DRAFT DETAILED REVIEW PAPER

for

AMPHIBIAN GROWTH AND REPRODUCTION ASSAY (Tier 2)

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1.0 EXECUTIVE SUMMARY

Concerns regarding both the presence of endocrine disruptors in food, water, or other environmental media and the potential risk they pose to humans and wildlife have been growing in recent years. Passage in 1996 of the Food Quality Protection Act (FQPA) and Amendments to the Safe Drinking Water Act (SDWA) reflected these concerns to screen chemicals found in drinking-water sources or food to determine whether they possess estrogenic or other endocrine activity *(21 U.S.C. §346a(p))*. In 1996, EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), charging the Committee to provide advice on how to design a screening and testing program for endocrine disrupting chemicals (EDCs). Following the recommendations made by EDSTAC in its final report (EDSTAC 1998), the EPA established the Endocrine Disruptor Screening Program (EDSP). The aim of this program is to develop a two-tiered approach, e.g., a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants.

Gray et al. (1997), Ankley et al. (1998a), EDSTAC (1998), and the National Academy of Sciences (NAS) (NAS, 1999) concluded that a tiered approach relying on a combination of *in vivo* and *in vitro* screens for Tier 1 was scientifically reasonable. This conclusion was based upon each group's assessment of the current state-of-the-science on the evaluation of agents affecting the endocrine system. Another consistent conclusion was the need to validate the individual screens and tests in the EDSP. Validation and peer review are prerequisites to the development and approval of test guidelines for regulatory use.

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

The recommended Amphibian Growth and Reproduction Assay (AGRA) is a chronic exposure test with morphological, biochemical, and molecular-based endpoints designed to evaluate the effects of EDCs on development, growth, and reproductive fitness. This may include a variety of different test method approaches. The Detailed Review Paper (DRP) will summarize, explain, and document decisions regarding the relevant principles, methods and techniques recommended for an initial protocol, and identify issues that may require prevalidation studies to adequately address potential concerns.

During advanced development in amphibians, certain tissues are resorbed, some are remodeled, and some are created to form an adult organism during metamorphosis capable of surviving in a different habitat. Endocrine control over sexual development and reproductive function is responsible for perpetuating populations of amphibians. Endocrine control of the

development, sexual differentiation, and ultimately reproduction in amphibians is highly complex and involves the CNS, hypothalamus, pituitary gland, thyroid gland, the gonads, various hormones including sex steroids, receptors, and transcriptional elements. Although highly complex, three principles remain constant, 1) steroid hormones are responsible for sexual differentiation and normal reproductive fitness, 2) metamorphic events are triggered by TH, and 3) tissue responsiveness to steroid hormones and TH are based on selective responses based on hormone interaction with specific receptors. Overall, the various endocrine axes are a potential target of EDC action.

In this case, the objective of this DRP is to develop a method to determine the effects of chemicals on growth and reproduction using an amphibian as a general vertebrate model. The AGRA could potentially consist of whole organism exposure tests, histological analysis, biochemical (hormone) analyses, or molecular assays designed to screen substances that might adversely disturb hypothalamo-pituitary-gonad (HPG) function. Whole organism tests reviewed in this paper include an adult reproductive test with *Xenopus sp.*, a chronic exposure model of varying lengths using *X. tropicalis*, a life cycle and multigenerational assay model using *X. tropicalis*, and an assay utilizing the sexual dichromatic reed frog, *Hyperolius sp*. Endpoints originally considered noteworthy included: gonadal development and differentiation and reproductive fitness endpoints, such as egg mass production, measures of ovarian cycle, and general ovary health, sperm count and dysmorphology, and measures of testis health. Methods for biochemical measurement of steroid hormones, biosynthetic enzymes (including aromatase and 5α-reductase), and receptor binding assays were evaluated. These methods included conventional radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) techniques, HPLC, electrophoretic measures, and radioreceptor methods. Finally, molecular techniques designed to biomark endocrine function, including transgenic whole animal lines, transfected amphibian cell culture lines, gene expression assays (reverse transcriptase-polymerase chain reaction [RT-PCR] and reporter gene assays), were reviewed. Recommendations are provided in the following DRP.

The recommended base exposure protocol consists of a ca. 35-45 chronic exposure from early embryo stage (Nieuwkoop and Faber [NF] stage 8) through the completion of metamorphosis and primary sexual differentiation using *X. tropicalis*. Although it is anticipated that data relevant to specific stages of development will be collected throughout the exposure, the primary focus should be placed on sexual differentiation and development of reproductive capacity. Thus, the majority of primary endpoints will be collected following the completion of metamorphosis. *X. tropicalis* cultured at 26-27˚C, complete metamorphosis generally at ca. 35-40 d, show signs of secondary sexual development at 60-75 d, and are generally capable of reproducing at d 150-180. Since some EDCs may differentially affect primary and secondary sexual differentiation, the base assay can be extended to 90 d to evaluate the onset of secondary sexual characteristics. Further, the base assay may be expanded to ca. 150-180 d if multigenerational data is sought at which time the specimens may be bred and the F1 progeny evaluated. However, limited data is currently available describing the length of time, and perhaps more importantly, the number of successive breeding required to produce a viable progeny under control conditions. Thus, a longer period of time may be required in order to accurately assess reproductive viability. Overall, it is anticipated that the base exposure protocol in combination with biochemical and molecular endpoints will be adequate for evaluating effects

of potential EDCs on development (growth) and reproduction. Static-renewal or flow-through exposure, with adequate test substance analysis based on the physicochemical properties of the test substance, is recommended. However, considering the length of the test, flow-through exposure will likely be the most practicable. Specimens should be randomly selected for histological examination of the gonads and samples should be collected for biochemical and molecular analysis of sex steroid hormone levels, estrogen/androgen receptor (ER/AR) binding, and vitellogenin (VTG) induction/inhibition, aromatase and 5α-reductase levels, and possibly ER/AR gene expression. It should be noted that if one of the biochemical or molecular endpoints demonstrates particular sensitivity, reliability, and speed, it could be chosen to represent either a single biochemical or molecular biomarker for the proposed assay. However, the use of multiple endpoints provides additional confirmation of the response and will help distinguish between EDC-based and no-EDC-based responses.

The primary data gaps that exist at this point include understanding of what responses may be induced at both organism and suborganism-levels by establishing estrogen, progestin, and androgen agonists and antagonists; which endpoints will link the effects induced as a estrogen-, progestin-, or androgen-based mechanism; the time course of the responses; the sensitivity of the measurement endpoints; and the point at which a molecular change constitutes a valid marker of HPG disruption. Finally, the dynamic range of HPG axis homeostasis and its relationship to gross morphological, molecular, biochemical, and histological endpoints need to be determined.

The path forward into prevalidation of the proposed assay should be divided into a phased-set of activities. The first phase should focus on final definition and development of the following recommended endpoints associated with the *X.tropicalis* AGRA protocol. The most significant work needs to be performed in the final development of the molecular endpoints. As for histological, morphological, and biochemical endpoints, preliminary protocols should be prepared for use in the second phase, which are prevalidation studies. A general protocol describing how the exposure is to be performed and how data from each endpoint is to be collected in an integrated format is also recommended. Once results with each endpoint are collected, analyzed, and reviewed, revised protocols should be prepared. Following analysis of the data, the revised protocol should again be reviewed and revised, if necessary, and a Final Protocol developed for use in interlaboratory Good Laboratory Practices (GLP) validation studies.

2.0 INTRODUCTION

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

Chemicals that are known or suspected of being endocrine disruptors (Kavlock et al., 1996), also referred to as hormonally active agents (NRC 1999), have received increased attention over the past decade. In 1996, the passage of the two laws, the Food Quality Protection Act (FQPA) and Amendments to the Safe Drinking Water Act (SDWA) mandated the United States Environmental Protection Agency (U.S. EPA) to screen substances found in drinking water sources of food to determine whether they possess estrogenic or other endocrine activity

(Federal Register, 1998a, 1998b). Pursuant to this goal, the U.S. EPA is required to "develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or other such endocrine effect..." (FQPA, 1996).

In 21 U.S.C. §346a(p)(3), the FQPA also states that in carrying out its screening program, the EPA: (a) shall provide for the testing of all pesticide chemicals; and (b) may provide for the testing of any other substance that may have an effect that is cumulative to an effect of a pesticide chemical if the Administrator determines that a substantial population may be exposed to such a substance.

Additionally, Congress amended the Safe Drinking Water Act (SDWA) (42 U.S.C. §300j-17), authorizing the EPA to provide for the testing, under the FFDCA Screening Program . . . any other substance that may be found in sources of drinking water if the Administrator determines that a substantial population may be exposed to such substance.

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

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

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

The EDSP's proposed statement of policy, including public comments, was reviewed by a joint panel of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) and the EPA Science Advisory Board (SAB) in May 1999. Gray et al.

(1997), EDSTAC (1998), and the National Research Council (NRC 1999) concluded that a tiered approach relying on a combination of *in vivo* and *in vitro* screens for Tier 1 was scientifically reasonable. This conclusion was based upon each group's assessment of the current state of the science on the evaluation of agents affecting the endocrine system. Another consistent conclusion was the need to validate the individual screens and tests in the EDSP. Validation and peer review are prerequisites to the development and approval of test guidelines for regulatory use. Many of the documents cited above and other EPA EDSP-related information may be found at http:\\www.epa.gov/scipoly/oscpendo.

In addition to the EPA's domestic EDSP validation program, a separate effort to validate certain screening assays and tests for international use is being conducted by the Organization for Economic Cooperation and Development (OECD) Test Guidelines Program. The EPA actively participates as a member of the OECD test guidelines program and its Endocrine Disruptor Testing and Assessment Task Force. The EPA is relying on the OECD effort to serve as the mechanism for validation of some of the components of its EDSP (OECD, 1996). Separate domestic and international activities are necessary in that laws and regulatory procedures differ in various countries. Although international activities are distinct from domestic activities, overlapping membership on various committees ensures appropriate liaison and communication, eliminates duplication of effort, and facilitates international harmonization.

2.2 The Validation Process

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

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

The validation process consists of four discrete phases: (1) initial protocol development, (2) prevalidation studies, (3) validation studies, and (4) external scientific peer review. The initial protocol, developed from existing information and experience (past and current research), serves as the starting point for initiating the validation process. Prevalidation studies consist of further development and optimization of specific initial protocols through targeted investigations. Either before or during prevalidation, a DRP addressing all critical areas outlined

in *Validation and Regulatory Acceptance of Toxicological Test Methods* (NIEHS, 1997) is prepared for each method to summarize, explain, and document decisions regarding the relevant principles, methods, and techniques recommended for the initial protocol. Targeted prevalidation investigations are designed to address questions necessary for completing an optimized, transferable protocol suitable for interlaboratory validation studies. Validation studies consist of comparative interlaboratory studies to establish the reliability and relevance of the protocols developed in the prevalidation stage. Validation requires the development of a detailed review paper to document what is known about the assay system proposed for validation.

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

2.3 Purpose of the Review

The purpose of Work Assignment 4-8 is to prepare a DRP for AGRA. The AGRA consists of chronic exposure approaches that utilize a battery morphological, biochemical, and molecular-based endpoints designed to evaluate the effects of EDCs on development, reproduction, and growth. The AGRA, which will potentially include partial life cycle, full life cycle, and/or multi-generational exposures accompanied by biochemical and/or molecular tests, will be evaluated for use as a Tier II test assay. The DRP will summarize, explain, and document decisions regarding the relevant principles, methods, and techniques recommended for one or more initial protocols, and will also identify issues that may require prevalidation studies to adequately address. Ultimately, a single AGRA method will be recommended for measuring the effects of endocrine axis pertubation during development, growth, and reproductive phases of the amphibian life cycle.

2.4 Rationale for and Objectives of the Amphibian Growth and Reproduction Assay

In 1996, the EPA formed the EDSTAC to provide guidance on how to design a screening and testing program to identify EDCs. In its final report, the EDSTAC recommended a two-tiered approach, i.e., screening (Tier 1) and testing (Tier 2), for the identification of these compounds (EDSTAC, 1998). The overall purpose of the testing is to determine whether a chemical or chemical mixture adversely affects the organism through endocrine-mediated pathways. However, the primary objective of the Tier 1 Screening Tests is to rapidly detect the

potential interaction of a given chemical with an endocrine system. The results of Tier 1 screening tests should enable EDSP to determine if Tier 2 testing is necessary to evaluate a potential hazard. Tier 2 tests, such as the AGRA method, will be developed to more specifically determine and characterize the endocrine disrupting effects. Upon completion of Tier 1 screening and Tier 2 testing, the EPA and other stakeholders will accept, both scientifically and as a matter of policy, the assessment of chemical substances or mixtures according to whether a chemical has the potential or little or no potential for having estrogen, androgen, or thyroid endocrine-disruptive effects. Through the EDSP, individual tests and screens will be selected based upon completion of each topical DRP. In the case of the present DRP, the AGRA is being considered for inclusion in the Tier 2 Test Battery to evaluate disruption of development, growth, and reproductive function though pertubation of various components of the amphibian endocrine system.

Premetamorphic larval amphibian growth rates depend on a subset of intrinsic and extrinsic factors. Intrinsic factors include macromolecule content, metabolic rate, and yolk reserve. In general, intrinsic control of larval development and growth is regulated by the hypothalamo-pituitary-thyroid (HPT) axis, as in most vertebrates. Temperature, food, and the presence of inhibitory substances are extrinsic factors that influence the rate of larval growth. Another factor that determines the extent to which a metamorphosing amphibian remains in a larval state is the size that must be reached at metamorphosis. Overall, growth rates in cohorts of are often highly variable. This variation may be primarily attributed to food availability, organism density, and the presence of exogenous growth inhibiting substances. The effects of organism density and growth trends have been well documented (Duellman and Trueb, 1994; Semlitsch and Caldwell, 1982). Runkova et al. (1974) and Stepanova (1974) identified a proteinaceous growth inhibiting substance secreted by larger larvae to inhibit growth of small larvae. Crowding effects have been primarily shown to be intraspecies specific (Duellman and Trueb, 1994; Semlitsch and Caldwell, 1982), however, some evidence supports that this effect may also be interspecific specific (Duellman and Trueb, 1994; Semlitsch and Caldwell, 1982). However, other laboratory experiments have shown that the natural interspecies inhibition of growth can be reduced in the laboratory by providing an excess of food. Semlitsch and Caldwell (1982) found that premetamorphic larvae raised that gained an early size advantage under high culture density conditions metamorphosed at a smaller size in order to avoid density stress. By removing the density stress from the system, the smaller larvae grew and metamorphosed at a larger size.

Metamorphosis is a period of substantial morphological change in which an organism alters its mode of living and occurs in all major chordate groups with the exception of amniotes (Dent, 1968; Just et al., 1981). In fact, metamorphosis is developmentally comparable to post-embryonic organogenesis in mammals (Tata, 1993). Three primary characteristics define metamorphosis, 1) change in non-reproductive structures between a post-hatch or larval state and sexual maturity, 2) form of the larvae enable it to occupy a unique ecological niche different from that used by the adult life stage, and 3) the morphological changes that occur at the conclusion of larval development depend on some environmental stimulus, either external (i.e., temperature or food supply), or internal (hormonal changes). Each of the three classes of amphibians, anurans, urodeles, and caecilians, undergo metamorphosis, although not all species

within each class metamorphose. For example, obligatory neotenic urodeles do not metamorphose, and reproduce as an aquatic "adult larvae".

Anuran metamorphosis is separated into three distinct periods, premetamorphosis, prometamorphosis, and metamorphic climax (Etkin, 1964; Etkin 1968; Dodd and Dodd, 1976). Premetamorphosis refers to a period of embryonic and early larvae development that takes place without thyroid hormone. Some advanced morphological developments occur during this stage including hind limb bud development. More specific morphogenesis, such as differentiation of the toes and rapid growth (elongation) of the hind limbs, occurs during prometamorphosis. Biochemically, prometamorphosis is characterized by rising concentrations of endogenous TH. The final period is metamorphic climax in which a surge of TH triggers the final processes associated with metamorphosis, including forelimb development and resorption of the tail. Drastic internal transformations at the organ system, tissue, and biochemical levels are also taking place during prometamorphosis and metamorphic climax.

Sexual development in anurans is strongly influenced by the HPG axis, as in most vertebrates. Sexual development in anurans can be generally divided into three phases: 1) primordial germ cell migration and formation of the primordial gonad (genital ridge), 2) primary sexual development (gonadal sex determination, and 3) secondary sexual development (phenotypic sex determination). Although the mechanisms of gonadal sex determination during primary sexual development are not fully resolved, specific factors from gonadal tissue and gonadal steroids are responsible for gonadal sex determination. Further, gonadal steroid play a major role in secondary sexual development. In the commonly studied Pipid, *Xenopus laevis*, reproductive maturity in females may require 1.5-2 years and 1-1.5 years for males. However, *Xenopus tropicalis*, male and females achieve reproductive maturity within 6 months post-hatch. In male anurans, the testis is structurally more similar to amniotes than to urodeles consisting of a homogeneous mass of seminiferous tubules with a permanent germinal epithelium and well-defined interstitial tissue. Endocrine control over reproductive behavior in amphibians is less understood. Several external factors are thought to influence the onset of mating behavior, including temperature, photoperiod, and rainfall. The diversity of reproductive modes utilized by amphibians is substantially greater than that found in other groups of vertebrates, particularly the amniotes. Further, reproductive modes and strategies between, and in some cases within, each of the three amphibian groups, anurans, urodeles, and caecilians are often quite different. As opposed to salamanders and caecilians, practically all anurans utilize external fertilization. However, reproductive cycles in each group of amphibians are subject to endocrine control which are influenced in by environmental factors to produce specific reproductive behaviors.

From an evolutionary standpoint, amphibians are distinctively separated phylogenetically from other vertebrates. However, the specific aspects of the endocrine axis are conserved amongst most chordates at both the morphological and molecular levels. Thus, the conserved nature of the anuran endocrine system enhances the ability to use an amphibian, particularly an anuran, as a general model for evaluating inter disruption that can be extrapolated to other vertebrate species.

The amphibian endocrine axis represents one potential target for environmental chemicals. Environmental agents, toxicants, natural products, and complex mixtures can alter

development, growth, metamorphosis, and reproduction by interacting with the various aspects of the amphibian endocrine axis, which include HPG and auxiliary axes. Further, the complexity of these systems yields many different possible mechanisms of inhibiting metamorphic processes in amphibians at differing biochemical and molecular levels. Thus, from this end, use of amphibians to screen for EDC or chemical mixtures as a representative chordate is not unreasonable.

This DRP considers the use of several potential amphibian species in the development of an AGRA model that will achieve the above-stated goals in the most effective and efficient manner possible. In the context of the present DRP, discussion of different species will not be limited to anurans; however, it should be noted that the majority of the currently available literature exists in the anuran domain. Considering the intended use of the AGRA model as a test method (Tier 2), the most substantial discussion will be given to those species whose life history and laboratory adaptability are most amenable to use in this light.

2.5 Methods Used in this Analysis

A detailed description of the methods employed for the literature search (e.g., key words, databases, and results) is provided in Appendix A. After key papers were identified, retrieved, and read for content, pertinent information was synthesized to create this DRP. In addition to the literature review, external peer review was solicited to critically review the present DRP and for development of the AGRA model to identify chemicals that affect (i.e., inhibit or enhance) thyroid activity. The results of the peer review are found in Appendix B.

2.6 Acronyms and Definitions

The following are acronyms and definitions of terms used in the DRP (Table 2-1).

Table 2-1. Acronyms and Definitions

ACTH	Adenocortropin Hormone
AGRA	Amphibian Growth and Reproduction Assay
AR	Androgen Receptor
ASTM	American Society for Testing Materials
AVT	Arginine Vasotocin
BRD	Background Review Document
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complimentary Deoxyribonucleic Acid
CNS	Central Nervous System
CORT	Corticosterone
CRF	Corticotropin Releasing Factor
CV	Coefficient of Variation
DHT	Dihydrotestosterone
DRP	Detailed Review Paper
E2	Estradiol
EACs	Endocrine Active Chemicals
EDCs	Endocrine Disrupting Chemicals
EDMVS	Endocrine Disruptor Methods Validation Subcommittee

EDs	Endocrine Disruptors
EDSP	Endocrine Disruptor Screening Program
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
ELISA	Enzyme-linked Immunosorbent Assay
ER	Estrogen Receptor
ERE	Estrogen Response Element
FETAX	Frog Embryo Teratogenesis Assay-Xenopus
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FQPA	Food Quality Protection Act
FR	Federal Register
FSH	Follicle Stimulating Hormone
GH	Growth Hormone
GHRH	Growth Hormone, Releasing Hormone
GnRH	Gonadotropin Releasing Hormone
HAES	Hyperolius Argus Endocrine Disruption Screen
HP	Hypothalamo-Pituitary
HPG	Hypothalamus-Pituitary-Gonad
HPT	Hypothalamus-Pituitary-Thyroid
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
LH	Lutenizing Hormone
MBS	Moderately Buffered Solution
mRNA	Messenger Ribonucleic Acid
MSH	Melanocyte Stimulating Hormone
NAS	National Academy of Sciences
NIEHS	National Institute of Environmental Health Sciences
NIST	National Institute of Standard and Technology
NOAEC	No-observed Adverse Effect Concentration
OECD	Organization for Economic Cooperation and Development
P	Progesterone
PCR	Polymerase Chain Reaction
PRL	Prolactin
RIA	Radioimmunoassay
Rnase	Ribonuclease
RPA	Ribonuclease Protection Assay
RXR	Retinoic Acid X Receptor
SAB	Science Advisory Board
SAP	Scientific Advisory Panel
SDWA	Safe Drinking Water Act
SS	Somatotropin
T	Testosterone
TH	Thyroid Hormone
TR	Thyroid Receptor
TRE	Thyroid Response Element
TRH	Thyrotropin Releasing Hormone
TSH	Thyroid Stimulating Hormone
TUNEL	Terminal Deoxynucleotidyl Transferase-mediated dUTP-biotin Nick-end Labeling
T3	3,3',5–triiodothyronine
T4	Thyroxine
VTG	Vitellogenin
XEMA	*Xenopus* Metamorphosis Assay

3.0 OVERVIEW AND SCIENTIFIC BASIS OF AMPHIBIAN GROWTH AND REPRODUCTION ASSAYS (ENDOCRINE CONTROL OF DEVELOPMENT, GROWTH, AND REPRODUCTION)

3.1 The Amphibian Endocrine System

The endocrine system, also referred to as the hormone system, consists of glands and secretory cells located throughout the body, hormones that are synthesized and secreted by the glands into the bloodstream, receptors in the various target organs, and tissues that recognize and respond to the hormones. Normal function of the endocrine system, therefore, contributes to homeostasis (the body's ability to maintain itself in the presence of external and internal changes) and to the body's ability to control and regulate reproduction, development, and/or behavior. The function of the system is to regulate a wide range of biological processes, including control of blood sugar (through the hormone insulin from the pancreas); growth and function of reproductive systems (through androgens, estrogens, and related components from the testes and ovaries); regulation of metabolism (through the hormones cortisol from the adrenal glands and thyroxin from the thyroid gland); development of the brain and the rest of the nervous system (estrogen and thyroid hormones); and development of an organism from conception through adulthood and old age. An endocrine system is found in nearly all animals, including mammals, nonmammalian vertebrates (e.g., fish, amphibians, reptiles, and birds), and invertebrates (e.g., snails, lobsters, insects, and other species).

As summarized by Hayes (2000), the function of the amphibian endocrine system is reasonably consistent with vertebrate hormonal axes, with several exceptions. As in most vertebrate endocrine systems, tropic hormones are released from the pituitary as the result of pituitary stimulation by releasing factors secreted by the hypothalamus (Hayes, 1997a; Hayes, 1997b). External environmental stressors and input from the central nervous system influence hypothalamic activity. A summary of the various hormones generally found within the amphibian endocrine system is provided in Table 3-1.

Anthropogenic compounds, as well as naturally occurring chemicals, have the potential to disrupt the endocrine system of animals, including humans (Colborn and Clement, 1992). Among the anthropogenic contaminants suspected to interfere with vertebrate and invertebrate endocrine systems are the persistent, bioaccumulative organic compounds including pesticides, industrial chemicals, as well as, some metals (Brucker-Davis, 1998). It is suspected that wildlife populations are already adversely affected by these compounds. Lister and Van der Kraak (2002) and McMaster et al. (2001) have summarized the potential impacts of EDCs in various wildlife which include, but may not be limited to: 1) thyroid dysfunction in birds, amphibians, and fish; 2) decreased fertility in birds, amphibians, fish, shellfish, and mammals; 3) decreased hatching success in birds, fish, alligators, and turtles; 4) gross birth defects in birds, amphibians, fish, and turtles; 5) metabolic abnormalities in birds, fish, and mammals; 6) behavioral abnormalities in birds; 7) demasculinization and feminization of male fish, amphibians, birds, and mammals; 8) defeminization and masculinization of female fish, amphibians, alligators, and birds; 9) and compromised immune system in birds and mammals.

Table 3-1. Overview of Amphibian Hypothalamic Releasing Peptides, Other Neuropeptides, Tropic Hormones, and Steroids

Source	Location	Substance(s)	Target
Hypothalamus	Preoptic Nucleus	GnRH, GAP, CRF, AVT, MST, TRH, SS, GHRH, MSH, ANP, NPY	Anterior Pituitary
Pituitary	Anterior, Posterior	TSH	Thyroid Gland
		LH, FSH	Gonads
		GH	Liver, Connective Tissues, Muscle
		PRL	Mammary Glands, Epididymus
		ACTH	Adrenal Cortex
		MSH	Melanin-Producing Cells
Thyroid Gland	-	T_4 and T_3	Most Tissues
Interrenal Gland	-	CORT	Most Tissues
Gonad	Ovary	Estrogens	Primary and Secondary Sexual Structures, Brain
		Progesterone	Uterus
	Testis	Androgens	Primary and Secondary Sexual Structures, Brain

The term "endocrine disruption" and the hypothesis that such agents exist in the environment that affect reproduction and development dates back to the late 1980s (Colborn and Clement, 1992; Kavlock et al., 1996). These authors described such effects in fish-eating birds, alligators, Great Lakes mink, frogs, invertebrates, and humans. They suggested that these chemicals served as agonists or antagonists to endogenous endocrine hormonal axes to disrupt the hormonal control of homeostasis, cellular differentiation, embryonic growth, and development, and notably included effects on reproductive organs and reproductive function. These agents were called endocrine active chemicals (EACs), EDCs, or most popularly "endocrine disruptors" (EDs) (EDSTAC, 1998).

Reduced growth, reproductive dysfunction, abnormal behavior, and abnormal development from exposure to a variety of natural and anthropogenic chemicals in invertebrates, fish, amphibian, reptilian, avian, and mammalian species have been recently demonstrated (Lister and Van der Kraak, 2002; McMaster et al., 2001). Although EDCs are now thought to adversely affect development, reproduction, and general homeostasis in a wide variety of different taxa, several other issues complicate the evaluation of EDCs in vertebrate animals: 1) the chemicals of concern may have entirely different effects on the embryo, fetus, or perinatal organism than on the adult; 2) the effects are most often manifested in offspring, not in the

exposed parent; 3) the timing of exposure in the developing organism is crucial in determining its character and future potential; and 4) although critical exposure occurs during embryonic development, obvious manifestations might not occur until maturity (Kavlock et al., 1996). It is also possible to have differing effects of the same compound in different species or tissues, presumably due to differences in receptors.

The primary intent of the present DRP is to derive a protocol for measuring the capacity of chemical substances to disrupt normal growth and reproduction (including sexual differentiation) of amphibians as a component of a larger EDC Screening Strategy. However, it should be noted that reproduction and growth can be influenced by a combination of other biotic and abiotic factors beyond the realm of chemical stressors. These factors include habitat, temperature, water availability, crowding, light, and diet (Dodd and Dodd, 1976). Amphibians respond to changes in these factors through high levels of plasticity in the phenotypes (Stearns, 1989). Some factors that inhibit growth when present during premetamorphic stages are also capable of inducing rapid metamorphosis when present during prometamorphosis. These factors include crowding, resource limitation, habitat desiccation, and predation (Denver, 1997a; Denver, 1997b; Denver, 1998). Temperature also affects reproduction as described in the preceding sections, as well as, growth. In the case of growth, greater temperatures stimulate the rate of metamorphosis (Hayes et al., 1993), whereas lower temperatures slow down TH-induced metamorphosis (Dodd and Dodd, 1976). The effects of temperature may be due to reduction in TH binding at the tissue level, changes in neuroendocrine control of TH synthesis, or more generalized effects on metabolism (Tata, 1972; Dodd and Dodd, 1976). Overall, it must be understood that the link between the HP-G, -thyroid, and -adrenal axes; and reproduction and growth (including metamorphosis) can be influenced by several different forms of extraneous factors as occurs in many other developmental processes.

The following sections provide information on a battery of potential AGRAs designed to screen for reproductive and developmental dysfunction. Initially, methods in four candidate amphibian species or groups (i.e., *X. laevis*, *X. tropicalis*, *R. pipiens, H. argus*, and urodeles) will be discussed. This document puts forward the relevant principles, methods, and techniques recommended for an initial protocol(s), and identifies issues that might require pre-validation studies to adequately address. The ultimate outcome will be a standardized transferable protocol that can be used to screen chemicals in a regulatory arena to determine their potential of being an EDC that could negatively affect the HP-G, -thyroid, and -adrenal axes in amphibians.

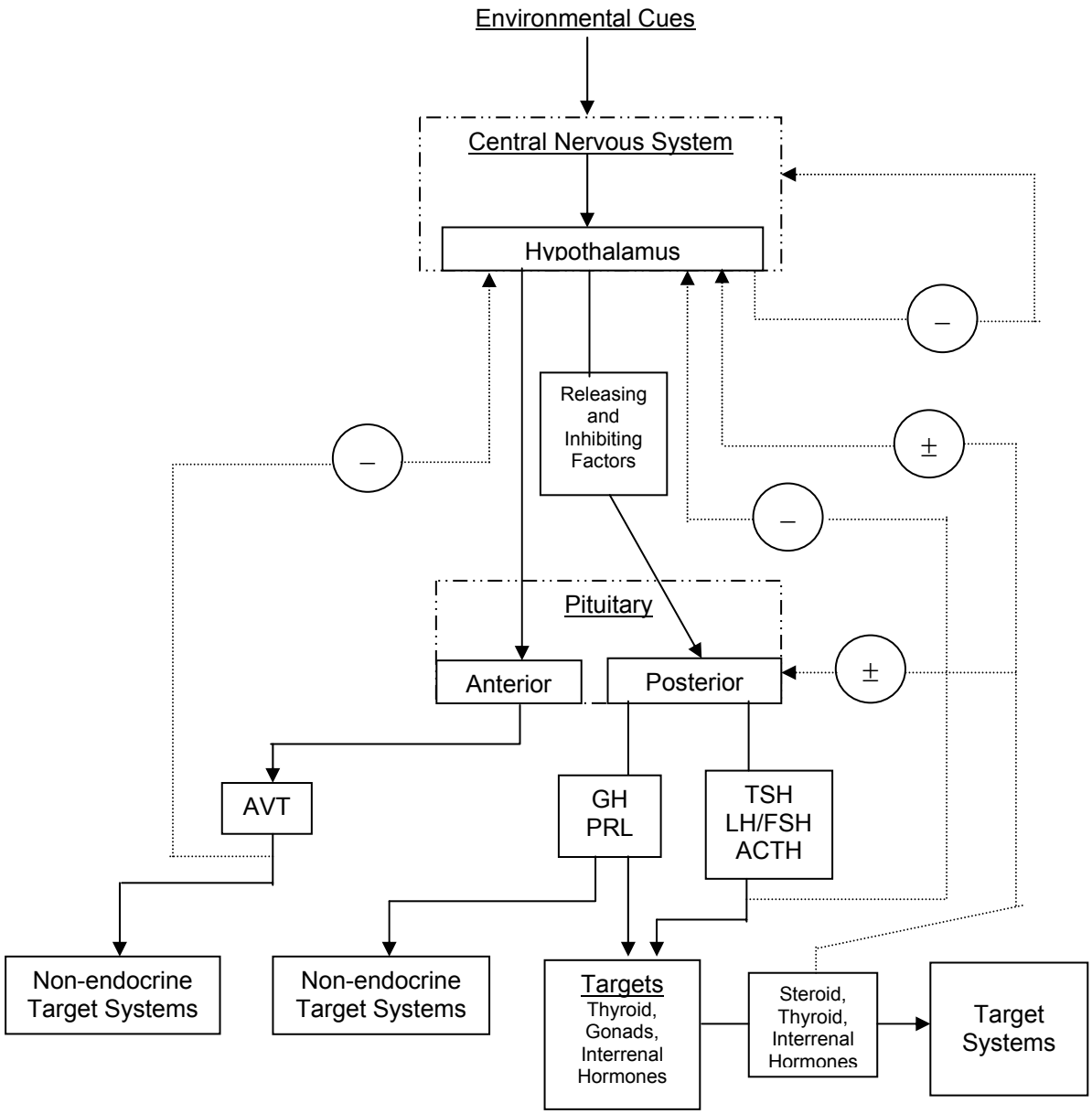
3.2 The Hypothalamo-Pituitary Axis

3.2.1 Organization

In terms of anatomical organization of the HP axis, amphibians have features that are generally characteristic of most tetrapods (Figure 3-1). Further, the tetrapod neuroendocrine system exhibit most of the general characteristics of mammals and lack the distinguishing structures that characterize the teleosts. In most tetrapods, the median eminence and the pars nervosa are well-developed and have distinct neurohemal function. In tetrapods, no vestige of a saccus vasculosus exists. The amphibian pars distalis displays marginal cellular regionalization, whereas, reptiles and birds demonstrate two reasonably distinct regions, however these zones are

not divided in to a rostal and proximal zone as in fishes. The pars tuberalis, which contains secretory cells, is a consistent feature amongst most tetrapods although a specific physiological function has not yet been defined.

