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for

AMPHIBIAN METAMORPHOSIS ASSAY

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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. 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 Metamorphosis Assay is a short-term test with morphological, biochemical, and molecular-based elements designed to evaluate the effects of EDCs on the thyroid axis. The 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 metamorphosis in amphibians, certain tissues are resorbed, some are remodeled, and some are created to form an adult organism capable of surviving in a different habitat. Thyroid axis control of the metamorphosis in amphibians is highly complex and involves the CNS, hypothalamus, pituitary gland, thyroid gland, thyroid hormone (TH) transport proteins, thyroid receptors (TR), and transcriptional elements. Although highly complex, two principles remain constant, 1) metamorphic events are triggered by TH, and 2) tissue responsiveness to TH

is based on selective response based on TH interaction with TR. Overall, the thyroid axis is 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 normal thyroid function using an amphibian as a general vertebrate model. The Amphibian Metamorphosis Assays could potentially consist of whole organism exposure tests, histological analysis, biochemical (hormone) analyses, or molecular assays designed to screen substances that might adversely disturb thyroid function. Whole organism tests reviewed in this paper include a short-term metamorphic climax test with *Xenopus sp.*, a complete metamorphosis test with *Xenopus sp*., a prometamorphosis test with *Xenopus sp.*, and an assay utilizing the sexual dichromatic reed frog, *Hyperolius sp*. Endpoints originally considered noteworthy included: limb development, skin maturation (and coloration), tail resorption, and thyroid morphology and pathology. Methods for biochemical measurement of TH, as well as deiodinase, were evaluated. These methods included conventional radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) techniques, and LC/GC-MS methods. Finally, molecular techniques designed to biomark thyroid function or dysfunction, including transgenic whole animal lines, transfected amphibian cell culture lines, receptor binding and carrier protein assays, and gene expression assays (differential display, RNase Protection Assay [RPA], real time-polymerase chain reaction [RT-PCR], and gene array technologies) were reviewed. Recommendations are provided in the following Detailed Review Paper (DRP). The recommended test protocol involves the use of *Xenopus laevis* as the test species since it was the only species reviewed that met the minimal criteria for the selection of a test species. A late pre- to early prometamorphic exposure (stages 51-54 [Nieuwkoop and Faber, 1975]) period is recommended during which time the following endpoints will potentially be monitored; thyroid gland histology, biochemical changes (THs), molecular changes in thyroid axis-related gene activity using either RT-PCR (single gene) or gene array (multiple genes) techniques, and apical morphological effects (i.e., limb differentiation). This exposure period is approximately 14-d in duration. Genes recommended as biomarkers include TR beta, TR alpha, and ST3, although gene arrays could account for a substantially greater number of relevant genes.

The primary data gaps that exist at this point include understanding of what responses may be induced at both organismal and suborganism-levels by establishing thyroid axis agonists and antagonists; which endpoints will link the effects induced as a thyroid-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 thyroid disruption. Finally, the dynamic range of thyroid 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. laevis* prometamorphosis assay 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. During this phase, a set of three known thyroid agonists (i.e., thyroxin) and antagonists (i.e., perchlorate,

propylthiouracil, and amiodarone) should be tested. Once results with each endpoint are collected, analyzed, and reviewed, revised protocols should be prepared (third phase). An additional set of three test chemicals which have anecdotal thyroid disruption capacity and perhaps one which has no information regarding thyroid axis activity should then be evaluated using the revised protocol (fourth phase). 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.

2.0 INTRODUCTION

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

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

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

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

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

The U.S. EPA established the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), to provide recommendations regarding a strategy for developing a testing paradigm for compounds that may have activities similar to naturally-occurring

hormones. Following the recommendations made by EDSTAC in its final report (EDSTAC, 1998), the U.S. EPA established the Endocrine Disruptor Screening Program (EDSP). The program's aim is to develop a two-tiered approach, e.g. a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial substances, and environmental contaminants (Federal Register 1998a, 1998b).

