ sex characteristics<br>Reproductive Behavior (optional)<br>Spawning activity<br>Fecundity<br>Fertilization success |
| F2 | 18 to 21 weeks | 50 embryos per replicate | Hatching success<br>Time to hatch<br>Normal/ abnormal |
| F1 | F1 Adult Termination | Survivors | Weight and length<br>Sex<br>GSI<br>Gonad Histopathology (optional)<br>Vtg and Sex steroids (optional) |
| F2 | 21 to 25 weeks | 25 larvae per replicate | Survival<br>Weight and length at 4 weeks |

**Table 9-8. Two-Generation Test with Sheepshead Minnow**

| Timeline | Test Day | # Organisms | Endpoints |
|---|---|---|---|
| Pre-test | 4 to 7 days<br>Pre-exposure | 5 females/2 males per replicate | $2^o$ sex characteristics<br>Reproductive Behavior (optional)<br>Spawning activity<br>Fecundity<br>Fertilization success |
| F0 | Initiate Exposure<br>0 to 14 days | 5 females/2 males per replicate | $2^o$ sex characteristics<br>Reproductive Behavior (optional)<br>Spawning activity<br>Fecundity<br>Fertilization success |
| F1 | Day 10 - 14 | 50 embryos per replicate | Hatching success<br>Time to hatch<br>Normal/ abnormal |
| F0 | F0 Adult termination | Survivors | Weight and length<br>Sex<br>GSI<br>Gonad Histopathology (optional)<br>Vtg and Sex steroids (optional) |
| F1 | Day 19 to<br>7 - 8 weeks<br>(post -hatch) | 25 larvae per replicate | Survival<br>Length at 4 and 8 weeks<br>Time to maturity<br>Sex ratio<br>$2^o$ sex characteristics<br>Pre-spawn condition |
| F1 | 8 -10 weeks | 5 females/2 males per replicate | Survival<br>$2^o$ sex characteristics<br>Reproductive Behavior (optional)<br>Spawning activity<br>Fecundity<br>Fertilization success |
| F2 | 8 - 10 weeks | 50 embryos per replicate | Hatching success<br>Time to hatch<br>Normal/ abnormal |
| F1 | F1 Adult Termination | Survivors | Weight and length<br>Sex<br>GSI<br>Gonad Histopathology (optional)<br>Vtg and Sex steroids (optional) |
| F2 | 10  - 14 weeks | 25 larvae per replicate | Survival<br>Weight and length at 4 weeks |

**Table 9-9. Two-Generation Test with Zebrafish**

| Timeline | Test Day | # Organisms | Endpoints |
|---|---|---|---|
| Pre-test | 4 to 7 days<br>Pre-exposure | 15 females/15 males per replicate<br><br>2 replicates per treatment | Reproductive Behavior (optional)<br>Fecundity<br>Fertilization success |
| F0 | Initiate Exposure<br>0 to 14 days | 15 females/15 males per replicate | Reproductive Behavior (optional)<br>Fecundity<br>Fertilization success |
| F1 | Day 10 to 31 | 100 embryos per replicate (encaged) | Hatching success<br>Time to hatch<br>Normal/abnormal<br>Weekly survival rate |
| F0 | F0 Adult termination | Survivors | Weight and length<br>Sex<br>GSI<br>Gonad Histopathology (optional)<br>Vtg and Sex steroids (optional) |
| F1 | Day 31 to 70 | 50 larvae/young fish per replicate | Survival<br>Length at 3 and 10 weeks |
| F1 | Day 70 to 105 | 30 fish per per replicate | Survival<br>Time to maturity<br>$2^0$ sex characteristics<br>Sex ratio (preliminary)<br>Time to first spawning<br>Reproductive Behavior (optional)<br>Fecundity<br>Fertilization success |
| F2 | Day 98 to 126 | 100 embryos per replicate (encaged) | Hatching success<br>Time to hatch<br>Normal/abnormal<br>Weekly survival rate<br>Weight and length after 4 weeks |
| F1 | F1 Adult Termination | Survivors | Weight and length<br>Sex, definite sex ratio*<br>GSI<br>Gonad Histopathology (optional)<br>Vtg and Sex steroids (optional) |
| * eggs per female and day recalculated based on the data at group termination | | | |

**Table 9-10. Two-Generation Test with Medaka**

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

| F1 | F1 adult termination | Survivors | Weight and length<br>Sex<br>GSI<br>Gonad Histopathology (optional)<br>Vtg and Sex steroids (optional) |
|----|----------------------|-----------|-----|
| F2 | 21 to 25 weeks | 25 larvae per replicate | Survival<br>Weight and length at 4 weeks |

Biological Methods

Table 9-7 provides a time line of the test utilizing fathead minnows, with shorter time lines resulting with the use of sheepshead minnow zebrafish and medaka, (Tables 9-8 through 9-10). The data endpoints to be collected in each significant life stage for all generations of the fish are presented in these tables (9-7 through 9-10). A general description of the biological methods is provided below.

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

- Mature F0 Adults Pre-exposure

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

- Mature F0 Adults Exposure

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

- F1 Embryo Exposure (hatching success)

Exposure is continuous; F1 embryos are maintained under exposure from fertilization onwards. Eggs will be collected from the F0 adult spawning groups near the end of the adult exposure period. For some species, subsamples can be microscopically examined to estimate fertilization success before incubation. The embryos will be contained in small incubation containers (groups within replicates) and observed daily until hatch.

- F1 Larval Exposure

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

- F1 Juvenile Exposure

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

- Reproductive F1 Adults

The F1 reproductive exposure will be conducted following the same procedures presented for the P reproductive exposure (except pre-exposure is not required).

- F2 Embryo Exposure (hatching success)

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

- F2 Larval Exposure

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

Statistical Analysis

The same procedures apply as for the partial life-cycle reproduction test described in Section 9.1

Reporting Requirements

The same requirements apply as for the partial life-cycle reproduction test described in Section 9.1.

**Table 9-11. Advantages and disadvantages of the candidate tests.**

| Test | Strengths | Weaknesses |
|---|---|---|
| **Partial Life-Cycle Reproduction Test** (F0 adult to F1 fry or juvenile) | • Relatively short and cheap<br>• For non-EDCs, predicts the NOECs of longer tests with reasonable accuracy<br>• Can test relatively high concentrations that might cause long-term mortality<br>• Reasonable track record with several species<br>• Uses relatively few test animals | • Not all life-stages are exposed<br>• Fails to evaluate sexual development fully<br>• Not sensitive to all modes of action (e.g. some EDCs)<br>• Requires use of relatively large uncertainty factors in risk assessment<br>• Cannot evaluate most potential effects of maternal transfer |
| **Full Life-Cycle Test** (F0 fertilized eggs to F1 juveniles) | • Shorter, less animal intensive and cheaper than the Two-Generation or Multi-Generation tests<br>• Long track record with several species<br>• Starts with eggs, thereby minimizing potential variability<br>• Requires smaller uncertainty factors in risk assessment than the Partial Life-Cycle test | • More expensive than the Partial Life-Cycle test<br>• Does not fully evaluate potential effects in the second generation<br>• Does not evaluate full potential effects of maternal transfer<br>• Long-term exposures may prevent testing of high concentrations<br>• Uses more test animals than the Partial Life-Cycle Test |
| **Two-Generation Test** (F0 adults to F2 juveniles) | • Cheaper than the Multi-Generation test<br>• Covers two cycles of reproduction and growth<br>• Maternal transfer is fully evaluated in the F2 generation<br>• Requires use of relatively smaller uncertainty factors in risk assessment than the shorter tests | • More expensive than the Partial Life-Cycle or Full Life-Cycle tests<br>• Potentially high variability caused by starting test with adults (particularly with the key population relevant endpoint of sex ratio)<br>• Long-term exposures may prevent testing of high concentrations<br>• Maternal transfer from the F0 generation may be limited<br>• Limited track record<br>• Uses more test animals than the Full Life-Cycle Test |

| Test | Strengths | Weaknesses |
|---|---|---|
| **Multi-Generation Test** (F0 fertilized eggs to F2 juveniles) | • Covers two cycles of reproduction and growth<br>• Maternal transfer from the F0 generation is maximal<br>• Potential effects of maternal transfer are evaluated in two generations<br>• Starts with eggs, thereby minimizing potential variability<br>• Requires use of relatively smaller uncertainty factors in risk assessment than the shorter tests | • Longer and more expensive than the other tests<br>• Long-term exposures may prevent testing of high concentrations<br>• Limited track record<br>• Uses the largest number of test animals of any life-cycle test |