Figure 3-1. Summary of the Amphibian Endocrine System



3.2.1.1 Hypothalamus. Of the tetrapods, the amphibian hypothalamus is less differentiated into specific nuclei than that found in reptiles, birds, and mammals, respectively. In most tetrapods, the control of the adenohypophysis is accomplished by neurovascular means than direct neuronal input. Amphibians, however, appear to have some direct neural control over the pars intermedia. The preoptic area contains several specific neurosecretory centers, including the lateral, medial, and the preoptic nucleus. In amphibians, the preoptic region of hypothalamus is responsible for secretion of gonadotropin releasing hormone (GnRH), growth hormone, releasing hormone (GHRH), somatotropin (SS), arginine vasotocin (AVT), thyrotropin releasing hormone (TRH), corticotropin releasing factor (CRF), and an oxytocin-like peptide referred to as mesotocin. The preoptic nucleus is further subdivided into the suprachiasmatic nucleus and the ventromedial nucleus in the posterior region. AVT has been found in the suprachiasmatic nucleus. The infundibular nucleus is located in the basal region of the hypothalamus and provides aminergic and peptidergic fibers to the median eminence. The infundibular nucleus is further subdivided into dorsal and ventral regions and is virtually homologous to the primary physiotropic region within the mammalian hypothalamus. TRH and SS-like peptides have been located in the dorsal regions and TRH and melanocytes stimulating hormone (MSH) in the ventral regions of the infundibular nucleus. A pituitary adenylate cyclase activating peptide which stimulates cyclic adenosine monophosphate (cAMP) production in the anuran pars distalis has also been found the infundibular nucleus. The neuropeptide appears to play a role in pituitary control by the hypothalamus. The influence of the hypothalamus on metamorphosis is mediated through induction of the release of thyroid stimulating hormones (TSH) from the pituitary. TRH is responsible for inducing the secretion of TSH from the pituitary in a similar pathway found in most mammals (Shi, 2000). Historically, the importance of the hypothalamus in the control of metamorphosis has been demonstrated by hypothalectomy, pituitary transplant to a remote part of the body, or providing an impermeable barrier between the hypothalamus and the pituitary gland in frogs (Dodd and Dodd, 1976; White and Nicoll, 1981; Kikuyama et al., 1993; Kaltenbach, 1996, Denver, 1996). High concentrations of TRH have been detected in the brain and skin of *R. pipiens* (Jackson and Reichlin, 1977). Further, in *X. laevis* and *R. catesbeiana* brain tissue, TRH levels have been found to increase throughout metamorphosis and metamorphic climax (King and Miller, 1981; Bray and Sicard, 1982; Millar et al., 1983; Balls et al., 1985; Mimnagh et al., 1987). However, a paradoxical relationships appears to exist between TRH and the rate of metamorphosis (Shi, 2000). More specifically, TRH is readily capable of inducing the release of TSH from the anuran pituitary. However, most experiments have not shown that administration of TRH accelerates metamorphosis (Dodd and Dodd, 1976; White and Nicoll, 1981; Denver and Licht, 1989; Kikuyama et al., 1993; Kaltenbach, 1996; Denver, 1993; 1996; 1998).

In mammals, CRF is responsible for inducing the secretion of adenocortropin hormone (ACTH). Further experimentation demonstrated that mammalian CRF is also capable of accelerating ACTH release from frog pituitaries (Tonon et al., 1986; Gracia-Navarro et al., 1992). Interestingly, ACTH does not induce the thyroid to produce TH (Sakai et al., 1991). CRF is now thought to act directly on the pituitary gland, stimulating the release of TSH (Denver 1988; Denver and Licht, 1989; and Jacobs and Kuhn, 1992). Because CRF is capable of raising TH levels in anurans and accelerating metamorphosis, and because the use of anti-CRF antibodies or CRF receptor antagonists slows metamorphosis; CRF appears to function as the mammalian surrogate of TRH and orchestrates regulation of the anuran pituitary at the

hypothalamic level (Rivier et al., 1984; Gancedo et al., 1992; Denver, 1993; 1997b). Anuran CRF genes in *X. laevis* are relatively homologous to mammalian CRF (ca. 93%) (Stenzel-Poore et al., 1992; Shi, 2000). CRF gene expression and the presence of CRF-expressing cells in the hypothalamus of *X. laevis* have not only been identified, but found to be TH-dependent (Verhaert et al., 1984; Olivereau et al., 1987; Gonzalez and Lederis, 1988, Carr and Norris, 1990; Stenzel-Poore et al., 1992). These findings generally agree with the suggestion by Denver et al. (1997) that a hypothalamic feedback loop at the pituitary level (Carr and Norris, 1990). Overall, the primary significance of this research is that CRF, not TRH, is the primary hypothalamic releasing hormone responsible ultimately for the induction of metamorphosis (Carr and Norris, 1990; Denver, 1996; Denver et al., 1997; Shi, 2000). Further discussion of the role of TRH, TSH, CRF, and TH in amphibian metamorphosis can be found in DRP 2-20 (4-5), "Amphibian Metamorphosis Assays" (Battelle, 2003).

3.2.1.2 Pituitary. The amphibian pituitary is generally divided into the neurohypophysis and the adenohypophysis. The pars nervosa of the neurohypophsis is innervated by peptidergic fibers originating from the preoptic nucleus. AVT and mesotocin are co-localized within the pars nervosa. Some evidence supports co-localization of TRH with mesotocin and has been demonstrated to induce MSH release. A set of ANP-reactive neurons have been shown to travel from the dorsal hypothalamus through the median eminence to the pars nervosa. The AVT-secreting cell bodies in the preoptic area and the axonal endings in the pars nervosa have been shown to also contain GHRH. Other sets of neurons following the same pathway form the hypothalamus to the pituitary contain MSH. The adenohypophysis contains three distinct regions including the pars tuberalis, pars intermedia, and pars distalis. Ultrastructural comparison of immunoreasctive cytoplamic granules suggest that two different cell types exist within the pars tuberalis and neural pathways have been identified. The pars intermedia has a poor vascular supply, but is enervated by aminergic neurons originating form aminergic nuclei of the hypothalamus. Secretion of MSH has been shown to be under the direct control of the aminergic, and possibly peptidergic, neurons. NPY- and ANP-reactive neurons also innervate the pars intermedia of most anurans. NPY and ANP have been shown to inhibit and stimulate MSH release, respectively. The pars distalis is generally not highly regionalized, although some localization based on cellular subtypes exists, particularly in urodeles. Much of the focus of amphibian pituitary function has focus on the activity of the pars distalis. Because of the extensive research focus on the pars distalis as the primary endocrine region of the pituitary and source of tropic hormones, considerable controversy over the source or sources of the tropic hormones exist (Norris, 1997). Thus, in summary, TSH, gonadotropin, ACTH, prolactin (PRL), and growth hormones (GH) are produced and released from the pars distalis.

3.2.2 Tropic Hormones

As in mammals, hormones in amphibians, as well as most nonmammals, are generally categorized as the gonadotropins (lutenizing hormones [LH], follicle stimulating hormones [FSH]) and TSH); growth hormones (GH and PRL); and ACTH, MSH, and other corticotropin-like substances.

3.3 Hypothalamo-Pituitary-Gonad Axis

3.3.1 Gonadotropes

Nonmammalian vertebrate gonadotropes are generally similar to those found in mammals. Generally, the hormonal control of reproduction is based on biomolecules with LH- and FSH-like activities. Typically, gonadotropin agonists, such as human chorionic gonadotropin (hCG); induce tropic responses in males including differentiation and development of seminiferous tubules, stimulation of spermatogenesis, multiplication and growth of interstitial cells, as well as, specific functions, including secretion of androgens from the interstitium and spermiation.

In most amphibians, normal ovarian, including vitellogenic growth depends on intact vascular connections with the brain. In male bufonids, ectopically-transplanted pars distalis is capable of maintaining normal spermatogenesis, but not the interstitial tissue. In ranids and urodeles, the same transplant of the pars distalis reduced spermatogenesis, as well as, reduced interstitium function. In *Rana esculenta* a pars distalis transplanted into a testis is capable of maintaining both spermatogenesis and the interstitium in the general vicinity of the graft presumably due to the low level of gonadotropins secreted by the pars distalis (Norris, 1997).

Two distinct gonadotropes have been isolated and purified from *R. catesbeiana*, *Ambystoma tigrinum*, and *R. pipiens*. Amphibian gonadotropes differ marked in structure and function when compared to both mammalian counterparts and among closely related amphibian species (Norris, 1997). Thus, a LH-active biomolecule in one species can not be assumed to have LH-activity in a closely-related species. In some cases, the LH-active substance actually exerts FSH-like activity. For example, purified bullfrog LH and FSH both stimulated speratogenesis and spermiation, however, only LH increased circulating plasma androgen levels. In *A. tigrinum*, purified LH was more effective than bullfrog LH in increasing plasma androgen levels. In bullfrogs, purified LH from *A. tigrinum* was less effective than bullfrog LH. Further, bullfrog LH was substantially more effective than *A. tigrinum* FSH. Bullfrog LH is also markedly more effective in stimulating P synthesis in amphibians than ovine LH. Anuran LH stimulates reptilian and avian thyroids implicating the structural similarities between LH and TSH biomolecules and their activities. The diversity in activity of gonadotropes may be due to differences at the receptor level or the result of subtle steric or molecular differences (Norris, 1997).

In summary, the phases of vitellogenic growth, maturation of ovarian follicles, spermatogenesis, and androgen secretion in the testicular interstitium proceed at low circulating levels of gonadotropins. In contrast, surges of gonadotropin secretion are necessary for spermiation and ovulation which effectively increases circulating gonadotropins by an order of magnitude (Jorgensen, 1992). The two gonadotropin-based processes may operate at different ranges to ensure that spontaneous spermiation and release of eggs does not occur prior to breeding.

3.3.2 Gonadal Steroids

Consistent with other vertebrates, the amphibian ovary produces and secretes steroid hormones (see Table 3-2) in response to LH stimulation. The ovarian sensitivity to LH is acquired following metamorphosis (Hsu et al., 1979). Seasonal hormone cycles are present in most female anurans (Licht et al., 1983; Pierantoni et al., 1984; Iela et al., 1986). Plasma 17β-estradiol (E2), androstenedione, testosterone (T), and P are associated with various ovarian changes that occur during the reproductive cycle (Pierantoni et al., 1984). Further, some ranids, including *R. catesbeiana*, T levels are greater in females than in males, and plasma T, but not estrogen, correlate with ovarian growth (Licht, 1983). As previously discussed, P and, possibly androgens, play a significant role in the maturation of the amphibian oocyte (Lutz et al., 2001; Pickford and Morris, 1999, 2003; and Blondeau and Baulieu, 1984). The liver of several anuran species has both cytosolic and nuclear estrogen receptors (ERs) (Paolucci and Botte, 1988). ERs in *R. esculenta* have been shown to have a high affinity for E2 and diethylstilbestrol, and a lesser affinity for estrone and estriol. Further biochemical evaluation indicates that the MW 48,000. Based on Scatchard analyses, the affinity of these steroids was unchanged throughout the reproductive cycle. Although both bound and unbound receptors were found throughout the cycle, the number of bound receptors increased during the vitellogenic oocyte growth phase (Paolucci and Botte, 1988). ER binding is affected by temperature such that binding to the nuclear receptor is low at lower temperatures (4°C) initially up to 4 h with stability with the cytosolic fraction achieved for 36 h. Greater nuclear receptor binding at greater temperatures (20°C) than the cytosolic fraction with both decreasing at 4 h.

The amphibian testis produces androgens under HPG control and testicular binding sites for LH and FSH are present (Ishii and Kubokawa, 1985). Endogenous local levels of E2 inhibit, and T and DHT enhance LH stimulated testicular androgen production (Pierantoni et al., 1986; Fasano et al., 1989). As with sex steroids levels in females, androgen levels in males fluctuate with season in many anuran and urodeles (Moore and Deviche, 1988). Circulating levels of T are typically greater in urodeles than anurans. Generally, the circulating levels of T and DHT are equivalent, however, in DHT levels are usually greater than T levels in *R. catesbeiana*. These physiological levels generally change with season and are coordinated with spermatogenic cycles (Licht et al., 1985). In males, androgen levels are low in the summer during the proliferation of secondary spermatogonia and spermatocyte development. During the winter months as the temperature decreases, androgen levels increase and spermatid formation begins. As previously discussed, androgens aid in the development of secondary sexual features, including the nuptial thumb pads. ARs have been located at these target locations (Ho et al., 1985). The brain is a primary site of androgen metabolism (Callard, 1985). As in many vertebrates, aromatase catalyzes the conversion of T to E2 and has high activity in the hypothalamic or limbic systems. 5α-reductase catalyzes the conversion of T to DHT. Administration of E2 directly to the brain acts similarly to androgens in terms of invoking sexual behavior in most anurans. However, inhibition of T aromatization blocks androgen-induced clasping of the female frog (Callard, 1985).

Table 3-2. Steroid Hormones Found in Amphibians

Category	Trivial Name	Chemical Name
Androgens	Testosterone	17β-Hydroxy-4-androsten-3-one
	Androstenedione	4-Androstene-3,17-dione
	Dehydroepiandrosterone	3β-Hydroxy-5-androsten-17-one
Corticoids	Aldosterone	11β,21-Dihydroxy-3,20-dioxo-4-pregnene-18-al
	Cortisol	11β,17,21-Trihydroxy-4-pregnene-3,20-dione
	Corticosterone	11β,21-Dihydroxy-4-pregnene-3,20-dione
	11-Deoxycorticosterone	21-Hydroxy-4-pregnene-3,20-dione
Estrogens	17β-Estradiol	1,3,5(10)-Estratriene-3,17β-diol
	Estrone	3-Hydroxy-1,3,5(10)-estratrien-17-one
	Estriol	1,3,5(10)-Estratriene-3,16α,17β-triol
Progestogens	Pregesterone	3β-Hydroxy-5-pregnen-20-one
	Progesterone	4-Pregnene-3,20-dione

Unlike corticoids, the role of gonadal steroids on metamorphosis is significantly less clear. Based on an early study by Frieden and Naile (1955) in *Bufo bufo,* estrone enhanced the effect of T4 on metamorphosis. However, the results of this study have not been demonstrated by other investigators. Rather, the majority of historical studies indicate that E2 and T antagonize the effects of T4 in *R. temporaria* (Roth, 1941; Roth, 1948) and inhibit larval development in *R. pipiens, X. laevis,* and *B. boreas* (Richards and Nace, 1978; Gray and Janssens, 1990; Hayes et al., 1993) *in vivo*. Hayes et al. (1993) found that at 22°C, T and E2 had no effect on growth or size at metamorphosis, although T induced precocious forelimb emergence. However, at 27°C, T and E2 inhibited growth and development, but did not alter the time to forelimb emergence. However, Gray and Janssens (1990) also found that gonadal steroids did not inhibit the resorption of cultured whole tails *in vitro*. These results suggest that an inhibitory action of gonadal steroids most likely does not occur at the TR level. Gray and Janssens (1990) and Hayes (1997a) suggest that gonadal steroids most likely act at the hypothalamic-pituitary-thyroid axis level. Hayes (1997a) further hypothesized that the most likely mechanism of gonadal steroid inhibition of metamorphosis occurs through the down-regulation of TH levels, and potentially by up-regulating PRL levels, which as described below also is capable of inhibiting metamorphosis.

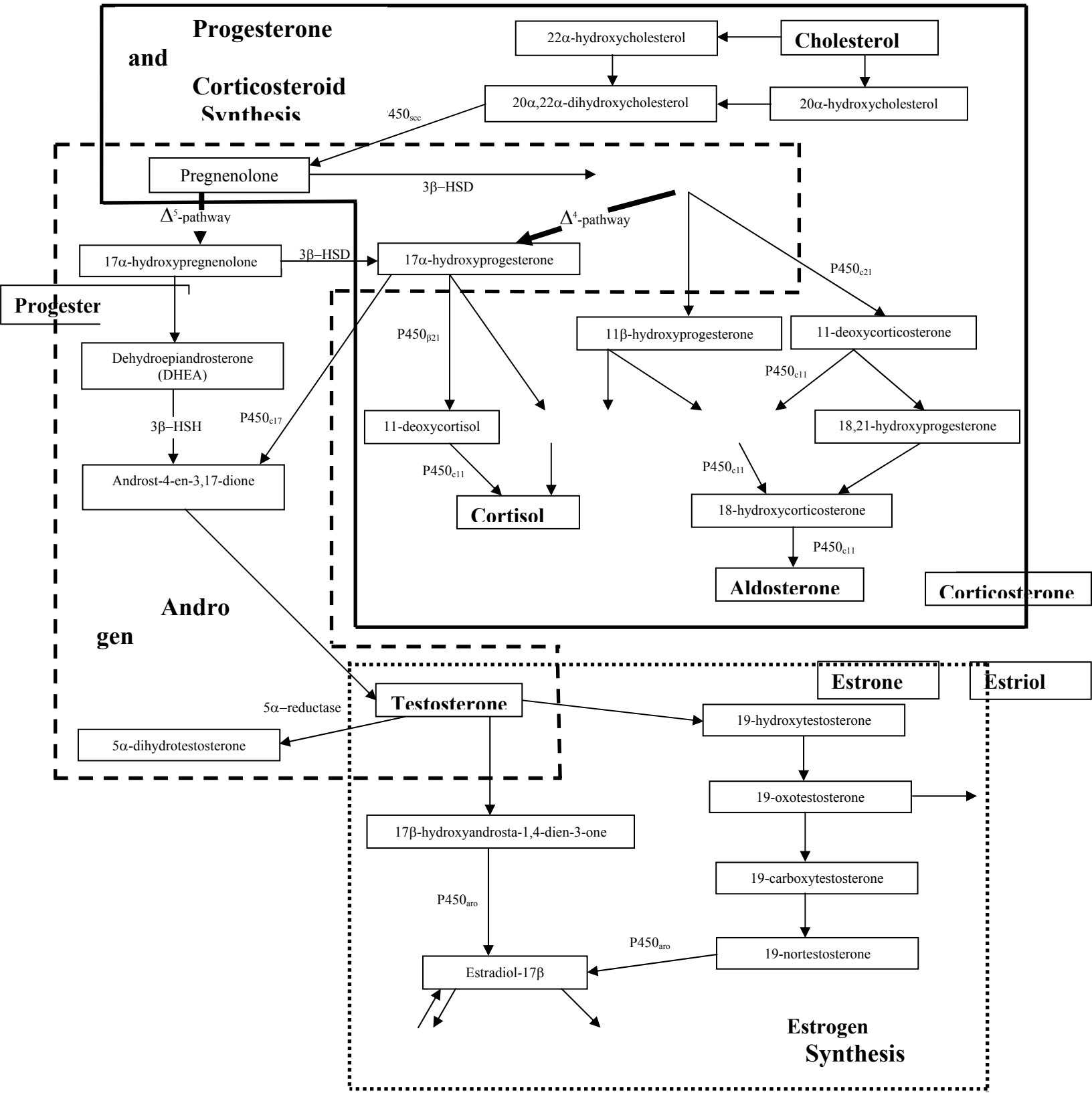
Several other investigators have evaluated the effects of gonadal steroids on thyroid axis homeostasis and function; and implications on larval growth, development, and metamorphosis (Jacobs et al., 1988; Vandorpe and Kuhn, 1989; Hayes et al., 1993). Jacobs et al. (1988) found that plasma concentrations of T4 were significantly raised following IV administration of synthetic lutenizing hormone-releasing hormone (LHRH) in ranids. These investigators concluded that this stimulatory effect was mediated through the hypophysis and suggested a possible correlation between the gonadal axis and thyroid axis. Vandorpe and Kuhn (1989)

evaluated the effect of E2 implants in female *R. ridibunda* on plasma TH levels and 5'-monodeiodination activity in kidney homogenates *in vitro*. These investigators found that plasma T3 and T4 levels, and the *in vitro* T3 production in kidney homogenates were significantly decreased, suggesting that E2 may repress the thyroid axis. Other investigators have evaluated the influence of TH on gonadal steroid activity during metamorphosis (Rabelo and Tata, 1993; Cohen and Kelley, 1996; Robertson and Kelley, 1996). Rabelo and Tata (1993) found that T3 enhanced the precocious activation of VTG genes by E2 in *X. laevis* during advanced metamorphosis between NF stages 58-64. Cohen and Kelley (1996) found that androgen-induced cell proliferation in the developing larynx of *X. laevis* is controlled by TH. These investigators determined that although TH was not required for androgen receptor (AR) mRNA expression is the larynx, cellular proliferation was enhanced by TH, both *in vitro* and *in vivo*. Further, Robertson and Kelley (1996) concluded that while gonadal differentiation is independent of TH, androgen-sensitive larangeal development, including sexual dimorphism, require exposure to endogenous TH.

3.3.2.1 Sex Steroid Synthesis. The biosynthetic pathways for the primary sex steroids, including E2, T, DHT, and progesterone (P) including the inter-relationships between the various pathways are illustrated in Figure 3-2. Enzymes important in sex steroid biosynthesis are provided in Table 3-1.

3.3.2.2 Sex Steroid Receptors. Diploid vertebrate animals, including *X. tropicalis*, possess two estrogen receptor (ER) genes (ER alpha and ER beta) (Lazar, 1993). *X. laevis*, which is oligotetraploid, possess four ER genes, two ER alpha and two ER beta (Mangelsdorf et al., 1995). ERs belong to the super family of nuclear hormone receptors, including glucocorticoid, estrogen, vitamin D, and retinoic acid receptors (Green and Chambon, 1988; Gaub et al., 1990; Evans, 1988; Tsai and O'Malley, 1994; Yen and Chin, 1994). In general, as with TRs (see Battelle, 2003 for review), ERs contains five different binding domains, A/B, C, D, E, F (amino to carboxy terminus) (Green and Chambon, 1988). Thus, these domains are reasonably consistent within this class of nuclear receptors (Evans, 1988; Beato, 1989; Shi, 2000). The amino terminus (A/B domain) of the ER alpha specifically contains the AF-1 domain, which appears to be involved in E2-independent recruitment of specific co-activators. Thus, ligand-independent activation of transcription may be mediated by the binding of specific co-factors to the AF-1 region of the A/B domain (Evans, 1988; Beato, 1989; Obertse-Berghaus et al., 2000; Yang and Privalsky, 2001). DNA binding occurs in domain C. The C domain is highly conserved amongst nuclear receptors. Domain D is the variable hinge region which contains a nuclear localization signal and influences both DNA binding and transactivation through co-repressor binding (Giguere et al., 1986; Godowski et al., 1988; Hollenberg and Evans, 1988; Picard and Yamamoto, 1987; Guiochon-Mantel et al., 1989; Zechel et al., 1994; Lee and Mahdavi, 1993; Uppaluri and Towle, 1995; Puzianowska-Zunicka et al., 1997). Domain E and F are the ligand, or hormone binding and transactivation domains. The carboxy terminus, or region F, contains the AF-2 domain. A hormone-inducible transcription activating domain, or AF-2, has been found to be a binding site for specific co-activators (Obertse-Berghaus et al., 2000; Heery et al., 1997; Langlois et al., 1997). Both AF-1 and AF-2 are required for optimal stimulation of transcription, but the relative contribution of the two in a cell- and promotor-specific manner (Lee et al., 1995; Tora et al., 1989; Tzukerman et al., 1994). Estrogen-mediated transcription changes are mediated by interaction between the ER and the estrogen

Figure 3-2. Amphibian Steroid Hormone Synthesis Pathways



response element (ERE) (Wood et al., 1998). EREs generally function as allosteric modulators of ER conformation.

The AR shares the same characteristics as other members of the steroid hormone gene super family, including the ER (Lubahn et al., 1988; He et al., 1990). Fischer et al. (1993) cloned a fragment of a *X. laevis* AR cDNA containing the DNA and ligand binding domains. Although AR alpha has been found in the testis, kidney, liver and the size and distribution are similar to that found in mammals; the expression of AR alpha in the developing larynx of males *X. laevis* is exceeds that of adult tissues including the testis. In addition, AR beta mRNA is specifically expressed during hormone-evoked differentiation of the larynx suggesting that this isoform is required for the masculinization program within the developing larynx of males. Thorton and Kelley (1998) identified a group of amino acids that characterize the ARs from other similar receptors. These investigators hypothesized that this unique region confers the functionality of the AR including the recognition of specific response elements. Although the four domains with the AR show marked evolutionary divergence, this conserved region appears to be essential to AR function.

3.4 Hypothalamo-Pituitary-Thyroid Axis

3.4.1 Thyrotropes

The pituitary hormone thyrotropin (or thyroid stimulating hormone [TSH]) produced and secreted by the par distalis region of the pituitary gland, is primarily responsible for inducing the production and release of TH from the thyroid gland (Shi, 2000). TSH production and release is controlled via negative feedback at the pituitary level (Dodd and Dodd, 1976; White and Nicoll, 1981; Kikuyama et al., 1993; Kaltenbach, 1996; Denver, 1996). Although traditional measures of plasma TSH have not been successful in amphibians due to a lack of sensitivity in the assay, Sakai et al. (1991) found that both purified frog and purified bovine TSH stimulated the release of T4 from the thyroid gland. In hypophysectomized *X. laevis* tadpoles, Dodd and Dodd (1976) estimated TSH levels in crude pituitary extracts during development using radioiodine uptake. This work suggested that TSH was detectable at trace levels during prometamorphosis (NF stage 56), but increased markedly at the onset of metamorphic climax (NF stage 59). In these studies, a decrease in pituitary TSH levels at stage 61 followed by a spike in pituitary TSH at stage 62 was found. Thus, increasing levels of TSH occur during metamorphosis when TH is required. Coincidently, the drop in pituitary TSH production occurs simultaneously with peak TH levels and appears to be the result of increased release of TSH from the pituitary. An understanding of this process at the molecular level has been achieved as the result of the production of complementary DNAs (cDNAs) coding for TSH in *X. laevis* (Buckbinder and Brown, 1993). Buckbinder and Brown (1993) essentially found that messenger RNA (mRNA) levels during metamorphosis indicated that TSH genes were activated around stage 53, immediately prior to the first stage in which pituitary TSH levels are detectable. TSH mRNA levels peak at approximately NF stages 58 or 59, and drop to appreciably lower levels toward the conclusion of metamorphosis (Dodd and Dodd, 1976; Shi, 2000). TSH gene repression subsequent to stage 59 coincides with high levels of plasma TH. This finding is consistent with a TH-induced negative feedback loop at the pituitary or hypothalamic levels. Interestingly, Dodd and Dodd (1976) and

Kikuyama et al. (1993) found a relatively high degree of homology between anuran TSH cDNA and mammalian species. Further discussion of the role of TRH, TSH, CRF, and TH in amphibian metamorphosis can be found in DRP 2-20 (4-5), "Amphibian Metamorphosis Assays" (Battelle, 2003).

Various inter-relationships between glucocorticoids, gonadal steroids, and the thyroid axis have been found to occur in developing amphibians (Roth, 1948; Frieden and Naile, 1955; Jaffe, 1981; Kobayashi, 1958; Kikuyama et al., 1982; Krug et al., 1983; Leatherland, 1985; Galton, 1990; Gray and Janssens, 1990; Leloup-Hatey et al., 1990; Hayes et al., 1993; Kikuyama et al., 1993; Hayes, 1995a; Hayes, 1995b, Hayes, 1997b, and Hayes, 2000). These endocrine pathway interactions are described in more detail in the following sections. In summary, TH interactions with gluctocorticoids include TH-induced production of corticoids by the interrenal gland and increased titers of T3 via conversion from T4. Both processes increase the activity of the thyroid axis. In contrast, sex steroids repress the activity of the thyroid axis directly opposite to the effect of the corticoids. TH interaction with gonadal steroid hormones include inhibition of T4 to T3 conversion and establishment of a negative feedback mechanism at the pituitary level, ultimately slowing the production and secretion of TH. In addition, numerous hormone interactions with the thyroid axis may occur at the receptor level, including: 1) corticoid enhancement of TH activity by facilitating binding to the TR (Niki et al., 1981; Suzuki and Kikuyama, 1983), 2) TH facilitation of steroid receptor induction in anurans (Hayes, 1997b), and 3) induction of TR synthesis by T3 (Rabelo and Tata, 1993; Tata, 1994; Ulisse and Tata, 1994). Two naturally-occurring TH, 3,5,3',5'-tetraiodothyronine (T4 or thyroxine) and 3,5,3'-triiodothyronine (T3) have been found in anuran species. Based on nearly one hundred years of research, the affect of TH on amphibian metamorphosis is no longer debated, although research in understanding the functional mechanisms and interaction with other hormonal pathways continues today (Gudernatsch, 1912; Allen, 1916; Allen, 1929; White and Nichol, 1981; Tata, 1968; Dodd and Dodd, 1976; Brown et al., 1995; Shi, 2000).

3.4.1.1 Prolactin and Growth Hormone. PRL has many different actions in vertebrates, including amphibians, which complicates a specific description of its role. A summary of the various actions of PRL is described in Table 3-3. In general, PRL plays a role in amphibian reproduction, growth and development, water and electrolyte balance, integumentary structure, and actions on steroid-stimulated processes.

Similar to the effect of corticoids on metamorphosis (Hayes, 1997a), PRL also appears to exert a bimodal effect on development and maturation of amphibians (Shi, 2000). However, in the case of PRL, the response is opposite that of corticoids, which are capable of inhibiting early development and potentiating TH-induced metamorphosis (Hayes, 1997a). In contrast, PRL is currently thought to stimulate development during embryogenesis and premetamorphosis, but inhibit the maturation events associated with metamorphosis. In fact, several investigators (Etkin and Lehrer, 1960; Dodd and Dodd, 1976; White and Nichol, 1981; Kikuyama et al., 1993; Denver, 1996) have elaborated on the capacity of PRL to serve as an apparent growth stimulator in amphibians during premetamorphosis, while also inhibiting metamorphosis in anuran species. Also, in contrast to the effect of corticoids on anuran metamorphosis, PRL is capable of exerting its inhibitory influence on metamorphosis *in vitro* (tail explants) (Dodd and Dodd, 1976; Tata et al., 1991). These results suggest that the inhibitory effects of PRL on metamorphosis could be

mediated at the TR level rather than endocrine regulatory level (Leloup and Buscaglia, 1977). In fact, Tata and coworkers demonstrated that PRL is capable of inhibiting induction of the TR beta genes by TH (Baker and Tata, 1992; Tata, 1997). Wakao et al. (1994) and Han et al. (1997) have also suggested that PRL inhibits the function of the TH-TR complex.

Table 3-3. Summary of PRL Actions in Amphibians

Function	**Action**
Reproduction	Water drive prior to reproduction Secretion of oviductal jelly Spermatogenic and/or antispermatognic Ovulation Stimulation of cloacal gland development
Growth and Development	Tail and gill growth Limb regeneration Proliferation of melanophores Structural changes accompanying water drive Brain growth in tadpoles Cloacal gland development Ultimobranchial stimulation
Water and Electrolyte Balance	Skin and electrolyte changes associated with water drive, metamorphosis
Integumentary	Skin changes associated with water drive Proliferation of melanophores Effects on toad bladder Skin yellowing in frogs
Steroid-dependent Targets or Actions with Steroids	Stimulation of oviductal jelly secretion (estrogens and progestogens) Na^+ transport across anuran bladder (aldosterone) Water-drive structural changes (sex steroids) Spermatogenesis (androgens) Cloacal gland development (androgens)

Anuran PRL, which was originally difficult to isolate due to the low plasma levels, was first isolated from bullfrogs (Shi, 2000). Cloned amphibian PRL was subsequently found to be relatively homologous to mammalian PRL (Yamamoto and Kikuyama, 1981; Yasuda et al., 1991; Takahashi et al., 1990; Buckbinder and Brown, 1993). PRL in anuran species is produced in the distal lobe of the pituitary gland (Yamamoto et al., 1986; Tanaka et al., 1991). PRL production and secretion is under tight stimulatory and inhibitory control at the hypothalamic level (Kaltenbach, 1996; Shi, 2000). PRL is transported to various target tissues through the plasma. Low plasma PRL levels have been detected during pre- and prometamorphic stages. However, PRL levels appear to rise to peak levels late in metamorphic climax (Clemons and Nicoll, 1977; Yamamoto and Kikuyama, 1982; Yamamoto et al., 1986). Interestingly, TRH serves as the primary PRL-releasing hormone in amphibians, whereas, dopamine serves as the primary neurological inhibitor of PRL release. Thus, rather than stimulating the release of TSH

(as in mammals), TRH induces the release of PRL and CRF induces the release of TSH. Further discussion of the role of PRL in amphibian metamorphosis can be found in DRP 2-20 (4-5), "Amphibian Metamorphosis Assays" (Battelle, 2003).

Like PRL, GH (also referred to as SS) is a simple peptide of approximately 200 amino acids and originating from the same gene family (Denver et al., 2002). Unlike, PRL, however, the role of GH in amphibian development is reasonably well established (White and Nicoll, 1981; Kikuyama et al., 1993; Denver, 1996). Many of the aforementioned studies using mammalian preparations of GH suggest that GH stimulates post-metamorphic development as in many other vertebrates (Harvey et al., 1995; Denver, 1996). These studies demonstrated that larval tissues have the capacity to respond to GH-like (or PRL-like) substances, and that functional receptors exist in either case that are capable of promoting growth and blocking TH-induced metamorphosis, most likely by inhibiting the auto-induction of TR as described in the preceding paragraphs (Tata et al., 1993). Huang and Brown (2000 a and b) developed transgenic lines of *X. laevis* larvae that over-expressed *X. laevis* GH and PRL. Since expression of the transgenes was promoted by the simian cytomegalovirus, all larval tissues actually expressed the transgene. These investigators found that over-expression of GH produced larval tadpoles and post-metamorphic frogs, but had no affect on the timing or duration of metamorphosis suggesting that GH activity in anurans in limited to growth regulation and not metamorphosis, unlike PRL.

3.5 Hypothalamo-Pituitary-Interrenal Axis

3.5.1 Corticotropin, Related Tropic Hormones, and Corticoids

In summary, corticoids are the primary stress hormones in vertebrates are produced as the result of a variety of environmental stressors (Seyle, 1976; Denver et al., 2002). The synthesis and secretion of endogenous corticoids are under the direct or indirect control of TH, ACTH, and CRF. Corticosterone and aldosterone function as the primary corticoids produced by the amphibian interrenal gland (Carstensen et al., 1961; Macchi and Phillips, 1966; Denver et al., 2002). The interrenal gland is relatively less active during prometamorphosis and progressively more active during metamorphic climax (Dodd and Dodd, 1976). The interrenal enzyme 3-HSD is present throughout development in *X. laevis* and *R. catesbeiana* (Hsu et al., 1979; Kang et al., 1995), and *A. tigrinum* (Carr and Norris, 1988), but is increases at metamorphic climax. RIA analysis of corticoid levels in both plasma (Jaffe, 1981; Krug et al., 1983; Kikuyama et al., 1986; Jolivet-Jaudet and Leloup-Hatey, 1984; Carr and Norris, 1988) and whole bodies (Kloas et al., 1997; Glennemeier and Denver, 2002b) has been monitored during development in a variety of species. With the exception of *X. laevis*, most of the anuran species studied and the tiger salamander showed a marked increase in corticoid production at metamorphic climax similar to the trends found with TH. However, Kloas et al. (1997) and Glennemeier and Denver (2000a, 2000b) suggested the whole body corticoid levels in X. laevis increases during premetamorphosis peaking at NF stage 48 and decreasing through the remainder of metamorphosis. Glennemeier and Denver (2002b) reported a slight spike of corticoids at metamorphic climax. Kloas et al. (1997) also found the same trends with whole body aldosterone, however the peak was observed later at NF stage 54 with decline thereafter. These

finding suggest that either tissue corticoid measurement is not reflective of plasma or that species differences exist (Denver et al., 2002).