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

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

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

2.2 The Validation Process

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

for test method validation that was applicable to conventional and alternate methods, and could be applied to the needs of different agencies and regulatory processes.

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

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

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

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

2.3 Purpose of the Review

The purpose of Work Assignment 2-20 is to prepare a Detailed Review Paper (DRP) for Amphibian Metamorphosis Assays. Several Amphibian Metamorphosis Assays consisting of potential short-term morphological, biochemical, and molecular-based tests designed to evaluate the effects of endocrine-disrupting chemicals (EDCs) on the thyroid axis will be evaluated for

use as a thyroid disruption screening 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 amphibian based assay will be recommended for measuring thyroid axis pertubation.

2.4 Rationale and Objectives of the Amphibian Metamorphosis Assays

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, in this case the thyroid axis. 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 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 Amphibian Metamorphosis Assays are being considered for inclusion in the Tier 1 Screening Test Battery to monitor disruption of normal thyroid function.

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, 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.

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

The thyroid axis represents one potential target for environmental chemicals. Environmental agents, toxicants, natural products, and complex mixtures can alter metamorphosis by interacting with the thyroid axis. Further, the complexity of the thyroid axis 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 thyroid disrupting chemicals or chemical mixtures as a representative chordate is not unreasonable.

To date, the debate on endocrine disruptors has mostly revolved around gonadal steroids including estrogens and androgens, because of controversy regarding their possible link to infertility, breast cancer, and lower sperm counts. Thus, the thyroid has received comparatively little attention. Brucker-Davis (1998) recently reviewed the effects of synthetic chemicals in the environment on thyroid function. This review confirms the hypothesis of thyroid disruption by environmental chemicals in wildlife and supports the need for human population and laboratory animal studies on compounds already identified as thyroid disruptors. In this review, Brucker-Davis (1998) described the effects of over 40 pesticides and 45 industrial chemicals on the thyroid axis.

This DRP considers the use of several potential amphibian species in the development of Amphibian Metamorphosis Assays 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 frogs; however, it should be noted that the majority of the currently available literature exists in the frog domain. Considering the intended use of Amphibian Metamorphosis Assays as a screening test (Tier 1), 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, interviews with experts were conducted to obtain the current views and opinions regarding promising assays, methods, procedures, and measurement endpoints that hold promise for developing Amphibian Metamorphosis Assays to identify chemicals that affect (i.e., inhibit or enhance) thyroid activity. The results of the interviews 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. Acronyms and Definitions

ACTH	Adenocortropin Hormone
ASTM	American Society for Testing Materials
BRD	Background Review Document
cDNA	Complimentary Deoxyribonucleic Acid
CNS	Central Nervous System
CRF	Corticotropin Releasing Factor
CV	Coefficient of Variation
DIT	Diiodotyrosine
DRP	Detailed Review Paper
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
FETAX	Frog Embryo Teratogenesis Assay-*Xenopus*
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FQPA	Food Quality Protection Act
FR	Federal Register
HAES	Hyperolius Argus Endocrine Disruption Screen
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
MBS	Moderately Buffered Solution

MIT	Monoicotyrosine
mRNA	Messenger Ribonucleic Acid
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
PCR	Polymerase Chain Reaction
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
TH	Thyroid Hormone
TR	Thyroid Receptor
TRH	Thyroid Receptor Element
TSH	Thyroid Stimulating Hormone
TUNEL	Terminal Deoxynucleotidyl Transferase-mediated dUTP-biotin Nick-end Labeling
T3	3,3',5–triiodothyronine
T4	Thyroxine
XEMA	*Xenopus* Metamorphosis Assay

3.0 OVERVIEW AND SCIENTIFIC BASIS OF AMPHIBIAN METAMORPHOSIS ASSAYS (ENDOCRINE CONTROL OF THE THYROID AXIS)

3.1 The 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 the hormones testosterone and estrogen and related components from the testes and ovaries); regulation of metabolism (through the hormones cortisol from the adrenal glands and thyroxin from the thyroid gland); development of the brain and the rest of the nervous system (estrogen and thyroid hormones); and 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).