## 10.0    RECOMMENDED PROTOCOL AND ADDITIONAL DATA NEEDS

235.    Table 10-1 summarises much of the published partial- and full life-cycle test data for the four OECD fish species (fathead minnow, sheepshead minnow, medaka, and zebrafish) exposed to a selection of EDCs, several of which have been tested with more than one of the species. A number of observations flow from this table:-

- Very few EDCs have been tested against sheepshead minnow
- There is significant experience in Partial Life-Cycle Testing of EDCs with fathead minnow and medaka, and some experience with the other two species
- As yet, there is relatively little experience with the partial life cycle fish sexual development test (FSDT), so far mainly employing zebrafish, but the limited available data shown in Table 10-1 suggest that it is at least as sensitive to estrogens as the partial life cycle reproduction test.
- There is also significant experience in Full Life-Cycle Testing of EDCs with medaka and zebrafish, but relatively little with fathead minnow
- There appears to be little experience, with any species, in operating the proposed Two-Generation or Multi-Generation test protocols with EDCs
- The EDCs for which test data exist for more than one species in a given test-type include only E2, EE2, NP, OP and BPA, giving a rather poor database for comparing sensitivities.
- However, the limited comparative data suggest that medaka, fathead minnow and zebrafish are of similar sensitivity to these substances, to within an order of magnitude. The single life-cycle test with sheepshead minnow exposed to EE2 suggests that this species is also of similar sensitivity to the other three species.
- A comparison of the Partial and Full Life-Cycle test results can be made for medaka exposed to E2, EE2, NP, OP, DNBP, o,p'-DDT, p,p'-DDT and BPA, and it  shows clearly that exposure of the Full Life-Cycle gives NOECs for reproductive endpoints that are often an order of magnitude (or more) below those resulting from Partial Life Cycle exposures.
- No single endpoint, whether reproductive or biomarker, appears to be consistently the most sensitive.
- There is insufficient experience with androgens, aromatase inhibitors, anti-estrogens or anti-androgens to enable any particular protocol to be preferred.

236.    After considering the strengths and weaknesses of the two candidate approaches for testing EDCs that have been employed extensively to date, it is concluded that the Full Life-Cycle is more sensitive than the Partial Life-Cycle reproduction test approach, and that no single species or endpoint

demonstrates consistently greater sensitivity than the others. However, it remains to be demonstrated whether the Fish Sexual Development Test will have reasonable predictivity of the fish full lifecycle response to EDCs as is the case for non-EDCs and the standard study designs (e.g. Mckin 1977). The primary question which requires an answer is, therefore, whether the Two-Generation or Multi-Generation protocols are likely to offer any greater sensitivity than the Full Life-Cycle protocol. This question can only be definitively answered by some comparative validation trials, but there are several theoretical reasons why the Full Life-Cycle protocol may not be maximally sensitive to some EDCs:-

- The Full Life-Cycle protocol only allows bioaccumulation over (at most) 1½ life cycles, whereas the Multi-Generation protocol allows bioaccumulation to proceed for 2½ life cycles. It is therefore possible that some bioaccumulative EDCs may only reach threshold levels in fish tissue during the Multi-Generation Test. The Two-Generation Test appears to offer no advantages in this respect, as it only proceeds for at most 1½ life cycles.
- A related issue concerns maternal transfer of EDC residues (e.g. Metcalfe et al., 2000). This can occur once during the Full Life-Cycle Test, but twice during the Two-Generation and Multi-Generation tests. Highly bioaccumulative EDCs may therefore potentially give lower NOECs in the latter two protocols.
- A possible reason for less-than-maximal sensitivity of the Full Life-Cycle protocol is that delayed effects on the F1 generation will not be detected. In other words, unless the F1 juveniles are raised to adulthood and allowed to breed, it is possible that the cumulative impact of abnormal development may not be fully realized. Only the Multi-Generation Test completely addresses this concern, although if the F1 juveniles show significant abnormalities (e.g. ovo-testis or poor growth) in the Full Life-Cycle Test, impacts on F1 breeding are implied. Furthermore, one would expect to see most, if not all, effects on breeding success in the F0 adults in the Full Life-Cycle protocol. It would also be possible to extend the full lifecycle design to assess endocrine effects in the F1 generation. Without necessarily extending the study to include reproduction of the F1. Thereby including a more detailed assessment of potential maternal transfer induced effects.

237.    In summary, the Two-generation and Multi-Generation protocols offer a few theoretical scientific advantages over the Full Life-Cycle protocol, but these remain to be demonstrated in published sources. Unpublished data for zebrafish and medaka exposed in Multi-Generation Tests to NP, OP or dehydroepiandrosterone (Thomas Braunbeck, pers. comm. 2007) provide little, if any, support for the possibility that the second complete generation may be more sensitive than the first, but perhaps such long-term effects can only be caused by more strongly bioaccumulated EDCs. One weakness of the multi-generation test starting with adult fish over the full life-cycle test starting with fertilized embryos is that the F1 has a different starting point and will represent an egg clutch from one specific parent pair. This might increase variability between the replicates by genetic or even clutch specific (e.g. sex ratio skew) differences. If the test is started with freshly fertilized eggs of unexposed parents, the eggs of a number of egg clutches can be randomly allocated to the test vessels and the test groups are more comparable.

238.    At this time, the only protocol that has been developed to a point that a standardized transferable protocol can be written without additional prevalidation studies is the Full Life-Cycle Test.  If the Two-Generation or Multi-Generation protocols are to be pursued, their sensitivity, reliability and reproducibility must be demonstrated and compared with the Full Life-Cycle Test. One possible problem

with the Two-Generation protocol, as indicated above, is that it begins with adult fish, and therefore may be associated with greater between-clutch variability (and hence need more replicates) than the Full Life-Cycle or Multi-Generation protocols. Issues such as differences in sensitivity among the species must be addressed if any test is to be used in risk assessment, although the limited available data suggest that none of the 4 species is consistently of greatest sensitivity.  Interlaboratory comparisons should be conducted with compounds that span the possible endocrine effects (strong and weak (anti-) estrogens, androgens, and aromatase inhibitors).

239.    The number of demonstration trials needed for full optimization of a method suitable for interlaboratory testing could be kept to a minimum if a single species is pre-selected. Before this occurred, practical as well as scientific considerations would have to be addressed. For example, would the use of a single species be acceptable to all jurisdictions, given the regulatory history of some species and protocols? Is the high cost of life-cycle testing with fathead minnows (compared with the other three species) justifiable? Irrespective of whether one or more species is chosen, if either the Two-Generation or Multi-Generation protocols do indeed show greater sensitivity than the Full Life-Cycle Test, is this advantage sufficient to justify the considerable numbers of animals and greater expected testing costs?