Based on the work of Hayes (1997a), CRF appears to have dual functions, stimulating the release of both TSH (thyrotropes) and ACTH (corticotropes) from two different regions of the pituitary (Denver and Licht, 1989). Tonon et al. (1986) found that both CRF and AVT are potent stimulators of ACTH secretion by cultured adult anuran pituitaries. Conversely, the role of TRH in metamorphosis, which is the primary thyrotrope in most mammals, is currently thought to be insignificant (Shi, 2000). Glennemeier and Denver (2002b) found that injection of ACTH into premetamorphic *R pipiens* and *X. laevis* larvae increased whole body corticosterone levels indicating that ACTH receptors are present prior to metamorphosis. Overall, physiological synthesis and secretion of corticoids play an important role in anuran metamorphosis.

In general, the relative importance and capacity of corticosteroids in enhancing TH-induced metamorphosis in amphibians has been purported by several sets of investigators (Kaltenbach, 1985; Kikuyama et al., 1993; and Hayes, 1997a). In amphibians, the interrenal gland is responsible for the production of corticosteroids and receives direct input from the hypothalamus via adrenocorticotropin (ACTH). In turn, two primary corticoids are produced and secreted by the anuran interrenal gland: 1) corticosterone, and 2) aldosterone (Cartensen et al., 1961; Macchi and Phillips, 1966; and Kikuyama et al., 1993; Shi, 2000). Interestingly, several investigators have demonstrated that the major corticoid levels in plasma in metamorphosing anurans follow the pattern of rising plasma TH levels in metamorphosing tadpoles (Jaffe, 1981; Krug et al., 1983; Jolivet-Jaudet and Leloup-Hatey, 1984; Kikuyama et al., 1986; Kikuyama et al., 1993; Hayes, 1997a). Experimental evidence supporting the role of corticoid hormones in the induction of metamorphosis range from basic fundamental studies to complex experiments. Denver et al. (2002) summarize the action of exogenously administered corticoids are influenced by dose, the stage of administration, and whether it is co-administered with TH. Further discussion of the role of corticoids in amphibian metamorphosis can be found in DRP 2-20 (4-5), "Amphibian Metamorphosis Assays" (Battelle, 2003).

Melatonin, produced by the anuran pineal gland, and somatostatin appear to be capable of altering anuran metamorphosis (Shi, 2000). Both factors inhibit TSH secretion and are capable of retarding metamorphosis (Denver, 1996). Melatonin may also inhibit metamorphosis through the induction of PRL pathways (Rose and Rose, 1998). On the contrary, GnRH has been found to increase TH levels in axolotls and frogs, thus has the potential of accelerating metamorphosis (Shi, 2000). Limited information is available on these and other hormones and the understanding of their roles in metamorphosis remains unclear.

3.6 Reproduction

The variety of life history traits of most amphibian species compared to other vertebrates makes them a unique target in the environment. Many amphibians utilize a water- and terrestrial-based environment for breeding and larval development and the adult life phase, respectively. These history traits effectively increase the number of potential exposure routes, and thus the probability of being exposure during the life of the amphibian. Early embryo-larval development has typically been considered the most sensitive stage of the vertebrate life cycle. For example,

in most fish species the embryo-larval stage of development is typically considered the most sensitive to chemical exposure (Nguyen and Janssen, 2002, 2001; Lowengart, 2001). However, considering the complexity of events that surround amphibian growth, metamorphosis, and reproduction; early embryo-larval development may not always be the most sensitive stage of development, particularly in the case of exposure to EDCs.

Exposure to environmental contaminants can adversely affect individuals, as well as meta-populations of amphibians (Carey and Bryant, 1995). Most studies have focused on effects at the individual level. Extrapolating toxicological effects observed in individual specimens in the laboratory to effects at the meta-population level is extremely difficult and requires an adequate evaluation of adverse responses in the field. Negative effects at the meta-population level may be the result of multiple responses including lethal responses, sub-lethal responses, and modest changes in biochemical homeostatis (Pickford and Morris, 1999). Sub-lethal responses include malformation, growth reduction and developmental delay. Changes in biochemical homeostasis in amphibians resulting from pertubation of critical aspects of the endocrine system, such as reproductive hormonal pathways and the thyroid axis also provide additional stress. Amphibian reproduction can be perturbed at a myriad of different sites within the body including, the brain, pituitary, thyroid, gonad, liver, and many of the effector tissues (Pickford and Morris, 1999 and 2003). Thus, not only are EDCs capable of disrupting reproductive function by perturbing endocrine systems in adults, but also by inducing abnormalities in critical reproductive tissues.

Overall, amphibians represent a suitable model for monitoring reproductive performance (Fort and McLaughlin, 2003; Fort et al., 2001a), early embryo-larval development (Fort et al., 2003b; Fort and McLaughlin, 2003; ASTM, 1998; Bantle et al., 1998; Dumont et al., 1983), and advanced development including metamorphosis (Carr et al., 2003; Fort and Stover, 1996, 1997; Fort et al., 2000, 2001b), and sexual maturation (Pickford et al., 2003; Hayes et al., 2002; Kloas et al., 1999; Tavera-Mendosa et al., 2002a and 2002b). Collection of concurrent information on the effects on the sensitivity of various developmental stages throughout the life cycle not only provides valuable hazard assessment information, but also provides mechanistic clues concerning the modes of action of reproductive toxicants.

The diversity of reproductive modes utilized by amphibians is substantially greater than that found in other groups of vertebrates, particularly the amniotes. Further, reproductive modes and strategies between, and in some cases within, each of the three amphibian groups, anurans, urodeles, and caecilians are often quite different. As opposed to salamanders and caecilians, practically all anurans utilize external fertilization. However, reproductive cycles in each group of amphibians are subject to endocrine control, which is influenced in by environmental factors, to produce specific reproductive behaviors. Micro-habitat, size, reproductive mode, and parental practices further influence reproductive behavior and productivity. In general, seasonal reproductive status is under control of the HP and associated axes. Amphibian ovaries are capable of producing P, E2, estrone, T, deoxycorticosterone, and DHT. Ovulation during breeding season is under the control of a LH-like gonadotropin and P. LH, as well as a wide variety of steroid hormones including P and androgens, are responsible for maturation of the oocytes (completion of meiosis and germinal vesicle breakdown [GVBD]) in preparation for breeding. Synthesis of P is under control of LH, and the action of P is indirect, operating

through stimulation of a maturation promotion factor. The role of androgens in oocyte maturation resulting from conversion of P to androstenedione in the oocyte has also been suggested recently (Lutz et al., 2001). PRL increases the sensitivity of oocytes to gonadotropins or P. Ovarian maturation is typically completed in autumn, and ovulation is delayed over winter until favorable environmental conditions exist for breeding. The exact control of diapause is currently unknown, although it is suspected that control is exerted by pituitary hormones. Growth and preparation of the oviducts are stimulated by either estrogens or androgens. Contraction of the oviducts during breeding is controlled by AVT, which apparently acts as the stimulus for oviposition. During the breeding season, the oviducts demonstrate increased sensitivity to AVT. P, but not estrogen, induces increased sensitivity of the oviducts to AVT. In response to LH stimulation, release of sufficient P from the pre- or post-ovulatory follicle may account for increased P levels during breeding. P then induces increased synthesis of AVT receptors in the muscle of the oviduct, which induce contraction and egg release. Androgens and estrogens apparently have not affect on the sensitivity of oviducts to AVT. It should also be noted that the interrenal gland may also serve as a source of P release and influence the responsiveness of the oviducts. Androgens appear to play a significant role in male reproductive behavior (Deviche and Moore, 1988; Herman, 1992). In many anuran species, castration reduces or eliminates sexual behavior and administration of exogenous androgens restores sexual behavior. In these cases, T, and not DHT, is responsible for the behavioral responses (Deviche and Moore, 1988). Exceptions to this general trend include the newt in which circulating levels of androgens do not correspond with sexual activity (Moore and Muller, 1977). In addition, T is not responsible for calling behavior in several hylids (Rastogi et al., 1986).

3.6.1 Female Characteristics

Historically, the production of oocytes and their development into mature eggs was considered to the a linear and sequential process, however, more recent studies suggest that rigid sequential coupling of stage from oogonial proliferation to final maturation of a complement of eggs may not exist. Although different methods of staging oocytes exist today (Dumont, 1972), two primary categories are evident in the amphibian ovary, small gonadotropin-independent oocytes. The number of small, non-vitellogenic oocytes increase rapidly following metamorphosis. In most anurans, the number of previtellogenic oocytes reaches a maximum by an early juvenile stage, and remains relatively constant throughout the life cycle at 20,000-50,000 oocytes. In most cases in bufonids, the influx of oocytes to the pool is generally in balance with the efflux of oocytes (recruitment for vitellogenic growth) (Billeter and Jorgensen, 1976). During an influx of oocytes very small oocytes (<0.08 mm) proliferate. Within a short period of time (ca. one month) in toads, the oocytes are capable of doubling in size and are incorporated in an existing pool of small oocytes. Generally, and influx event is followed by a period of rest which may last for several years. Further, oogenesis is not coordinated with either ovulation or recruitment of oocytes for vitellogenic growth, and it occurs in the ovaries of sexually immature toads. In *R. temporaria*, oogenic episodes are not correlated with seasons and may occur at low temperatures during hibernation (Jorgensen, 1984). Oogenesis proceeds synchronously in both ovaries suggesting that initiation of an oogenic event is under systemic control. However, many of the factors that control oogenesis are poorly understood and it has been historically thought that gonadotropin is not directly involved. (Billeter and Jorgensen, 1976; Jorgensen, 1973). Since unilateral ovariectomy induces oogenesis in many anuran species

which appears to be a compensatory mechanism to restore a viable population of small oocytes, control of oogenesis and the pool size of small previtellogenic oocytes are under a somatic-type growth control process (Jorgensen et al., 1975). The nutritional status of the to organism also appears to influence oogenic episodes (Jorgensen, 1984). The reserve of small oocytes serves as a pool from which oocytes can be recruited for vitellogenic growth. This process normally occurs at sexual maturation, following ovulation, and following necrosis of unused vitellogenic oocytes. Overall, the reserve of small oocytes and the mechanisms that control pool size and fecundity is a highly dynamic system that is relatively unaffected by environmental conditions, as opposed to many of the other intricacies of amphibian oocyte development, growth, maturation (vitellegenesis), ovulation, and the ovarian cycle which are profoundly influenced by environmental conditions and seasonal events (Vijayakumar et al., 1971 and Jorgensen, 1992).

The recruitment of a set of oocytes for vitellogenic growth marks the onset of an ovarian cycle and proceeds until the oocytes are fully developed (Jorgensen, 1973, 1975). As with oogenesis, vitellogenic oocyte growth is a synchronous process, such that a reasonably consistent size-frequency distribution exists. Further, the consistency of distribution is maintained in anurans from both temperate and tropical climates (Jorgensen, 1974, 1984; Jorgensen, 1984). However, whereas synchronous vitellogenic growth occurs in individual female anurans, patterns of vitellogenic growth within a population may vary substantially with the environmental conditions (asynchrony in tropical populations to near synchrony in temperate populations). The degree of asynchrony within a population is often associated in species with wide latitudinal distribution, such as the toad, *B. viridus*. In amphibians from the colder temperate regions, the vitellogenic growth phase is typically complete prior to estivation for species that breed relatively soon after emerging from hibernation. In species from the warmer temperate zones, vitellogenic growth of oocytes can proceed during periods of estivation. Seymour (1973) found that the majority of vitellogenic growth occurred during dormancy in spadefoot toads (*Scaphiopus*). Long (1989) found no change in ovary mass during dormancy (ca. 6-7 months), however, an increase in oocyte diameter was recorder during this period. Generally, the vitellogenic growth phase correlates with an increase in ovary weight since the accumulation of yolk (VTG) adds weight to the developing oocyte. This relationship can be expressed by the following equation developed by Long (1989): oocyte volume (mm^3)= -19.3+1.07(ovary mass [mg]) [r^2=0.79]. In temperate zone mammals, birds, reptiles, fish (teleosts), seasonal variation in day length is vitally important in the timing of reproductive cycles (Laming, 1984). In contrast, photoperiod appears to have no direct affect on vitellogenic growth patterns in amphibians. Normal vitellogenic oocyte growth was observed in *Xenopus laevis* and *R. pipiens* maintained in complete darkness. However, artificial light regimens, such as those used in the laboratory, are capable of altering normal vitellogenic growth. In female toads, exposed to differing photoperiods (i.e., LD 14:10 and 6:18), the oocyte recruitment process for vitellogenic growth was unaffected, however, an increase in oocyte atresia was observed, most notably at LD 6:18 (Jorgensen, 1992). These effects were also found in the bullfrog (*R. catesbeiana*) (Horseman, 1978) and *X. laevis* (Fort et al., unpublished data), where exposure to artificial light for >14 and <8 h dramatically increased oocyte necrosis compared to LD12:12 which is normally used in the laboratory (Fort et al., 2001a).

Growth and maintenance of vitellogenic oocytes is dependent on circulating gonadotropins. Administration of hCG in anurans has been used to evaluate the ovarian cycle.

Generally, in females without vitellogenic oocytes (immature females, female devoid of vitellogenic oocytes following breeding, and in hypophectomized females with degenerated oocytes) administration of hCG initiated an ovarian cycle (Jorgensen, 1975). In toads, a constant dose of hCG throughout the ovarian cycle induces recruitment and vitellogenic growth with a period of several weeks after which point no further recruitment occurred. In females with a full complement of vitellogenic oocytes, hCG has no affect on oocyte recruitment or vitellogenic growth patterns. Thus, the transition from the recruitment phase to the synchronous vitellogenic growth phase of the ovarian cycle is not dependent on gonadotropin, but appears to be the result of intraovarian regulatory factors that inhibit further recruitment (Jorgensen, 1974; 1978). The vitellogenic growth phase proceeds until a normal complement of full-grown oocytes is achieved and at constant levels of circulating gonadotropin titers. This suggests that intraovarian mechanisms control the normal physiological pattern of the vitellogenic growth phase (Jorgensen, 1975; 1982). The vitellogenic growth phase is highly sensitive to stress including, starvation and light, which are capable increasing atresia and altering normal vitellogenic growth patterns.

The final phases of the ovarian cycle are oocyte maturation of vitellogenic oocytes and ovulation. During the maturation phase, the follicle and oocyte are prepared for ovulation and eventual fertilization. Sensitivity during this time, the sensitivity to hCG gradually increases (Rugh, 1948; Jorgensen, 1978) in preparation for ovulation. The process of oocyte maturation in *Xenopus* has been well studied. The maturation of the amphibian oocyte represents the final stage of oogenesis, which ultimately prepares the oocyte for fertilization. Oocyte maturation is marked morphologically by GVBD (Bondeau and Baulieu, 1984; Hausen and Reibesell, 1991; Pickford and Morris, 1999), and is induced by P via an oocyte plasma membrane receptor, and androgens via a classical intercellular AR (Lutz et al., 2001). Thus, maturation of the oocyte could potentially be disrupted by EDCs. Disruption of oocyte maturation events in *R. pipiens* (Lin and Schuetz, 1983) and *X. laevis* (Baulieu et al., 1978) by E2 and a synthetic estrogen has been demonstrated previously. Thus, maturation of the oocyte could potentially be disrupted by EDCs. It has been previously hypothesized that P-induced maturation of amphibian oocytes could be disrupted by environmental pollutants with anti-progestin activity (Pickford and Morris, 1999), and subsequently evaluated by Fort et al. (2002).

Ovulation is marked by the rupture of the follicles and extrusion of mature eggs into the oviducts which complete the normal ovarian cycle. As in most vertebrates, natural ovulation is induced by a surge of gonadotropin in anurans (McCreery and Licht, 1983). A concominant and resultant surge of P also occurs prior immediately prior to ovulation and serves as the actual inducer of ovulation. Pickford and Morris (2003) have subsequently demonstrated that the process of ovulation and oviposition can be altered by anthropogenic chemicals, including EDCs, in *X. laevis*.

3.6.2 Male Characteristics

In male anurans, the testis is structurally more similar to amniotes than to urodeles consisting of a homogeneous mass of seminiferous tubules with a permanent germinal epithelium and well-defined interstitial tissue. Essentially, spermatogenesis is consistent amongst most vertebrate classes (Lofts, 1974 and 1984). Spermatogenesis with mitotic divisions

of spermatogonia and progresses through the meiotic spermatocyte stages to the maturation stages referred to as the spermatogenic wave, terminating in the insertion of the spermatozoan bundles into the Serotoli cells. In anurans, the spermatogenic wave from proliferation of the nests of secondary spermatogonia to the maturation of spermatozoans requires 5-6 weeks (Kalt, 1976; Guha and Jorgensen, 1978; Rastogi et al., 1983; Toyoshima and Iwasawa, 1984). During the spermatogenic wave the stages of development proceed synchronously. In juvenile male anurans, spermatogenesis begins with the differentiation of the first sets of seminiferous tubules. Spermatogenesis proceeds simultaneously in the newly diffentiated tubules and creates a wave throughout the entire organ. However, with increasing age and additional tubules differentiate spermatogenesis becomes increasingly asynchronous with seminiferous tubules with all stages of spermatogenesis throughout the testis (Jorgensen and Billeter, 1982). Although asynchronous continuous spermatogenesis is generally thought to be a characteristic of male anurans, and possibly other male amphibians living in constant environments, however, cyclical spermatogenesis is observed in some species, including *R. tigrina* (Basu and Mondal, 1961; Saidapur and Kanamadi, 1982; Saidapur and Nadkarni, 1975) and *B. marinus* (Saidapur, 1983). At least in the case of *R. tigrina*, spermatogenesis becomes discontinuous during the dry season in the winter and early Spring. Most species with continuous spermatogenesis are capable of breeding throughout the year, whereas species that possess discontinuous spermatogenesis breed during short, discrete periods. Cyclicity is spermatogenesis typically becomes more pronounced in species from increasing latitudes, and is associated with climatic cycles. In the cold temperate zones, spermatogenesis ceases during hibernation and is resumed following spermiation following breeding. In many species, including *R. esculenta* (Galgano, 1936), *R. graeca* and *R. lastei* (Cei, 1944), *R. iberica* (Crespo and Cei, 1971), *R. catesbeiana* (Japan) and *Hyla japonica* (Toyoshima and Iwasawa, 1984; Yoneyama and Iwasawa, 1985), and several South American leptodactylids (Cei, 1961) the process is discontinuous with the capacity to follow a continuous pattern if conditions warrant. An additional spermatogenic pattern has emerged from the cold temperate zone in which cyclicity is inherent and independent of the climatic cycle (van Oordt, 1960). In this pattern which has been evaluated in detail in *R. temporaria*, normal spermatogenesis is not resumes following breeding in the early Spring, but is resumed after a lag period. This type of inherent cyclicity may also be observed in some urodele (Werner, 1969). Overall, precipitation and temperature are the only two environmental factors which have any consistent influence on spermatogenic cycles, although photoperiod may influence spermatogenesis in some species (vanOordt, 1956; Werner, 1969; Toyoshima and Iwasawa, 1984). Light has no real influence on spermatogenesis. Further, the influence of nutritional status on spermatogenesis is appreciably less marked than on gonadal function in female amphibians (Guha et al., 1980). Extreme starvation following emergence from hibernation in *B. bufo* reduced the number of secondary spermatogonial cysts. However, depression of spermatogenesis was hypothesized to be the result of decreased gonadotropin section. Thus, the primary environmental factors controlling spermatogenesis under normal environmental conditions are rainfall in the tropical species and temperature in the temperate species (Jorgensen, 1992).

Spermatogenesis is typically identified with sexual maturity. However, in several anuran species, spermatogenic waves occur during early development prior to the secondary sexual development characteristics. In some cases, early spermatogenesis in larval or newly metamorphosed frogs can be considered precocious (Iwasawa and Kobayashi, 1976; Kobayashi

and Iwasawa, 1988) and typically ends with degeneration of spermatogenic nests prior to the completion to the spermatogenic cycle. However, in some species, such as *R. catesbeiana* (Swingle, 1921), *R. esculenta* (Rastagoli et al., 1983), and several bufonids (Jorgensen and Billeter, 1982) the juvenile spermatogenic cycle may proceed to the formation of spermatozoans with no discernable difference between the adult spermatogenic cycle. In some bufonids, the early phases of tubule formation proceed directly into normal spermatogenesis.

In adult male amphibians, normal testis function depends on the secretion of gonadotropins. However, true dependence on gonadotropins appears to develop over time with sexual maturation. The testis of hypophysectomized juvenile toads contain nearly normal numbers of secondary speratogonial nests nearly two months following surgery, whereas in sexual mature specimen activity ceases within one month following the surgical procedure (Jorgensen and Billeter, 1982). Generally, in several anuran and urodele species hypophysectomized adult males eventually only display primary spermatogonia in the tubules (Guha and Jorgensen, 1978). In *R. temporaria* (van Oordt, 1956) and *B. bufo* (Guha and Jorgensen, 1978), only the premeiotic spermatogonial stages are dependent on gonadotropin. Thus, once primary spermatogenesis is reached the remaining cycle proceeds in a relatively normal fashion without significant degeneration or gonial nests or cells. In hypophysectomized male toads (*Bufo*), daily injections of 5 IU hCG/100 g body weight (relatively equivalent to a 10 ng/mL plasma level of LH) maintains normal levels of spermatogenesis and interstitial cell function, as well as, the development of full development of secondary sex characteristics, including the thumb pads (Jorgensen 1984, 1992). This effect of hCG has been well noted in other anuran species including, *Xenopus sp.* (Norris, 1997; Fort et al., 2003).

Spermatogenesis and interstitium activity operated independently with development of the interstitium proceeding in the autumn (Lofts, 1964; Jorgensen et al., 1979) in cold temperate zone anurans exhibiting an annual cycle in testis function regardless of whether the cycle is continuous or discontinuous (vanOordt, 1960; van Oordt and Lofts, 1963). Thus, testicular function depends on the temporal pattern of the secretion of the two primary endogenous gonadotropins, LH and FSH. Both LH and FSH, act with greater specificity and potency than hCG (Jorgensen 1984, 1992; Fort et al., unpublished data).

Interstitial cells in anurans are similar to their mammalian counterparts in terms of ultrastructure and in 3β-HSD activity. Lipid metabolism in the interstitial cells and 3β-HSD activity prior to breeding season closely follows trends in male androgen-induced secondary sexual accessory development, such as the enlarged nuptial pads in ranids. During the post-spawning period, the interstitial cells demonstrate extensive lipid accumulation, but little 3β-HSD activity. During this period, the nuptial pads regress. During the winter, the Sertoli cells lack lipid content. However, as breeding nears, the Sertoli cells elongate and a transient increase in lipid granules occurs. Cytologically, changes in the cellular ultrastructure of the Sertoli cells include a well-developed and active smooth endoplasmic reticulum. Increased 3β-HSD activity is also found in the Sertoli cells of breeding male anurans. After sperimiation occurs, the Sertoli cells detach from the tubule wall and degenerate. New Sertoli cells with arise from fibroblasts that differentiate prior to the subsequent breeding season. In male anurans, T, FSH, LH vary throughout the year, but E2 levels are typically low. In anurans in which gametogenesis and the development of accessory structures is associated with breeding (*R. catesbeiana*), the greatest

levels of reproductive hormones are detected during mating. In other amphibians, including *R. esculenta* and *A. tigrinum*, in which gametogenesis is disassociated from breeding, the greatest levels of reproductive hormones are not typically detected during the breeding season.

Endocrine control over reproductive behavior in amphibians is less understood (Norris, 1998). Several external factors are thought to influence the onset of mating behavior, including temperature, photoperiod, and rainfall. Breeding may divided into four general patterns in anurans, 1) continuous (throughout the year when appropriate conditions exist), 2) opportunistic, 3) sporadic wet, and 4) sporadic dry. Among anurans, two primary reproductive patterns are utilized. Most tropical and subtropical species are capable of reproducing throughout the year. In this case, the primary reproductive trigger is rainfall. However, most temperate species breeding is cyclical and dependent on several extrinsic factors, including temperature and rainfall. The mechanism of endocrine control of reproductive behavior is less understood. Studies involving castration, hypophysectomy, and injections of pituitary hormones suggest that androgens are involved in calling, courtship, and clasping behavior. However, in most amphibian species, mating behavior can not be induced by directly administering androgens indicating that the process is more complicated. In some species, increased brain and gonadal aromatase activity is observed during courtship and mating in males. Neural peptide including, AVT, GnRH, and ACTH, induce mating behavior in androgen-primed specimens. Generally, female anurans, which are not receptive, emit a release call, whereas, females which are receptive do not emit call. A female that is receptive generally retains water as the result of AVT for ovulation and oviposition. Administration of AVT results in the inhibition of the release call in female specimens. Although most anurans do not utilize chemical communication mechanisms during breeding, urodeles utilize phermones produced and released from hedonic or cloacal glands. Anurans typically rely on auditory, tactile, and visual cues.

3.7 Metamorphosis

A thorough review of amphibian metamorphosis and thyroid hormone assays are provided in DRPs 2-20 (4-5) (Battelle, 2003) and 4-7 (Battelle, in preparation), respectively. Thus, discussion of metamorphosis provided is limited to that which pertains to a general understanding of amphibian endocrinology, reproduction, and growth.

3.7.1 Morphology and Biochemistry

Morphological changes that occur during amphibian metamorphosis have been extensively described and various reviews exist regarding these drastic changes in anatomy (Dodd and Dodd, 1976; Hourdry and Dauca, 1977; Gilbert and Frieden, 1981; Fox, 1983; Balls et al., 1985; Yoshizato, 1989). Essentially, three primary changes in tadpoles take place during metamorphosis in order to transform almost all of the tadpole organs to their adult form (Shi, 2000). The first change involves complete destruction or digestion of tadpole-specific organs. The most obvious example of such a resorption process is the loss of the tail during metamorphic climax. The second change involves *de novo* development of new tissues from newly produced and proliferated cells. As with many embryological processes these newly produced, but unspecified cells, subsequently differentiated cell lines ultimately leading to tissue morphogenesis (i.e., digits of the hind limbs). Finally, restructuring of existing organ systems,

such as the liver, lungs, and intestine into their adult forms occurs. These processes occur to allow the metamorph to adapt to a new terrestrial environment or adulthood in species remaining aquatic. For the sake of brevity, only morphological features that are relevant to the development of AGRA will be discussed in this DRP.

The role of TH in primary and secondary sexual differentiation has only recently been investigated (Hayes, 1997a). Administration of goitrogens, such as thiourea, which block TH production, resulted in skewed sex ratios (100% female) in *X. laevis* (Hayes, 1997a; Hayes, 1998). In addition, TH has also been shown to directly induce the T receptor in the larynx of developing male *X. laevis* (Cohen and Kelley, 1996; Robertson and Kelley, 1996). In the sexually dichromatic anuran, *H. argus*, administration of E2 induces female coloration in both male and female specimens (Hayes, 1997a). However, when E2 is administered concurrently with thiourea, the skewing toward female coloration characteristics does not occur. Hayes (1997a) found that when thiourea, classical TH synthesis inhibitor, is co-administered with T, induction of gular pouch development does not occur. However, gular pouch development is induced when T is administered alone (Hayes, 1997a).

The cellular and biochemical changes that occur in anurans during metamorphosis can be divided into at least six general areas: 1) molecular and biochemical activities associated with programmed cell death or apoptosis, 2) shift from ammonotelism to ureotelism, 3) increase in serum protein levels, 4) changes in hemoglobin isoforms, 5) alterations in digestive enzymes, and 6) changes in the respiratory system (Shi, 2000). The role of apoptosis in the selective resorption of tadpole tissues was discussed in the previous section. Many of the genes involved in programmed cell death have been isolated and characterized largely due to genetic studies in *Caenorhabditis elegans*. At least three execution genes, seven engulfment genes, and one degradation gene are involved in apoptosis in the nematode *C. elegans*, which represent the three primary processes in selective cell death (Yuan et al., 1993; Alnemri et al., 1996; Cryns and Yuan, 1998). Genes participating in execution of apoptosis and subsequent steps are most likely common in biological organisms where cell death takes place (Ellis and Horovitz, 1986). Homologs to the *C. elegans* and mammalian genes are currently being studied in amphibians. On the other hand, signal transduction genes participating in the early steps leading to apoptosis, such as induction by TH, may vary in different species. A primary feature of apoptosis involves fragmentation of chromatin, which is exploited as a means of evaluating apoptosis by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling). TUNEL is capable of marking apoptosis prior to the fragmentation of the nucleus and the cytoplasm (Gavrieli et al., 1992). The initiation of apoptosis is dependent on TH, and increasing concentrations of T3 (5 to10 nM) induce an increasing response, demonstrating dose-dependence. Classical inhibitors of apoptosis, including ATA and Z-VAD, are capable of inhibiting epithelial apoptosis (Su et al., 1997; Shi et al., 1989). Apoptotic bodies in the tail can be observed as early as NF stage 59 in *X. laevis* (Shi, 2000).

3.7.2 Thyroid Hormone and Thyroid Hormone Receptors

The primary active THs, T4 and 3,3', 5–triiodothyronine (T3), are synthesized directly in the thyroid gland (Shi, 2000). Metabolic conversion of T4 to T3, however can occur in other tissues (Fox, 1983; Dodd and Dodd, 1976). TH synthesis is initiated by up-regulation of the

thyroglobulin gene in the thyroid, which consequently produces thyroglobulin, the precursor of T4. An intricate set of post-translational modifications, including iodination and condensation of the tyrosine residue to produce T4, is then required. T4 can either be secreted into the plasma from the thyroid gland, or directly converted to T3 in the thyroid by 5'-deiodinase. Both T4 and T3 can be selectively inactivated by 5-deiodinases by converting either TH to T2 or reverse T3, respectively. This allows different tissues to possess different ratios of T3 to T4 depending on their specific requirements. St. Germain and Galton (1997) located two different 5-deiodinases in anurans have different enzymatic properties and tissue distributions. Differing deiodinases have been isolated and cloned in *R. catesbeiana* (Davey et al., 1995; Becker et al., 1995) and *X. laevis* (St. Germain, 1994). Each different isoform was found to have distinctly different regulation patterns in different tissues, thus supporting the hypothesis of TH level regulation at the tissue level. The action of TH during development is regulated at many different levels, due in part to the presence of numerous TH binding proteins in the plasma, cytosol, and nucleus. Since many of the proteins with which TH interacts are cytosolic and many of the effects occur at a non-genomic level, it was originally thought that TH acted through cytosolic actions (Davis and Davis, 1996). However, today more evidence exists that TH acts at the nuclear level mediating gene regulation via nuclear-based TR (Tata and Widnell, 1966; Tata, 1967; Oppenheimer, 1979). TH secreted from the thyroid is carried in the plasma to various tissue by various serum TH hormone binding proteins. At least nine cytosolic and plasma proteins are known to transport TH, although several are more significant factors (Shi, 2000). In the serum, TH immediately encounters serum binding proteins, the most notable of which is transthyretin (Yamauchi et al., 1993), which transport TH to the target tissues where TH enters the cytosol (Jorgensen, 1978; Barsano and DeGroot, 1983; Galton, 1983; Benvenga and Robbins, 1993). Cellular uptake mechanisms are not well understood. T3 and T4 are relatively hydrophobic at physiological pH (Shi, 2000). Thus, passive diffusion through the cell membrane is a possible route. However, some evidence suggests that a carrier-mediated transport process involving translocation of both the TH transporter and TH is possible (Blondeau et al., 1988; Oppenheimer et al., 1987; Robbins, 1992; Ribeiro et al., 1996; Benvenga and Robbins, 1993). Within the cytoplasm, TH interacts with a separate group of multifunctional proteins, collectively referred to as CTHBP (cytoplasmic TH binding proteins) (Cheng, 1991). It is presently unclear whether the TH-CTHBP complex is required for activation of the nuclear TRs, or whether it only provides a means of transport to the TR.

Diploid vertebrate animals, including *X. tropicalis*, possess two TR genes (TR alpha and TR beta) (Lazar, 1993). *X. laevis,* which is oligotetraploid, possess four TR genes, two TR alpha and two TR beta (Mangelsdorf, et al., 1995). Alternative splicing of the TR beta transcripts gives rise to two different isoforms in higher vertebrates and four different isoforms in *X. laevis* (Brooks et al., 1989; Yaoita et al., 1990). TRs belong to the super family of nuclear hormone receptors, including glucocorticoid, estrogen, vitamin D, and retinoic acid receptors (Evans, 1988; Tsai and O'Malley, 1994; Yen and Chin, 1994).

3.8 Sexual Development and Maturation

The potential influence of EDCs on sexual development, most notably secondary sexual development, has come under critical study and review within the last several years. Studies by Tavera-Mendosa (2002a, 2002b), Hayes et al. (2000, 2003), and Carr et al.(2002) have

demonstrated that the estrogenic herbicide atrazine is capable of causing a variety of abnormal gonad development profiles, including hemaphrodism in developing *X. laevis* at low concentrations. Further, several investigators have demonstrated that exposure to different toxicants prior to and during sexual development are capable of altering gonadal development, including alteration of phenotypic gender ratios (Fort et al., in press a and b).

The chronic toxicity of methoxychlor to the South African clawed frog, *Siluria* (*Xenopus) tropicalis* was evaluated using a life cycle approach (Fort et al., in press b). The chronic exposure period ranged from mid-cell blastula stage (NF stage 8) to 90-d of exposure during which time the organisms generally completed metamorphosis and emerged as juvenile frogs. Methoxychlor concentrations ranged from 1 to 100 μg/L. Methoxychlor concentrations >10 μg/L caused delayed development. No increase in mortality or external malformation was observed at any of the test concentrations. A concentration-dependent increase in internal abnormalities of the liver, gonads, and thyroid was noted, however. Skewing of sex ratio toward the female gender, decreased ovary weight and number of oocytes, increased oocyte immaturity and necrosis were noted at methoxychlor concentrations of 100 μg/L. Reduction in testis weight and sperm cell count, and an increase in the frequency of sperm cell dysmorphology were detected at 100 μg/L methoxychlor. Results from these studies suggested that methoxychlor was capable of altering the rate of larval development, but did not adversely affect embryo-larval development as manifested in external malformations. Internal malformations, including feminization, were induced by chronic methoxychlor exposure. In addition, reproductive endpoints, most notably in the female specimens, were adversely affected by methoxychlor exposure.