The endocrine system of amphibians is similar to that of most vertebrates; the peripheral endocrine tissues are controlled by tropic hormones from the pituitary, which are controlled by releasing hormones from the hypothalamus. The hypothalamus is, in turn, controlled by the central nervous system and affected by various environmental stimuli (Hayes, 1997a). However, there are some unique features in amphibian hormonal axes. In larval frogs thyroid hormone secretion and glucocorticoid secretion are ultimately stimulated by a single hypothalamic releasing hormone –corticotropin-releasing hormone (CRH)- that stimulates both thyrotropin and adrenocorticotropin (ACTH) (Denver, 1993; 1997; 1998; Denver and Licht, 1989; Gancedo et al., 1992). In addition, corticosterone (CORT) may negatively feed back on the pituitary, or possibly the hypothalamus, and decrease both thyrotropin- and ACTH-secreting cells of the pituitary (Hayes, 1997a). This relationship is not observed in adult amphibians in which thyrotropes and adrenocorticotropes are controlled by thyrotropin-releasing hormone (TRH) and CRH, respectively. Also, a single population of cells synthesize the gonadotropins and thyrotropins in larvae of a t least one species (*Bufo boreas*), but distinct populations of cells secrete these two hormones in adults (Pearson et al., 1998).

In addition to the unique features described above, there are a number of interactions between thyroid hormones, sex steroids, and glucocorticoids in amphibians (Roth, 1942; Roth, 1948; Frieden and Naile, 1955; Kobayashi, 1958; Jaffe, 1981; Kikuyama et al., 1983; Krug et al., 1983; Leatherland, 1985; Galton, 1990; Gray and Janssens, 1990; Leloup-Hatey, 1990; Hayes et al., 1993; Kikuyama et al., 1993; Hayes, 1995a; 1995b; 1997a; 1997b). Glucocorticoids interact with the thyroid axis in several ways: The TH, thyroxine (T4), stimulates corticoid production by interrenal tissue. In turn, corticoids accelerate the conversion of T4 to the more potent triiodotyronine (T3). The THs and the corticoids may have a negative feedback effect on the pituitary (Hayes, 1997a).

Sex steroids inhibit the thyroid axis (Roth, 1942; 1948; Leatherland, 1985; Maclatchy et al., 1986; Gray and Janssens, 1990; Hayes, 1997a), and both androgens and estrogens can inhibit TH function. Sex steroids inhibit the conversion of T4 to T3 (Leatherland, 1985; Maclatchy et al., 1986), and, in a recent study, we showed that estrogen treatment resulted in the complete involution of the thyroid gland (Hayes, unpublished data). This involution was hypothesized to result from inhibition of stimulation from the pituitary. Other studies showed that the same cells synthesized gonadotropin and thyrotropin in *B. Boreas* (Hayes, 1997a; Pearson et al., 1998) and that negative feedback on these cells by sex steroids may simultaneously down-regulate gonadotropin and thyrotropin secretion.

THs and steroids also interact at the receptor/molecular level. Corticoids enhance thyroid hormone activity by increasing the binding of T3 to its receptor (Niki et al., 1981; Suzuki and Kikuyama, 1983). Also, T3 auto-induces its receptor (Rabelo and Tata, 1993; Rabelo et al., 1994; Tata, 1994; Ulisse and Tata, 1994; Tata, 1996; Shi et al., 1998) and induces the estrogen receptor (Rabelo and Tata, 1993; Rabelo et al., 1994). Similar data suggest that THs may be

required for steroid receptor induction in all frogs, but salamanders may express steroid receptors in the absence of THs (Hayes, 1997b).