**Table 10-1.   OVERVIEW OF LIFE-CYCLE AND PARTIAL LIFE-CYCLE FISH TESTS USING THE FOUR STANDARD TEST SPECIES AND SOME EDCs**

| Fish species | Method used | Duration | Generations exposed | Chemical tested | Most sensitive endpoints  (biomarkers and reproduction) | Reference |
|---|---|---|---|---|---|---|
| Medaka | Partial life-cycle reproduction test | 21-d (+ fry 60-d in clean water) | F0 (adult) to F1 | 17-beta estradiol (E2) | **F0:** NOEC (VTG) <29.3 ng/l      NOEC (fertility; fecundity) 227 ng/l **F1:** NOEC > 463 ng/l (all endpoints) | Kang et al. (2002b) |
| Medaka | Partial life-cycle reproduction test | 21-d | F0 (adult) to F1 (hatching) | 17-beta estradiol (E2) | **F0:**  NOEC (testis-ova) 7.4 ng/l      NOEC (VTG) < 0.91 ng/l | Anon (2006) |
| Medaka | Fish sexual development test | 0-60 Dph (0-38 Dph for VTG) | F0 (fertilized egg to sexual differentiation) | 17-alpha-ethynylestradiol (EE2) | **F0**: NOEC (Sex ratio) 10 ng/l      NOEC: (VTG) 10 ng/l | Örn et al. (2006) |
| Medaka | Full life-cycle test | 101+59 d | From F0 (embryos to 101 dph) to F1 (59 dph) | 17-beta estradiol (E2) | **F0:** NOEC (VTG) 2.86 ng/l      NOEC (fertility) 2.86 ng/l **F1:** NOEC (all endpoints) ≥ 8.66 ng/l | Seki et al. (2005) |
| Medaka | Full life-cycle test | 100-d | From F0 (embryos to 100 dph) to F1 (fert.) | 17-beta estradiol (E2) | **F0:**   NOEC (sex ratio; fecundity) <126.5 ng/l **F0:**  NOEC (GSI) <126.5 ng/l | Hirai et al. (2006) |
| Medaka | Partial life-cycle reproduction test | 21-d | F0 (adult) | 17-alpha-ethynylestradiol (EE2) | **F0:** NOEC (VTG; testis-ova) 32.6 ng/l      NOEC (fecundity) 261 ng/l | Seki et al. (2002) |
| Medaka | Partial life-cycle reproduction test | 21-d | F0 (adult) to F1 (hatching) | 17-alpha-ethynylestradiol (EE2) | **F0:**  NOEC (testis-ova; HSI) 12.2 ng/l      NOEC (VTG) 12.2 ng/l | Anon (2006) |
| Medaka | Full life-cycle test | 104-d | From F0 (embryos to 101 | 17-alpha-ethynylestradiol | **F0:** NOEC (VTG) 3.07 ng/l      NOEC (fertility) 3.07 ng/l | Anon (2006) |

| Fish species | Method used | Duration | Generations exposed | Chemical tested | Most sensitive endpoints (biomarkers and reproduction) | Reference |
|---|---|---|---|---|---|---|
| | | | dph) to F1 (61 dph) | (EE2) | **F1:** NOEC (VTG) ≤ 0.99 ng/l<br>NOEC (other endpoints) ≥ 9.26 ng/l | |
| Medaka | Full life-cycle test | 120-180d | From F0 (embryos to 120 dph) to F1 (13+ dph) | 17-alpha-ethynylestradiol (EE2) | **F0:** NOEC (testis-ova) 0.2 ng/l<br>**F0:** NOEC (behaviour) 2 ng/l<br>**F1:** NOEC (fertilization success; time to hatch) > 10 ng/l | Balch et al. (2004) |
| Medaka | Full life-cycle test | 90-d | F0 (eggs or fry to 14 dph, and again at adulthood)<br>F1 (fert.) | 17-alpha-ethynylestradiol (EE2) | **F0:** NOEC (fecundity; egg size) 20 ng/l<br>**F0:** NOEC (VTG; E2) 20 ng/l<br>**F1:** NOEC (fert.success) 2000 ng/l | Foran et al. (2002) |
| Medaka | Full life-cycle test | 104-d | From F0 (embryos to 101 dph) to F1 (60 dph) | methyl-testosterone (MT) | **F0:** NOEC (sex ratio; $2^o$ sex) 9.98 ng/l<br>NOEC (other endpoints) ≥ 27.75 ng/l<br>**F1:** NOEC (female VTG) <0.35 ng/l<br>NOEC (other endpoints) ≥ 9.98 ng/l | Seki et al. (2004) |
| Medaka | Partial life-cycle reproduction test | 21-d | From F0 (adults) to F1 (hatch) | 4- nonylphenol (4-NP) | **F0:** NOEC (growth) 11.6 µg/l<br>NOEC (testis-ova; VTG) 6.08 µg/l | Anon (2006) |
| Medaka | Full life-cycle test | 104-d | From F0 (embryos to 101 dph) to F1 (60 dph – sexually differentiated) | 4- nonylphenol (4-NP) | **F0:** NOEC (testis-ova) 8.2 µg/l<br>NOEC (mortality) 8.2 µg/<br>**F1:** NOEC (testis-ova) 8.2 µg/l<br>NOEC (other endpoints) >183 µg/l | Yokota et al. (2001) and Anon (2006) |
| Medaka | Full life-cycle test | 90-d | From F0 (embryos to 90 dph) to F1 (fert.) | 4-*tert*-octylphenol (OP) | **F0:** NOEC (growth) 20 µg/l<br>NOEC (ovo-testis; sex ratio) < 2 µg/l<br>**F1:** NOEC (fert.; mortality) < 2 µg/l | Knörr & Braunbeck (2002) |
| Medaka | Full life-cycle test | ? | From F0 (embryos) to F1 (hatch) | 4-*tert*-octylphenol (OP) | **F0:** NOEC (testis-ova) 9.92 µg/l<br>NOEC (VTG) 4.27 µg/l<br>**F1:** NOEC (testis-ova) 9.92 µg/l<br>NOEC (VTG) 4.27 µg/l | Anon (2006) |

| Fish species | Method used | Duration | Generations exposed | Chemical tested | Most sensitive endpoints (biomarkers and reproduction) | Reference |
|---|---|---|---|---|---|---|
| Medaka | Partial life-cycle reproduction test | 21-d | From F0 (adult) to F1 (hatch) | 4-*tert*-octylphenol (OP) | **F0:** NOEC (testis-ova) 11.4 µg/l<br>NOEC (VTG) 6.94 µg/l | Anon (2006) |
| Medaka | Full life-cycle test | 104-d | From F0 (embryos to 101 dph) to F1 (61 dph) | 4-*tert*-pentylphenol (4-PP) | **F0:** NOEC (VTG) ≤ 51.1 µg/l<br>NOEC (fertility) 100 µg/l<br>**F1:** NOEC (testis-ova) 100 µg/l<br>NOEC (growth; sex ratio) 100 µg/l | Seki et al. (2003) |
| Medaka | Partial life-cycle reproduction test | 21-d | From F0 (adult) to F1 (hatch) | di-n-butyl phthalate (DNBP) | **F0:** NOEC (mortality; testis-ova) 21.9 µg/l<br>NOEC (VTG) ≥ 235 µg/l | Anon (2006) |
| Medaka | Full life-cycle test | ? | From F0 (embryo) to F1 (hatch) | di-n-butyl phthalate DNBP) | **F0:** NOEC (testis-ova) 74.5 µg/l<br>NOEC (other endpoints) ≥ 233 µg/l<br>**F1:** NOEC (time to hatch) 23.9 µg/l<br>NOEC (other endpoints) ≥ 233 µg/l | Anon (2006) |
| Medaka | Partial life-cycle reproduction test | ? | From F0 (adult) to F1 (hatching) | o,p'-DDT | **F0:** NOEC (testis-ova) < 0.19 µg/l<br>NOEC (VTG) 0.37 µg/l<br>NOEC (GSI) 0.83 µg/l | Anon (2006) |
| Medaka | Full life-cycle test | ? | From F0 (embryo) to F1 (hatching) | o,p'-DDT | **F0:** NOEC (testis-ova; fertility) 0.145 µg/l<br>NOEC (VTG) 0.145 µg/l<br>**F1:** NOEC (time to hatch) 0.015 µg/l<br>NOEC (testis-ova) 0.145 µg/l | Anon (2006) |
| Medaka | Full life-cycle test | 56-d | From F0 (embryos to 56 dph – then clean water) to F1 (hatching) | o,p'-DDT | **F0:** NOEC (VTG) < 0.5 µg/l<br>NOEC (sex ratio)_2.5 µg/l<br>**F1:** NOEC (fertility; hatching success) < 0.5 µg/l | Cheek et al. (2001) |
| Medaka | Partial life-cycle reproduction | 21-d (?) | From F0 (adult) to F1 hatching | p,p'-DDT | **F0:** NOEC (testis-ova; mortality; time to hatch) 11.1 µg/l<br>NOEC (VTG) 11.1 µg/l | Anon (2006) |