Sexual development in anurans is strongly influenced by the HPG axis, as in most vertebrates. Sexual development in anurans can be generally divided into three phases: 1) primordial germ cell migration and formation of the primordial gonad (genital ridge), 2) primary sexual development (gonadal sex determination), and 3) secondary sexual development (phenotypic sex determination). Although the mechanisms of gonadal sex determination during primary sexual development are not fully resolved, specific factors from gonadal tissue and gonadal steroids are responsible for gonadal sex determination. Further, gonadal steroid play a major role in secondary sexual development. Primordial gonadal development may be observed as early as NF stage 46 (ca. 4-5 d) in *X. laevis* and the formation of primordial germ cells noted by NF stage 40 (ca. 2-3 d). During early development (ca. NF stages 46-55), the gonad remains undetermined. During primary sexual development, differentiation of the indifferent gonad into an ovary or testis occurs and gonadal sex is, thus, determined. Postmetamorphic sexual development includes follicular growth and oocyte maturation in females, and enlargement of gonophores, adult-type testis formation, production of spermatocytes, and eventually spermatozoa occur. The process of secondary sexual development establishes phenotypic sex and results in the reproductive maturation of the gonads. This process is typically complete, yielding reproductive maturity, within 1 year postmetamorphosis in males and 1-2 years postmetamorphosis for most anurans. The greatest amount of time required to reach reproductive maturity in ranids are species residing at high altitudes or latitudes in which approximately 4 years for males and 6 years for females is required. However, in tropical ranids, such as *R. erytharea*, reproductive maturity may be obtained in 6-7 months in males and in 9 months for females. Small hylid and hyperoliid species typically mature more quickly. In the commonly studied pipid, *X. laevis*, reproductive maturity in females may require 1-2 years and

1-1.5 years for males. However, *X. tropicalis*, male and females are capable of achieving reproductive maturity within 6 months post-hatch (Norris, 1996).

Since sexual development and differentiation has been studies more extensively in *X. laevis* than other anurans, the remainder of this section will focus on a more specific discussion of sexual differentiation in *X. laevis* (see Kelley, 1996 for review). Developing gonads are first discernible in *X. laevis* at NF stage 46 as a thickened ridge of tissue, commonly referred to as the genital ridge, on both sides of the dorsal root of the dorsal mesentary (Iwasawa and Yamaguchi, 1984). In accordance with Nieuwkoop and Faber (1994), the primordial germ cells are first visible prior to the formation of the ridge at NF stage 40. The germ cells are encased within the primitive gonad by NF stage 49. Until ca. NF stage 56 or so, the primitive gonad is undifferentiated and determination of phenotypic sex morphologically is impossible. During the period of indifference, non-germ cells collect to the proximal portion of the primordial gonad, while the germ cells, which undergo active mitotic division, congregate in the distal region. Eventually, the gonad enlarges forming a separate medulla in the interior and a peripheral cortex. In females, epithelial tissue forms in the middle of the gonadal medulla and the germs cells are confined to the cortex region. In males, no medullary cavity is formed and the germ cells migrate the center of the primordial gonadal medulla. Newly differentiated oocytes marked by follicular encasement develop during the onset and during metamorphic climax in females (NF stages 56-66) (Nieuwkoop and Faber, 1994). During this same general stage of development, seminiferous tubules develop and the germ cells differentiate marking the onset of spermatogenesis.

The genus *Xenopus* is generally polyploid and *X. laevis* is oligotetraploid with many duplicated gene copies (Graf and Kobel, 1991; Kelley, 1996). *X. tropicalis* is a diploid species (see discussion of cytogenic advantages and disadvantages in Section 4) (Fort et al., in press c). Much of the understanding of the control of primary sexual development has arisen from experiments with sex-reversed specimens. Young larvae exposed to E2 during gonadal differentiation develop as reproductively fertile female specimens (Chang and Witschi, 1956; Kloas et al., 1999). On the contrary, tadpoles exposed to testicular grafts, but not androgens alone during primary sexual development have been shown to develop as phenotypic males (Mikamo and Witschi, 1963). However, the role of androgens in development of the male gonad is still not fully elucidated. Further studies by Mikamo and Witschi (1963) and Witshi (1971) led these investigators to determine that the female gender was heterogametic (ZW) and the male homogametic (ZZ). The conclusions derived from a series of sex reversal studies by these investigators indicated that the ultimate gender fate of primordial germ cells is not determined by cytogenetics, but rather the constitution of the gonad in which they locate (Kelley, 1996). Thus, gamete phenotype is not determined by gonad genotype, but rather gonad phenotype. The importance of gonad phenotype in primary sexual determination was provided by Blackler (1965), who transplanted undifferentiated germ cells from a genotypic male and female into a genotypic female and male, respectively. Ultimately, Blackler (1965) found that the host gonad determined the phenotypic fate of the gametes. The relationship between chromosomal sex and the sexual orientation of the gonad has been extensively studied in mammals (Koopman et al., 1991; Giese et al., 1992; Harley et al., 1992; Griffiths, 1991; Lovell-Badge, 1993). However, the specific mechanism by which genetic sex relates to gonadal sex is still not completely resolved (Kelley, 1996). Several testis-specifying genes have been isolated and cloned in mammals,

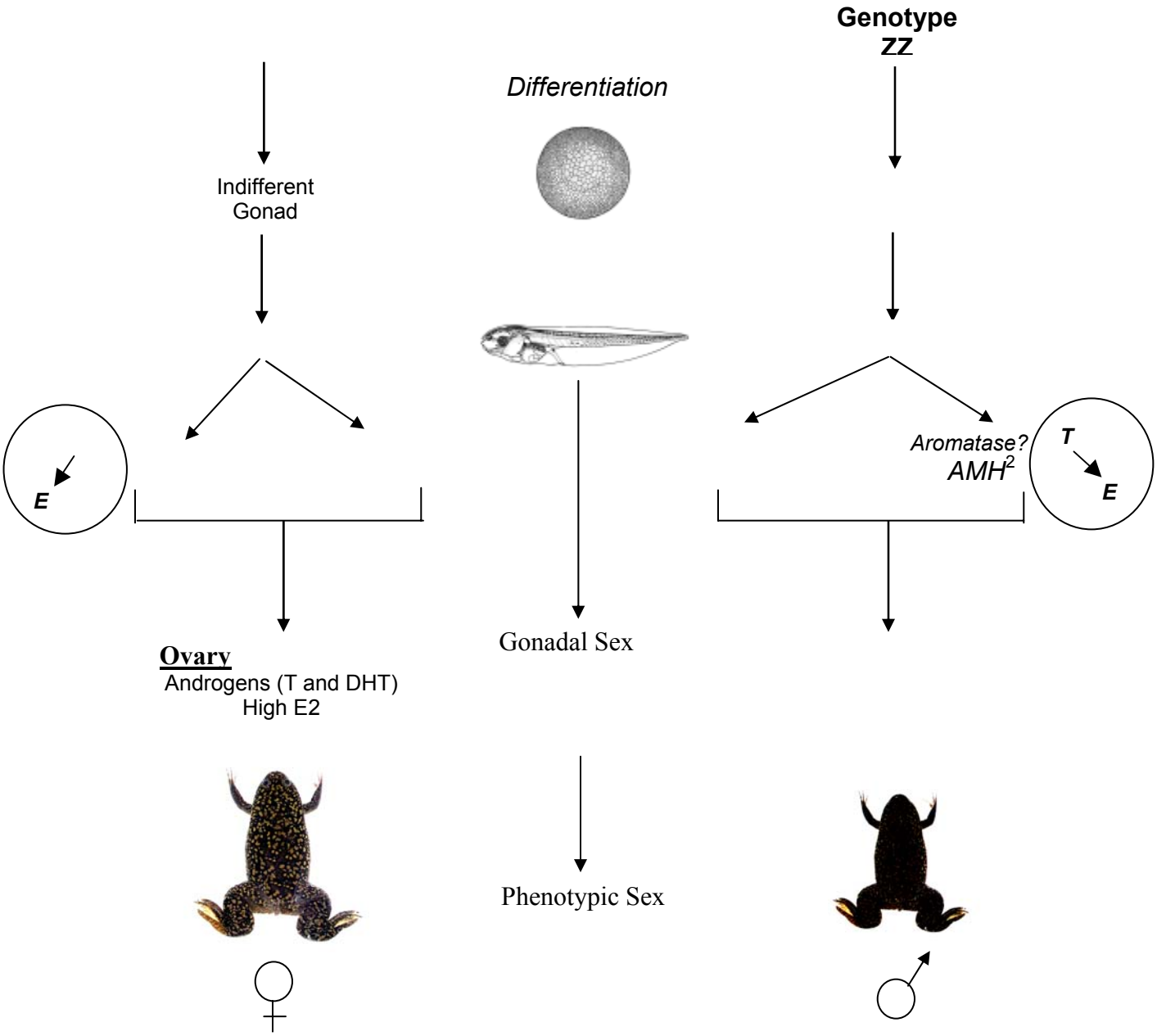
including TDF and SRY in humans and *Tdy* and *Sry* in mice. The *Sry* gene family, which includes *Sry*-like genes, collectively, are referred to as SOX (Lovell-Badge, 1993; Denny, 1992; Giese et al., 1992; Harley et al., 1992). Several members of the SOX gene family exist within *X. laevis*. However, based on the lack of female-specific copies of the SOX gene family in other species in which the female is the heterogametic sex, Griffiths (1991) determined that SOX gene family was not likely involved in the specification of ovarian development. Kelley (1996) suggested a potential role for the H-W antigen in the ovaries of ZW females (associated with the female phenotype), which is the homolog of the H-Y antigen found in several strains of mice and correlated with testis formation. In mammals, the specific role of the H-Y antigen in testis formation is not clear since testis determination and H-Y antigen expression can be separated and because other testis specifying genes have been isolated (Goldberg et al., 1971; Wachtel et al., 1975; Ohno et al., 1979; Goldberg et al., 1991). In *X. laevis*, the H-W antigen is specifically expressed in ZW females, but not in ZZ males. Sex-reversed males (phenotypic female) express the H-W antigen in the ovary. Kobel (1992) further determined that the quantity of H-W antigen produced was inversely related to the number of copies of the Z chromosomes, which is coincidently the probability of an animal expressing the female phenotype (Engel and Kobel, 1980). However, as is the case with the H-Y antigen in most mammals, it is not yet entirely clear whether the H-W antigen determines gonadal sex. Overall, the link between genetic sex and gonadal sex is becoming somewhat better understood in amphibians (Kelley, 1996; Hayes et al., 1998; Bogi et al., 2002).

Until recently, the role of endogenous steroids, particularly androgens, in determination of gonadal sex was not fully elucidated in amphibians. Although the ovary in adult *X. laevis* is the primary source of circulating E2, the developing gonad is not capable of producing estrogen and the capacity of other extra-gonadal tissue to produce estrogen at this period of development has not been fully addressed (Witschi et al., 1971; Iwasawa and Yamaguchi, 1984). Bogi et al. (2002) found that E2 and androgens may have maternal origins in amphibians with high concentrations maternal transferred to the oocytes and accumulated in early post-hatch larvae. These steroid levels dropped drastically during the onset of metamorphosis and at the conclusion of metamorphosis. In *Xenopus* species, as opposed to many reptilian species, temperature has no direct effect on sex ratios. Because of this, much of the studies evaluating the effects of endogenous estrogens on gonadal sex determination have been performed in reptilian species, such as turtles (Crews et al., 1989; Dorizzi et al., 1991). In the case of these species, anti-estrogens are capable of blocking the feminizing actions of specific temperature ranges (Wibbels and Crew, 1992). Since the ER is absence from developing gonads (Gahr et al., 1992), it would appear superficially that extra-gonadal tissues may be estrogen competent and responding by producing a feminizing factor that acts directly on the gonad to induce ovarian development. As observed in some of the more recent studies (Hayes et al., 2001; 2003; Carr et al., 2002) in *X. laevis*, aromatase inhibition could effectively produce the same consequence in the developing undetermined gonad. However, the relationship between intersexual development and aromatase inhibition has yet to be determined in *Xenopus* sp. In *X. laevis* masculinizing factors may play a role in gonadal development, similar to the anti-Mullerian hormone found in mammals that induces regression of the Mullerian ducts resulting in testicular development (Vigier et al., 1987, Kelley, 1996). The capacity of testicular grafts to confer the development of the testis can not be accomplished directly by androgen administration (Chang and Witschi, 1956; Gallien, 1962). Kelley (1996) developed a general model for the determination of gonadal sex in *X. laevis*

(Figure 3-3). In this model, Kelley (1996) suggested that ovarian development is the default pathway based on the assumption that the developing gonad is subject to circulating E2 levels regardless of genotypic sex. In genetic males, however, the presumptive testis produces a masculinizing factor that disrupts the default ovarian development, possibly through inhibition of aromatase. In a broader context, if this model is at least partially correct, EDCs, which disrupt the production or action of the masculinizing factor or act as aromatase inhibitors during gonadal determination, should skew gonadal development toward the male phenotype.

Based, in part, on the apparent "paradoxical literature" regarding the role of androgens in sexual differentiation (Witschi, 1971; Rastogi and Chieffi, 1975; Hayes, 1998; Wallace et al., 1999), Bogi et al. (2002, 2003) revisited the conceptual model described in the preceding paragraph by Kelley (1996) regarding sexual differentiation in amphibians and proposed a revised model based upon functional genomics (Figure 3-3). As originally discussed, the conceptual model proposed by Kelley (1996) and described by Hayes (1998) suggested that during primary sexual differentiation in amphibians, genetic sex is translated to gonadal sex during larval development. Following establish of gonadal sex steroids are produced which, in turn, induce secondary sexual development. The Bogi et al. (2002) model was based on two keys historical assertions. First, the female genotype may preferentially and systematically express aromatase at early larval stages in *Xenopus,* which would effectively convert T to E2 under normal conditions (Kelley, 1996). Second, stage-specific responsiveness to E2 exists during larval development in *X. laevis*. Based on the work of Hayes and Menendez (1998), Kloas et al. (1999), and Bogi et al. (2003), it is now generally accepted that estrogens consistently induce feminization. Villalpando and Merchant-Larios (1990) determined that stage-sensitivity in *X. laevis* suggested that the stage of exposure to E2 benzoate affected gonadal development in *X. laevis*, such that complete sex reversal of presumptive genetic males was induced when E2 as administered before migration of the primordial germ cells from the gonadal epithelium to medulallary region (ca. NF stages 44-50), ambiguous gonads were formed when treatment was initiated during NF stages 51-54, and normal testis differentiation occurred when E2 treatment was administered after the primordial germ cells had completed their translocation to the medulallary area (NF stage 55-56). Bogi et al. (2002) proposed that the functionality of steroids at the genomic level was directly linked to their respective receptors. The time course of ER and AR expression as measured by mRNA indicated that expression of both receptors began immediately following hatching. Both ER- and AR-mRNA increased rapidly until NF stage 50 at which time mRNA levels flatten and are relatively consistent until the conclusion of metamorphosis. Bogi et al. (2002) found that the marked increase in ER-mRNA between hatching and NF stage 50 is not as rapid as for AR-mRNA and ER-mRNA levels were greater in genotypic females. Little difference in AR-mRNA levels between genotypic male or female

Figure 3-3. Sexual Differentiation in *Xenopus laevis*[1]



[1] Modified from Kelley (1996) and Bogi et al. (2002).
[2] Kelley (1996).
[3] Bogi et al. (2002).

specimens were observed, which suggests that both receptors are regulated by different pathways, and that increased ER-mRNA expression relative to AR-mRNA expression may help confer estrogen sensitivity promoting feminization as the default gender. Bogi et al. (2002) also found that E2 was capable of more significantly auto-inducing both ER- and AR-mRNA compared to T which was weakly capable of inducing receptor expression.

Kloas et al. (1999) demonstrated that E2, the antiestorgen tamoxifen, T, MT, and DHT induced feminization, neutralization, no effect on sex ratio, and masculinization, respectively in *X. laevis*. The response observed with the androgens could potentially be attributed to aromatase activity, which effectively would convert T, but not MT and DHT, to E2. Maternally supplied T must be converted by 5α-reductase to DHT. Therefore, Bogi et al. (2002) suggested that genotypically controlled expression of 5α-reductase could be a means of testicular differentiation. On the contrary, the role of aromatase in female gonadal development is questionable based on several findings. First, substantial increase in aromatase expression was found near the end of larval development and treatment of *X. laevis* with aromatase inhibitors has not been shown to significantly alter sex ratio (Miyata and Kubo, 1999 and Miyashita et al., 2000). Bogi et al. (2002) suggested that increased expression of aromatase most likely occurs to late to influence female sexual differentiation based on the stage sensitivity findings of Vallalpando and Merchant-Larios, 1990). However, Bogi et al. (2002) have proposed an updated model presented in Figure 3-3. In this model, genetic females possess low 5α-reductase activity during primary sexual development and thus, ineffectively convert T to DHT producing a low DHT/E2 ratio. Greater levels of E2 resulting from metabolic conversion of T by aromatase than DHT confer ovarian development. In genetic males, greater 5α-reductase activity is proposed resulting in greater efficiency in conversion of T into DHT ultimately yielding a greater DHT/E2 ratio than found in females. The greater levels of DHT and relatively low levels of E2 confer testicular development. More work is needed to evaluate the time course of 5α-reductase expression during amphibian development and validate this conceptual model.

Secondary sexual development in *X. laevis* is appreciable better understood than many of the mechanisms of primary sexual development. As in most vertebrates, secondary sexual differentiation is controlled by gonadal steroids. Responsiveness of a tissue to gonadal steroids can be determined by following the expression of specific receptors. Further, continual secretion of gonadal steroids is required to maintain the secondary sexual characteristics. These patterns are closely followed in secondary structures, including the oviducts in females and the forelimb nuptial pads in males. Oviducts grow in response to estrogen and regress in the absence of estrogen (or in the case of ovariectomy). Clasping behavior and thickening of the nuptial pads are the result of a specific response to androgens. However, both are lost following castration. Some structures or behaviors do not present themselves simply because they appropriate steroids are not present. Alternatively, some characteristics can not be expressed in adults since the structure was lost during development. For example, the oviduct in developing males regresses presumably as the result of secretion of an "anti-Mullerian hormone". If castration is performed prior to this developmental process, the oviducts are retained. Generally, the determination of phenotypic sex is capable of proceeding to a point without gonadal influence. Further most species have a default phenotypic sex, female in mammals, male in birds, and female in *X. laevis*. Observations from the former two classes of vertebrate animals led to the assumption that the homogametic sex was the driven default (XX females in mammals and ZZ males in birds)

(Adkins, 1975). However, as previously discussed the male is the homogametic sex in *X. laevis*. Therefore, the homogametic sex is not necessarily the default phenotype in all vertebrates. As previously discussed, secondary ovarian development involves differentiation of the follicles and oocyte maturation. In *X. laevis*, oocytes are generally divided into six sequential stages ranging from stage I-III which are previtellogenic, stage IV in which vitellogenic growth occurs, stage V and VI in which final maturation and GVBD occurs in preparation for eventual ovulation and fertilization (Dumont, 1972). Further discussion of GVBD and induction by P and/or androgens will be provided later in this DRP. Although typically dictated by environmental conditions, female *X. laevis* become sexually mature between 12 and 24 months. In male X. laevis, spermatogenesis may occur as early as NF stage 59 (Nieuwkoop and Faber, 1994), although this finding has not been confirmed microscopically (Kelley, 1996). Witski (1971) identified spermatocytes two to three months post-metamorphosis. Production of C19 gonadal steroids occurs between stage 59-62 (Kelley and Dennison, 1990; Robertson et al., 1991; Kang et al., 1994).

The development of gonadal steroid response competence is initiated by TH. May and Knowland (1980) determined that the capacity of larvae to respond to estrogen with induction of the VTG gene begins at NF stage 62 and requires TH secretion. Kawahara et al. (1987) subsequently determined that TH did not directly induce the VTG gene or establish inducibility by estrogen, but rather produced a morphological change in the population of competent hepatocytes in the liver. Further study by Roberson and Kelly (1992) demonstrated that several male secondary sexual characteristics, including development of the larangeal morphology required TH sensitization for responsiveness to DHT. Further discussion of the role of TH in conferring gonadal steroid responsiveness during secondary sexual development in *X. laevis* is provided in DRP 4-7, "Thyroid Hormone Assays" (Battelle, in preparation). In short, TH does not appear to act directly on the gonads based on several lines of evidence. First, no TR exists in the gonad (Kawahara et al., 1991) at this stage of development. Second, TH is not required for continued sensitivity to gonadal steroids or secondary sexual development (Leloup and Buscaglia, 1977).

3.9 Anticipated Sites of EDC Impact

Based on the previous discussion, EDCs could potentially affect reproduction and growth (development) at six levels: 1) CNS, 2) hypothalamus, 3) pituitary, 4) thyroid gland, 5) interrenal gland, and 6) gonad. More specifically, specific modes of actions of reproductive and growth (advanced development) disruptors could potentially include alteration of neuroendocrine regulation of the various axes; tropic hormonal synthesis, release, and regulation; thyroid and interrenal axis pertubation; and interference with gonadal development and function, including interference with the HPG axis and aspects of feedback control. Bogi (2002) proposed that four principal endocrine activities should be considered including estrogenic, antiestrogenic, androgenic and anti-androgenic effects. Thyroid axis agonists and antagonists should also be included in this list of potential modes of activity. Overall, the effect at any of these levels is complicated by the inter-relationships between each level of control. The effect at the pituitary level is complex since it may involve gonadotropes (LH and FSH), thyrotropes (TSH), corticotropes (ACTH), and lactotropes (PRLs). In addition to the gonad, the thyroid gland and the interrenal gland may also be a site of EDC action, which could potentially impact advanced

development, growth, and reproduction. Thus, the impact of potential EDCs on reproduction and growth may occur at multiple different levels. In addition other physical environmental factors may also alter metamorphosis. Biochemical factors outside the thyroid axis, such as the corticotropes, may also affect metamorphosis. The complexity of reproduction and growth; as well as, control by the neuroendocrine system and associated axes must be strongly considered in the design of appropriate test methods. Since the objective of the test method is to provide a test for reproductive dysfunction and impact on advanced development and growth, a thorough, definitive testing will be more advantageous. It is likely that incorporation of high throughput biochemical or molecular assays within a morphologically based method will be advantageous. It is crucial that the methodology used demonstrate diagnostic power by distinguishing between endocrine-based effects on reproduction and growth and non-specific effects on these processes.

4.0 CULTURE AND HANDLING OF TEST SPECIES

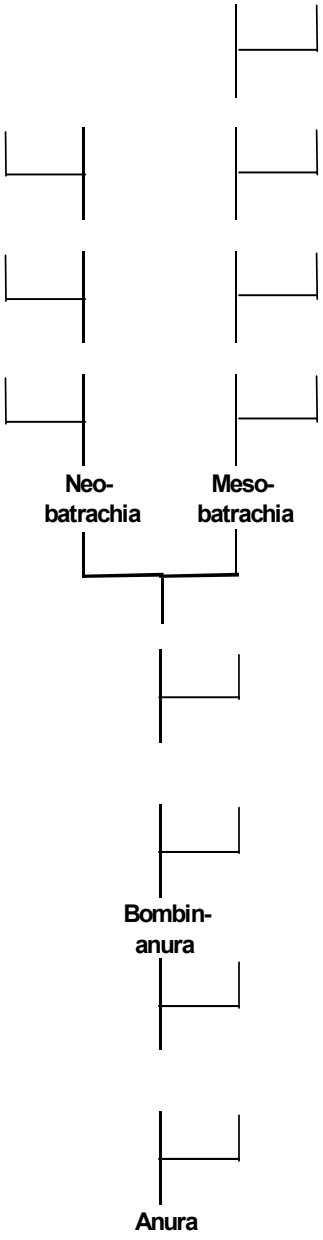
4.1 Anurans

4.1.1 Pipids

4.1.1.1 ***Xenopus laevis***. The primitive family pipidae of the order Anura consists of only three genera, two of which are native to Africa and one native to South America. *Xenopus* fossils have been dated back to the Cretaceous period. Pipidae are characterized as being purely aquatic and having tongues completely attached to the floor of the mouth. The phylogenetic relationship between the species discussed in this section are described in Figure 4-1. The South African clawed frog (*X. laevis*) represents the most widely distributed species of the fourteen species in the Genus *Xenopus*. *X. laevis* is native to Africa south of the Sahara desert. Their natural habitat in Africa primary includes murky ponds and puddles. *X. laevis* is known to move from pond to pond during the rainy season and is capable of burrowing in the mud for several months at a time during droughts.

Adult *X. laevis* males and females are ca. 5 to10 cm and 10 to 15 cm in length, respectively. Metamorphic aged tadpoles are approximately 2 to 3 cm in length. *X. laevis* is sexually dimorphic. Sexually mature females are substantially larger than males of the same general age. In addition, females possess an enlarged cloaca from which eggs emanate during breeding. Males possess thick black nuptial pads on their forearms. Cytogenetically, *X. laevis* is oligotetraploid.

Figure 4-1. Phylogenetic Relationships Between *Xenopus* and *Rana*[1]



[1] Based on Ford et al. (1993).

Adult *Xenopus*, embryos, and tadpoles can be obtained from several commercial vendors who specialize in the rearing and distribution of these frogs. To block outdoor lighting cues, *Xenopus* are best maintained in rooms with no external light sources using a 12 hour laboratory light/dark cycle. Adults are housed separately by sex in tanks in either a static or flow-through system. Since *Xenopus* live naturally in static environments, care is required when using flow-through culture systems so that the flow does not disturb the frogs. Grates cover the tanks to prevent the frogs from jumping out of the aquaria. Generally, a minimum water depth of 5 to10 cm is required. At this depth, the optimal number of adults that could be housed together is four per 5 to10 L of water. Prior to use, adult culture water is dechlorinated using activated carbon filters. Standard measures of water quality are routinely checked to ensure adequacy and consistency and include temperature (18 to 20°C), pH, alkalinity, hardness, specific conductance, residual chlorine, ammonia, heavy metals, and total organic carbon. Several diets have been used to culture *Xenopus* adults including ground beef liver, frog brittle (Nasco, Ft. Atkinson, WI), trout starter, and salmon starter diet (Zeigler Brothers, Inc., Gardners, PA). A comparison of the nutritional breakdown of each standardized diet is provided in Table 4-1. Although beef liver is the classic diet for *Xenopus*, the salmon or trout starter diets have received more attention lately because it is a standardized diet, and the frogs consume it similar to the liver. Adults should be fed at least three times per week. Within 2-4 hours after feeding the culture tanks should be cleaned to remove uneaten and regurgitated food. Each of the diets proposed for use are relatively high in protein with fish meal contributing the primary source of protein. Soy meal is present in a relatively low quantity in each diet. The influence of soy products that potentially contain phytoestrogens will need to be examined further (Kupferberg, 1997). However, years of culture data documenting normalcy in development and growth, sexual maturation, sex ratios, and reproductive performance supports the use of any one of these diets (Fort, personal communications). Although low soy diets are not generally available for amphibians, customized diets may be obtained from several different vendors.

Adult *X. laevis* are susceptible to several diseases in culture. The most noteworthy diseases are the skin invading capillarid, *Capillaria xenopodus*, and the bacteria *Aeromonus* (red leg). Capillarids include parasitic round worms, including nematodes (Tinsley, 1996). Adults may be treated with ivermectin or tetracycline for these diseases. Ivermectin should be administered by sc injection into the dorsal lymph sac at a dose of ca. 2 g/g body weight once per week until the infection has been treated. Tetracycline may be administered once or twice daily for 7d by either parenteral (sc) or enteral routes (intubation). In either case, a dose of ca. 0.2 mg/g body weight has been relatively effective in controlling early stages of bacterial infections (Bantle et al., 1998).

Breeding of the adult frogs is induced by injecting commercially obtained human chorionic gonadotropin (hCG) into the dorsal lymph sac. Injecting the frogs with two doses of hCG ranging 1 to 5 hours apart is performed. Male frogs receive 500 to 800 U (two equal injections) and the female frogs receive 750 to 1,000 U (primer dose of ca. 200 U in first injection) hCG injected into the dorsal lymph sac. Once the injections are complete the frogs are placed in a breeding tank with a false bottom to allow collection of the deposited embryos. Frogs may be bred in dechlorinated tap water or Frog Embryo Teratogenesis Assay – *Xenopus* (FETAX) Solution, a reconstituted water medium for the culture of *Xenopus*. *X. laevis* is capable of producing 1,500 to 2,000 fertilized embryos per breeding (Dawson et al., 1992; ASTM, 1998;

Bantle et al., 1998). Adults that have been bred require a one to two month refractory period. Females are typically productive in the lab for 2 to 3 years, whereas the males are productive for 3 to 5 years under normal healthy conditions.

Embryos are collected by gently flushing them from the bottom of the breeding tank. Samples of the embryos can be removed and examined under a dissecting microscope to ensure fertilization indicated by cleavage and the formation of blastomers. The remaining organisms are placed in a flask or beaker for sorting. To ease in handling, some investigators use 2% (w/v) cysteine (pH 8.1) to remove the jelly coat. Embryos and tadpoles are reared in dechlorinated tap water or FETAX Solution (see Section 10.1.1). After the embryos have reached stage 47 (ca. 4.5 d), feeding is required. Strained baby food green beans or peas, boiled Romaine lettuce (organically grown), a well-mixed slurry of salmon or trout starter (Zeigler Bros., Inc., Gardners, PA), or a blended mixture of TetraFin (Tetra Sales, Blacksburg, VA,), *Spirulina* algae discs (The Wardley Corporation, Secaucus, N.J.), Silver Cup Trout Starter (Nelson & Sons Inc., Murray Utah), along with live brine shrimp (Bio-Marine Brand, Bio-Marine Inc., Hawthorne CA) is used to feed the developing tadpoles. Generally, ca. 250 mg food per tadpole daily is provided in a slurry. As tadpoles reach metamorphosis, the rate of feeding is increased. Feeding is temporarily terminated during metamorphic climax when tail resorption is occurring, as food is not required during this stage. For static cultures, only partial replacement of culture water, including excess food material, is recommended, especially during metamorphosis, as the larvae become sensitive to more drastic environmental changes. Flow-through culture systems may also be used effectively to culture tadpoles and may be more effective in controlling buildup of waste products. Care must be taken with flow-through systems that adequate feeding time is maintained and food is not substantially lost. These factors must be evaluated on a case-by-case basis, depending on the rate of system flow. Essentially, feeding practices should be as close to ad libitum as possible, which can be determined by the presence or absence of feeding behavior. A comparison of nutritional constitution of standardized *Xenopus* diets is provided in Table 4-1. Measurement of developmental rates should be performed and generally follow the time frame established by Nieuwkoop and Faber (1994). Culture densities are a critical factor in successfully raising tadpoles through metamorphosis in a normal period of time (2 to 3 months). Generally, tadpoles grow faster and more successfully in small groups. The ideal density is 2-3 metamorphic-age tadpoles per 500 mL per culture water. Since this is often not practicable, slightly greater densities can be used during earlier development, followed by segregation of the tadpoles by stage into smaller groups at a later stage of development prior to metamorphosis. *Xenopus* larvae are transparent until metamorphosis is complete which allows visualization of the developing and changing organ systems.

4.1.1.2 ***Xenopus tropicalis***. X. (Silu*rana*) *tropicalis* is a close relative to *X. laevis* and resides naturally in the southern tip of Africa. Compared to *X. laevis*, which has been studied for over 100 years, use of *X. tropicalis* in research has been fairly recent. However, two primary aspects of *X. tropicalis* development have attracted researchers: a diploid genome, and a relatively short life cycle. The life cycle for *X. tropicalis* is roughly 4 to 5 months, whereas, the life cycle in *X. laevis* can run from 1.5 to 2 years. *X. tropicalis* is capable of producing 1,500 to 3,000 fertilized embryos per breeding. Because of the closeness in the family relationship between these two *Xenopus* species, many techniques of animal husbandry, breeding, and

Table 4-1. Comparison of Nutritional Constitution of Standardized *Xenopus* Diets

Diet	Nutritional Breakdown (%)					
	Protein[2]	Fat	Fiber	Moisture	Ash	Soy[3]
Salmon Starter[4]	≥55.0	≥15.0	≤1.0	≤12.0	≤10.0	2.8-5.5
Frog Brittle[5]	44.2	6.0	2.0	NR	11.0	8.4-8.8
Tetra Fin[6]	≥42.0	8.0	2.0	6.5	NR	NA
Trout Starter[3]	≥50.0	≥15.0	≤1.0	≤12.0	≤10.0	2.5-5.0

NR = Not reported.
NA = Not available or proprietary information.

[1] Major source of protein = pesticide free fish meal.

[2] Expressed as % of total protein.

[3] Ziegler Brothers, Gardners, PA. Ingredients: Fish meal, dehulled soybean meal, corn gluten meal, fish oil, wheat flour, blood meal, brewers dried yeast, soy lecithin, yeast culture, vitamin A acetate, vitamin D3 supplement, dl-alpha tocopheryl acetate (vitamin E supplement), vitamin B12 supplement, riboflavin supplement, niacin, calcium pantothenate, menadione sodium bisulfite complex (source of vitamin K activity), folic acid, thiamine mononitrate, pyridoxine hydrochloride, biotin, choline chloride, manganese, proteinate, zinc proteinate, copper proteinate, calcium iodate, iron proteinate, cobalt proteinate, calcium carbonate, sodium selenite, L-ascorbyl-2-polyphosphate (source of vitamin C), ethoxyquin.

[4] NASCO, Fort Atkinson, WI. Ingredients: Fish meal, meat meal, soybean meal, corn meal, wheat flour, dried yeast, distillers solubles, whey, wheat germ meal, salt, diacalcium phosphate. Vitamin supplements: Vitamin A (14,000 IU/Kg), vitamin D (5,000 IU/Kg), vitamin E (88 IU/Kg), vitamin B12 (0.04 mg/Kg), folic acid 1.5 mg/Kg), thiamine (4.0 mg/Kg), riboflavin (9.0 mg/Kg), pantothenote acid (11.0 mg/Kg), niacin (60.0 mg/Kg), choline (1,550 mg/Kg), calcium (2.0%), phosphorus (1.6%), sodium chloride (1.2%), magnesium (2.0%), linoleic acid, (2.8%), iron (300 mg/Kg), copper (10.0 mg/Kg), cobalt (2.2 mg/Kg), manganese (9.0 mg/Kg), zinc (100 mg/Kg), iodine (48.0 mg/Kg), pyridoxine (8.8 mg/Kg).