A large number of anthropogenic chemicals released into the environment, as well as a few natural ones, have the potential to disrupt the endocrine system of animals, including humans (Colborn and Clement, 1992). Among these are the persistent, bioaccumulative, organohalogen compounds, which include some pesticides (fungicides, herbicides, and insecticides), industrial chemicals along with other synthetic products, and some metals (Brucker-Davis, 1998). Many wildlife populations are already affected by these compounds. The impacts include thyroid dysfunction in birds, amphibians, and fish; decreased fertility in birds, amphibians, fish, shellfish, and mammals; decreased hatching success in birds, fish, alligators, and turtles; gross birth defects in birds, amphibians, fish, and turtles; metabolic abnormalities in birds, fish, and mammals; behavioral abnormalities in birds; demasculinization and feminization of male fish, amphibians, birds, and mammals; defeminization and masculinization of female fish, amphibians, alligators, and birds; and compromised immune system in birds and mammals (Lister and Van der Kraak, 2002; McMaster et al., 2001).

The origin of the term "endocrine disruption" and the proposition that these are agents in the environment that affect, via alteration of the internal hormonal milieu, reproduction and development dates back to the late 1980s (Colborn and Clement, 1992; Kavlock et al., 1996). The authors described effects in fish-eating birds, alligators, Great Lakes mink, frogs, invertebrates, and humans. These agents were proposed to act, even at very low environmentally relevant doses, as agonists or antagonists to endogenous endocrine hormones to disrupt the hormonal control of homeostasis, differentiation, growth, and development, including effects on reproductive structures and functions. These agents were called endocrine-active chemicals (EACs), endocrine-disrupting chemicals (EDCs), or most popularly "endocrine disruptors" (EDs) (EDSTAC, 1998).

Numerous studies (Lister and Van der Kraak, 2001; McMaster et al., 2001) provide evidence of compromised growth, reproduction, altered behavior, and abnormal development from exposure to a variety of natural and anthropogenic chemicals in invertebrate, fish, amphibian, reptilian, avian, and mammalian species. In addition to the potential to affect a wide variety of taxa, the following four attributes further complicate the study and regulation of EDCs: 1) the chemicals of concern may have entirely different effects on the embryo, fetus, or perinatal organism than on the adult; 2) the effects are most often manifested in offspring, not in the exposed parent; 3) the timing of exposure in the developing organism is crucial in determining its character and future potential; and 4) although critical exposure occurs during embryonic development, obvious manifestations might not occur until maturity (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 thyroid disruption capacity of chemical substances as a component of a larger EDC Screening Strategy. However, it should be noted that metamorphosis and, in some cases, thyroid function can be influenced by a combination of other biotic and abiotic factors beyond the realm of chemical stressors. These factors include temperature, water availability, crowding, light, diet, and

environmental iodine levels (Dodd and Dodd, 1976). Amphibian larvae 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, 1997; 1998). Temperature also affects the rate of metamorphosis such that higher 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). Biotic factors, which alter the rate of metamorphosis, such as the synergistic effects of corticosteroids on TH-induced metamorphosis, must also be considered. Overall, it must be understood that the link between the thyroid axis and 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 amphibian metamorphosis assays designed to screen for thyroid disruption. Initially, methods in four candidate amphibian species (i.e., *X. laevis*, *X. tropicalis*, *R. pipiens*, and *Hyperolius sp*.) 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 prevalidation 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 thyroid axis in amphibians.

3.2 The Thyroid and Thyroid Hormone (TH)

Understanding of the role of the thyroid in inducing metamorphosis dates back to circa 1912-1915 (Gudernatsch, 1912; Kendall, 1915; 1919). These studies led to the isolation of two endogenous THs and demonstration that TH is the causative agent of amphibian metamorphosis. However, like most developmental processes, metamorphosis is under the control of other hormonal systems, including hormones from the pituitary and the adrenal glands. In addition, a complex neuroendocrine pathway appears to be involved in amphibian metamorphosis.