| Fish species | Method used | Duration | Generations exposed | Chemical tested | Most sensitive endpoints (biomarkers and reproduction) | Reference |
|---|---|---|---|---|---|---|
| | test | | | | | |
| Medaka | Full life-cycle test | ? | From F0 (embryo) to F1 (hatching) | p,p'-DDT | **F0:** NOEC (fertility; mortality) 0.30 μg/l <br> NOEC (VTG) 1.0 μg/l <br> **F1:** NOEC (mortality) 1.0 μg/l <br> NOEC (testis-ova) 3.06 μg/l | Anon (2006) |
| Medaka | Partial life-cycle reproduction test | 81-d | F0 (adult) to F1 (60 dph) | bisphenol A (BPA) | **F0:** NOEC (testis-ova) <837 μg/l <br> NOEC (other endpoints) >3120 μg/l <br> **F1:** NOEC (all endpoints) >3120 μg/l | Kang et al. (2002) |
| Medaka | Partial life-cycle reproduction test | 21-d (?) | F0 (adult) to F1 (hatching) | bisphenol A (BPA) | **F0:** NOEC (testis-ova) 470 μg/l <br> NOEC (VTG) 220 μg/l | Anon (2006) |
| Medaka | Full life-cycle test | ? | F0 (embryos) to F1 (hatching) | bisphenol A (BPA) | **F0:** NOEC (mortality) 248 μg/l <br> NOEC (HSI) 248 μg/l <br> **F1:** NOEC (testis-ova) 248 μg/l <br> NOEC (VTG) 248 μg/l | Anon (2006) |
| Fathead minnow | Full life-cycle test | 305 + 28-d | From F0 (embryos to 305 dph) to F1 (28 dph) | 17-alpha-ethynylestradiol (EE2) | **F0:** NOEC (VTG) 1.0 ng/l <br> NOEC (fecundity; growth; testis-ova)1.0 ng/l <br> **F1:** NOEC (growth) 1.0 ng/l (effect at 0.2 ng/l in control range) <br> NOEC (other endpoints) ≥ 1.0 ng/l | Länge et al. (2001) |
| Fathead minnow | Full life-cycle test | 150-d | F0 (embryos to 60-dph) | 17-alpha-ethynylestradiol (EE2) | **F0:** NOEC (fertilization success) <0.32 ng/l <br> NOEC (sex ratio; GSI) 1.0 ng/l | Parrott et al. (2000) |
| Fathead minnow | Full life-cycle test | 150-d | F0 (embryos to 150-dph) | 17-alpha-ethynylestradiol (EE2) | **F0:** NOEC (fert. success; sex ratio) <0.32 ng/l <br> NOEC ($2^o$ sex characteristics) 0.32 ng/l | Parrott & Blunt (2005) |
| Fathead minnow | Partial life-cycle | 145-d | F0 | 17-alpha-ethynylestradiol | **F0:** NOEC (sex ratio; fecundity) < 10 ng/l | Parrott et al. (2004) |

| Fish species | Method used | Duration | Generations exposed | Chemical tested | Most sensitive endpoints (biomarkers and reproduction) | Reference |
|---|---|---|---|---|---|---|
| | reproduction test | | | (EE2) | | |
| Fathead minnow | Partial life-cycle reproduction test | 19-d | F0 | 17-beta estradiol (E2) | **F0:** EC10 (fecundity) 6.6 ng/l<br>EC10 (VTG) 36 ng/l | Kramer et al. (1998) |
| Fathead minnow | Partial life-cycle reproduction test | 21-d | F0 | 17-alpha-trenbolone | **F0:** NOEC (male T and VTG) 10 ng/l<br>EC50 (fecundity) 11 ng/l | Jensen et al. (2006) |
| Fathead minnow | Partial life-cycle reproduction test | 21-d | F0 | 17-beta-trenbolone | **F0:** NOEC (fecundity; $2^o$ sex) 5 ng/l<br>NOEC (female VTG) 5 ng/l | Ankley et al. (2003) |
| Fathead minnow | Partial life-cycle reproduction test | 21-d | F0 | methyl-testosterone (MT) | **F0:** NOEC (fecundity) < 200 μg/l<br>NOEC (GSI, T, E2, VTG) < 200 μg/l | Ankley et al. (2001) |
| Fathead minnow | Partial life-cycle reproduction test | 21-d | F0 | 4- nonylphenol (4-NP) | **F0:** NOEC (fecundity) 10 μg/l<br>NOEC (no. spawnings) 1 μg/l<br>NOEC (VTG) 1 μg/l | Harries et al. (2000) |
| Fathead minnow | Partial life-cycle reproduction test | 164-d | F0 | bisphenol A (BPA) | **F0:** NOEC (growth; GSI) 160 μg/l<br>NOEC (VTG) 16 μg/l<br>**F1:** NOEC (hatchability) 160 μg/l | Sohoni et al. (2001) |
| Fathead minnow | Partial life-cycle reproduction test | 21-d | F0 | fadrozole | **F0:** NOEC (fecundity) < 2 μg/l<br>NOEC (female VTG) < 2 μg/l | Ankley et al. (2002) |
| Fathead minnow | Partial life-cycle | 21-d | F0 – F1 hatch | flutamide | **F0:** NOEC (fecundity) 62.7 μg/l<br>NOEC (VTG; T) 62.7 μg/l | Jensen et al. (2004) |