[5] Tetra, Blackburg, VA. Ingredients: Fish meal, ground brown rice, dried yeast, shrimp meal, feeding oat meal, wheat gluten, soybean oil, fish oil, corn gluten, algae meal, sorbitol, potato protein, lecithin, ascorbic acid, inositol, niacin, 1-ascorbyl-2-polyphosphate (vitamin C), A-tocopherol-acetate (source of vitamin E), d-calcium pantothenate, riboflavin-5-phosphate, thiamin, mononitrate, menadione sodium bisulfite complex, folic acid, pyridoxine hydrochloride, vitamin A, cyanocobalamin, cholecalciferol, manganese sulfate, zinc sulfate, ferrous sulfate, cobalt sulfate, artificial colors, ethoxyquin and citric acid as preservatives.

tadpole rearing are similar. Further, many of the molecular probes developed historically for *X. laevis* can be also used with X. tropicalis. Cytogenetically, *X. laevis* is tetraploid, containing duplicated gene copies of which many are non-functional. This cytogenetic organization complicates creating transgenic lines and analyzing gene regulation. *X. tropicalis* utilizes a smaller diploid genome comprised of twenty chromosomes, with about 1.7×10^9 bp, compared to *X. laevis* which has thirty-six chromosomes with ca. 3.1×10^9 bp. *X. tropicalis* is the only diploid species in the *Xenopus* genus and a re-evaluation of morphological data and molecular evidence has conclusively shown that *X. tropicalis* is monophyletic with the rest of the *Xenopus* family. The remainder of this section will be devoted to a discussion of fundamental differences between the two *Xenopus* species in culturing techniques.

Grainger et al. (2000) have identified three critical factors in successful *X. tropicalis* husbandry: diet, density, and temperature. Salmon pellets as described for *X. laevis*, except smaller in size (1/32") serve as a standardized diet, although live food, including blackworms can be used in combination with the salmon diet. The recommended density for *X. tropicalis* is one frog per liter of water. Froglets and adults are cultured at temperatures of roughly 24 to 25EC. Prolonged exposure to water temperatures below 22EC may cause an increased susceptibility to disease. Recent findings by Reed et al. (2000) have isolated both chytrid and *Chlamydia pneumoniae* fungal infections in *X. tropicalis*. The former pathogen has been identified in many native anuran species and represents a serious problem for laboratory cultures.

X. tropicalis do not display the extreme sexual dimorphism observed in *X. laevis*. Thus sexing *X. tropicalis* can be challenging. Several criteria can be used to sex these frogs including 1) cloacal protrusion, 2) body shape, 3) body size, and 4) nuptial pads. However, none of the characteristics are inherently obvious. Breeding is induced by hCG using a small primer dose of ca. 15 U 1 to 2 days prior to mating followed by a final injection of 100 U 3 to 4 hours prior to mating. Although the embryos are smaller in size, they are collected and treated similarly to methods used with *X. laevis*. Removal of the jelly coat typically requires a 1 min. incubation in 2% (w/v) cysteine (pH 8.1). Since *X. tropicalis* generally require lower salt concentrations in their media than do *X. laevis*, Grainger et al. (2000) recommend the use of 1/9 modified Barth's saline (MBS). 100% MBS consists of 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 15 mM Hepes NaOH (pH adjusted to 7.6), 0.3 mM $Ca(Na_3)_2$, 0.41 mM $CaCl_2$, and 0.82 mM $MgSO_4$ per L of solution. After several days it is recommended that the MBS be diluted 1:20, and the tadpoles finally placed into water with no additional salt supplementation. FETAX Solution is also effective in culturing *X. tropicalis* embryos and tadpoles (Song et al., 2003; Fort et al., in press).

Raising tadpoles appears to be the most challenging aspect of *X. tropicalis* husbandry. Minor changes in temperature, salinity, or pH can rapidly kill large quantities of growing tadpoles. Grainger et al. (2000) recommend an initial tadpole density of 12 tadpoles/L. Partial removal and full renewal practices effectively double the water volume, and thus decrease the culture density. Tadpoles are fed four times daily with small amounts of SERA Micron (Sera USA, Poulsbo, WA).

4.1.2 Ranids

4.1.2.1 *Rana pipiens*. The Northern Leopard Frog (*R. pipiens*) is native to a large portion of North America, including Canada (Canada's Aquatic Environments, 2002; Northern Leopard Frog, *R. pipiens* , 2002). The only regions within the U.S. where *R. pipiens* is not located are in the southeastern, south-central, and western portions of the country. *R. pipiens* is found in marshes, meadows, and ponds in the U.S. and Canada. Adult *R. pipiens* often migrate far from water; however, they must return to water to breed. Breeding season ranges from mid-March to the first part of April. Egg masses may be as large as 6,000. In the laboratory, metamorphosis is complete within 3-4 months post-fertilization, and sexual maturity is achieved in roughly 2 years (Duellman and Trueb, 1994).

Adult *R. pipiens* may be maintained in the laboratory under appropriate conditions (Ankley et al., 1998b). Unlike the pipidae, which are purely aquatic, a protected outdoor aquatic and terrestrial environment needs to be provided for longer-term cultures of ranid species (>4 weeks). Short-term adult cultures (<4 weeks) can be maintained in smaller exposure chambers. Small enclosures (ca. 1 or 2 m^2) containing a small tub of water and moistened sphagnum moss can be used to house adult *R. pipiens*. Alternatively, a stainless steel culture rack in which the housing chambers slide in and out of the rack can be used. Since ranids feed primarily on living food, their diet consists of crickets, larval flies, or black worms. Food should be provided daily at an amount that is commensurate with consumption by the specimen. Fresh water should also be provided daily. Sexual dimorphism is not readily apparent in *R. pipiens*, thus other factors including gravidity should also be considered. The primary dimorphic characteristic is the length of the second toe of the forelimb.

Breeding *R. pipiens* currently requires artificial fertilization techniques, although methods for laboratory simulated natural breeding are under investigation (Ankley et al., 1998b). Artificial fertilization requires the injection of a large dose of female pituitary extract or fresh reconstituted pituitary into the dorsal lymph sac of the female a day or two prior to artificial fertilization. Some investigators have used LHRH to induce super-ovulation, but in general, the use of pituitary extract has been most successful. Males are sacrificed immediately prior to the artificial fertilization, and the testes are quickly removed, minced, and homogenized to produce a concentrated sperm solution. Eggs can be stripped from the properly prepared female, by gently squeezing the specimen along the dorsal flanks in an anterior-posterior direction. The stripped eggs are placed directly into a glass Petri dish and the concentrated sperm solution is poured over the eggs. Fertilization is monitored, and the developing embryos are separated into different test dishes. Newer techniques are currently being developed and evaluated which involve the use of simulated forced hibernation. These techniques involve the use of temperature-controlled incubators which drop the temperature over a set period of time to induce hibernation in the winter (over-wintering) for a predetermined amount of time (ca. 30 days), followed by a ramp of increasing temperatures to simulate the onset of Spring or breeding season. Although hormonal treatment including dopamine (prior to over-wintering) and LHRH (immediately prior to breeding) are required, this process is designed to induce amplexus. Thus, artificial fertilization is not required by this technique. More work will be required to fully demonstrate this process and standardize the methods.

R. pipiens embryos develop slower than their pipid counterparts. Developing embryos (unhatched) are protected by a thick jelly coat and do not hatch for 7-10 days. Embryos are cultured in spring water or dechlorinated tap water with the optimal pH near 8 at a temperature of 18-20< C. Larvae are generally fed an algal diet, although the larval diets previously described for the other anuran species have also been used. Developing ranid larvae can be maintained in either static or flow-through systems although close attention to water quality is required.

4.1.3 Hyperoliids

4.1.3.1 *Hyperolius sp.* Native to Kenya, *Hyperolius* sp., commonly referred to as the reed frog, is unique in that it undergoes ontogenic color change during juvenile maturation (Hayes, 1997b). Generally, ontogenic color change in maturing anurans and sexually-based dichromaticism is unusual in most anuran species. Male and female *H. argus* metamorphose with a bright green dorsum. However, females ultimately develop brownish dorsal coloration with white spots (Hayes, 1997b). Mature males are also distinguished by the development of gular pouches.

In accordance with the work of Hayes (1997b), *Hyperolius* can be maintained in captivity. However, currently there are no commercial cultures of *Hyperolius* are available to the scientific community, which represents a significant disadvantage. It might be possible to directly acquire this species from Africa by a commercial source, which currently imports other anuran species from Africa. In accordance with husbandry methods established by Hayes (1997b) and Hayes and Menendez (1999), adults are maintained in groups of ten per chamber (five males and five females) on a reverse 12-hour light cycle. Adults are typically fed crickets dusted with vitamins and calcium similar to that used for other terrestrial anuran species, including ranids. *Hyperolius* spontaneously breed approximately every two weeks (Hayes and Menendez, 1999). In the laboratory of Hayes (Hayes, 1997b), larvae are raised in 10% Holtfreter's solution (Holtfreter, 1931), at a density of 30 tadpoles per 5 L of solution. 100% Holtfreter's solution consists of 3.5 g NaCl, 0.2 g $NaHCO_3$, 0.05 g KCl, 0.2 g $MgSO_4$@7 H_2O, and 0.1 g $CaCl_2$ per L of solution adjusted to pH 7.0-7.5. Tadpoles' food includes boiled spinach. It is recommended that the culture water is changed daily. The water temperature is maintained at 27°C under a 12-hour photoperiod cycle.

4.2 Urodeles

The urodeles (e.g., salamanders, newts, axolotls) are indigenous to many locations in North America and other locations across the globe. Breeding season for the spotted and tiger salamander (*Ambystoma maculatum* and *A. tigrinum*) is typically in January and February in the warmer regions and March and April in the colder regions (Carolina Biological Supply Company, 1993; Canada's Aquatic Environments, 2002). *A. maculatum* and *A. tigrinum* egg masses rarely contain greater than 100 to 200 embryos. These eggs are large (ca. 2.5 to 3 mm) for amphibian eggs and are protected by a massive jelly coat. Hatching typically requires 3 to 4 weeks with metamorphosis occurring in about three months. Other than the resorption of external gills, morphological changes during metamorphosis in urodeles are reasonably minor. Following metamorphosis, *Ambystoma* initiates a terrestrial lifestyle and returns to the water 1 to

two years later to breed. Newts utilize a similar life strategy, although reproductively mature newts that return to water from terrestrial life often remain aquatic for the remainder of their lives. Some urodeles, such as *Necturus*, are neotenic (obligatory) and retain their larval characteristics throughout adulthood.

Little reasonably standardized information is currently available on urodele husbandry, breeding, and culture. Studies using these species have been conducted using the techniques described for the anuran species (Caldwell, et al., 1980; Petranka and Sih, 1987). Adult terrestrial salamanders require cool temperatures (18-20°C) and high humidity, which may be provided in a terrestrial aquarium (Caldwell, et al., 1980; Petranka and Sih, 1987). A water source and daily misting of the natural substrates (leaves, rocks, moss, small tree branch) maintain high humidity. Salamanders are fed mealworms, crickets, whiteworms, earthworms, and some soft-bodied insects. Unlike frogs, salamanders consume water orally and require fresh drinking water. Embryos and larvae can be cultured in spring water, pond water, or dechlorinated tap water using partial renewal techniques. Larvae are fed the same food described for the anuran species. Although less information is available on the culture requirements for urodeles, it is necessary to evaluate this order of amphibians, since urodeles represents the "non-frog" amphibians.

4.3 Strengths and Weaknesses of the Test Species

In summary, *Xenopus sp.* represent a significant advantage in the wealth of information available ranging from culturing to molecular biology, and their ease of use in the laboratory. Its ubiquity in use and acceptance in the scientific research community as a model for development makes it an excellent candidate for the AGRAs. It should be noted, however, that substantially more information is available for *X. laevis* than for *X. tropicalis*. The primary advantages of using *X. tropicalis* lie primarily in the potential for developing transgenic lines (see section 4.1.1.2), the rate of development, and the robust developmental kinetics. Of these attributes, the rate at which metamorphosis and sexual maturity is achieved is most distinct from the standpoint of chronic and/or life cycle test method development However, it should be noted that from a practical standpoint, the smaller egg size of *X. tropicalis* imparts greater difficulty in creation of founder transgenic animals (F_0) than in *X. laevis*. Rowe et al. (2002) suggest that *X. tropicalis* display extremely consistent kinetics associated with metamorphic change. On the contrary, *X. laevis* show greater kinetic variability. Since the objective of the assay is to screen for disruption of growth and reproductive process through pertubation of the endocrine system, the origin of the species is not a primary factor in selection. Rather, it is most important that the species serve as a representative vertebrate model. Next to *Xenopus sp*., there is some literature available on the native American *Rana sp*. developmental processes and culturing practices. However, the use of *Rana* in the laboratory (husbandry, breeding, and rearing tadpoles) is more difficult than for *Xenopus*. The most literature available on reproduction and growth is for *R. catesbeiana* (bullfrog). However, use of *R. catesbeiana* in the laboratory for reproduction and growth studies is impractical due to the long length of development, thus warranting the consideration of *R. pipiens*. However, evaluation of reproduction and growth in both species is difficult in the laboratory. The use of *Hyperolius sp*. as described by Hayes (1997b), represents an apparent advantage in the ease of use and external visualization of endpoints. It also integrates an evaluation of potential effects on the thyroid with the expression of primary and secondary

sexual characteristics that may also be influenced by gonadal steroids. However, little is known about this genus in the toxicology field, including sensitivity, repeatability, and versatility, as well as the similarities in reproductive and growth processes to the other anurans.

The only potential advantage to the inclusion of the urodeles sp. to the list of potential candidate species for the AGRA, are that they represent a "non-frog" species and some differences in sensitivities may exist between salamander and frogs. However, little literature is available on the use of urodeles, and they are generally more difficult to work with in the laboratory. A description of the general strengths and weaknesses of each species is provided in Table 4-2.

5.0 EXPERIMENTAL DESIGN CONSIDERATIONS FOR AMPHIBIAN METAMORPHOSIS ASSAYS

5.1 Exposure Period

The primary objectives of the present DRP is identify an assay approach or approaches that will a suite of amphibian reproductive toxicity and growth endpoints using one of the model species previous described as a model species for use as a T2T. It will be advantageous to incorporate endpoints including, morphological, histological, and biochemical components into a primary model system. In which the capacity for short-term complementary, or spin-off assays, can be effectively utilized. Within the context of growth, development and maturation will be primarily considered since separate assay are being developed to monitor effects specifically on metamorphosis (see DRP 2-20 [4-5], "Amphibian Metamorphosis Assays" [Battelle, 2003]). Generally, the maintenance of longer-term exposures is costly and can result in unexpected interruptions in exposure as a result of test-substance behavior in water or equipment malfunction. Chemical analysis of the exposure solutions and cleaning the exposure system to maintain high dissolved oxygen concentrations, especially in the presence of organic solvents used as carriers, add significantly to the time and effort in maintaining a long-term exposure. Therefore, the exposure duration of a study needs to encompass an appropriate time of exposure necessary to elicit an effect, but not beyond this necessary time frame, thereby controlling costs and potential exposure interruptions. In the case of the AGRA, several options are available including: 1) a suite of partial life cycle tests evaluating specific periods of development, including sexual maturation; 2) an adult exposure protocol evaluating adult reproductive fitness and reproductive outcome, 3) long-term chronic exposure model spanning early embryo-larval development to various points post-metamorphosis, including completion of primary sexual development or secondary sexual development (reproductive maturity); and 4) multi-generational evaluation in which exposure is initiated at an early embryo-larval stage and completed following breeding of the exposed individuals and evaluation of the fecundity of the F1 progeny.

Table 4-2. Comparative Larval Anuran Stages

Specie Stages			Morphological Landmarks	Metamorphic Event[4]
X. laevis[1]	*R. pipiens*[2]	Anuran[3]		
46	I	26	Limb Bud Growth	Premetamorphosis
47/48	II	27		
49/50	III	28		
51	IV	29		
52	V	30		
53	VI	31		
53	VII	32		
53	VIII	33		
54	IX	34	Digit Differentiation	Prometamorphosis
55	X	35		
55	XI	36		
55	XII	37		
56	XIII	38		
57	XIV	39		
57	XV	40		
58	XVI	40	Rapid Hind Limb Growth	Climax
59	XVII	40	and	
60	XVIII	41	Tail Resorption	
60	XIX	41		
61	XX	41		
62	XXI	42		
63	XXII	43		
64	XXIII	44		
65	XXIV	45		
66	XXV	46		

[1] Nieuwkoop and Faber (1994).
[2] Taylor and Kollros (1946).
[3] Gosner (1960).
[4] Dodd and Dodd (1976).

5.1.1 Partial Life Cycle

The length of each assay described above increases in length in the order presented.. The short-term battery approach represent the shortest assays which could range from several d to one month. However, this approach would require several different assays with a test battery to provided the desired information which ultimately increase the length of time need to complete an evaluation. Partial life cycle tests evaluating short-term embryo-larval development; metamorphic-based growth, including limb development; primary sexual development; secondary sexual development; and adult reproduction could potentially be utilized separately or as a battery of assays. The advantages of such an approach is the ability to focus on a certain aspect of the life cycle in a cafeteria-like format, and the potentially the time- and cost-effectiveness of the tests. The disadvantage is the ability to piece the battery data together to form a basis for interpreting the results. This process also requires the selection of which phases of the life cycle need to be incorporated in the test battery. Thus, incongruity within the data sets can make interpretation difficult.

5.1.2 Full Life Cycle

Overall, the practicability of using a full life cycle approach with many amphibian species is relatively low based on the length of the life cycle and difficulty of long-term culture in the laboratory. As will be described in the following sections, few anuran species could effectively be used in a life cycle test format. The primary advantage of a continuous chronic exposure model is that it provides access to development, growth, and reproductive endpoints in one continuous process. Thus, it is possible that the connection between development, growth, and reproductive effects can be elucidated. Further, evaluation of effects in subsequent generations in a multi-generation format may be possible. However, a multi-generational assay format would only be practicable with a rapidly developing and maturing species, such as *X. tropicalis*. In the case of *X. tropicalis*, metamorphosis (completion of primary sexual development) and reproductive maturity can be effectively reached in approximately 35 and 150 d, respectively. Within the context of a multi-generational test format, a continuous breeding design could be considered to evaluate effects on reproduction and the developing progenies.

5.2 Route of Administration

5.2.1 Water

Water exposure is the most common route to expose larval, metamorphic, and aquatic-dwelling amphibians to EDCs. The delivery of a toxicant in water at different concentrations is reasonably well established for most aquatic test species, although considerations for static-renewal and flow-through exposures must be made. Although many amphibians, including those previously discussed in this review paper, prefer a static environment (Dawson et al., 1992), the difficulties associated with longer-term static renewal exposure studies necessitate the use of a flow-through design (ASTM, 1998; Ankley et al., 1998b; Kloas et al., 1999). Other than the cost of maintaining a long-term static renewal exposure study, the primary problem associated with the static-renewal approach is maintaining a consistent aqueous concentration during the study. Flow-through exposure systems, using a variety of mechanical approaches, have been successfully used by many laboratories (Greenhouse, 1976; Benoit et al., 1981). The greatest

challenge in an aqueous exposure system is solubilizing the test substance in water. Organic solvents are often used as co-solvents to assist in delivering a test substance into water, which requires the maintenance of a solvent control. Organic solvents also enhance bacterial growth in the test system, which increases maintenance time during the exposure. Organic solvents may also act interactively or non-interactively with the toxicant to increase (additivity, synergism or potentiation) or decrease (antagonism) its effect. In some cases, saturator columns have been used to eliminate the use of solvents.

5.2.2 Oral (Food)

Patyna et al. (1999) recommends that hydrophobic compounds with log P values >5 should be administered via food. Although dosing adult amphibians via food has been accomplished, little work has been performed using this route of administration in tadpoles (Fort et al., 2001a). Dosing the commercial salmon diet is practicable, although homogenization of the food after spiking the test substance is challenging and obtaining a homogeneous mixture is often quite difficult. Dosing live food items, such as worms, has been performed, but it is also quite difficult to obtain a consistent diet and differentiate between effects from the diet and effects from toxicants that leach from the diet into the culture water (Fort et al., 2001a). In a large-scale testing program like EDSP, oral dosing is probably not as practicable as aqueous exposure, unless required due to limitation in aqueous solubility.

5.2.3 Parenteral

Intramuscular or intravenous administration of a toxicant in a premetamorphic tadpole (stage 47-48) is possible. The technique is technically difficult and the environmental relevance is questionable since it is difficult to determine or estimate effective environmental concentrations. However, parenteral dosing (sc, im, or iv) of post-metamorphic specimens and adults is a potentially practicable method of dosing (Fort et al, 2000). Thus, selection of this route of administration would be based on the age of the organism used and the study format. In the case of a life cycle-type format, a parenteral route of exposure would be counter-productive.

5.3 Dose Selection

Dose levels for AGRA should be selected with the use of range-finding data, unless other reliable data are readily available. Selected concentrations should be less than lethal levels and no greater than the maximum water soluble concentration. Exposure concentrations should be measured no less than weekly during the exposure under flow-through conditions. Under static conditions analysis should be performed no less than every 48 h. However, the exposure method and rate of the test substance analysis will be dependent on the degradation rate of the substance. Test substances with rapid degradation rates will require flow-through exposure with more frequent analysis of test substance concentration. Since test substance concentrations tend to decrease over time in mature culture environments, careful attention to maintain a consistent concentration will also be required.

For screening purposes, at least three widely spaced concentration levels would be appropriate depending on the assay endpoints considered. The treatment levels can be separated

by up to an order of magnitude. It is not necessary for a partial life cycle study to have a treatment level that causes no effect. A definitive AGRA study should be conducted with at least four treatment levels, and the treatment levels should be separated by approximately a factor of two. At least one of the treatment levels should be below the no-observed adverse effect concentration (NOAEC). Concentrations should be selected to produce an adequate concentration-response curve for the endpoints measured during the study.

5.4 Stages of Exposure

As previously indicated, partial life cycle tests could effectively evaluate early life stages, metamorphic stages, as well as, reproductively mature adults. However, in the case of a life cycle-based approach, organisms could be exposed as embryos (i.e., NF stage 8) through the completion of primary sexual development (metamorphosis) (post-NF stage 66), or to sexual maturity (approximately 150 d in *X. tropicalis*). In the case of a multi-generational evaluation, the progeny would most likely not be exposed, but examined at least through early larval development (i.e., NF stage 46+).

5.5 Statistical Considerations

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

Design-associated variability can be reduced by minimizing the variability in the exposure dose and chemical purity through the route and duration of exposure. Chemical analysis of the exposure tanks' water and/or food samples over time should be considered. Species with shorter metamorphic periods or experimental designs using shorter exposure periods reduce the length of the test and by default reduce the possibility of 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 test's duration.

Ideally, an experimental design incorporates randomness, independence, and replication (Cochran and Cox, 1957). Randomness is used to remove noise, independence is used to extend the inferences made, and replication provides a measure of variability for testing (Chapman et al., 1996). Randomization of 1) experimental containers within a testing environment, 2)

treatment application to experimental containers, and 3) application of organisms to experimental containers allows one to incorporate the variability associated with the environmental conditions, the containers, and the organisms equally across all treatments. Thus, when the difference between treatments, meaning the variability associated with experimental environment, experimental containers, and organisms being treated, are removed, only the effect of the treatment remains.

Independence of treatment application, including the creation of the treatment, incorporates the variability associated with someone else, somewhere else making and applying the same treatment. Thus, the inferences associated with the treatment response are extended to someone else repeating the experiment. The random sample of organisms from a given population actually limits the inference to that population. However, one can evaluate the stability of the inherent variability of the population over time. An experimental unit is defined as the group of material to which a treatment is applied independently in a single trial of the experiment (Cochran and Cox, 1957). Replication of experimental units for each treatment provides a measure of all the necessary sources of variability needed to extend the inference across time and space. A reduction in the sources of variability that are truly independent constrains the inference (Hurlbert, 1984). Thus, if only one solution of each treatment is made and then divided between replicates, the source of variation associated with making the treatment is not included in the variability for testing, and the inference is limited. It can be argued that this variability is nuisance noise, too small to be of concern, and costly to include. Therefore, if this source of variability is not included, it should at least be acknowledged. The variability between replicate experimental units may also include noise that was not randomized out due to a poor randomization or variable measurement error. These sources of variability can be reduced without loss to inference.

Statistical power is the probability of rejecting the null hypothesis (of equal means) when the alternative is true (i.e., detecting a difference when there is a difference). Statistical power is a function of the variability between replicate experimental units (i.e., within a treatment), the number of replicate experimental units, the size of the type I error, and the percentage of difference one wishes to detect. The latter three components can be controlled; however, the variability in response is inherent in the organism being tested. Thus, the choice of which species should be tested and the relevant endpoints measured should include a comparison of inherent variability or CVs (coefficient of variation=standard deviation/mean x 100%). In terms of power, high CVs have low power for detecting small-scale differences. Increasing the number of replicates can increase power. The choice of the test species and endpoints with the least inherent variability, by default, requires the least replication for a given level of power and, thus, are more cost effective.

5.5.1 Sample Size: Ensuring Adequate Test Specimens

Typical practical considerations for sample size are based on the number of endpoints to be collected and whether the specimen must be sacrificed to collect the data. With long-term 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 specimens). Additionally, it is assumed that at some dose there will be no difference between the

dosed and un-dosed specimen. In order to statistically determine the appropriate sample size, the inherent variability of the endpoint must be measured, according to the desired statistical resolution, and the power of predictability determined. No current guidelines are available for longer-term exposure assays with amphibians. However, the length of the prospective assays and the natural variability associated with longer term growth, development and reproduction will require larger sample sizes than used for short-term partial life-cycle amphibian tests. As a useful guide, 100 larvae per replicate has been a standard sample size for starting a long-term exposure for regulatory purposes. Due to density issues when raising amphibians, a greater number of replicates with fewer specimens will be required (i.e., 5 replicates of 20 specimens per concentration). However, before sample size and replicate requirements can be determined for the AGRA, formal statistical power analysis will be required.

5.5.2 Statistical Considerations - Endpoints

The NOAEC has been used to evaluate data from longer-term amphibian exposure studies, typically when the differences between exposure levels are no greater than a factor of approximately 3. An AGRA battery will generate multiple NOAEC values if multiple endpoints are selected.

Even though the NOAEC is widely used, it should not be relied on as the sole indicator of low toxicity. The largest dose for which statistical differences have failed to be detected is a direct function of the power of the test. It is also conceivable that short-term range finding experiments will have difficulty in predicting the location of an NOAEC. Using ECx calculations are an appropriate alternate for estimating doses associated with low toxicity. However, care must be taken not to estimate an ECx value that is more sensitive than the data and experimental design will allow. Ultimately, the data may simply indicate impairment of growth, sexual differentiation, or reproduction.

6.0 DESCRIPTION OF ASSAY ENDPOINTS REFLECTIVE OF DEVELOPMENTAL ANOMALIES AND GROWTH INHIBITION

6.1 Description of Developmental Phases

General amphibian developmental stages include: 1) early embryo-larval, 2) premetamorphic, 3) prometamorphic, 4) metamorph/juvenile, and 5) adult stages (reproductively mature). Early embryos larval stages generally encompass development from fertilization to premetamorphosis during which time the organism starts and completes most of the major stages of larval organogenesis. In *X. laevis*, this process spans ca. 4.5 days post-fertilization from NF stage 1 to 46. Metamorphosis is a period of substantial morphological change in which an organism alters its mode of living and occurs in all major chordate groups with the exception of amniotes (Dent, 1968; Just et al., 1981). In fact, metamorphosis is developmentally comparable to post-embryonic organogenesis in mammals (Tata, 1993). Three primary characteristics define metamorphosis, 1) change in non-reproductive structures between a post-hatch or larval state and sexual maturity, 2) form of the larvae enable it to occupy a unique ecological niche different from that used by the adult life stage, and 3) the morphological changes that occur at the

conclusion of larval development depend on some environmental stimulus, either external (i.e., temperature or food supply), or internal (hormonal changes). Each of the three classes of amphibians, anurans, urodeles, and caecilians, undergo metamorphosis, although not all species within each class metamorphose. For example, obligatory neotenic urodeles do not metamorphose, and reproduce as an aquatic "adult larvae".

Three primary morphological changes occur during metamorphosis, 1) resorption or regression of tissue or organ systems that have primary function only in the larval life stage, 2) the remodeling of larval organ systems to their adult form, which are suitable only for the adult, and 3) *de novo* development of tissues in the adult that are not required by the larvae. These changes are most marked in anuran species, and less obvious in urodeles and caecilians. In each of the three classes of amphibians, metamorphosis is controlled by thyroid hormone (TH), although less is currently known about the role of TH in the metamorphosis of caecilian species. Amphibian metamorphosis has been most widely studied in anurans, primarily due to the dramatic nature of metamorphosis and the ease in use of anuran species in research. However, within the anurans, of which are nearly 4,000 species (Stebbins and Cohen, 1995) metamorphosis has only been reasonably well studied in three species*, Xenopus laevis* (South African clawed frog), *Rana catesbeiana* (bull frog), and *R. pipiens* (Northern Leopard frog).

Anuran metamorphosis is separated into three distinct periods, premetamorphosis, prometamorphosis, and metamorphic climax (Etkin, 1964; Etkin 1968; and Dodd and Dodd, 1976). Premetamorphosis refers to a period of embryonic and early larvae development that takes place without thyroid hormone. Some advanced morphological developments occur during this stage including hind limb bud development. More specific morphogenesis, such as differentiation of the toes and rapid growth (elongation) of the hind limbs, occurs during prometamorphosis. Biochemically, prometamorphosis is characterized by rising concentrations of endogenous TH. The final period is metamorphic climax in which a surge of TH triggers the final processes associated with metamorphosis, including forelimb development and resorption of the tail. Drastic internal transformations at the organ system, tissue, and biochemical levels are also taking place during prometamorphosis and metamorphic climax. Following the completion of metamorphosis, second sexual development continues for a period of time at which point reproductive maturity is achieved. The time to achieve sexual maturity is variable in most amphibians, even with a given genus. *X. laevis* may not become sexually mature for 1.5-2 years, whereas *X. tropicalis* become sexually mature between 4-6 months post fertilization. Most anuran species require at least 1 year and typically 2 to 4 years to reproduce. Further, males are typically reproductive viable before females in many anuran species.

6.2 Growth and Morphological Alternations

6.2.1 Embryo-Larval Malformation and Growth

Potential morphological impact of EDCs on early embryological development could be measured through several different morphological and submorphological endpoints. General morphological endpoints including, lethality, malformation, and inhibition of growth are generally considered. Standardized and well-validated methods for performing the Frog Embryo Teratogenesis Assay – *Xenopus* (FETAX) are available (ASTM, 1998; Bantle et al., 1998).

Further, validation of FETAX is well documented (see Fort et al. [2003] for review). In addition, other amphibian toxicity methods have been developed, including AMPHITOX (Herkovits and Perez-Coll, 1999; Herkovits et al., 2002; Herkovits and Perez-Coll, 2003), for assessing the developmental toxicity of various agents to *B. arenarum* using different exposure lengths. However, the sensitivity of short-term early developmental toxicity assay endpoints to the effects of EDCs has been questioned (Veldhoen and Helbing, 2000; Pickford and Morris, 2003; Fort et al., in press a and b). Little information regarding the capacity of early embryos to respond to EDCs using the morphological endpoints described is currently available, although it has been suggested (Veldhoen and Helbing, 2000; Pickford and Morris, 2003) that these morphological endpoints might not be as sensitive to EDC exposure as other molecular or biochemical endpoints. Bevan et al. (2003) suggest, on the contrary, that the majority of EDC effect focus has been on sexual differentiation (Hayes et al., 2001; Carr et al., 2002; Hayes et al., 2003) and reproductive development (Sharara et al., 1998; Fort et al., 2001), however early embryological processes may also be sensitive. These investigators found that environmental estrogens (nonylphenol, octylphenol, and methoxychlor) exposed to *X. laevis* for ca. 48-h at concentrations ranging from 10 nM to 10 μM induced morphological deformities, increased apoptosis, and altered the deposition and differentiation of neural crest melanocytes in tail bud stage embryos (ca. NF stage 37). Since expression of *X. slug*, a factor that regulates both induction and migration of neural crest cells, was not altered, these investigators suggested that the effects on the neural crest derived melanocytes occurred during the latter stages of melanocytes differentiation. It should be noted that Fort et al. (in press a) found no adverse gross morphological effects of methoxychlor at concentrations as great as 1 mg/L in a FETAX-like assay. Bevan et al. (2003) ultimately suggested that differentiation of neural crest-derived melanocytes in *X. laevis* may serve as a sensitive early indicator of the effects of EDCs. More work will be needed to determine the specific sensitivity of amphibians to EDCs during early embryological development.

6.2.2 Metamorphic Growth and Malformation

Specific discussion regarding the potential endpoints potentially useful for monitoring the effects of EDCs during metamorphosis is provided in more detail in DRP 2-20 [4-5], "Amphibian Metamorphosis Assays" (Battelle, 2003). Morphological measures of metamorphosis and thyroid status include tail resorption, limb emergence and development, skin development, and skin coloration (*Hyperolius*). Protocols ultimately developed to morphologically mark thyroid impairment may include any applicable endpoint discussed in the following sections, and should not be limited to one endpoint if possible.

6.2.2.1 Tail Resorption. Tail resorption occurs in anuran species during metamorphic climax and is triggered by a surge of TH. Metamorphic climax in *X. laevis* occurs roughly over a 16-18 day period and incorporates stages 58 to 66. Tail resorption can be monitored during culture using digital photography and measuring the tail lengths with a scanning digitizer. A rate of tail resorption can be determined from the mean length data obtained. Abnormal tail resorption can also be monitored during this process. Specimens can be selectively preserved throughout the process of tail resorption and evaluated morphologically. In terms of culture, different approaches may be considered. For example, the specific process of tail resorption can be monitored by culturing tadpoles at relatively low densities such as in multiple replicates for

16 to 18 d from stage 58 to 66 during which time the rate of tail resorption is measured (Fort et al., 2000). Low densities of ca. one organism per 500 mL of test solution are required to achieve consistent maximum growth rates in culture. However, this approach does not consider the metamorphic events that lead up to metamorphic climax. In addition, natural inherent variation in tail resorption can be observed due to differing levels of endogenous TH between organisms. As an alternative approach, a German team has developed a longer term (28 days) "*Xenopus* Metamorphosis Assay" (XEMA) that is designed to measure biochemical and morphological changes, including tail resorption, during metamorphosis. In this assay, *X. laevis* are exposed to test materials from stage 48 to stage 66. Based on discussion with the investigators (OCCD, 2001), it appears that this modified assay is set in a static-renewal format. This assay is currently being reviewed by OECD (OECD, 2001). Although the investigators indicated that the assay can be completed in 28 days, the assay will require a 50-day exposure length based on our experience. Tail resorption can also be monitored in the discussed anuran species using the general approach developed in *Xenopus*.