The thyroid gland in most amphibians develops during late embryogenesis (Dodd and Dodd, 1976; Regard, 1978). In *X. laevis*, the thyroid develops from a thickening of the pharyngeal epithelium around Nieuwkoop and Faber stage 35 (hatching) (Nieuwkoop and Faber, 1975). For ease of discussion, a comparison between *Xenopus* development and development in *Rana* is provided in Table 3-1. Following division of the thyroid, follicular development is present by stage 44. A functional thyroid gland with approximately 20 follicles is present by stage 53. As the prometamorph matures, follicular development continues, resulting in growth of the gland. Concurrently, TH synthesis and secretion into the circulatory system increase in

Table 3-1. Comparison of Larval Anuran Stages

	Stages																							
Species	Limb Bud Growth								Foot Differentiation							Rapid Hind Limb Growth						Tail Resorption			
Xenopus laevis[a]	46	47/48	49/50	51	52	53	53	53	54	55	55	55	56	57	57	58	59	60	60	61	62	63	64	65	66
Rana pipiens[b]	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX	XXI	XXII	XXIII	XXIV	XXV
	Premetamorphosis[c]								Prometamorphosis[c]							Climax[c]									

[a] Nieuwkoop and Faber (1956)
[b] Taylor and Kollros (1946)
[c] Dodd and Dodd (1976)

preparation for metamorphosis. After metamorphosis is complete, the thyroid gland regresses in size, which may be responsible for the reduced levels of circulating TH at that time. Two naturally occurring THs, 3,5,3',5'-tetraiodothyronine (T4) and 3,5,3'-triiodothyronine (T3) have been identified. Based on years of experimental evidence, there is little question today concerning the causative effect of TH on amphibian metamorphosis (Gudernatsch, 1912; Allen, 1916; Allen, 1929; Leloup and Buscaglia, 1977; White and Nichol, 1981; Tata, 1968; Dodd and Dodd, 1976; Brown et al., 1995).

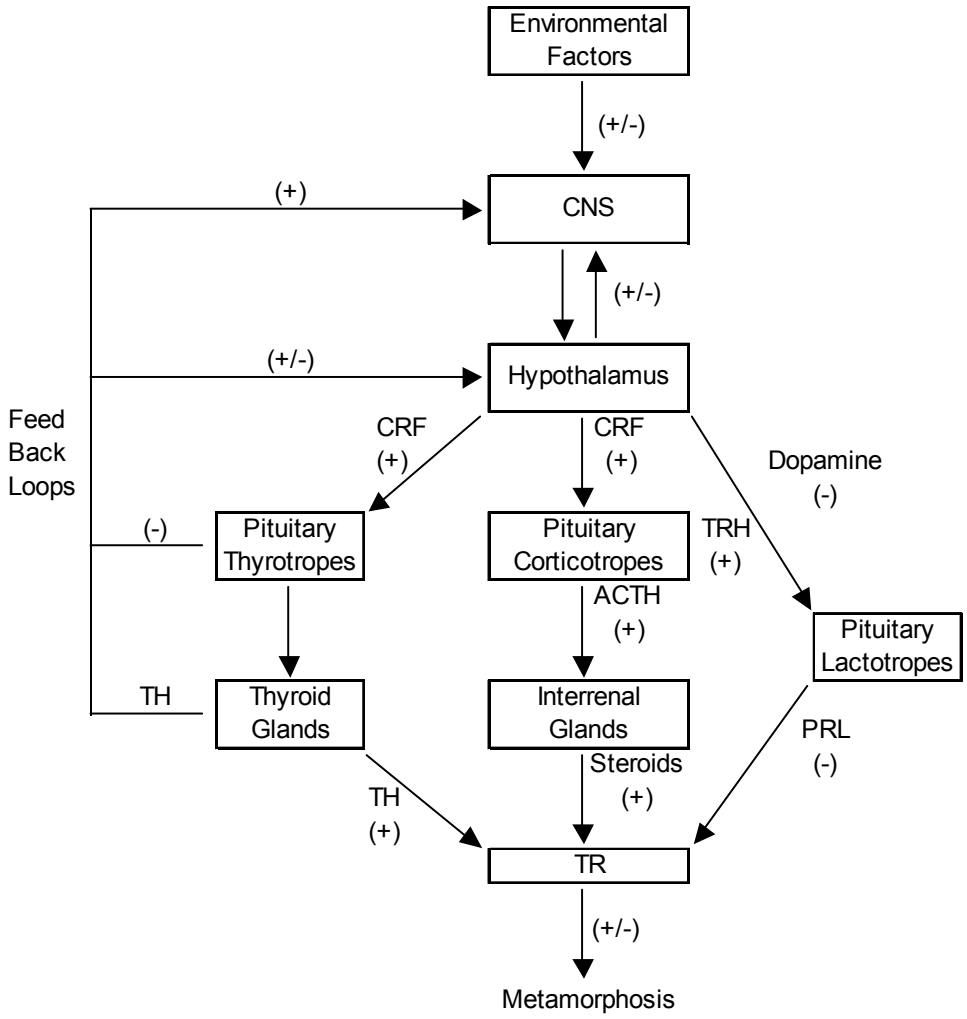
3.3 Neuroendocrine Control of the Thyroid