| Fish species | Method used | Duration | Generations exposed | Chemical tested | Most sensitive endpoints (biomarkers and reproduction) | Reference |
|---|---|---|---|---|---|---|
| | reproduction test | | | | **F1:** NOEC (hatching) 62.7 µg/l | |
| Fathead minnow | Fish sexual development test | 0-60 Dph | F0 (fertilized egg to sexual differentiation) | prochloraz | **F0**: NOEC: (Sex ratio) 96 µg/l<br>NOEC: (VTG) <29 µg/l | FSDT Phase 1 validation |
| Fathead minnow | Fish sexual development test | 0-60 Dph | F0 (fertilized egg to sexual differentiation) | 4-tert pentylphenol | **F0**: NOEC: (Sex ratio) 36 µg/l<br>NOEC: (VTG) 36 µg/l | FSDT Phase 1 validation |
| Sheepshead minnow | Partial life-cycle reproduction test | 59-d | F0 – F1 hatch | 17-alpha-ethynylestradiol (EE2) | **F0:** NOEC (fecundity) 20 ng/l<br>NOEC (testis-ova) 2 ng/l<br>**F1:** NOEC (hatching) 20 ng/l | Zillioux et al. (2001) |
| Sheepshead minnow | Full life-cycle test | 180-d | F0 (embryos) to F1 (30 dph) | Tributyltin oxide (TBTO) | **F0:** NOEC (mortality) 0.41 µg/l<br>NOEC (fecundity) ≥ 3.2 µg/l<br>**F1:** NOEC (mortality) 1.3 µg/l | Manning et al. (1999) |
| Zebrafish | Fish sexual development test | 20-60 Dph (20-38 Dph for VTG) | F0 (fertilized egg to sexual differentiation) | Estrone (E1) | **F0**: NOEC: (sex ratio) 35 ng/l<br>NOEC: (VTG) <14 ng/l | Holbech et al. (2006) |
| Zebrafish | Partial life-cycle reproduction test | 63-d | F0 (adults) to F1 (hatching) | 17-beta estradiol (E2) | **F0:** NOEC (VTG) 27.2 ng/l<br>NOEC (other endpoints) ≥ 272 ng/l<br>**F1:** NOEC (sex ratio) < 27.2 ng/l | Van der Ven et al. (2007) |
| Zebrafish | Fish sexual development test | 20-60 Dph (20-38 Dph for VTG) | F0 (fertilized egg to sexual differentiation) | 17-beta estradiol (E2) | **F0**: NOEC: (Sex ratio) 24 ng/l<br>NOEC: (VTG) 24 ng/l | Holbech et al. (2006) |
| Zebrafish | Fish sexual development test | 20-60 Dph (20-38 Dph for VTG) | F0 (fertilized egg to sexual differentiation) | Estriol (E3) | **F0**: NOEC: (Sex ratio) <6.7 µg/l<br>NOEC: (VTG) <300 ng/l | Holbech et al. (2006) |
| Zebrafish | Fish sexual | 20-60 Dph | F0 (fertilized | 17-alpha- | **F0**: NOEC: (sex ratio) <0.6 ng/l | Örn et al. |

| Fish species | Method used | Duration | Generations exposed | Chemical tested | Most sensitive endpoints (biomarkers and reproduction) | Reference |
|---|---|---|---|---|---|---|
| | development test | (20-38 Dph for VTG) | egg to sexual differentiation) | ethynylestradiol (EE2) | NOEC: (VTG) <0.6 ng/l | (2003) |
| Zebrafish | Full life-cycle test | 78 d (++) | F0 (embryos) to F1 hatching | 17-alpha-ethynylestradiol (EE2) | **F0:** LOEC (fecundity; growth, time to spawn) 1.7 ng/l<br>**F0:** LOEC (VTG) 1.7 ng/l<br>**F1:** LOEC (fertilization success) 1.7 ng/l | Segner et al. (2003 a&b) |
| Zebrafish | Full life-cycle test | 174-d | F0 (embryos) to F1(36 dph) | 17-alpha-ethynylestradiol (EE2) | **F0:** NOEC (growth; time to spawn; fecundity) 0.3 ng/l<br>**F1:** NOEC (fertilization success) 0.3 ng/l | Wenzel et al. (2001) |
| Zebrafish | Full life-cycle test | 118-d | F0 (embryos) to F1 (fert.) | 17-alpha-ethynylestradiol (EE2) | **F0:** NOEC (fecundity; sex ratio) < 3 ng/l<br>**F0:** NOEC (VTG) < 3 ng/l<br>**F1:** NOEC (fertilization success) < 3 ng/l (no eggs produced, so no fertilization) | Fenske et al. (2005) |
| Zebrafish | Full life-cycle test | 60 d (+ 150 -d recovery phase) | F0 (embryos to egg production) | 17-alpha-ethynylestradiol (EE2) | **F0:** NOEC (fecundity) 1 ng/l | Van den Belt et al. (2003) |
| Zebrafish | Partial life-cycle test | 60-d | F0 (embryos to 60 dph) – remaining test in clean water | 17-alpha-ethynylestradiol (EE2) | **F0:** NOEC (sex ratio) 1 ng/l<br>**F0:** NOEC (VTG) 1 ng/l<br>**F1:** NOEC (fert. success; hatching success; swim-up success) 1 ng/l | Hill & Janz (2003) |
| Zebrafish | Fish sexual development test | 0-60 Dph (0-38 Dph for VTG) | F0 (fertilized egg to sexual differentiation) | Fadrozole | **F0**: NOEC: (Sex ratio) <10 µg/l<br>NOEC: (VTG) <10 µg/l | Andersen et al. (2004) |
| Zebrafish | Fish sexual development test | 0-60 Dph (0-38 Dph for VTG) | F0 (fertilized egg to sexual differentiation) | ZM 189, 154 | **F0**: NOEC: (Sex ratio) <100 µg/l<br>NOEC: (VTG) <100 µg/l | Andersen et al. (2004) |
| Zebrafish | Fish sexual development | 20-60 Dph (20-38 | F0 (fertilized egg to sexual | Methyl-testosterone | **F0**: NOEC: (Sex ratio) <22 ng/l<br>NOEC: (VTG) 42 ng/l | Örn et al. (2003) |

| Fish species | Method used | Duration | Generations exposed | Chemical tested | Most sensitive endpoints (biomarkers and reproduction) | Reference |
|---|---|---|---|---|---|---|
| | test | Dph for VTG) | differentiation) | | | |
| Zebrafish | Fish sexual development test | 20-59 Dph (20-38 Dph for VTG) | F0 (fertilized egg to sexual differentiation) | Trenbolone | **F0**: NOEC: (Sex ratio) <5 ng/l  NOEC: (VTG) 23 ng/l | Holbech et al. (2006) |
| Zebrafish | Fish sexual development test | 0-60 Dph | F0 (fertilized egg to sexual differentiation) | Prochloraz | **F0**: NOEC: (Sex ratio) 64 µg/l  NOEC: (VTG) <16 µg/l | Kinnberg et al. (2007) |
| Zebrafish | Partial life-cycle reproduction test | 63-d | F0 (adults) to F1 (hatching) | Tamoxifen (TMX) | **F0:** NOEC (gonad histology) 15.2 µg/l  NOEC (VTG) 27 µg/l  **F1:** NOEC (hatching; survival; growth) 15.2 µg/l  NOEC (VTG) 48.4 µg/l | Van der Ven et al. (2007) |
| Zebrafish | Partial life-cycle test | 60-d | F0 (embryos to 60 dph) – remaining test in clean water | 4- nonylphenol (4-NP) | **F0:** NOEC (sex ratio) 30 µg/l  **F0:** NOEC (VTG) 10 µg/l  **F1:** NOEC (fert. success; hatching success; swim-up success) 30 µg/l | Hill & Janz (2003) |
| Zebrafish | Full life-cycle test | 78 d (++) | F0 (embryos) to F1 hatching | 4-*tert*-octylphenol (OP) | **F1:** EC50 (fertilization success) 28 µg/l | Segner et al. (2003 a&b) |
| Zebrafish | Full life-cycle test | 174-d | F0 (embryos) to F1(36 dph) | 4-*tert*-octylphenol (OP) | **F0:** NOEC (growth; time to spawn; fecundity) 12 µg/l  F1: NOEC (fertilization success) 12 µg/l | Wenzel et al. (2001) |
| Zebrafish | Full life-cycle test | 78 d (++) | F0 (embryos) to F1 hatching | bisphenol A (BPA) | **F0:** LOEC (fecundity; growth, time to spawn) 1500 µg/l  **F0:** LOEC (VTG; gonad hist.) 375 µg/l  **F1:** LOEC (fertilization success) 1500 µg/l | Segner et al. (2003 a&b) |

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

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

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

243.    The exposure is all these tests is generally, but not necessarily, continuous throughout all life stages and generations.  Although this exposure may not reflect real-world exposure conditions, it will establish a level at which a continuous exposure will be a minimal risk.  Additional testing might be needed on individual EDCs if real-world exposure levels are intermittent but at levels greater than a continuous-exposure level that causes significant effects.