Based on this information, several factors relating to the measurement of tail resorption and the interpretation of the results require consideration. First, the rate of tail resorption is naturally variable in whole organism culture (Fort et al., 2000; Fort et al., 2001b), which reduces the sensitivity and predictability of this endpoint. Second, this process occurs in the later stage of metamorphosis, when the thyroid is fully active and at its peak early in the climatic period. However, when used with other morphological and biochemical or molecular endpoints, such as TH measurement and TR gene expression, this endpoint is one which could be considered. Issues regarding exposure design, including the use of flow-through systems, need to be addressed.

6.2.2.2 Limb Emergence and Differentiation. As previously discussed, early hind limb bud development (emergence) occurs prior to thyroid activity in the developing anuran tadpole. However, hind limb differentiation and forelimb development occur during the prometamorphic phase of metamorphosis. Thyroid dysfunction impairs the process of limb differentiation, but not limb bud emergence. Selecting an exposure window that encompasses hind limb differentiation would require exposure from stages 54 to 60. The longer-term test format described by the German investigators incorporates limb differentiation as an endpoint. The same limitations in use based on the design expressed in Section 6 apply, however. The only standardized test method that evaluates limb development was evaluated by Fort and Stover (1996, 1997) using *X. laevis*. However, this modified FETAX assay (ASTM, 1998) evaluated only hind limb development, initiating exposure at an early blastula stage and completing exposure around 30 days at stage 54. Thus, this design did not address the effects of thyroid dysfunction on limb differentiation and is longer than necessary since it incorporates a substantial period of premetamorphosis. However, a modification of the assay might be considered that expresses *X. laevis* from stage 51 (limb bud stage) to stage 54, at which time the hind limb is reasonably well differentiated.

The morphology, rate, and extent of limb differentiation can be monitored in a similar manner as described for tail resorption. However, in this case the occurrence of abnormal limb development, including asymmetrical differentiation, should be monitored, thus requiring additional specimens for histological examination, CAT scan, and x-ray analysis.

6.2.2.3 Skin Development. During metamorphosis, substantial changes to the skin in terms of protein structure, keritinization, and pigmentation occur. Changes in skin structure have already been discussed; however, changes in pigmentation also occur that change a transparent tadpole such as *X. laevis* to a frog with pigmented, non-transparent skin. Classical thyroid inhibitors, such as thiourea, also inhibit pigmentation by blocking melanin synthesis. An evaluation of melanin distribution in skin structure can be evaluated in *Xenopus* under normal light microscopy. Unlike larval skin, metamorph skin possesses well-formed melanocytes containing a relatively dense distribution of melanin. Staining is only required to evaluate the neurological status of the pigmentation process. For evaluation of epidermal structure and keritinization, a standard eosin or hemotoxylin/eosin can be effectively used. Immuno-histochemical techniques can be used to distinguish the presence of specific proteins that mark the newly developing frog skin. Therefore, skin maturation is a potential valuable endpoint. Like the endpoints previously discussed, this endpoint is best served with a battery of other metamorphosis-based morphological endpoints in a longer-term exposure design.

Use of the *Hyperolius argus* endocrine disruption screen (HAES) model developed by Hayes and Menendez (1999), which uses the sexually dichromatic reed frog, *H. argus*, provides a novel means of evaluating thyroid function simultaneously with primary and secondary sexual development. In this case, evaluation of skin coloration and patterning determine the potential impact of goitrogens on metamorphosis because the thyroid exerts at least some control over sexual maturation in this species.

6.2.3 Early Gonad Development

The various stages of gonad development are illustrated in a generalized conceptual model developed for *Xenopus* (Kelley, 1996; Bogi, 2002) in Figure 3-3. Various endpoints, which could be measured during any given point of gonad development or sexual differentiation, are provided in the following sections.

6.2.3.1 Histological Techniques. A series of endpoints reflective of gonadal development prior to sexual differentiation (ca. prior to NF stage 56) could potentially be considered. Specific endpoints could include, development and migration of primordial germ cells, and formation of the indifferent gonad (ca. NF stage 49). The development and fate of the primordial germ cells and formation of the primitive gonad is reasonably well understood in *Xenopus*. The majority of techniques used to evaluate early gonadal development will require some form of histology, histopathology, and or histochemical analysis. Although light and electron microscopic procedures have been established to examine formation of the genital ridge using a variety of different histological and immuno-histochemical staining procedures (Wylie and Heasman, 1976), most of the procedures used presently involve molecular probes. As previously discussed, primordial germ cell development, migration, and differentiation depend on the maternally provided cytoplasmic factors that constitute the germ plasm (Weidinger et al., 2003). Little is actually know regarding the role and mechanisms by which the germ plasm acts to induce development and differentiation of the primordial germ cells, and eventual gonad. However, several putative markers have been used to evaluate early gonadal development in amphibians, including anti-SSEA-1 (Gomperts et al., 1994) and RNA probes from the novel

putative germ plasm RNA binding protein, dead end (Weidinger et al., 2003). Dead end protein is localized in the perinuclear germ granules within the primordial germ cells and can be used to track the migration of the germ cell to the presumptive gonad. Thus, the production of promodial germ cells and the organization of the primitive gonad (migration of the primordial germ cells) can be assessed.

6.2.3.2 Evaluation of Gamete Production. Gamete production can be measured in the newly differentiated gonad around NF stage 56 at which time gonadal sex can be determined (Kelley, 1996). The viability and normalcy of development of new gametes could potentially be assessed as early as the onset of metamorphic climax through histological or immuno-histochemical procedures. However, from a practical standpoint, evaluation of gamete production would be most easily measured in post-metamorphic (juvenile or adult) specimens in which various stages of gametogenesis can be observed (Fort et al., 2001). As with early evaluation of the indifferent gonad, most of the techniques utilized today involve molecular approaches. However, histological examination of the differentiated gonad using both light and electron microscopic process can be measured as described in the following sections.

6.2.4 Sexual Differentiation

The most practicable period of evaluating gonad and gamete production from a developmental standpoint is in the differentiated gonad (see Figure 6-1). Since primary sexual development is complete following, evaluation of gonadal development, including germ cell fecundity, could be assessed in newly metamorphosed specimens. Although earlier assessment is feasible using molecular techniques, it is not necessarily practicable from a morphological standpoint. However, Travera-Mendoza et al. (2002a, 2002b) performed quantitative histological analysis of both ovaries and testes in NF stage 56 *X. laevis* larvae exposed to atrazine for 48-h. These investigators (Travera-Mendoza et al., 2002a) evaluated testicular volume; and primary spermatogonical cell nests and nurse cell fecundity. Testicular aplasia and the completeness of testicular development were also examined. In females (Travera-Mendoza et al., 2002b), oogonial viability and the frequency of secondary oogonia, and primary and secondary oogonial resorption were evaluated using quantitative histology. Evaluation of newly post-metamorphic organisms allows for gross examination of gonad formation with histological procedures used to assess gonad and gamete viability (Hayes et al., 2002 and 2003; Carr et al., 2003). These endpoints are described in the following section discussing inter-sexual development.

6.2.4.1 Characterization of Sex Reversal and Inter-Sexual Development. Characterization of intersexual development in amphibians, particularly anurans, has emerged as a point of interest with recent findings that contaminants, and potential EDCs, may alter gonadal development resulting in various forms of sex reversal or intersexual development in *X. laevis* (Fort et al., 2001; Hayes et al., 2002; Goleman et al., 2002; Carr et al., 2003), *R. pipiens* (Hayes et al., 2002 and 2003), *R. catesbeiana* (Chang et. al., 1996), *R. esculenta* (Zaffagnini, 1968), and *Pleuorodeles waltl* (Chardard and Dournon, 1999). Further, examination of organisms for sex reversal and intersexual development is relatively straightforward from a morphological and histological standpoint although consistency amongst the types of abnormal development nomenclature is needed. As previously described, sex reversal involves the expression of a

gonadal (phenotypic) sex that is inconsistent with genotypic sex. Exposure of sexually undifferentiated anurans to E2, estrogenic compounds, or aromatase inducers results in the development of females based on gonadal sex as marked by the presence of developed ovaries (see previous discussion). In males, testicular grafts [*X. laevis* (Mikamo and Witschi, 1963 and Kelley, 1996)] aromatase inhibitors [*X. laevis* (Villalpando and Merchant-Larios, 1990, Miyata and Kubo, 2000), *X. tropicalis* (Fort et al., in press b), and androgens [*R. curtipes* (Saidapur et al., 2001), *H. argus* (Hayes and Menendez, 1999); *X. laevis* (Bogi et al., 2002); and *X. tropicalis* (Fort et al., in press b)]; have been shown to masculinize developing amphibians. As previously discussed, the androgens found to induced masculinization *in vivo* include those which are not aromatized to E2, including DHT and MT. Thus, the effects of various agents on sexual development are highly species-specific. In some amphibian species, like reptiles, sexual determination can be determined or influenced by environmental factors, such as, temperature. However, in most amphibian models in gonadal research used today, temperature does not act as a sex determinant facilitating their use as model systems. Morphological and histological endpoints that could be used to evaluate sexual development could include measurement of sex ratios using direct visual inspection (necropsy) and the development of intersexual gonads using both morphological and histological techniques. Molecular endpoints, including measure of target gene expression (Sugita et al., 2001; Shibata et al., 2002; Aoyama et al., 2003; Ohtani et al., 2003) described in the following section could be combined with morphological, histological, and biochemical endpoints to form a battery of endpoints used to monitor effects on gonadal development and sexual differentiation.

6.3 Secondary Sexual Characteristics

In addition to monitoring primary sexual development, effects of EDCs on secondary sexual development at the morphological, biochemical, and molecular levels could be measured. Morphological indicators of secondary sexual development might include development of sexually dichromatic skin coloration in *H. argus*, as proposed by Hayes (Hayes, TB, 1999, Hyperolius argus endocrine screen test: Regents of the University of California, Oakland, CA: Published under Patent Cooperation Treaty, International publication No. WO 99/47181), and the moor frog (*R. arvalis*) (Sheldon et al., 2003); development of nuptial or thumb pads in males (Emerson et al., 1997; Harvey and Propper, 1997); changes in ovarian (Dumont, 1972; Fort et al., 2001) and testicular cycles (Fort et al., 2001; Woodley, 1994); oviduct development and cloacal swelling; and development of the larnyx (*Xenopus*) (Watson et al., 1993; Robertson and Kelley, 1996). Biochemical and molecular measures of secondary sexual development are described in the following sections.

6.4 Biochemical Measures

Biochemical indicators of endocrine pertubation of reproductive and growth in amphibians could potentially include measure of plasma and tissue steroid and gonadotropin levels, as well as, THs, PRL, corticoids, and ACTH and CRF.

6.4.1 Plasma and Tissue Steroid Concentrations

Plasma and tissue steroid levels throughout various developmental windows and the reproductive cycle could potentially be monitored. The type of sample collected (plasma vs. tissue and whole body vs. gonadal samples) will be determined by the practicability of sample collection. Thus, samples collected in premetamorphic larvae will be limited to whole body tissue, whereas, plasma may be collected in small quantities from metamorphic age larvae. Gonadal samples may also be collected in metamorphosed and adult specimens. In amphibians, the primary steroids include, P, DHEA, androstenedione, T, DHT, E2, estrone, and estriol. Although may of the intermediates described in Section 3-3 are not included in aforementioned list, these steroids encompass the primary active progestins, androgens, and estrogens. Of these hormones, P, T, DHT, and E2 have been the most routinely measured (Bogi et al., 2003; Yang et al., 2003; Carr et al., 1993; Hayes et al., 2003; Hayes et al., 2002; Bogi et al., 2002; Chang et al., 1996; Hayes and Menendez, 1996; Hayes and Licht, 1992; Kloas et al., 1990).

6.4.1.1 Plasma and Tissue Gonadotrophs. Plasma and/or tissue gonadotropin levels may also be measured as an indicator developmental or reproductive dysfunction. In terms of evaluating developmental dysfunction, measurement of gonadotropin levels in prometamorphic larvae may not necessarily be indicative of downstream gonadal steroid production and levels since anuran oocytes may contain maternal sex steroids for use by the developing larvae later in development (Bogi et al., 2003; Lutz et al., 2003). However, measurement of LH and FSH could potentially be useful, particularly in metamorphic and post-metamorphic anurans species. Since the specific roles of LH and FSH are not as defined in amphibians as in mammals resulting in high inter-species variability, specifically measure the individual gonadotropins may not be essential. In reproductive-age specimens, measure of endogenous gonadotropin levels particularly during the reproductive cycle would potentially be valuable as a biochemical endpoint. Thus, the use of gonadotropin measurement as a biochemical indicator of endocrine disruption would likely be most useful in adult reproductive age animals.

6.4.1.2 Steroid Biosynthetic Enzymes. Measurement of enzyme activity important in steroid biosynthesis may be potentially useful as an indicator endocrine disruption. Specific enzymes which could potentially be utilized include the P-450s, CYPscc, CYPc21, CYPβ21, CYPc17, CYPc11 (Lutz et al., 2001; Hammes, 2003; Yang et al., 2003), aromatase (Kuntz et al., 2003a and b; MacKenzie et al., 2003; Ohtani et al., 2003; Hayes et al., 2002; Petrini and Zaccanti, 1998); 3β-HSD, 17β-HSD (Mensah-Nyagan, 1996; Martel et al., 1992), and 5α-reductase (Bogi et al., 2002; Petrini and Zaccanti, 1998). Of these enzymes, aromatase has been the most widely studied. Co-measurement of aromatase, 17β-HSD, and 5α-reductase could provide significant information regarding sex steroid biosynthetic pathway activity when combined with the morphological endpoints described previously.

6.4.2 Plasma and Tissue Thyroid Hormone, Prolactin, and Corticoids

Measurement of plasma and tissue THs, PRLs, corticoids may be potentially useful in evaluating effects of EDCs on larval growth and metamorphosis (Denver et al., 2002; Denver, 1998). As previously discussed in the present DRP, as well as, DRPs 2-20 (4-5), "Amphibian Metamorphosis Assays" (Battelle, 2003) and 4-7, "Thyroid Hormone Assays" (Battelle, in

preparation), each of these hormones provided some form of regulation of larval growth and metamorphosis. Also, THs have been shown to be important in sensitization of presumptive male amphibians to androgens (Roberson and Kelley, 1992; Kelley, 1996)

6.4.2.1 Plasma and Tissue TSH, ACTH and Corticotropin Releasing Factor. Measurement of plasma and tissue TSH, ACTH, and possibly CRF may be potentially useful in evaluating effects of EDCs on larval growth and metamorphosis (Denver et al., 2002; Denver, 1998). As previously discussed in the present DRP, as well as DRPs 2-20 (4-5), "Amphibian Metamorphosis Assays" (Battelle, 2003) and 4-7, "Thyroid Hormone Assays" (Battelle, in preparation), each of these stimulating hormones or releasing factors provided indirect control of larval growth and metamorphosis.

6.4.3 Vitellogenin

The yolk protein precursor, VTG, has been extensively used as a biochemical means of marking estrogenic compounds since production of vitellogenic production in the liver is induced by endogenous estrogen and exogenously by exposure to substances with estrogenic activity (Pickford and Morris, 2003; Hutchison and Pickford, 2002; Hurter et al., 2002; Kloas et al., 1999; Lutz and Kloas, 1999). Further, the process of vitellogenesis in amphibians (Dumont and Brummett, 1978), the methodology for measuring VTG production *in vivo* and *in vitro* are well established Pickford and Morris, 2003; Hurter et al., 2002; Kloas et al., 1999; Lutz and Kloas, 1999).

6.5 Molecular Measures

6.5.1 Complementary cDNA and mRNA Techniques

Several techniques are currently available to measure single gene and multiple gene activity, including RPA and RT-PCR and differential display and gene arrays, respectively. One approach to screening differential gene activity is differential display (Liang and Pardee, 1992). This process of genetic screening involves synthesizing cDNA from a subset of the compared mRNA population by using a partially degenerate primer. In this case, the subset might include mRNA specifically expressed during critical phases of development, such as sexual differentiation. A subset of the cDNA is amplified using polymerase chain reaction (PCR) with the appropriate 5'-primer. The amplified products from two samples are then displayed on a sequencing gel. Bands of differing intensities between the two samples are derived from mRNAs of different quantities. These bands can be cut out of the gel, eluted into buffer, and PCR amplified. The amplified products can then be used for *in situ* hybridization to confirm the regulation of the specific genes. Crump et al. (2002) evaluated the effects of octylphenol, an estrogenic EDC, and uv-B radiation on hypothalamic gene expression, using differential display, as diagnostic tools to screen for candidate genes that were differentially expressed in *R. pipiens*. These investigators found that NcK, ash, phospholipase C gamma banding protein, brain angiogenesis inhibitor-3 in tadpoles; and GAD 67, cytochrome C oxidase, and brain angiogenesis inhibitors -2 and -3 in metamorphs were differentially expressed. Overall, differential display analysis is a reasonably well developed technique for measuring multiple gene activity. However, results from differential display analysis can be difficult to interpret.

A more recently developed technique for evaluating multiple gene activity involves the use of gene arrays. DNA micro- and macro- arrays are powerful tools in the analysis of differential gene expression. The cDNA micro-arrays are capable of profiling gene expression patterns of tens-of-thousands of genes in a single experiment. In this technique, DNA targets, in the form of 3' expressed sequence tags (ESTs), are arrayed onto glass slides or membranes and probed with fluorescent- or radio-labeled cDNAs (Duggan et al., 1999). Although microarray analysis of early developmental gene expression has been performed in *X. laevis* (Altmann et al., 2001) and Denver et al. (1997) evaluated differential gene expression during metamorphosis to evaluate TH-responsive genes using cDNA expression arrays and a slightly different technique, PCR-based subtractive hybridization, use of microarrays in amphibians is limited. Gene array constructs using E2- or androgen-responsive genes could be developed to evaluate the effect of potential EDCs on multiple TH-dependent gene expression. Thus, although the gene array technology offers advantages over differential display, it is not yet clear how interpretable and technically feasible the gene array analysis approach will be in a testing format.

An alternative technique would be to consider several specific individual genes that are activated or inactivated during metamorphosis. Using a single gene expression assay called the ribonuclease protection assay (RPA) (see below for description of methodology), the specific activity of selected genes can be monitored during development and various phases during reproduction. For example, estrogen- or ARs or response gene expression, VTG gene expression, aromatase and 5α-reductase expression, or specific proteins differentially-expressed during gonadal development. Gene activities for developmental proteins, such as the germ plasm marker, DAZL (Johnson et al., 2001), dead end protein (Weidinger et al., 2003), SSEA-1 (Gomperts et al., 1994), germ cell proteins Xmeg and Xtr (Ikema et al., 2002) have been measured. Likewise, TH responsive genes that could be evaluated during prometamorphosis are TR beta or stromelysin-3 (ST3). Like TR beta, ST3 is also a strong a candidate for use in molecular screening of thyroid function. RPA techniques are currently being used in *X. laevis* and are being adapted for use in *X. tropicalis*. Although no one has investigated the use of these specific molecular approaches for evaluating the impact of EDC exposure on growth or reproductive function, both approaches show substantial promise for use with the AGRA.

Essentially, the RPA detects and quantitates mRNA, maps mRNA termini, and determines the position of introns within the corresponding gene. This is accomplished by initially generating a high specific activity ^{32}P-UTP-labeled single stranded complementary RNA (cRNA). The cRNA is hybridized with the target mRNA to ensure that most of the mRNA is hybridized to the cRNA probe. A combination of Rnase A and Rnase T1 is then used to digest the unhybridized, single-stranded RNA, and the digestion products are analyzed by denaturing, using polyacrylamide gel electrophoresis, and autoradiography (PhosphorImaging). The undigested cDNA probe will contain a stretch of plasmid sequence and is therefore larger than the original mRNA, which protects it from the action of Rnase by duplex formation. Thus, it will migrate slower than the protected fragments and is used for identification purposes. As with differential display, RPA is somewhat outdated and has been recently over-shadowed by RT-PCR technology.

Thus, one of the most promising single gene molecular biomarker assays described in this section is RT-PCR. RT-PCR methodologies for specifically measuring estrogen (Bogi et al., 2002 and 2003; Lutz et al., 2001; Kloas et al., 1999; Weiler et al., 1987) and AR (Kelley et al., 1989; Dostal et al., 1994; Dosch et al., 1997), 5α-reductase (Luo et al., 2003), aromatase (Kuntz et al., 2003a, 2003b; Ohanti et al., 2003), and VTG (Lorenzen et al., 2003; Rotchell and Ostrander, 2003; Islinger et al., 2002; Lattier et al., 2001) gene expression have been developed. However, it should be pointed out that the most common method of measuring VTG is by enzyme-linked immunosorbent assay (ELISA) as described by Pickford and Morris (2003), Hurter et al. (2002), Kloas et al. (1999), and Palmer and Palmer (1995). Measurement of retinal binding protein (RBP) mRNA as a biomarker of potential endocrine disruption has been proposed and evaluated (Levy et al., 2003). These investigators suggested that RBP could serve as molecular biomarker for many different modes of EDC action since it is regulated by sex steroid hormones. Further this model was capable of evaluating anti-estrogenic and anti-androgenic compounds using a *X. laevis* primary heptocyte culture system.

RT-PCR methodologies for specifically measuring TR beta gene expression changes, as the result of exposure to potential EDCs in *X. laevis* tail biopsies, were developed by Veldhoen and Helbing (2001). RT-PCR analysis of ST3 or other relevant gene activity during prometamorphosis could also be considered. RT-PCR technology is based on the construction of cDNA from isolated RNA using reverse transcriptase. The cDNA and cDNA primer fragment (i.e., TR beta) are then amplified. The amplified DNA products are then separated on an agarose gel and the amplified DNA bands quantitatively analyzed using densitometry. RT-PCR techniques have been used to measure the induction of VTG genes in *Xenopus* as the result of exposure to the weakly estrogenic compound bisphenol A (Kloas et al., 1999), as well as ER and AR gene expression (Bogi et al., 2002). Work by Veldhoen and Helbing (2001) demonstrates that quantitative analysis of single gene activity, such as TR beta, is feasible. If the TH-response genes selected for evaluation are ubiquitous, other tissues could be sampled besides the tail, including the hind limb, using a similar biopsy approach. In summary, based on the current state of technology available, RT-PCR, RPA, and differential display techniques will likely be the most useful at the present time. However, the potential power of gene array technology as a diagnostic tool in the future cannot be over-looked.

6.5.2 Transgenic Strains

A physiological means of studying gene function is through gene knockout and transgenic lines. To date, no gene knockouts have been developed in amphibians. However, two methods of developing transgenic lines have been developed using amphibian species. The first approach involves the nuclear transplantation of somatic nuclei into an enucleated oocyte. Once the transplantation is complete, the oocyte is fertilized. Kroll and Gerhart (1994) used this approach to transfect a gene of interest into a *X. laevis* tissue culture cell line. Successfully transfected nuclei are then microinjected into newly fertilized embryos. However, this approach has not been largely successful in growing embryos beyond a young larval stage. Thus, the use of transfected somatic nuclei transgenesis is not well suited for studying metamorphosis. The inability to raise the transgenic specimen beyond early larval stages prompted Kroll and Amaya (1996) to develop the second approach that uses undifferentiated sperm cell nuclei. This approach entails the insertion of a gene or genes with appropriate promoters in a plasmid. The

plasmid is then linearized using restriction enzymes. The linearized plasmid and *X. laevis* sperm cell nuclei are mixed in a high-speed extract made from *X. laevis* eggs. A short incubation period allows decondensation of the nuclei to occur, allowing plasmid incorporation into the chromatin. The transfected sperm nuclei are then microinjected into oocytes at a rate of one nucleus per egg. Although the efficiency is still rather low, the techniques compare favorably to similar approaches developed for the mouse and zebrafish. The availability of many genes involved with development, including sexual differentiation, in combination with tissue-specific promoters, will eventually allow construction of a transgenic lines that model the expression of a series of genes important for successful development and metamorphosis. Adaptation to *X. tropicalis*, a diploid organism with a shorter life cycle, further increases the feasibility of these studies. Transgenic lines constructed with specific reporter genes and exploiting activation of the ERE and specifically, ER expression, have been developed (Matthews et al., 2002; Hiroi et al., 1999; Mattick et al., 1997; Weirden et al., 2001; Nardulli, et al., 1991). A similar approach could be used for evaluating AR activity (Huang and Wong, 2002).

Activation of EREs or TREs by the action of an exogenous EDC could be marked by a marker protein. For example, Oofusa et al. (2001) evaluated the activity of the thyroid responsive element (TRE) using *X. laevis* carrying a transgene containing the 5' upstream region of the TR beta A1 gene and a green fluorescent protein (EGFP) gene. EGFP expression was then monitored throughout the entire premetamorphic, prometamorphic, and metamorphic climax periods. TR beta expression was found as early as neurula stage at low levels, with low activity during the remainder of premetamorphosis, culminating at metamorphic climax. A similar transgenic *Xenopus* line could be developed to measure TR beta expression and the influence of potential EDCs on gene expression. Furlow and Brown (1999) identified a novel lencine zipper transcriptional factor (TH/bZIP) that is induced by TH during metamorphosis in transgenic *X. laevis*. Two genomic TH/bZIP genes regulated by an adjoining DR+4 TRE have been found in *X. laevis*. The effect of potential EDCs on up and down regulation of TH/bZIP could also be measured using an EGFP marker.

Overall, it is possible that a reporter gene assay created from either somatic or germinal transgenesis could be used to mark ER, AR, VTG, or other noted gene expression in a quantifiable, rapid process. Further, this assay could be used in conjunction with a broader-based morphological assay.

6.5.3 Estrogen and Androgen Receptor Binding Assays

Since EDC binding to ER and AR represents an additional process, which may be affected by EDCs, a receptor-binding assay could also be considered. Currently, ER and AR binding assays, which measure the relative binding affinities of potential EDCs to the ER or AR, are available and are being evaluated (Bogi et al., 2003; Bogi et al., 2002; Kloas et al., 1999; Lutz and Kloas, 1999).

7.0 DESCRIPTION OF ASSAY ENDPOINTS REFLECTIVE OF REPRODUCTIVE DYSFUNCTION

7.1 Measure of Reproductive Fitness

An evaluation of reproductive status fitness could potentially include measures gonad health and function, and gamete production and viability. Ovary pathology, oocyte count, oocyte maturity and maturation capacity (as measured by GVBD), and necrosis were evaluated in the female, while testis pathology, sperm count, dysmorphology, and motility were studied in the male.

7.1.1 Gonad Health and Gamete Fecundity

In terms of gonad health, ovary pathology; measure of normalcy of the ovarian cycle including, oocyte count, vitellogenesis, oocyte stage distribution and maturation capacity (GVBD); and oocyte necrosis could be evaluated in the female (Pickford and Morris, 2003; Fort et al., 2002; Bogi et al., 2003; Fort et al., 2001; Pickford and Morris, 1999; Kloas et al., 1999). In turn, testis pathology; measures of testicular cycle including, sperm count, sperm cell stage distribution; sperm cell dysmorphology, and sperm cell motility could be evaluated in the male (Bogi et al., 2003; Fort et al., 2001). Each of these endpoints could be evaluated in recently metamorphed specimens (Bogi et al., 2002, 2003; Kloas et al., 1999) or adult specimens (Pickford and Morris, 1999, 2003; Fort et al., 2001, 2002).

7.2 Measures of Reproductive Performance

7.2.1 Changes in Breeding Behavior/Breeding Success

Induction of breeding response either naturally or induced by gonadotropin administration as indicated by promotion of secondary sexual characteristics, courtship, oviposition, and egg mass production could be measured as an indication of breeding success. Overt gravidity, expansion of the oviducts, swelling of the cloaca, GVBD, and formation of nuptial spots (pigment spots) could be measured in females as an indication of breeding status using secondary sexual characteristics as a morphological indicator. In males, induction of nuptial pads, larangeal muscle differentiation, vocalization, and production of motile sperm cells could potentially be used as endpoints indicative of breeding behavior. The rates of fertilization and viability of the progeny could serve as indication of breeding success.

7.2.1.1 Fertilization. The rate and normalcy of fertilization can be measured either by induced breeding or through artificial (*in vitro*) means. The induced breeding can be performed in at least several anuran species, but most easily in *Xenopus sp.* through the administration of gonadotropin (Pickford and Morris, 2003, Fort et al., 2001; ASTM, 1998). Induction of natural breeding in *R. pipiens* has been accomplished using incubators that simulate the emergence from hibernation. Artificial fertilization has been performed in a variety of ranid species, as well as *Xenopus sp.*

7.2.1.2 F1 Generation Viability. As previously indicated, the viability of the F1 generation could also be used as indication of reproductive success. Measures of viability could include abnormal development, hatching success, mortality, developmental kinetics (stage of development and developmental delay), and growth. Viability may be assessed during early embryo-larval development as measured by a FETAX-like model (Fort et al., in press a; Song et al., 2003; Fort and McLaughlin, 2003; Fort et al., 2001; ASTM, 1998; Bantle et al., 1998) or over a long term developmental period, including a full life cycle (Fort and Stover, 1996, 1997; Fort et al., in press b).

7.3 Biochemical Measures

Several biochemical measures of reproductive dysfunction, including endogenous steroid levels and gonadotropin profiles; VTG induction or repression; steroid receptor binding characteristics; and aromatase, 17□-HSD, and 5□-reductase activities could be used.

7.3.1 Sex Steroid Concentrations

E2, T, DHT, and P levels in both plasma and various tissues can be measured through the reproductive cycle. Measures of sex steroid concentrations will most likely be useful in combination with other morphological and molecular tests of reproductive function. Further, to evaluate the effects of potential EDCs on the reproductive cycle, conventional sex steroids levels may be measured following reproductive stimulation with exogenously administered gonadotropins (Pickford and Morris, 2003).

7.3.2 Gonadotropin Levels

Aside from measuring sex steroid levels in response to gonadotropin stimulation, endogenous gonadotropin levels can be measured. These measurements could entail a combination of LH and FSH, or as individual gonadotrophs.

7.3.3 Other Hormones

Other hormones including PRL, TH, ACTH, AVT, and corticosterone could also be measured along with sex steroids. However, aside from AVT, which induces oocyte release by acting on the oviducts, less is known regarding the role of these hormones in reproductive function.

7.3.4 Vitellogenin Induction/Inhibition

A now standard means of evaluating response to estrogenic and antiestrogenic EDCs is through the measure of VTG induction (estrogenic) in male specimen and inhibition of VTG induction in females (antiestrogenic) (Bogi et al., 2003 and 2002; Pickford and Morris, 2003; Kloas et al., 1999; Palmer et al., 1998; Palmer and Palmer, 1995). Although measure of VTG induction (mRNA) will be described in the following section 7.4, “Molecular Measures”, production of VTG and other yolk precursors can be directly measured in the plasma (Palmer and Palmer, 1995).

7.3.5 Steroid Receptor Binding

7.3.5.1 Estrogen/Androgen Receptors. Measure of sex steroid or EDC binding characteristics to the steroid receptors (ER and AR) could be measured as a biomarker of EDC agonism and antagonism of the ER or AR. Measurement could be performed *in vivo*, or in an *in vitro* model (Bogi et al., 2003; Kloas et al., 1999; Lutz and Kloas, 1999).

7.3.5.2 Progesterone Receptor. Agonism or antagonism of the P receptor by potential EDCs could also be measured. As previously described, the P receptor in the *Xenopus* oocyte is plasma membrane-bound. The membrane fraction can be isolated and used to evaluate binding characteristic *in vivo* (Lutz et al., 2001) and *in vitro* (Fort et al., 2002; Lutz, 2001; Pickford and Morris, 1999; Lui and Patino, 1993; Lin and Schuetz, 1983; Blondeau and Baulieu, 1984). It should also be noted that the amphibian oocyte could also serve as a useful model for evaluating AR agonism and antagonism by potential EDCs.

7.3.6 Aromatase, 17β-HSD, and 5α-Reductase Activities

Concentrations of steroid biosynthetic enzymes including, 17β-HSD, 5α-reductase, and aromatase could be measured as an indication of the influence of EDCs on pathway predominance. These activities could effectively be measured in gonadal tissue, or a variety of other tissues including the liver and brain (Pozzi et al., 1992; Mensah-Nyagan et al., 1996; Tata et al., 1993; Andersson et al., 1989: Jurman et al., 1982).

7.4 Molecular Measures

7.4.1 Steroid Receptor Expression

ER, AR (Sato et al., 2004; Bogi et al., 2003), and PR gene expression have been measured by several investigators as means of evaluating the endocrine disrupting activities of various test materials. Since ER and AR are autoinduced by E2 (Tata et al., 1993) and non-aromatizable androgens in some species such as *R. esculenta* (Varriale, 1996), measurement of ER and AR expression are at least partially indicative of exposure to estrogenic substances (Bogi et al., 2003). Further, Sumida et al. (2003) have shown limited species difference in ERα mediated reporter gene transactivation by estrogenic xenobiotics. Induction of brain and pituitary PRs by estrogens has also been demonstrated in *X. laevis* (Roy et al., 1986). Thus, measurement of steroid receptor expression could serve as a useful biomarker of EDC exposure.

7.4.2 Aromatase, 17β-HSD, and 5α-Reductase Expression

Measurement of aromatase (Assisi et al., 2000; DiFiore et al., 1998), 17β-HSD (Mensah-Nygan et al., 1996), and 5α-reductase (Andersson et al., 1989; Jurman et al., 1982) gene expression could be used to evaluate the impact of EDCs on steroid biosynthesis (Bogi et al., 2003).

7.4.3 Vitellogenin Expression

Induction of VTG gene activity (Bogi et al., 2003; Carnevali et al., 2000; Palmer and Palmer, 1995; Tata et al., 1993) in male specimens or inactivation in females could also be used as a potential biomarker of estrogenic EDC activity.

8.0 RESPONSE TO ESTROGEN AGONISTS AND ANTAGONISTS

The response of the various potential endpoints described in the previous sections to estrogen agonists and antagonists are described throughout the DRP. However, a summary of the anticipated response to developmental and reproductive endpoints in described in the following section.

8.1 Endpoint Sensitivity to 17β-Estradiol or Synthetic Estrogen Exposure

As previously indicated, generally, EDCs exerting effects on developmental and reproductive processes may act via estrogenic, antiestrogenic, androgenic, and antiandrogenic processes. However, progestin, antiprogestin and anti-thyroidal effects may also alter development and reproduction in amphibians. The complex inter-relationships within the HPG axes are responsible for sexual differentiation and maintenance of reproductive viability. For both estrogens and antiestrogens, it is anticipated that the order of endpoint sensitivity will be molecular>biochemical>morphological. However, unlike the morphological endpoints used in measuring thyroid axis disruption (see DRP 2-20 [4-5], "Amphibian Metamorphosis Assays" [Battelle, 2003]) in which a dichotomy in sensitivity was anticipated between the morphological endpoints and the biochemical/molecular endpoints, the morphological endpoints associated with estrogenic or antiestrogenic effects on gonadal differentiation and reproductive development/fitness are remarkably sensitive. Thus, less separation in sensitivity exists between the morphological endpoints and the molecular and biochemical endpoints.