Although TH is the primary factor that controls metamorphosis, synthesis of TH in the thyroid is under complex neuroendocrine control. TH, in turn, completes a complicated feedback loop at the central nervous system (CNS), hypothalamus, and pituitary levels. These interactions form the hypothalamus-pituitary-thyroid axis. This axis is illustrated in Figure 3-1.

3.3.1 Pituitary Regulation of the Thyroid

The primary effect of the pituitary gland on the thyroid gland is mediated by thyrotropin (or thyroid stimulating hormone [TSH]), which is produced and secreted by the par distalis region of the pituitary gland. Thus, TSH induces the production and release of TH from the thyroid gland. TH provides feedback to the pituitary to control the release of TSH (Dodd and Dodd, 1976; White and Nicoll, 1981; Kikuyama et al, 1993; Kaltenbach, 1996, and 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, however, the purified frog TSH was more potent. Dodd and Dodd (1976) estimated TSH levels in crude pituitary extracts during development using radioiodine uptake in hypophysectomized *X. laevis* tadpoles. These investigators founds that TSH was present in relatively low quantities during prometamorphosis (stage 56), but rose significantly at the onset of metamorphic climax (Nieuwkoop and Faber [NF] stage 59). A subsequent decrease in pituitary TSH level at stage 61 followed by a spike in pituitary TSH at stage 62 was found. Thus, increasing levels of TSH occur during metamorphosis when T4 release from the thyroid gland is required. The drop in pituitary TSH coincides with peak T4 levels and is thought to be the result of increased secretion of TSH from the pituitary to stimulate TH release from the thyroid gland. Because complementary DNAs (cDNAs) coding for TSH in *X. laevis* have been cloned, an understanding of this process at the molecular level is now available (Buckbinder and Brown, 1993). In essence, analysis of messenger RNA (mRNA) levels during metamorphosis indicated that TSH genes were activated around stage 53, just prior to the first stage in which pituitary TSH is detectable. TSH mRNA levels rise to a peak around NF stages 58 or 59, and subsequently drop to lower levels toward the conclusion of metamorphosis (Leloup and Buscaglia, 1977). The repression of TSH genes after stage 59 coincides with high levels of plasma TH and is consistent with negative feedback on the pituitary gland or hypothalamus by TH. A high degree of homology exists between frog TSH cDNA and mammalian counterparts (ca. 60 to 70% at the amino acid sequence level).