244.    Regulatory and historical recognition should be considered in the selection of a test species. Fathead minnow and sheepshead minnow have wide USEPA acceptance as test species, whereas the zebrafish and medaka are accepted in Europe and Japan, respectively.  It should also be noted that many research scientists are using the zebrafish and medaka, which offer advantages, including smaller size and shorter life cycles compared to fathead minnows.  Each of the test species under consideration in this review has specific regulatory strengths and weaknesses, although none of these are considered to be sufficiently important to rule out a species at this stage.  Important to the development of the protocol is the consideration that the selected test species should provide definitive data endpoints necessary to identify relevant population effects covering a wide range of EDC modes of action. Another regulatory consideration is whether the uncertainty factors used in risk assessment might have to be larger when applied to data from the Full Life-Cycle than the Two-Generation or Multi-Generation Tests, in recognition of the greater information available from the latter.  It is also important that the standardized transferable protocol can be readily followed and executed by participating test laboratories in several countries.

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

blood plasma samples for steroid and protein analyses, although this is also possible but more difficult for zebrafish and medaka.

## 10.1 Appropriateness of the Various Reproductive Endpoints

246. Endpoints such as the number of eggs/female/day, fertilization, hatching success, and GSI should be collected at every opportunity. These endpoints are clear and direct measurements of potentially significant population-level effects. Endpoints such as histopathology and biochemical analyses can demonstrate exposure to EDCs and provide information on modes of action, but they are not considered essential in a life-cycle test for EDCs as MoA's should already be known, and such endpoints should therefore be included optionally in a test guideline, especially if it is to be used with non-EDCs as well. The most practical approach to the use of histopathology and biochemistry might be to take and archive blood and tissue samples routinely during life-cycle tests, but only to process them for these endpoints if mechanistic information is required in the light of the subsequent apical results.

247. The interpretation of histological material requires experience in identifying normal and abnormal structures in organs and tissues. It is recommended that histology interpretation be conducted by a certified fish pathologist with training and experience in veterinary and fish pathology. For histopathological analysis to be especially useful in reproductive screening tests, it is important to ensure maximum control of the subjective nature of the interpretations, and to limit the interpretation to repeatable results that can be verified by different investigators. As an aid toward to this end, the following steps should be implemented:

- Staging and abnormalities are best defined by incremental changes (i.e., presence or absence of particular structures) and in all cases, need to be codified in a standardized definition that can be used by multiple investigators.
- Measurement of the size of structures is subject to a high degree of measurement error as a result of variations in planes of sections and must, therefore, be compared using appropriate statistical tests.
- Methods for fixation, embedding, sectioning, and staining need to be standardized.
- Samples should be read and interpreted in a blind fashion.

248. Detailed guidance on the best gonad histopathological procedures to use with the four recommended species can be found as an appendix to the protocol used for the Phase 1B of the validation of the 21-day fish endocrine screening assay, and obtained upon request to the OECD Secretariat. The guidance document is not yet declassified as a stand alone document and requires further work for its completion..

249. Impacts of EDCs on the sex determination of larval fish could be an endpoint that provides important information with the potential to detect endocrine disruption in the early life stages of fish. Raising fish to sexual maturity could provide a cost-effective analysis of this endpoint. At this time, relatively little information is available on the impact of EDCs on sex-determination effects of embryonic or larval stages of fish. However, see http://www.rivm.nl/fishtoxpat, as well as data from the Nordic countries on the responses of zebrafish to EDCs (TemaNord, 2001).

**10.2    Preferred Methods for Quantification of Biochemical Endpoints**

250.    The most widely used assays for individual sex steroids based on competitive binding with a specific antibody include RIA and ELISA.  Both are equally suitable, sensitive, and specific for detecting the common sex steroids of interest (E2, T, 11-KT) in fish blood plasma or other tissue. 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 is the ELISA.

251.    For quantification of VTG, ELISA offers ease of use, sensitivity, and specificity for measuring VTG. ELISA methods to quantify VTG in plasma and tissue homogenates include the competitive antibody-capture and sandwich ELISA, with slightly greater sensitivity achieved with sandwich ELISAs. Antibody-capture ELISAs require larger amounts of purified VTG to perform the assay, whereas sandwich ELISAs require larger amounts of multiple VTG antibodies. Highly sensitive VTG-ELISA kits for zebrafish and medaka are commercially available. 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.  However, new mass spectrometry methods offer exciting possibilities for VTG analysis and in the future might prove to be the preferred method.

252.    Finally, recent developments have made it possible to detect induction of the genes coding for VTG (e.g. Tong et al., 2004), and a useful comparison of gene- and protein-based methods has been published by Wheeler et al. (2005). These methods are highly sensitive and species-specific, but it should be noted that VTG mRNA is only briefly induced by a transient exposure to estrogen, whereas VTG protein remains present for much longer (e.g. Kirby et al. 2003).

**10.3    Significant Data Gaps for EDCs**

253.    It is clear that several aspects of the basic biology of the chosen test species require further research. This should include work to identify the most sensitive life stages of each species to a range of EDCs, and to enable a clear distinction to be made between primary and secondary responses to endocrine disrupters.

254.    The general techniques for the long-term exposure and evaluation of reproductive potential are fairly well established for all four species.  However, for the sheepshead minnow, additional assessment of growth and reproductive performance in different salinities should be conducted. Testing and culture of sheepshead minnow at lower salinities will offer distinct cost-controlling advantages and will assist the solubilization of some potential EDCs during testing.  There is a need for basic endocrinology studies in this species, as little is known on sex-steroid levels.

255.    For all species, further testing is required using anti-estrogenic, androgenic and anti-androgenic substances, as well as aromatase inhibitors. In addition, the determination of sexual differentiation for the preferred fish species might be important information in evaluating the results of a life-cycle test. Methods of sexual differentiation are established for zebrafish and medaka, but are not published for fathead minnow or sheepshead minnow.

256.    If the Two-Generation or Multi-Generation Tests are to be taken further with the species examined in this review, it will first be necessary to demonstrate that they can be significantly more sensitive than the Full Life-Cycle Test with certain EDCs, and secondly, to measure the reliability and reproducibility of each test with the candidate species.  Specific protocol variables, such as the size of the spawning groups for a particular species, must be determined for the candidate species under consideration for each test.  The individual elements of the protocol must be optimized and the required number of interlaboratory comparisons must be determined.

257.    One aspect which has received little attention to date concerns the reversibility of effects in life-cycle tests. In other words, to what extent do adverse effects subside if the test animals are returned to clean water? It is already known that some abnormalities (e.g. ovo-testis or skewed sex ratio) appear to be irreversible, while others (e.g. VTG induction in males) eventually disappear if exposure ceases (e.g. Cheek et al., 2001). However, it would be useful to conduct systematic studies of reversibility in both the F0 and F1 generations, with a view to the possible development of guidelines for such work.

258.    Finally, although the standard Full Life-Cycle Test with fathead or sheepshead minnows ends simply with assessment of F1 hatching success and fry survival, there are some indications that greater sensitivity might be obtained by extending the test slightly, to the point where important abnormalities in gonad development (e.g ovo-testis) can be detected. For example, Yokota et al. (2001), conducting a Full Life-Cycle Test with medaka exposed to NP, showed that induction of ovo-testis in the F1 fish was the most sensitive endpoint of the whole experiment.  This possibility requires further investigation, because it would be cheaper to run a slightly extended test of this type than to conduct Multi-Generation or Two-Generation Tests.