8.1.1 Morphological Endpoints

Although the general modes of action of sexual differentiation is still not completely resolved (Wallace et al., 1999; Hayes, 1998; Dohler and New, 1989), it is now well established that estrogens (both E2 and synthetic estrogens) lead to feminization during sexual differentiation (Bogi et al., 2002). Generally, estrogens are capable of altering changes in sexual differentiation in at concentrations, which generally range from 10^{-6} to 10^{-9} M. E2 induces feminization in developing *X. laevis* at concentrations ranging from 10^{-7} to 10^{-8} M in a concentration-dependent manner (Bogi et al., 2002; Carr et al., 2002; Hayes, 2001; Kloas et al., 1999). A similar sensitivity in response has been observed recently with E2 in *X. tropicalis* (Fort et al., unpublished data). However, the response and sensitivities of xenoestrogens, such as nonylphenol, bisphenol A, and atrazine during sexual differentiation in anuran species vary from study to study (MacKenzie et al., 2003; Hayes et al., 2003; Pickford et al., 2003; Carr et al., 2002; Bogi et al., 2002; Hayes et al., 2001; Miyata and Kubo, 2000; Iela et al., 1975; Rastagoli and Chieffi, 1975). MacKenzie et al. (2003) found that leopard frogs (*R. pipiens*), and to a lesser extent, wood frogs (*R. sylvatica*) exposed to estrogenic compounds (E2, ethinyl estradiol, and

nonylphenol) induced feminization or an inter-sex condition at concentrations between 1 and 10 μg/L for E2 and ethinyl estradiol and 100 μg/L for nonylphenol in *R. pipiens*. These effects were less dramatic in *R. sylvatica*. Kloas et al. (1999) found that static-renewal exposure to E2, nonylphenol, bisphenol A, bultylhydroxyanisol, and octylphenol in the 10^{-8} M (E2) and 10^{-7} M (other substances) concentration range in *Xenopus*. However, Pickford et al. (2003) found that although exposure of developing *X. laevis* larvae (NF stage 43) through stage 66 (completion of metamorphosis in a flow through exposure system to E2 induced feminization gonad differentiation at 2.7 μg/L, bisphenol A did not induce skews in sex ratios at concentrations as great as ca. 500 μg/L. Overall, morphological response to estrogen and synthetic estrogens are relatively sensitive.

8.1.2 Biochemical and Molecular Endpoints

As with the morphological endpoints, many of the biochemical and molecular endpoints discussed are sensitive to estrogen agonists. Palmer and Palmer (1995), Palmer et al. (1998) demonstrated that E2 concentrations in the low μg/L range in were capable of inducing vitellogenin using an ELISA- based test. Using an ELISA-based method, Tada et al. (2004) established detection limits of VTG of ca. 4 μg/L in *Chinemys reevesii*. Kloas et al. (1999) found that significant VTG induction using RT-PCR methods to detect gene expression at E2 concentrations of 10-5 to 10-10 M. Further, Kloas et al., (1999) found that the dose-dependent potency of several estrogenic EDCs were: E2>nonylphenol>bisphenol A. Methodological differences will ultimately confer differences in sensitivity, such that methods measuring of VTG gene expression through RT-PCR will likely be more sensitive than measure of VTG by ELISA methods. However, ELISA-based methods are generally more simplistic and more easily performed under routine laboratory settings. Folmar et al. (2002) found that the sheepshead minnow VTG assay was markedly (at least 1,000 times) more potent in determining estrogenic potency of E2, ethinyl estradiol, and a diverse series xenobiotics than conventional *in vitro* tests such as yeast estrogen screen (YES) and the MCF-7 breast tumor cell proliferation assay (E-Screen).

One of the few disadvantages of VTG as a biomarker of estrogenic activity is that VTG mRNA is not detectable during larval development. Generally, in juvenile *Xenopus*, plasma VTG mRNA has been reported to be a more sensitive biomarker of estrogenic activity than either ER or AR mRNA expression (Bogi et al., 2002 and 2003). Increased ER and AR expression induced by E2 concentrations of 10^{-8} M have been demonstrated (Bogi et al., 2003; Bogi et al., 2002: Lutz et al., 2001). Competitive ER, AR, and PR binding using radioreceptor assays (Lutz et al., 2003; Bogi et al., 2003; Lutz and Kloas, 1999) are capable of measuring E2 equivalents in the 10-8 to 10-9 M range. Matthews et al. (2002) found that a series of diverse natural and synthetic chemicals induced ER expression in various species, including *Xenopus*. These investigators found that E2 induced reporter gene expression with effective concentrations ranging from 0.05 to 0.7 nM. Measure of the autoinduction of other estrogen biosynthetic liver enzymes is possible although little information is currently available. It is more likely that xenobiotic exposure may itself induce changes in estrogen biosynthesis pathways.

8.2 Endpoint Sensitivity to Anti-Estrogen Exposure

Anti-estrogens include direct-acting estrogen antagonists (i.e., tamoxifen; ICI 164,384; ICI 182780; and ICI 46474). indirect-acting aromatase inhibitors (i.e., flavone, CGS 16949A, and 4-hydroxyandrostene dione, RU486) (MacKenzie et al., 2003; Bogi et al., 2002; Miyata and Kubo, 2000; Schmidt and Loffler, 1997). Thus, anti-estrogens may exert activity by blocking estrogen biosynthesis or by antagonizing ER binding.

8.2.1 Morphological Endpoints

In general, antiestrogenic compounds neutralize sexual differentiation (Dohler and New, 1989). However, the response of various amphibian species to antiestrogenic substances is currently still variable (Bogi et al., 2002; Rastogi and Chieffi, 1975). Much of the differences in response can be attributed to differences in species, experimental conditions, compounds evaluated, and concentrations tested. Further, there is substantially less information available regarding the effects of antiestrogens on sexual differentiation and reproductive development (Bogi et al., 2002). Bogi et al. (2002) showed that the direct acting antiestrogen tamoxifen caused no change in gender ratio, but rendered the gonads non-functional in *X. laevis* as the result of sever underdevelopment at concentrations of 10^{-8} M. MacKenzie et al. (2003) found that ICI 182780 induced intersexual development in which the gonadal tissue was predominantly testicular with the presence of one or several oocytes at 1 μg/L in *R. pipiens*. At 10 μg/L, ICI 182780 induced at slightly greater incidence of intersexual development, although the sex ratio was unexpectedly skewed toward the female gender. Evaluation of this paradoxical response associated with the direct acting anti-estrogen ICI 182780 from MacKenzie et al. (2003) is difficult since steroid hormone levels were not concurrently measured. However, these investigators suggested that at the lower dosing range a compensatory mechanism may exist in which MacKenzie et al. (2003) found the same general response in leopard frogs (*R. pipiens*) using the aromatase inhibitor flavone at concentrations ranging from 10 to 100 μg/L. However, in the case of the flavone treatment, gender ratios were not altered. In wood frogs (*R. sylvatica*), treatment with flavone only increased the skewing of the sex ratio toward the female gender slightly (MacKenzie et al., 2003). Few intersex *R. sylvatica* were found.

8.2.2 Biochemical and Molecular Endpoints

The effects of anti-estrogenic substances on the various biochemical and molecular endpoints previously described, including ER binding, ER gene expression, VTG expression, and aromatase activity are generally less understood in amphibians. In most cases, anti-estrogens, such tamoxifen or the ICI series, are biological active antagonists at similar concentrations as ER agonists which is not surprising since tamoxifen, ICI 164,384, and ICI 182,780 compete directly with E2 for binding sites on the ER at sub-μM concentrations (Mahfoudi et al., 1995; Fuqua et al., 1993; Wakeling and Bowler, 1988; Borgna and Rochefort, 1980). Lorenzen et al. (2003) found that inhibition of both VTG and ER transcription by tamoxifen, methoxychlor, and o, p-DDT as measured by quantitative RT-PCR occurred at concentrations ranging from 100 to 1,000 nM, 10-50 μM, and 1,000-10,000 nM, respectively. Tamoxifen induced low-density lipoprotein synthesis. Using ELISA, Pickford and Morris (2003) found that 500 μg/L methoxychlor also inhibited vitellogenesis in *X. laevis*. Riegel et al.

(1987) demonstrated that 4-hydroxytamoxifen was readily capable of binding to ER reducing the number of estrogen binding sites in *Xenopus* male hepatocytes from nearly 800 sites/cell in hormonally naïve specimens to 250 sites per cell. Although administration of E2 24 h following hydroxytamoxifen administration resulted in the induction of ER to levels found in unchallenged control animals, E2 was not able overcome antiestrogen inhibition of VTG gene transcription suggesting that different mechanisms are associated with induction of ER and VTG gene transcription. Other studies (Riegel et al., 1986) have suggested that antiestrogens may have effects on post-translational processing or secretion of VTG in addition to transcription effects. Similar results were found by Perlman et al. (1984) in the nM concentration range. Perlman et al. (1984) further hypothesized that the effect of antiestrogens was at least in part due to decreased ER expression and that receptor number is rate-limiting in VTG gene transcription.

8.3 Strength and Weaknesses of Test Species

Some ranid and bufonid species that undergo a semi-differentiated state of sexual differentiation will pass through a period of juvenile hermaphroditism. This state of natural hermaphroditism occurs normally prior to completion of metamorphic climax, however may persist in juveniles. Thus, several investigators have reported hermaphroditic metamorphic specimens in laboratory controls (MacKenzie et al., 2003; Richards and Nace, 1978; Witschi and Chang, 1950; Humphrey et al., 1950). Finding of variable natural hermaphoditism within a given natural population of *R. pipiens* has also been described (MacKenzie et al., 2003;. These findings underscore the complexity of sexual differentiation and reproductive development in some species. Ultimately, in the context of developing laboratory models for testing EDCs, background incidence of juvenile hermaphroditism confounds interpretation of sexual differentiation and inter-sexual development caused by exposure to xenobiotics with potential EDC activity (Hayes et al., 2003). On the contrary, in the pipids (*X. laevis* and *X. tropicalis*), gonadal development is direct which strengthens their use as a laboratory model system.

9.0 RESPONSE TO ANDROGEN AGONISTS AND ANTAGONISTS

The response of the various potential endpoints described in the previous sections to androgen agonists and antagonists are described throughout the DRP. However, a summary of the anticipated response to developmental and reproductive endpoints in described in the following section. For both estrogens and antiestrogens, it is anticipated that the order of endpoint sensitivity will be molecular>biochemical>morphological. However, as noted with for the estrogenic agonists, the morphological endpoints associated with androgenic or antiandrogenic effects on gonadal differentiation and reproductive development/fitness are remarkably sensitive. Thus, less separation in sensitivity exists between the morphological endpoints and the molecular and biochemical endpoints.

9.1 Endpoint Sensitivity to Androgenic Steroid Exposure

9.1.1 Morphological Endpoints

Unlike estrogens in which it is now well-established exposure leads to feminization during sexual differentiation (Bogi et al., 2002), the response to androgen exposure is more complex. For example, DHT and MT are capable of inducing testicular development in *Xenopus* at concentrations of 10^{-8} M (Bogi et al., 2002), but T does not efficiently masculinize gonadal development at similar concentrations. This response may in part be explained by aromatization of T to E2. Thus, current models suggest that non-aromatizable androgens, such as DHT (produced from T by 5α-reductase), are responsible for male sexual differentiation, including the development secondary sexual characteristics (see Figure 3-3 for conceptual model). Of the endpoints potentially considered, measurement of gonadal development, gamete production, and secondary sexual development including differentiation of the nuptial pads on the forelimb and differentiation of the larynx (Carr et al., 2002; Thorton and Kelley, 1998; Perez and Kelley, 1996; Fischer et al., 1995). Overall, morphological response to androgens are less characterized in standardized model systems as the estrogens or anti-estrogens, however, are relatively sensitive endpoints. Androgens have been shown to be potent inducers of GVBD in amphibian oocytes (Lutz et al., 2001). Lutz et al. (2001) found that ca. 50 nM androstene dione and ca. 5-10 nM T were capable of inducing GVBD in *X. laevis in vitro* which are similar to effective concentrations found by Fort et al. (in press).

9.1.2 Biochemical and Molecular Endpoints

As with the morphological endpoints, many of the biochemical and molecular endpoints discussed are sensitive to androgen agonists. Measures of AR gene expression have been measured by a multitude of investigators (Bogi et al., 2003 and 2002; Thorton and Kelley, 1998; Perez and Kelley, 1996; Fischer et al., 1995; Varriale and Serino, 1994; Kelley et al, 1989) Bogi et al. (2003) found that one month old juvenile *X. laevis* exposed to 10-8 M E2 , but not 10-8 T, significantly elevated both ER- and AR-mRNA levels. These investigators hypothesized that T was substantially less efficient in autoinducing AR mRNA which supports a regulatory role of other androgens, such as DHT. Varriale and Serino (1994) found that AR mRNA was upregulated by T in the Harderian gland and the thumb pad of *R. esculenta*. AR binding kinetics have also been evaluated (Bogi et al., 2003; Paolucci et al., 2003; Di Forre et al., 1998) and are sensitive to androgen agonists similar to that of the ER. Further, since the AR is molecularly distinct from the ER or PR in amphibians (Thorton and Kelley, 1998; Fischer et al., 1993) specific AR binding studies can be performed. Although less literature is available regarding the sensitivity of these endpoints to AR agonists, it can be generally assumed to nearly as sensitive as for the AR agonists.

9.2 Endpoint Sensitivity to Anti-Androgen Exposure

The majority of anti-androgens evaluated include direct-acting AR antagonists (i.e., cyproterone acetate and flutamide). In amphibians, indirect-acting anti-androgens, which act by inducing aromatase, include vinclozlin, atrazine, and IBMX (Sanderson et al., 2002) or inhibitors of 5α-reductase, such as finasteride (Lambright et al., 2000). Thus, anti-androgen may exert

activity by inhibiting androgen biosynthesis, increasing androgen conversion to estrogen, or by directly antagonizing ER binding or synthesis of AR.

9.2.1 Morphological Endpoints

In general, administration of antiandrogenic substances causes feminization, as in the case of cyproterone acetate which was administered to developing *Xenopus* tadpoles at 10-8 M (Bogi et al., 2002). Haider (1980) found that administration of 1mg/wk cyproterone acetate to adult *Rana* temporaria caused disruption of the formation of spermatids, however, did not alter the early stage of spermatogenesis. Further, interstitial cell degeneration and shrinkage of the Leydig cells was noted ultimately resulting in a reduction in testicular weight. Similar effects on testicular function were noted by Minucci et al. (1992), Kurian and Saidapur (1985), Saidapur et al. (1981), Chieffi et al. (1974), and Rastagoli and Chieffi (1971). However, the response of various amphibian species to antiandrogenic, like antiestrogenic, substances is currently still variable (Bogi et al., 2002; Rastogi and Chieffi, 1975). Di Matteo et al. (2000) found that treatment of adult *R. esculenta* testis with E2 caused massive proliferation of mast cells, while T had little effect on mast cell number. Cyproterone acetate also caused a massive increase in mass cell number, as well as, substantial interstitial and Leydig cell degeneration. Administration of antiandrogens has been shown to ameliorate secondary male sexual differentiation of the nuptial pads and larynx (Kang et al., 1995; Varriale and Serino, 1994; Saidapur et al., 1981). As with the anti-estrogens, differences in response can be attributed to differences in species, experimental conditions (time and duration of administration), compounds evaluated, and concentrations tested. Further, there is generally less information available regarding the effects of antiandrogens on sexual differentiation and reproductive development in amphibians than for the estrogens or antiestrogens (Bogi et al., 2002).

9.2.2 Biochemical and Molecular Endpoints

The effects of anti-androgenic substances on the various biochemical and molecular endpoints previously described, including AR binding, AR gene expression, and aromatase and 5α-reductase activities. In male rats, flutamide, procymidone, and viclozolin acts as AR antagonists, inhibit AR-DNA binding, and alter androgen-dependent gene expression both *in vitro* and *in vivo* (Lambright et al., 2000). The 5α-reductase inhibitor, finasteride and some phthalate esters inhibit androgen synthesis. The anti-androgenic effects of other environmental chemicals including, bisphenol A and nonylphenol (Lee et al., 2003); o, p-DDE (van Wyk et al., 2003); diethlyhexyl phthalate (Moore et al, 2001); and the AR antagonistic herbicide Linuron (Lambright et al., 2000) have been documented recently. For example, Lee et al. (2003) have recently shown that bisphenol A and nonylphenol inhibited androgen induced AR transcription in a yeast detection model. Generally, the direct acting antiandrogenic compounds are effective in the mid-to-low nM concentrations (Bogi et al., 2002). Environmental antiandrogens, such as those mentioned above, are typically active in the μM concentration range. Luo et al. (2003) described highly sensitive methods for evaluation 5α-reductase mRNA expression in the human prostate using RT-PCR techniques. These techniques could be broadly applicable to anuran species considering the highly conserved nature of 5α-reductase in vertebrates.

9.3 Strength and Weaknesses of Test Species

A similar case for the strengths of the pipids over the ranids based on the direct nature of gonadal development can be made here as was done for the estrogens and antiestrogens.

10.0 RESPONSE TO THYROID AGONISTS AND ANTAGONISTS

The sensitivity of the whole organism developmental endpoints, including tail resorption, limb emergence, and skin development, will likely be differentially sensitive to thyroid stimulation or inhibition. It is possible to speculate which of the morphological endpoints will be the most and least sensitive as the result of EDC exposure. It is anticipated that tail resorption would be relatively less sensitive to pertubation than other tissues remodeled during metamorphosis. The rationale for this hypothesis is that endogenous TH is at its peak during metamorphic climax. Thus, alteration of metamorphic events during this period would require substantial change in TH homeostasis compared to prometamorphosis when TH concentrations are appreciably lower. Of these morphological events and corresponding TH levels, hind limb differentiation is more sensitive to TH-induction than resorption of the tail, as the hind limb reaches full differentiation well in advance of the tail at the completion of the prometamorphic phase under lower endogenous TH levels. Likewise, the biochemical and molecular endpoints will confer a different degree of sensitivity compared to the morphological endpoints. This sensitivity depends on the biochemical, molecular, and morphological endpoints selected. The anticipated order of endpoint sensitivity would most likely be expressed as molecular tests (greatest sensitivity)$\geq$ biochemical measurements>histological endpoints>> morphological endpoints. Although the morphological endpoints tend to be less sensitive than the other tests, they provide an understanding of an actual physical outcome in the organism and how that relates to changes in biochemical molecular activities. The apical nature of the morphological tests suggest that they might demonstrate a greater propensity to detect positive response than the more specific molecular and biochemical approaches. However, the diagnostic capability of the single morphological endpoint is generally poor as they are not capable of categorizing chemicals as to their mode of action and whether or not the changes are the result of thyroidal or non-thyroidal mechanisms.

10.1 Gender Differences

Although metamorphosis in amphibians is not dependent upon the sex of the animal, general endocrinological differences between male and female specimens may confer a different sensitivity to the EDCs. Gonadal steroids and PRL could potentially be factors. However, sexual development (some cases primary, and all cases secondary) in anurans occurs after metamorphosis is complete and is at least partially dependent on successful TH-induced metamorphosis (Hayes, 1997a). Therefore, the influence of gender differences on the induction of TH disruption will need to be evaluated further, particularly in the case of simultaneous precocious sexual development and the requirement of TH sensitization of gonadal tissue prior to male sexual differentiation (Kelley, 1996).

10.2 Species Sensitivity

Since the mechanisms involved in metamorphosis are reasonably well-conserved, especially among anurans, a large difference in response is not necessarily expected due to phylogenetic differences. Variance in sensitivity may result from differences in life history strategies that confer differing lengths of metamorphosis, and thus, different exposure regimes. Little is currently known about species responsiveness and sensitivity differences amongst species.

11.0 RESPONSE TO OTHER HORMONAL DISTURBANCES

The effect of other hormones on development and reproduction is specifically described in section 3.0. The most significant steroid hormone not discussed previously is P. P plays may roles in the reproductive cycle aside from serving as a precursor in androgen biosynthesis. One of the more notable roles of P in the amphibian oocyte is in the induction of GVBD during oocyte maturation (Dumont, 1972; Pickford and Morris, 1999; Fort et al., 2002; Lutz et al., 2001). One notable difference is that the oocyte P receptor is membrane bound rather than intracellular, making directly accessible to external factors, such as EDCs, that could modulate its activity (Pickford and Morris, 1999). The corticosteroids and PRL can potentially have a great effect on regulating larval development, which can impact sexual differentiation. Due to the complex nature of amphibian endocrinology, undiscovered hormonal influences or disturbances may also affect the reproduction and development.

12.0 CANDIDATE PROTOCOLS

12.1 USEPA Guidelines for Reproductive Toxicity Assessment (To be added)

12.2 OPPTS Reproductive Toxicity Test Guidelines(To be added)

12.3 Morphological Tests

Morphological test protocols for assessing the effects of EDCs on reproduction in growth using *Xenopus sp.* (*X. laevis* and *X. tropicalis*), ranids (*R. pipiens*), and *H. argus* are described in the following sections. *H. argus* is discussed in a separate section.

12.3.1 Partial Life cycle Tests

It is anticipated that a series or battery of potential partial life cycle test protocols to assess reproductive and growth effects of EDCs could be utilized.

12.3.1.1 Developmental and Growth Endpoints. Developmental test protocols could potentially focus on early embryo-larval development and larval development including metamorphosis.

12.3.1.1.1 Short-Term Embryo-Larval Assays. Standardized methods of conducting early embryo-larval developmental exposure are well established and include the ASTM-standardized Frog Embryo Teratogenesis Assay – *Xenopus* (FETAX) (Fort et al., 2003; ASTM, 1998; Bantle et al., 1998; Dumont et al., 1983) and the AMPHITOX assay battery using *B. arenarum* (Herkovits and Perez-Coll, 2003; Herkovits et al., 2002). The FETAX model as described in ASTM E1439-98 (ASTM, 1998), Bantle et al., 1998, and by Fort et al. (2003, in press c) exposes blastulae stage embryos from either *X. laevis* or *X. tropicalis* for ca 4-d until control NF stage 46 at which point the major stage s of organogenesis are complete, but metamorphosis has not begun. Provisions for a variety of exposure delivery methods including static, static-renewal, and flow-through; as well as provisions for the use of alternative species are available (ASTM, 1998; Fort et al., in press c). The primary advantage of FETAX is the extensive validation database available (Dumont et al., 2003; Fort et al., 2003) and the simplicity of the methods used. However, the primary disadvantage, as for any early embryo-larval life phase test in amphibians surrounds questions as to the sensitivity of this stage of development to EDC activity. Although a few reports, including recent work by Bevan and co-workers (2003), suggest that early embryo-larval development may be affected adversely by EDCs, the majority of reports indicate that this stage of development is not sensitive to EDC action. More advance endpoints, such as gonadal development and sexual differentiation could be included and will be discussed with the longer-term partial life cycle test approaches.

In the FETAX method (ASTM, 1998), groups of embryos 20 are placed in covered Petri dishes (10-25 mL) with varying concentrations of the respective test material. The test materials are dissolved in an appropriate volume of FETAX Solution, a reconstituted water media suitable for the culture of developing *Xenopus* embryos (Dawson and Bantle, 1997). Carrier solvents, such as, DMSO (not to exceed 1%v/v) may be used as a co-solvent. Test concentrations are generally tested in duplicate, and four separate dishes of 20 embryos each were exposed to FETAX Solution and designated as FETAX Solution controls. Each treatment vessel contained a total of 10-25 mL of solution depending on the dish volume. An exogenous metabolic activation system (MAS) that includes a liver microsomal preparation may also be used with FETAX. Each metabolically activated treatment receives 0.4 U/dish of N-demethylase activity, an NADPH generating system, and a penicillin-streptomycin mixture to control bacteria contamination. Controls (including FETAX Solution), uninhibited MAS, each inhibited MAS, cyclophosphamide or acetylhydrazide (FETAX reference proteratogens) (17), and unactivated toxicant (no MAS) are tested simultaneously with each experiment. All control treatments also receives antibiotics. *X. laevis* embryos are cultured at 23.5 ± 0.5ºC and *X. tropicalis* are cultured at 26.5 ± 0.5ºC . All solutions were changed every 24 h of the test, dead embryos removed, and fresh solutions added. Following 96 h of exposure for *X. laevis* or 48 h of exposure for *X. tropicalis* of exposure, embryos are anesethized in MS-222 and fixed in 3.0% (w/v) formalin (pH 7.0), and the number of live malformed embryos is ascertained using a dissecting microscope. The median lethal (LC50), teratogenic (EC50) concentrations, and respective 95% confidence intervals of two separate definitive concentration-response tests are determined. Head-tail length of surviving embryos is measured as an index of growth computer-based digitizing software. The length data are then used to calculate the minimum concentration to inhibit growth (MCIG or NOAEC [growth]) value for each experiment.

The AMPHITOX assay has many of the attributes of FETAX, although it incorporates a variety of different life stage exposures (Herkovits and Perez-Coll, 2003). Thus, it this sense if offers some advantages over the FETAX model. However, limited information is available regarding the effects of known EDCs in this model. The protocol as described by Herkovits and Perex-Coll (1999) incorporates a 7-d early embryo-larval method (like FETAX) using neurula stage organisms. Ten organisms are placed in three replicates containing 40 mL of test solution at 20 ± 1°C.

12.3.1.1.2 Larval and Complete Metamorphosis Assays. Although many investigators have described various intermediate length exposure studies with amphibians, few have been standardized and considerable variability in methodologies exists. Longer-term larval exposure assays evaluating limb development have also been described by Fort and Stover (1996) using methods similar to that for FETAX with decreased organism density flow-through methodology and a 21-d exposure regime. Herkovits and Perez-Coll (2003) also describe a 4-d acute embryo-larval exposure, 7-d short-term chronic, 14-d chronic each using Gosner stage 23-25 organisms within the AMPHITOX framework. Much recent attention to gonadal development (Bogi et al., 2003 and 2002; Hayes et al., 2003; MacKenzie et al., 2003; Carr et al., 2002; Hayes et al., 2001) has prompted inclusion of gonadal development as an assessment endpoint. Such assays practicably require exposure through metamorphosis requiring ca. 60 d for *Xenopus* and 75 d of exposure for other ranid species. Many of the aforementioned studies incorporating sexual differentiation were performed under static renewal conditions. Overall, in terms of developmental tests, inclusion of sexual differentiation regardless of whether the assay is based on a partial life cycle format or a full life cycle format, inclusion of sexual differentiation is desirable.

Fort et al. (in press b) described exposure of embryonic *X. tropicalis* through advanced development, metamorphosis, and sexual maturity. Metamorphosis and sexual differentiation in *X. tropicalis* is complete within 35 d post-fertilization. An evaluation of growth kinetics, normalcy of development, metamorphosis, and sexual development was performed at 60 and 90 d. Based on the results of these studies, an evaluation of primary sexual development could be performed using a 45 d exposure protocol. Aquatic phase culturing practices were conducted in accordance with the methods described in the ASTM Standard Guide (ASTM, 1998) with the modifications to adapt for use with *X. tropicalis* (Fort et al., 2003, Bantle et al., 1998). For life cycle tests initiated with young embryos, groups of 50 embryos were be placed in each of two 70 L exposure chambers with varying concentrations of the toxicant. Constant renewal of each toxicant dilution was provided by a flow-through diluter system. Complete test vessel volume replacement was set at 24 hours depending on the half-life of the test material in solution. Total test solution volume was 50 L. The test materials were dissolved in FETAX solution, a reconstituted water media designed for the culture of *Xenopus larvae* modified for use with *X. tropicalis*. One percent DMSO (v/v) was used as a co-solvent as needed. Five separate exposure vessels with 20 embryos each were exposed to modified FETAX solution alone or 1% (v/v) DMSO and designated FETAX solution or DMSO controls, respectively. It should be noted that use of natural water including dechlorinated tap water was also acceptable and reduced the demands of producing high quantities of reconstituted synthetic dilution water.

The pH of all stock solutions was strictly maintained between 7.0-7.2, if possible. *X. tropicalis* embryos were cultured at 27±1°C. Dead embryos were removed daily during observations. Following exposure, a subset of surviving specimen was fixed in 3.0% formalin, pH 7.0 for necropsy and pathological examination. A final separate subset of the metamorphosed specimens and larvae from the bred specimens were frozen for analytical analysis of tissue residues. The number of specimens and the stage of development were then be ascertained externally using a dissecting microscope. Additional, necropsy was performed to evaluate internal pathology of the primary organs and review gonadal development.

12.3.1.2 Reproductive Endpoints. A reproductive toxicity assay using *X. laevis* (Fort et al., 2001) and more recently using *X. tropicalis* (Fort et al., in press b) has been developed using a similar protocol. Following 14 d of acclimation, sets of four female and four male frogs were administered either the toxicant at varying concentrations or culture water. Test substances were administered via the culture water or food depending on the nature (solubility and probable route of exposure).

Eight female frogs per treatment group were super-ovulated by injecting 750 IU of human chorionic gonadotropin (hCG) into the dorsal lymph sac s.c. Within 3-4 hours post injection, oviposition occurred. For breeding, the super-ovulated females were paired with males injected with ~500 IU hCG s.c. Each mating pair was placed in a polyethylene breeding chamber and allowed to breed overnight. In order to ensure that each female fully discharged the majority of mature oocytes, each female was squeezed gently along the flanks at the anterior portion of the ovary with posterior movement down the oviduct so as to strip the ovary of oocytes not yet released. Each female was then placed into her respective treatment for 45 d, at which time a thorough evaluation of reproductive status will be performed. Test concentrations were based on a range of sub-lethal concentrations determined from a preliminary experiment evaluating acute lethality. Five separate concentrations were tested in each study. Four of the eight females were paired with unexposed males to evaluate breeding response as previously described. The females were weighed and anesthetized with 1% w/v MS-222. The ovaries, oviducts and livers were then surgically removed prior to euthanasia. A 10% solution (w/v) of MS-222 at a dose of 0.2 mL per 50 g of body weight was injected into the dorsal lymph sac of the animal to be euthanized. The weight of each organ was determined, and the ovary and liver further processed for pathological evaluation.

Oocyte staging was performed in which stages are based on maturity and numbered from I to VI, with I representing the most immature oocytes and VI representing fully mature, banded oocytes. A thorough count of all oocytes will then performed. Once this information is collected, this process was repeated twice to verify the data collected. The number of necrotic oocytes were also be determined.

An in vitro GVBD assay developed by Pickford and Morris (1999) was used to evaluate impact on oocyte maturity. Ovaries were excised and placed in sterile, chilled OR2 buffer, pH 7.6 (Fort et al., 2002; Pickford and Morris, 1999; Dumont, 1972). Ovarian tissue was then cut into strips, rinsed, and incubated overnight at 4°C in OR2. Tissue was then digested for 75 min. in a 0.2% solution of collagenase D in OR2 until the follicular cell layer was removed from the

oocytes. After sufficient digestion, the oocytes were rinsed again in fresh OR2 and transferred to a disposable 100-mm Petri dish.

Large, banded, pre-ovulatory stage VI oocytes were selected by hand using a sterile Pasteur pipette under a dissecting microscope and plated 20/well in sterile 6-well Pyrex glass culture plates in 10 mL OR2. P (250 nM) was added and the plates were incubated in a shaker at 20°C for 24 h. The medium was then aspirated and the oocytes were fixed in 5% (w/v) trichloroacetic acid. Maturation was visible externally as a white "Roux" spot that indicates where the spindle has anchored to the plasma membrane at the animal pole of the oocyte. GVBD was verified by cracking open representative samples from the fixed oocyte. The maturational response of 20 oocytes in each well was expressed as the percentage exhibiting GVBD, and the mean maturational response for each treatment combination represents a minimum of four replicate wells. It should be noted here that Pickford and Morris (2003) describe methodology in *X. laevis* for evaluating the effects of potential EDCs on oviposition, which could be adapted into the protocols described in the preceding paragraphs.

Following 30 d of exposure, four male adults were collected and used to breed with respective females. The remaining four adult males were anesthetized at 30 d of exposure. At this time the testes and liver were removed and the specimen euthanized. One testis was used exclusively for determining sperm counts, while the other was sectioned and used to determine rates of dysmorphology. The frogs were anesthetized as described previously for the females. The testes were removed and any remaining fat was trimmed from the testis. Each testis was weighed and the information recorded. For the total sperm count, saline-merthiolate-Triton (SMT) [1 mL/10 mg tissue] [0.9% (w/v) NaCl, 0.01% (w/v) merthiolate, 0.05% (v/v) Triton X-100] was used to maintain the tissues during homogenization. Testes were placed in a clean scintillation vial with SMT, minced with scissors, and homogenized (Powergen® 125, Fisher Scientific, Houston, TX) for two min. A sample was then placed into a hemacytometer and the spermatids counted. At least three chambers were counted for each sample. If the totals were not within 10%, the samples were recounted. Dysmorphology was assessed by fixing the testis in 10% (w/v) formalin, pH = 8.0. The preserved sperm cells were then surveyed. The total number of abnormal sperm was counted and the types of abnormalities recorded. Several tissue sections were surveyed per testis to ensure that the rates fell within 10% of one another. Sections were also evaluated for overt pathology.

Head to tail length was measured as an indicator of embryo growth using Sigma Scan digitizing software (Jandel Scientific, Corte Madera, CA) and a personal computer. Concentrations inducing growth inhibition (MCIG) was then calculated using ANOVA (Bonferroni t-test, $P<0.05$). Reproductive status, including ovary and testis weights and pathology, total egg count, oocyte necrosis, oocyte stage distribution, maturation capacity, sperm counts and sperm dysmorphology rates were determined for each adult. Breeding success, fertilization rates, and embryonic viability were also determined. Comparisons of reproductive fitness evaluations were performed using ANOVA (Dunnett's Test, $P < 0.05$).

12.3.2 Full Life cycle and Multi-Generational Tests

Conducting full-life cycle tests in most amphibian species is impracticable due to the length of time required for sexual maturity. Most ranid species require at least 1 year and typically 2-3 years to become reproductively mature (Duellman and Trueb, 1996). Further, *X. laevis* require 1.5 to 2 years to become reproductively mature (Kelley, 1996). Fort et al. (in press b) described an approach that involved exposure of embryonic test organisms through advanced development, metamorphosis, and sexual maturity. Specimens surviving through sexual maturity were then bred in a cross over design (exposed female with unexposed male and exposed male with unexposed female) and the progeny cultured under standard conditions to evaluate trans-generational effects. Sexual maturity in *X. tropicalis* was achieved in 4-5 months and the progeny evaluated for an additional week. An evaluation of growth kinetics, normalcy of development, metamorphosis, sexual maturation (primary and secondary sexual development), reproductive fitness (see reproductive endpoints in following section), and developmental fitness in the resulting progeny (F1 generation) was performed.