## 10.4    Testing Non-EDCs

259.    It would clearly be desirable to develop a single fish life-cycle test guideline that could be applicable to both EDCs and non-EDCs. Non-EDCs have not previously been given consideration in this DRP, but the use of life-cycle tests for this (large) group of chemicals will now be briefly considered. Table 10-2 provides a summary of life-cycle tests conducted mainly with non-EDCs, although it should be noted that the list is not exhaustive. Several observations can be made about these data:-

- Almost all the available information is derived from experiments conducted using the Full Life-Cycle Test protocol (or variations of it).
- Experience with variants of this protocol goes back to the early 1970s, so it can be considered reliable.
- There are, however, three examples in which the Two-Generation Test has been used (with brook trout and zebrafish), and one with fathead minnows using the Multi-Generation Test.
- There are no comparative data that would allow an assessment of which of the three protocols (if any) is the most sensitive.
- Most experience is with the fathead minnow, but there is some information from flagfish (*Jordanella floridae*) and brook trout (*Salvelinus fontinalis*), and a little from sheepshead minnow, medaka and zebrafish.
- An assessment of the older data made by McKim (1977) showed that Full Life-Cycle MATCs can be estimated with early-life stage tests to within a factor of 2 in most cases. This suggests that, for non-EDCs, the Full Life-Cycle protocol is likely to offer little advantage over the Partial Life-Cycle approach.

260.    On the basis of this brief assessment, there is little doubt that the Full-Life-Cycle Test protocol works reliably with non-EDCs, although it probably offers little increased sensitivity compared with the Partial Life-Cycle approach. There are insufficient data to decide whether the Two-Generation or Multi-Generation protocols would offer greater sensitivity to non-EDCs, but the limited available information (Holcombe et al., 1976; McKim et al., 1976; Bresch et al., 1990) suggests that the F1 generation is no more sensitive to non-EDCs than the F2 generation in the two-gen protocol, but that both are more sensitive than the (partial) F0 generation. The single published test using the Multi-Generation protocol (Smith & Oseid, 1975) did not, however, indicate that the complete F1 generation was any more sensitive than the complete F0 generation.

261.    It is conceivable that highly persistent and bioaccumulative non-EDCs that experience strong maternal transfer could be more toxic in the Two-Generation or Multi-Generation protocols than in the Full Life-Cycle protocol, but as with the EDCs, this could only be assessed after considerable pre-validation work.

262.    On balance, therefore, there appears to be no sound reason for developing a life-cycle test guideline for non-EDCs that differs from a putative guideline for EDCs (except that the mechanistic endpoints for EDCs will, of course, not be needed). However, it is probable that the Full Life-Cycle protocol is sufficient to establish a genuine chronic threshold for most non-EDCs, whereas it is possible that some EDCs will prove to be more potent in the Two-Generation and Multi-Generation protocols.

263.    Due to these uncertainties, it is recommended that some model non-EDCs are included in any programme of pre-validation and validation of life-cycle test methods.

**Table 10-2  OVERVIEW OF LIFE-CYCLE FISH TESTS OF SOME GENERAL CHEMICALS (MAINLY NON-EDCs)**

| Fish species | Method used | Chemical tested | Most sensitive endpoints | Reference |
|---|---|---|---|---|
| Fathead minnow | Full life-cycle test | cadmium | NOEC (fecundity) 37 µg/l | Pickering & Gast (1972) |
| Fathead minnow | Full life-cycle test | zinc | NOEC (egg fragility) 78 µg/l | Benoit & Holcombe (1978) |
| Fathead minnow | Full life-cycle test | mercury | NOEC (growth) <0.31 µg/l | Snarski & Olson (1982) |
| Fathead minnow | Full life-cycle test | methyl mercury | NOEC (fecundity) 0.07 µg/l | Mount & Olson – cited in McKim (1977) |
| Fathead minnow | Full life cycle test | PCB-1254 | NOEC (mortality) 1.8 µg/l | Nebeker et al. (1974) |
| Fathead minnow | Full life cycle test | PCB-1242 | NOEC (mortality) 5.4 µg/l | Nebeker et al. (1974) |
| Fathead minnow | Full life cycle test | PCB-1248 | NOEC (mortality) 1.1 µg/l | DeFoe et al. (1978) |
| Fathead minnow | Full life cycle test | PCB-1260 | NOEC (mortality) 2.1 µg/l | DeFoe et al. (1978) |
| Fathead minnow | Full life cycle test | carbaryl | NOEC (mortality) 210 µg/l | Carlson (1971) |
| Fathead minnow | Full life cycle test | azinphosmethyl | NOEC (fecundity) 0.33 µg/l | Adelman et al. (1976) |
| Fathead minnow | Full life cycle test | lindane | NOEC (growth) 9.1 µg/l | Macek et al. (1976a) |
| Fathead minnow | Full life cycle test | heptachlor | NOEC (mortality) 0.86 µg/l | Macek et al. (1976b) |
| Fathead minnow | Full life cycle test | chlorpyrifos | NOEC (growth) < 0.12 µg/l | Jarvinen et al. (1983) |
| Fathead minnow | Full life cycle test | hydrogen cyanide | NOEC (fecundity) 13 µg/l | Lind et al. (1977) |
| Fathead minnow | Full life cycle test | ammonia | NOEC (hatching success) 0.19 mg/l | Thurston et al. (1986) |

| Fish species | Method used | Chemical tested | Most sensitive endpoints | Reference |
|---|---|---|---|---|
| Fathead minnow | Multi-generation test | hydrogen sulfide | NOEC (growth) < 0.4 µg/l | Smith & Oseid (1975) |
| Sheepshead minnow | Full life-cycle test | endrin | NOEC (hatchability; growth; mortality) 0.12 µg/l | Hansen et al. (1977) |
| Sheepshead minnow | Full life-cycle test | chlordecone | NOEC (growth) 74 ng/l | Goodman et al. (1982) |
| Flagfish | Full life-cycle test | cadmium | NOEC (fecundity) 4.1 µg/l | Spehar (1976) |
| Flagfish | Full life-cycle test | lead | NOEC (fecundity) 31.3 µg/l | Anderson – cited in McKim (1977) |
| Flagfish | Full life-cycle test | zinc | NOEC (growth) 26.0 µg/l | Spehar (1976) |
| Flagfish | Full life-cycle test | diazinon | NOEC (hatchability) 54 µg/l | Allison (1977) cited in McKim (1977) |
| Flagfish | Full life-cycle test | endrin | NOEC (growth) 0.22 µg/l | Hermanutz (1978) |
| Flagfish | Full life-cycle test | malathion | NOEC (growth) 8.6 µg/l | Hermanutz (1978) |
| Brook trout | Full life-cycle test | cadmium | NOEC (growth) 1.7 µg/l | Benoit et al. (1976) |
| Brook trout | Full life-cycle test | chromium | NOEC (mortality) 200 µg/l | Benoit (1976) |
| Brook trout | Two-generation test | lead | NOEC (growth; scoliosis) 39 µg/l | Holcombe et al. (1976) |
| Brook trout | Two-generation test | methyl mercury | NOEC (growth) 0.29 µg/l | McKim et al. (1976) |
| Medaka | Full life-cycle test | cadmium | NOEC ≥ 10 µg/l (2 week exposure from fertilized eggs, and 2 week re-exposure as adults) NOEC (VTG) < 1 µg/l | Foran et al. (2002b) |
| Zebrafish | Two-generation test | 4-chloroaniline | NOEC (fecundity) < 40 µg/l | Bresch et al. (1990) |

## 11.0    IMPLEMENTATION CONSIDERATIONS

### 11.1    Animal Welfare

264.    It is expected that the optimized fish reproduction assay protocol will obtain the maximum amount of information from the minimum number of animals in the least amount of time necessary to detect a potential endocrine active substance.  The incorporation of several viable endpoints to detect disruption of male and/or female reproductive systems will ensure 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.

#### *11.1.1 Rationale for the Need to Use Animals*

265.    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.

#### *11.1.2 Relative Pain or Distress for Animals*

266.    It is not anticipated that the animals would suffer prolonged pain or distress. The study protocols necessitate that great care be given to test animals to induce spawning. Because the goal of the assay is to evaluate the effects of EDCs on reproduction, it is anticipated that the doses will be chosen such that there is little or no overt parental toxicity and mortality. Animals will be sacrificed humanely by using MS-222, a proven and effective fish anaesthetic.

### 11.2    Recommended Protocol

267.    Before a particular test protocol is chosen for full optimization, it will be necessary to demonstrate the reliability and reproducibility of the test with the four targeted species. Additionally, inter-laboratory comparison of results of the method must address issues such as differences in sensitivity among the species, so as to support their use in a risk assessment.  All the life-cycle protocols require significant cost and time commitments, and the expense of demonstration trials will be increased if multiple species are used. The pre-selection of a species would limit the number of demonstration trials for full optimization of a method suitable for interlaboratory testing, although there are regulatory reasons why this may not be advisable or possible – in particular, the need for mutual acceptance of data which is a cornerstone of OECD testing guidelines.  Validation of the protocol through interlaboratory comparisons should be conducted with compounds that span the possible endocrine effects for which fish are sensitive test species, including strong and weak androgen receptor agonists and antagonists, estrogen receptor agonists and antagonists, and aromatase inhibitors.

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

268.     Relatively few testing laboratories have the necessary experience to conduct life-cycle tests with fish to the standards of Good Laboratory Practise (GLP) required for regulatory purposes. Although those able to conduct the Full Life-Cycle Test may not find too much difficulty in conducting the Two-Generation or Multi-Generation Tests. However, capacity within those few qualified laboratories will increasingly become an issue as test durations increase with the number of generations exposed.. Probably even fewer laboratories have in-house fish culture facilities, which are not pre-requisites for running life-cycle tests, but are certainly desirable. Before completion of a fish life-cycle testing guideline, it would therefore be useful to find out the number and global distribution of laboratories which consider themselves able to perform such procedures, and with which species. It would clearly be unfortunate and counter-productive to develop a guideline which very few testing laboratories felt competent to conduct, and for which there may be little market demand to stimulate increased supply.

**11.4     Confounding Factors**

269.     It is likely that there are many factors which could confound interpretation of the data derived from fish life cycle tests. Some of these will be discussed briefly here, but the pre-validation and validation trials of chosen protocols should investigate and document them systematically. Some known and suspected confounding factors include:-

- **Large variability in fecundity of fathead minnows**. This might mask subtle effects which could nevertheless have importance at the population level, although presumably this could be counter-balanced by increased numbers of replicates.
- **Low fertilization rate in sheepshead minnows**. This might also be addressed by increasing the numbers of eggs/fish per replicate.
- **Variability in sensitivity of different cultures of a given species**. This is probable, but its magnitude is not well understood at present.
- **Differences in sensitivity between species**. Most available data do not show systematic differences in sensitivity between the four targeted species, but relatively few other species (e.g. flagfish; brook trout) have a large life-cycle testing database. Without more information of this type, extrapolation of test data in risk assessment must rely on the assumption that EDCs will affect other fish species in a similar way.
- **Measurement accuracy**.  Many measurements in fish life-cycle experiments (e.g. fecundity; fertilization success) are relatively simple to make accurately, although are often quite time-consuming. Others, however, can give rise to significant inaccuracies. For example, zebrafish are quite hard to sex by their external appearance, low levels of ovo-testis in any species can be missed, and the presence and degree of induction of secondary sexual characteristics is subject to a degree of interpretation. These difficulties can be minimized by suitable training, but the reliability of tests would benefit through the development of supporting guidance documents.
- **Season**.  Although the chosen species reside in warm water and will breed all year round, it is to be expected that they may still have a degree of seasonality in their reproductive output, particularly if the stock has been recently derived from wild fish. It may therefore be desirable to investigate whether there is any seasonality in the sensitivity of the chosen test species to various model EDCs.

**11.5    Data Interpretation**

270.    Although largely outside the scope of this DRP, the issue of data interpretation is a crucial one for risk assessment. It should always be remembered in this context that the primary purpose of life-cycle testing is to provide definitive apical data on sexual development, growth, and reproductive output and quality. All these endpoints have a direct bearing on possible (but not inevitable) effects at the population level, while biochemical (and many histopathological) endpoints can only provide an explanation for possibles modes of action.

271.    The key issue for data assessors is for them to have a sound understanding of the links between the various apical effects described above and their possible consequences for fish populations and aquatic communities. For example, it is clear that many fish species possess considerable redundancy in their reproductive output, and that a given level of reproductive inhibition may have no impact on population size. Reproductive output is also often density-dependent, so a predicted pollutant-related drop in output may be compensated for by the population. Yet another issue concerns the relationship between results obtained under conditions of constant exposure, and impacts that might occur when exposure in the field is transient or intermittent. To some extent, these problems apply to all toxicity data and can be addressed through a good understanding of fish biology and the sensible use of exposure and population modeling, but it is worth remembering that even the NOEC obtained in a Multi-Generation Test does not necessarily represent the 'safe' level for a wild population. The true 'safe' concentration may be either higher or lower than that obtained in the test. These issues of risk assessment are much more fully dealt with elsewhere (e.g. Calow, 1998; Hutchinson & Pickford, 2002; Rand, 2003).

272.    Perhaps the most intractable issue of all concerns the need to extrapolate from results obtained with one or a few test species, to all species in the ecosystem at risk. Unfortunately, fish life-cycle testing has only been conducted with a handful of species to date, so such extrapolations require considerable expert knowledge. For example, the four species chosen in this DRP for further protocol development are all asynchronous spawners, and we do not know whether synchronous spawners (e.g. roach *Rutilus rutilus*) are likely to be of similar sensitivity under field conditions, given that their most sensitive life stages may be present at a time of year when contamination is low or absent.

273.    Another aspect of species-species extrapolation concerns the taxonomic representativeness of the chosen fish – none are cartilaginous (elasmobranchs), and there are no representatives from many economically and ecologically important orders of bony fish (actinopterygians) such as the salmoniformes, the perciformes and the pleuronectiformes (to name but a few). Some groups (e.g. salmonids) tend to be more sensitive than many other species to acute toxicants, but that does not necessarily imply that the same processes operate over long-term exposures.

274.    A third concern is the fact that all four species are warmwater fish. This is largely unavoidable given the practical need to use species with short life-cycles, but it may well be that coldwater species have systematically different sensitivities to some chemicals. For example, a recent review by Kwok et al. (2007) has shown that temperate freshwater species tend to be more acutely sensitive to metals than equivalent tropical species, while the reverse is true for un-ionized ammonia, phenol and some pesticides. These authors recommend the use of an uncertainty factor of 10 when extrapolating from temperate water quality criteria to equivalent tropical values, but there are insufficient data to decide whether this is appropriate for extrapolating from life-cycle data based on the four chosen (warmwater) species.

275.    These and other extrapolation issues will require further examination, but the practicalities and financial implications of life-cycle testing will inhibit the rapid development of a suitable database with which           to           underpin           recommendations           about           uncertainty           factors.

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