A separate subset of metamorphosed sexually mature specimen were bred in a cross over format, such that exposed females were bred with unexposed males, and unexposed females were bred with exposed males. We anticipate that at least four breeding per cross were required to obtain an adequate number of samples for analysis based on preliminary power analyses. A final separate subset of the metamorphosed specimens and larvae from the bred specimens were frozen for analytical analysis of tissue residues. The number of specimens and the stage of development were then be ascertained externally using a dissecting microscope. Additional, necropsy was performed to evaluate internal pathology of the primary organs and review gonadal development. In order to utilize *Xenopus sp*., particularly *X. tropicalis*, in a life cycle or multigenerational assay format, additional work is needed to assess when the frogs reach a state of reproductive viability. This point may not be as much temporality related beyond a certain point as it is related to the number of induced breedings required to obtain viable clutches of fertilized eggs.

12.3.3 *Hyperolius argus* Endocrine Screening (HAES) Assay

The HAES assay is a patented method (International Patent Publication No. WO 99/47181, 1999) focused on skin color and pattern change in juvenile maturation. Although it may be a useful model for assessing advanced sexual development, no further discussion of this assay is provided in the DRP since it does not appear to meet the objectives for developing a comprehensive ESTP Tier 2 Reproduction and Growth test.

12.4 Histological Analyses

Specimens for histological examination can be selectively preserved in Bouin's Solution prior to preparation and stored in 70% ethanol. Following tissue processing, including decalcification if needed, the tissue sample can be embedded in paraffin. For gonad assessment, the dorsal wall of the abdominal cavity with kidneys and gonads attached can be processed for standard paraffin embedding. Microtome sectioning at 8-10 μm, hemotoxylin-eosin staining, and evaluation using standard light microscopy can be performed.

For thyroid examination, microtome sectioning (4-5 μm) or step sectioning (30-32°μm between steps) can be performed prior to hemotoxylin-eosin staining. The histological examination could include changes in the gland, including hypertrophy of follicular cells, hyperplasia of thyroid follicles, size of the follicle, and degree of colloid accumulation. Use of digital photographs can be used to illustrate changes and provide a means for outside peer examination. In addition to traditional light microscopic procedures, electron microscopy (EM), particularly scanning EM (SEM) or scanning transmission EM (STEM), can be considered as a potentially useful diagnostic tool. As for light microscopy described previously, EM procedures are readily available and can be adapted for evaluation of thyroid pathology.

12.5 Biochemical Tests

Methods for assessing steroid hormone concentrations; VTG levels; sex steroid receptor binding; and aromatase, 5α-reductase activities, and 3β-HSD activities are described in the following sections.

12.5.1 Measurement of Steroids and Thyroid Hormones

E2 can be effectively measured using radioimmunoassay (RIA). Generally, detection limits for simple aqueous matrices are in the order of 20-50 ng/L (Carr et al., 2002). Tissues are prepared by homogenization in distilled water, extraction with dichloromethane, and drying. The dried steroid extracts can be stored at -80˚C. The steroid extracts are then redissolved in 5% (v/v) ethanol, and both E2 and androgens (T and DHT) assayed by RIA (Bogi et al., 2002; Kloas and Hanke, 1990). Bogi et al. (2002) determined that the recovery rates for E2 and the androgens were between 60 and 70% using [^{3}H] labeled steroids. The cross-reactivity was determined to be no greater than 1%. Detection limits for E2 and the androgens were 2 and 5 pg/g tissue. ELISA methods are available currently in humans and rodents (Taherianfard and Shariaty, 2004), but not amphibians. Steroid hormones, both endogenous and radioactive, have also been analyzed by reversed phase HPLC (Mensah-Nyagan, 1996) following tissue extraction. Methods of capillary column GC-MS analysis with detection limits in the low ng/mL range for HPLC-purified tissue fractions have also been developed (Mensah-Nyagan, 1996). Immuno-detection techniques have been described by Mensah-Nyagan (1996) in which pulse-chase fractions purified by HPLC are incubated with anti-serum for estrogens or androgens. Following centrifugation, the antibody-bound radioactive steroids are counted using a liquid scintillation counter.

TH (T3 and T4), precursors (MIT and DIT), and deiodinase activities can be analyzed by the following methods. Since these methods have not been extensively used in amphibians, multiple methods will need to be considered. Further, biochemical measurement of thyroid activity can be measured in plasma obtained from cardiac puncture and whole body tissue. Three primary methods are available, although limited data are available on each in terms of sensitivity and reliability (Moller et al., 1983; Galton et al., 1991; Ekins, 1999; De Brabandere et al., 1998; Baiser et al., 2000). These methods include RIA, ELISA, and LC/GC-MS. Of these methods, only RIA techniques have been used to measure amphibian TH (Galton et al., 1991). Currently, ELISA and LC/GC-MS test methods have been developed for mammals, in human

tests of thyroid function. In order for ELISA to be routinely used, an ELISA kit would need to be developed for amphibian TH and deiodinase. In addition, new chromatographic methods developed for human TH analysis need to be adapted for amphibian samples. At this point, conventional RIA analysis methods of TH are adequate. Regardless of method, quality assurance (QA) measures associated with ELISA and RIA analyses should include an evaluation of cross-reactivity with other hormones or similar substances, evaluation of linearity using standard curves, and the use of standard additions to assess recoveries.

12.5.2 Measurement of Vitellogenin

Several techniques have been used including ELISA (Pickford and Morris, 2003; Huthinson and Pickford, 2002; Hurter et al., 2002; Lutz and Kloas, 1999; Kloas et al., 1999; Palmer et al., 1998; Palmer and Palmer, 1995; Riegel et al., 1986), RIA (Stanchfield and Yager, 1980; Kato et al., 1986); chromatographic (Shi et al., 2003); immuno-histochemical (Herbener, 1989), and electrophoretic methods (Allner et al., 1999). Of these methods, the ELISA techniques are most extensively developed and more widely used within the research community replacing the previously established RIA methods. Pickford and Morris (2003), Palmer and Selcer (1998), and Palmer et al. (1998) describe ELISA method to detect plasma VTG in *X. laevis* (*x*VTG), which utilizes anti-*x*VTG rabbit polyclonal antibody and *x*VTG standard using microtitre plates. Following antigen absorbance, washing and reaction blockage, and primary (rabbit anti-*x*VTG) and secondary (perioxidase labeled goat anti-rabbit IgG) antibody incubation; color development is performed using a peroxidase-based EIA substrate kit. Absorbance is read at 450 nm (655 nm as a reference wavelength) using a microtitre plate reader. Pickford and Morris (2003) determined the MDL of this assay to be 78 ng/well in their study. Kato et al. (1986) described RIA methods in which purification was achieved using ammonium precipitation, DEAE-Sephadex/Sephadex G-200 chromatography and RIA using rabbit anti-*x*VTG. The cross-reactivity with newt and chick VTG was ca. 65% and 6%, respectively. Stanchfield and Yager (1980) described an *in vitro* method of measuring VTG in cultured bullfrog hepatocytes using immuno-precipitation of labeled protein using a specific antiserum against VTG. The investigators also used indirect immuno-fluorescence microscopy to identify cells producing VTG in response E2 stimulation. Shi et al. (2003) developed a rapid membrane chromatographic method of separating VTG from plasma using a multi-step gradient elution process. The VTG peak was confirmed using SDS-PAGE and gel-permeation chromatography. These investigators suggest that this method is more rapid and technically simple than conventional HPLC and FPLC columns used for VTG separation. Allner et al. (1999) described a similar electrophoretic approach as described by Shi et al. (2003). The plasma volume requirements for electrophoretic analysis are reported to be ca. 1 μL (Shi et al., 2003; Allner et al., 1999).

12.5.3 Measurement of Estrogen and Androgen Receptor Binding

Assays designed to measure ER and AR binding are described by several investigators (Lutz et al., 2003; Bogi et al., 2003; Lutz and Kloas, 1999). These radioreceptor assays measure the binding of agonists or antagonists to the cytosolic receptors. Cytosol was prepared by homogenizing adult *Xenopus* liver followed by repeated centrifugation to achieve a lip-free preparation. This lipid-free fraction containing the cytosolic ER and/or AR was incubated with

labeled E2 or T and various dilutions of the test material. A reference consisting of unlabelled E2 in a concentration series ranging from 10-9 to 10-3 M was concurrently incubated and served as a reference. Separation of free and bound fractions was performed using dextran-coated activated charcoal treatment and centrifugation. Radioactivity was then measured using a liquid scintillation counter.

12.5.4 Measurement of Aromatase

Methods of evaluating aromatase activity in fish and mammals are better characterized than in amphibians. However, methods used in fish and mammals are generally applicable to amphibians. Established methods include, product isolation/isotope dilution (Prefontaine et al., 1990; Timmers and Lambert, 1987), and radiometric $[^3H]H_2O$ release (Prefontaine et al., 1990). The product isolation method quantitatively measures the conversion of $[^3H]$T in the presence of $[^{14}C]$estradiol to $[^3H]$estradiol using isotope dilution (Prefontaine et al., 1990). A similar approach was used by Timmers and Lambert (1987) using 7-$[^3H]$androstenedione and 19-$[^3H]$androstenedione. The $[^3H]H_2O$ release method measure the production of $[^3H]H_2O$ as the result of aromatization of $[^3H]$androstene dione (Prefontaine et al., 1990).

12.5.5 Measurement of 5α-Reductase

Measurement of 5α-reductase activity can be performed by similar methods as described for aromatase in the preceding section. In this case, the conversion of $[^3H]$T to $[^3H]$DHT is measured using thin layer chromatography and HPLC to separate the metabolites (DHT) from T (Lazier et al., 2004). In methods described by Nakae et al. (2002), the 5α-reductase is expressed as the sum of the $[^3H]$ metabolites generated from $[^3H]$T after chromatographic separation.

12.5.6 Measurement of 3β-Hydroxy-Δ^5-Steroid Dehydrogenase (HSD)

An immuno-fluorescence procedure for the detection of HSD activity in male *R. ridibunda,* was developed by Mensah-Nyagan et al. (1996). In this method, tissue samples were processed for indirect immuno-fluorescence with antiserum raised against purified human type I placental 17β-HSD. The specificity of this antiserum was verified using immunoblot analysis and found not to cross-react with other steroid biosynthetic enzymes, including 5α-reductase.

12.6 Molecular Tests

Assays evaluating the VTG, ER, AR, aromatase, and 5α-reductase gene expression have been developed primarily using various RT-PCR techniques.

12.6.1 Measurement of Vitellogenin Gene Expression

Detailed RT-PCR methods for measuring VTG gene expression in *X. laevis* are described by Bogi et al. (2003 and 2002), Lutz et al. (2003), and Kloas et al. (1999). These methods, including primer sequences and amplification procedures, are similar to those described in more detail for ER and AR mRNA expression described in the following section.

12.6.2 Measurement of Estrogen and Androgen Receptor Expression

Bogi et al. (2002) provides a detailed description of the RT-PCR method used to measure both ER and AR gene expression. Tissue total RNA was extracted using Trizol and chloroform followed by centrifugation (12,000 x g) to separate phases. Isopropanol was then added to the RNA-containing supernatant and recentrifuged. Ultimately, the RNA-rich pellet was re-dissolved in RNA-stabile water and the total RNA quantified at 260 nm using a spectrophotometer.

For the ER expression analysis, reverse transcriptase was used to transcribe RNA into cDNA using the RT-primer 5'CCTGATTCTAGAGCTATTTTTTTTTTTTTTTT-3'. Following the addition of the RT mix, the samples were incubated at 37˚C for 60 min. and terminated by heating to 90°C for 2 min. The *X. laevis* ER gene (Weiler et al., 1987) cDNA containing samples were subsequently amplified in accordance with the methods of Lutz et al. (2001). The ER primers used were forward: 5'-ATTTCGCATGATGAGGTTACG-3' and reverse: 5'-TAGTGGTAGGTGGACTCCGG-3'. Use the prescribed PCR reaction premix, amplification was conducted using a thermocycler following an initial step at 94˚C for 1 min. The amplification cycle (37 cycles) included 94˚C for 1 min., 60˚C for 1 min., 72˚C for 1 min., and 10˚C for 2 min. The PR products were electrophoresed using agarose gels with ethidium bromide staining, and densitometric measurement using an image analyzer.

Similar methods were used for amplification of *X. laevis* AR gene amplification as outlined by Kelley et al., 1989). In this case, the AR primers used by Bogi et al. (2002) were forward: 5'-CAGGAAATGTTAT-3' and reverse: 5'-ACGGTCATTTGGTCGCTTAC-3'. The same PCR reaction and cycles described for the ER cDNA was used.

Matthews et al. (2002) have described the use of reporter gene expression mediated by ERs from different species including *Xenopus*. In these studies, MCF-7 cells were transiently transfected with a Gal4-regulated luciferase reporter gene (17m50G-Luc) and Gal4-ER chimeric receptors containing Gal4-xERd for *Xenopus*.

12.6.3 Measurement of Aromatase Gene Expression

Measurement of aromatase gene expression has been described by Ohtani et al. (2003) in *R. rugosa* and by Miyashita et al. (2000) in *X. laevis*. The latter investigators cloned aromatase cDNA and evaluated mRNA expression. CDNA clones were isolated from the Xenopus ovarian cDNA library. An open reading frame of 1500 bp from the ATG start to TAA stop codons encoding for five predicted (vertebrate) amino acids as identified. A high degree of homology in the aromatase genes in *Xenopus* and human exists. RQ-RT-PCR was then used to quantify aromatase gene expression in the ovary.

12.6.4 Measure of 5α -Reductase Gene Expression

Torres and Ortega (2004) have recently described RT-PCR methodology for detecting 5α-reductase gene expression in rat using one-step RT-PCR combined with separation by capillary electrophoresis. The procedure, although not specifically developed using amphibians,

combined RT-PCR with laser-induced fluorescence capillary electrophoresis. The 5' forward primers were labeled with 6-carboxyfluorescein. Fluorescence-based quantification of separated mRNAs provided a reasonably sensitive and precise means of measurement (Torres and Ortega, 2004). Similar methods (Luo et al., 2003) have been developed microarrays in humans to evaluate the impact of 5α-reductase inhibitor on prostate cancer.

13.0 RECOMMENDED PROTOCOL AND ADDITIONAL DATA NEEDS

13.1 Preferred Test Species

13.1.1 Species Selection Criteria

Considering that the objectives in selecting an amphibian species are to: 1) develop a short-term Tier I screening assay for thyroid disruption as described in DRP 2-20 (4-5) (Battelle, 2003), and 2) develop a longer term Tier II assay that includes advanced developmental and reproductive endpoints as described in the present DRP (4-8), the minimal attributes of a test species are described in the following eight criteria:

1. The species must be amenable to continuous culture in the laboratory;

2. Reproduction in the laboratory must be routine throughout the year, using either naturally occurring reproduction or through the use of some type of hormonal induction;

3. Larvae must be able to be routinely reared to predetermined developmental stages;

4. The developmental rate for the interval included in any test must be relatively fast so that the effects are observed quickly, thus minimizing test duration and test costs;

5. The endpoints which constitute the test data that will be used for regulatory or pre-regulatory action need to be supported by a sufficient knowledge base that indicates that they are relevant to the question at hand.

In addition to the minimal criteria noted above, it would be highly beneficial for the subject species to have additional information relevant to the following areas:

6. Genetic information, including gene sequences of developmentally-relevant genes and some knowledge of the genetic programs associated with developmental and reproductive processes;

7. Biochemical information on the endocrine axis, particularly of the HPG axis; and

8. Metabolic information, especially as it relates to steroid biosynthesis and homeostasis and receptor expression as it relates to sexual development and reproduction.

13.1.2 Test Species

It is not absolutely essential that the same test species is selected for use in the Tier I Amphibian Metamorphosis Assay and the Tier II AGRA. However, selection of species that are closely related and work well within an integrated laboratory program is advantageous. The only anuran species which meets the minimal criteria established above are *X. laevis* and *X. tropicalis*. However, for the Tier II Amphibian Reproduction and Growth Test, *X. tropicalis* offers significant advantages over X. laevis, and is thus the recommended species. This species is routinely cultured in laboratories worldwide and is easily obtainable through commercial suppliers. Reproduction can be easily induced in this species throughout the year using hCG injections and the resultant larvae can be routinely reared to selected developmental stages in large numbers to permit the use of stage-specific test protocols. The developmental rate for *X. tropicalis* is rapid compared to *X. laevis* and especially the two ranid species commonly used in biological and toxicological research, *R. pipiens* and *R. catesbeiana*. In terms of post-embryonic development and reproduction at morphological , biochemical, and molecular levels, more is known about *Xenopus* than any other anuran species. In addition to meeting the minimal requirements stated above, the genetic information regarding *Xenopus sp.* is more extensive than other anurans where numerous publications have detailed the genes and the genetic program involved in early development, metamorphosis, sexual differentiation, and reproduction. Finally, the information on the biochemical and metabolic control of sexual differentiation and reproduction in this species is well-developed, and includes information on all of the typical HPG modulators, as well as, receptor expression and steroid biosynthetic pathways.

The only alternative species that should be considered is *X. laevis*. This species is similar in terms of ease of culture and reproduction. The primary disadvantages of this species compared to *X. tropicalis* are: 1) relatively longer rate for sexual differentiation and reproductive viability that could shorten test protocols, and 2) the genome of this species is oligo-tetraploid which will potentially complicates the use of some molecular endpoints. In terms of a Tier II screen that includes advanced developmental (e.g., gonadal development) and reproductive endpoints, *X. tropicalis* is superior to *X. laevis*. The primary advantage is that sexual maturity occurs in *X. tropicalis* in about 4 to 5 months, whereas *X. laevis* requires 1 to 2 years. One possible problem common to both species is that spontaneous reproduction cannot be reproducibly achieved in the laboratory and amplexus must be induced by hCG injections. However, spontaneous reproduction of other amphibian species in the laboratory also does not generally occur and breeding in the laboratory is substantially more complex.

13.2 Exposure Protocol

The recommended base exposure protocol will consist of a ca. 35-45 chronic exposure from early embryo stage (NF stage 8) through the completion of metamorphosis and primary sexual differentiation using *X. tropicalis*. Although it is anticipated that data relevant to specific stages of development of development will be collected throughout the exposure, the primary focus should be place on sexual differentiation and development of reproductive capacity. Thus, the majority of primary endpoints will be collected following the completion of metamorphosis. *X. tropicalis* cultured at 26-27˚C, complete metamorphosis generally at ca. 35-40 d, show signs of secondary sexual development at 60-75 d, and are generally capable of reproducing at d 150-

180. Since some EDCs may differentially affect primary and secondary sexual differentiation the base assay can be extended to 90 d to evaluate the onset of secondary sexual characteristics. Further, the base assay may be expanded to ca. 150-180 d if multigenerational data is sought at which time the specimens may be bred and the F1 progeny evaluated. However, limited data is currently available describing the length of time, and perhaps more importantly, the number of successive breeding required to produce a viable progeny under control conditions. Thus, a longer period of time may be required in order to accurately assess reproductive viability. Overall, it is anticipated that the base exposure protocol in combination with biochemical and molecular endpoints will be adequate for evaluating effects of potential EDCs on development (growth) and reproduction.

Static-renewal or flow-through exposure, with adequate test substance analysis based on the physicochemical properties of the test substance, is recommended. However, considering the length of the test, flow-through exposure will likely be the most practicable. On at least d 15, completion of metamorphosis (ca. d 35-45) for the base assay; and 45, 60, 75, and 90, 120, 150 and 180 for the advanced exposure tests; specimens should be digitally photographed to document morphological development and stage. Specimens should be randomly selected for histological examination of the gonads and samples should be collected for biochemical and molecular analysis of sex steroid hormone levels, ER/AR binding, and VTG induction/inhibition, aromatase and 5α-reductase levels, and possibly ER/AR gene expression. It should be noted that if one of the biochemical or molecular endpoints demonstrates particular sensitivity, reliability, and speed, it could be chosen to represent either a single biochemical or molecular biomarker for the proposed assay. However, the use of multiple endpoints provides additional confirmation of the response and will help distinguish between EDC-based and no-EDC-based responses. More research will be required to survey this issue and will be addressed in the Data Gaps Section (Section 13.5).

13.3 Appropriateness of Developmental, Growth, and Reproductive Endpoints

Morphological endpoints including abnormal development, developmental rate, completion of metamorphosis, gonadal development, sex ratio, and induction of reproductive behavior (advanced base test only) are both appropriate and sensitive endpoints. Further, incorporation of biochemical endpoints including, plasma or tissue steroid levels (at least E2, T, DHT), ER/AR receptor binding, and VTG induction/inhibition, aromatase and 5α-reductase activities; and molecular endpoints including, ER/AR gene expression are appropriate regardless of whether the base assay extended based assay is used.

13.4 Preferred Methods of Quantification of Biochemical and Molecular Endpoints

The preferred methods of biochemical endpoint analysis include RIA for steroid levels, although ELISA methods should be considered if available, radioreceptor assays for ER/AR binding, ELISA for VTG, and radiometric methods for enzyme analysis, including the $[^3H]H_20$ method for measuring aromatase activity. RT-PCR methods for evaluating ER/AR mRNA expression similar to those described by Lutz et al. (2001 and 2003), Bogi et al. (2002), and Kloas et al. (1999) are recommended.

13.5 Significant Data Gaps

The primary objective in identifying data gaps is to prioritize and apply resources to areas of uncertainty so as to reduce this uncertainty through research. As the EDSP process moves closer to implementation of this assay, several critical questions must be addressed. First, what responses, both organismal and sub-organismal, to established estrogen or androgen agonists and antagonists are currently known? The effects of classical estrogen or androgen agonists and antagonists on apical morphological changes during anuran sexual differentiation are reasonably well understood. However, the relationship between changes in HPG axis homeostasis and apical morphological changes are not as obvious. Before the effects of unknown chemicals on sexual differentiation and reproduction can be assessed, the response of known estrogen- or androgen-based disruptors in the recommended model system must be identified.

Second, which of the proposed endpoints will provide confidence that the observed effects are due to estrogen- or androgen-based mechanisms? At this point, it is understood that disruption of apical morphological changes during sexual differentiation may or may not be the result of alteration of HPG function. More work will be required to assess confidence in the histology, biochemical, and molecular endpoints in terms of predictability of endocrine impairment. As additional data are collected, an assessment of whether these endpoints could stand alone or require other complementary or confirmation endpoints can be made.

Third, what is the time course of these responses? As research continues in these previously discussed areas, it will also be important to determine the time frame required to observe the effects of EDC exposure based on the selected endpoints. For example, observation of molecular and biochemical changes may be observed more rapidly than histological or morphological changes. However, the estimated time course in which one would expect to observe these responses and changes in sensitivity relative to the time course, need to be elucidated. Morphological effects on sexual differentiation may be markedly more sensitive than the morphological effects observed on the thyroid axis.

Fourth, what is the sensitivity of the measurement endpoints? The relative sensitivities of each endpoint recommended can be estimated at this point. However, information on the specific sensitivities of each endpoint needs to be determined.

Fifth, when does a molecular change constitute a valid indication of HPG, sexual differentiation, or reproductive fitness perturbation? To understand with confidence at what point molecular changes are an indicator of endocrine disruption, the results must somehow be shown to be related to an upstream or downstream response within the respective endocrine axes. If molecular changes, such as inhibition of ER or AR mRNA synthesis, can be linked to a histological, biochemical, or possibly even an apical morphological change in gonadal development, this relationship can be addressed. Work will be required to determine the threshold of molecular change that results in a physiological change in gonadal status. However, once this relationship and set-point are determined, the molecular assays, like gene arrays and RT-PCR techniques, will be extremely valuable.

Finally, and most importantly, what is the dynamic range of endocrine homeostasis and its relationship to gross morphological, molecular, biochemical, and histological changes? The degree to which HPG homeostasis can be changed without adversely affecting the organism needs to be determined. In addition, the relationship between the sensitivity of HPG axis homeostasis and the measurements also requires understanding. To bridge these data gaps, further studies during prevalidation will need to be specifically directed toward these areas.

14.0 IMPLEMENTATION CONSIDERATIONS

14.1 Animal Welfare Considerations

Although *in vitro* and molecular test methods of evaluating specific aspects of HPG axis disruption, including reporter gene assays, ER and AR binding assays, gene arrays, and other gene expression techniques are emerging and appear promising, an *in vivo* based assay is considered to be the most sensitive, reliable, and practicable approach for use in a screening battery at the present time. Additional discussion of the attributes and short-comings of current *in vitro* and molecular approaches in relation to the *in vivo* methods is provided in the previous sections of the present DRP.

In accordance with current animal welfare guidelines, the test protocols developed and research conducted throughout prevalidation phases will comply with requirements stipulated in the Animal Welfare Act and documented in the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH, 1985). Under this animal welfare program, consideration should be given to the reduction, replacement, or refinement in the use of animals in research. Use of amphibians in research does reduce the use of higher vertebrate animals, including mammals. Care is required to ensure that the number of organisms used and pain and distress induced in research animals are minimized. Laboratories participating in prevalidation and validation studies should have an "Assurance of Compliance with Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals" on file with PHS and EPA. This process requires the establishment of an active Institutional Animal Care and Use Committee (IACUC) and the use of Animal Care and Use Protocols (ACUPs) for the studies performed.

14.2 Recommended Equipment/Capabilities

14.2.1 Laboratory Capabilities

Laboratories generally qualified to perform research and conduct testing associated with the prevalidation and validation of the AGRA should have staff toxicologists, chemists, biologists, and biochemists that specialize in freshwater ecotoxicology, trace-contaminant analysis/metals chemistry, organic chemistry, environmental forensics, ecological risk assessment support, bioassay method development, endocrine activity assessment, and ecotoxicological research in amphibians.

14.2.1.1 Amphibian Laboratory. General aquatic toxicology laboratory facilities are required to conduct amphibian bioassays. Bioassay laboratories should provide adequate space for studies requiring flowing and static freshwater. Aquatic animal isolation facilities should be available to conduct moderate and high hazard work, with space for a variety of tank configurations for physiological, biochemical, and molecular studies on amphibians. Large tubs can be used to maintain pathogen-free, genetically characterized aquatic animals for studies of this nature.

A freshwater distribution system should be available to supply purified freshwater to the laboratories. In addition to freshwater, high-quality, ASTM Type II purified reagent-grade water can be obtained from a separate reverse osmosis-deionization polishing system. A system of pumps can be used to provide a continuous supply of dechlorinated freshwater to experimental tanks, as needed. Since some amphibian studies involve static exposure, freshwater carboys can be stationed throughout the laboratories for these studies. An emergency generator should be available to ensure continuous freshwater supply and other essential services in the event of electrical failure. Reserve tanks are required to provide freshwater to the wet laboratories for up to 72 h in the event of failure of the pumps.

Holding and breeding facilities for amphibians at a variety of different ages should be provided in these laboratories. All water leaving the laboratory from experimental test systems should be passed through a treatment system prior to release into the sewer system. Static water can be collected and analyzed for test substances prior to release into the sewer system. This includes pathogen disinfection and hazardous waste collection for the controlled access rooms.

Animal test facilities should be generally organized and operated following PHS guidelines on care and use as previously described in Section 12.1. In addition to the general requirements, flow-through diluter systems, solid phase concentrators, and other relevant delivery systems may be required. Aeration and dissolved oxygen and pH monitoring equipment may also be necessary.

14.2.1.2 Analytical Laboratories. Additional laboratories, including general chemistry, biochemistry/molecular biology, and histopathology are useful and may be required. Instrumentation and supplies might include an array of chromatography and metals instrumentation, digestion systems, analytical balances, sonicators, freezers, refrigerators, drying ovens, ash furnaces, centrifuges (ultra and low/mid-low speeds) with rotors, microfuges, spectrophotometers (double beam) with spectroscopy software, spectrophotometers with gel scanner, absorbance monitors with flow cells, fraction collectors and syringe pumps for fractionating gradients, power supplies, UV transilluminator and digital camera system, micro injection equipment (with pipette puller) for frog oocyte injection experiments, fume hoods, laminar flow hoods, Polytron tissue homogenizers, hot-cold circulator baths, water baths, incubators, and aquatic monitoring equipment (including pH, conductivity, and dissolved oxygen meters, thermometers, and titration systems) for measuring routine water quality (pH, conductivity, D.O., temperature, alkalinity, hardness, ammonia-nitrogen, and residual chlorine).

The following are descriptions of the specialized laboratories that might be required or considered useful: 1) QA Laboratory for receipt of samples and sample preparation for analysis;

2) General Wet Chemistry Laboratory for general water quality analysis, analysis of physical properties for water and sediment, and preparation of sample extracts; 3) Chromatography and Mass Spectroscopy Laboratory for conducting specialized cleanup procedures and analyses of test materials and organic contaminants using microprocessor-controlled high-resolution gas chromatographs (GC) with FID/PID, N/P, ECD, and mass selective (GC-MS) detectors and high-performance liquid chromatograph/ion chromatograph (HPLC/IC) with variable-wavelength ultraviolet detector, and pulsed electrochemical detector, and fraction collector for specialized sample preparation; 4) Metals Laboratory for preparation and analysis of samples for metals using atomic absorption (AA) spectrophotometers equipped with graphite furnace and flame capabilities, and autosamplers and inductively coupled plasma (ICP) spectroscopes; and 5) Biochemical/Molecular Laboratory for evaluation of gene activity, creation of transgenic lines, analysis of hormone levels, and histopathology using water baths, thermocyclers (PCR), blotting and DNA imaging equipment, electrophoresis equipment, power supplies, transfusion equipment, ELISA and RIA equipment (including scintillation counters), and histological equipment (including microtomes, fluorescence and light microscopes, and electron microscopes).

14.2.2 Standards and Reference Materials

Standards and reference materials should be traceable to the National Institute of Standards and Technology (NIST) or other nationally recognized standard (e.g., American Society for Testing and Materials or ASTM). The traceability should be documented by a certificate or label that verifies this traceability.

14.2.3 Reagents, Chemicals, and Solutions

The procurement of reagents, chemicals, and solutions should include requirements for shipping stocked inventory materials with the longest period to the expiration date (i.e., the freshest material) possible, with specified lot numbers. When large quantities of materials are purchased, procurement should require obtaining materials from the same lot to minimize variability. In some cases where extremely high purity material is requested, a request for purity documentation may be necessary

Procurement procedures should require that a manufacturer's recommended expiration date be provided with every standard material. If manufacturer's expiration dates are not provided, the laboratory should assign an appropriate expiration date, based on professional judgment and in consideration of the shelf life for similar materials at similar concentrations.

A chemical inventory system-lab information management system (CIS-LIMS), with requirements for logging in reagents, chemicals, and solutions into the associated chemical management system of the company-wide health and safety program, should also be considered.

The procurement of animals and feed should include requirements for chain of custody of animals during shipping and documentation of any available feed analysis, feed storage recommendations, and expiration dates so that feed quality can be monitored. Animal shippers should be requested to document conditions of animals and environmental parameters

(temperature) at the time of shipping for comparison with conditions encountered at the time of receipt. In some cases, it might be important to include QA requirements for a minimum/maximum thermometer or temperature strip in the cooler at the time of shipping.

14.2.4 Sample Tracking Capabilities and Criteria

Chemicals should be immediately coded and entered into appropriate logs. A sample custodian should supervise the chain of custody log-in. All items under chain of custody should be inventoried, and the sample custodian should examine the sample container(s) to ensure that the sample seals are intact and the sample containers have not been damaged.

14.2.5 Data Handling (Database and Statistical Capabilities)

The following areas associated with data handling and management should be considered by participating laboratories: 1) statistical design and measurement – experimental designs, statistical models, statistical analysis strategies, and measurement protocols; 2) data collection and management – automated and manual data collection planning, scheduling, and implementation systems and protocols and data acquisition, data transfer, data processing, and data storage and retrieval systems and protocols; and 3) data analysis and presentation – statistical analyses, systems, and methodologies and data visualization systems and techniques, and QA review.

14.2.6 Specimen and Data Storage Facilities

Sample specimens should be stored in appropriate locations (freezers, refrigerators, walk-in coolers, etc.) based on temperature and light requirements, until time for disposal. Repository chemicals should be stored as per manufacturer's recommendation prior to purity testing.

Data storage while tests are in progress should be maintained by the Quality Assurance Unit (QAU), under "restricted access" conditions. These areas include record file cabinets, record storage rooms, and the GLP data archive areas. Post-project data archives should be maintained in a locked, limited access room, free from environmental or pest damage or degradation. Since validation studies will be conducted under the GLP guidelines, post-project files should be retained for 10 years (5 years for non-GLP studies).

14.2.7 Facility GLP Requirements (Validation Phase Only)

The QAU, under the direction of the QAU manager, should maintain all GLP QA documents, including the GLP guidance document, health and safety plan, IACUC animal welfare plan, master schedule log, standard operating procedures (SOPs), study protocols, training records, staff experience information, and GLP document forms.

14.3 Recommendations for Prevalidation Studies

One of the primary objectives of the prevalidation studies will be to address the Data Gaps identified in Section 11.6. In order to address these gaps, prevalidation studies should be

divided into a phased-set of activities. The first phase should focus on final definition and development of the following recommended endpoints associated with the *X. tropicalis* chronic exposure protocol for assessing advanced developmental and reproductive effects of EDC exposure. The most significant work needs to be performed in the final development of the molecular endpoints. Use of ER and AR gene expression from tissue samples using RT-PCR analysis is reasonably well developed.

As for histological, morphological, and biochemical endpoints, preliminary protocols should be prepared for use in prevalidation studies. A general protocol describing how the exposure is to be performed and how data from each endpoint are to be collected in an integrated format is also recommended. During this phase, a set of three known estrogen agonists and antagonists, as well as, androgen agonists and antagonists should be tested each utilizing different mechanisms of action. Once results with each endpoint are collected, analyzed, and reviewed, revised protocols should be prepared in the third phase. An additional set of three test chemicals which have anecdotal estrogenic/antiestrogenic or androgenic/antiandrogenic capacity and perhaps one that has no information (fourth phase) regarding EDC activity should then be evaluated using the revised protocol. Following analysis of the data, the revised protocol should again be reviewed and revised, if necessary, and a Final Protocol developed (fifth phase) for use in interlaboratory GLP validation studies.

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APPENDIX A LITERATURE SEARCH

APPENDIX B EXPERT REVIEW (Dr. Brent Palmer, University of Kentucky)