FIGURE 3-1
HORMONAL PATHWAYS INVOLVED IN AMPHIBIAN METAMORPHOSIS



3.3.2 Hypothalamic Regulation of the Pituitary

The stimulatory action of the hypothalamus on metamorphosis is primarily due to its ability to stimulate the release of TSH from the pituitary. As in mammals, TRH is responsible for inducing the secretion of TSH from the pituitary. 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, and 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, and Denver, 1996). High concentrations of TRH have been detected in the brain and skin of *R. pipiens* (Jackson and Reichlin, 1977). Further, TRH concentrations in the brain of *X. laevis* and *R. catesbeiana* 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, although TRH has been shown to induce the release of TSH from the pituitary in anurans, 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, and Denver, 1993; 1996; 1998).

Denver and co-workers (Denver, 1988; and Denver and Licht, 1989) found that mammalian corticotropin-releasing factor (CRF) stimulates the release of TSH from the pituitary. In mammals, CRF is responsible for inducing the secretion of ACTH, and mammalian CRF is also capable of accelerating ACTH release from frog pituitaries (Tonon et al., 1986; Gracia-Navarro et al., 1992). However, ACTH has no thyroid stimulating activity (Sakai et al., 1991). CRF is now known 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 equivalent of TRH and mediates the regulatory role of the hypothalamus on the anuran pituitary (Rivier et al., 1984; Gancedo et al., 1992; Denver, 1993; 1997b). Two genes encoding for anuran CRF have been cloned in *X. laevis* and the homology to mammalian CRF was determined to be greater than 93%. *In situ* hybridization studies have demonstrated the presence of CRF-expressing cells in the hypothalamus of *X. laevis* and expression of the CRF genes has been found to be TH-dependent (Verhaert et al., 1984; Olivereau et al, 1987; Gonzalez and Lederis, 1988, Carr and Norris, 1990; and Stenzel-Poore et al., 1992). The hypothalamic feedback loop was demonstrated by Denver et al. (1997b): the CRF genes were up-regulated by a 4-hour treatment of TH and down-regulated following a 24 hour treatment with TH in *X. laevis*. CRF mRNA levels increase significantly during prometamorphosis and peak at metamorphic climax (Carr and Norris, 1990), which is consistent with CRF being the primary metamorphic hypophysiotropin (Denver, 1996).

3.4 Impact of Other Hormones on Metamorphosis

3.4.1 Corticoids

Corticoids are capable of accelerating TH-induced metamorphosis in amphibians (Kaltenbach, 1985; Kikuyama et al., 1993; and Hayes, 1997). Both corticosterone and aldosterone, the major corticoids secreted by the interrenal gland of amphibians (Cartensen et al., 1961; Macchi and Phillips, 1966; and Kikuyama et al., 1993), 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, 1993; Hayes, 1997). Direct evidence for the role of corticoids in metamorphosis comes from several critical findings. Investigators found that exogenous corticoids accelerate tail resorption of premetamorphic tadpoles when directly added to culture water (Kaltenbach, 1985; Kikuyama et al., 1983). Similar effects of exogenous corticoid administration, including aldosterone, corticosterone, and deoxycoritcosterone or tail

reduction in organ culture has also been observed (Kikuyama et al., 1983; Hayes et al., 1993; Hayes and Wu, 1995; Hayes, 1997). Other investigators have demonstrated the role of corticoids in the regulation of metamorphosis, including the presence of corticoid binding sites in the tail (Woody and Jaffe, 1984 and Yamamoto and Kikuyama, 1993) and the potentiating effect of exogenous corticoids on TH-induced metamorphosis. The synergistic effects of corticoids on TH action have been observed in several different tissues including the limbs (Galton 1990; Kikuyama et al., 1993; and Hayes, 1997) and skin (keratin gene expression) (Shimizu-Nishikawa and Miller (1992). However, corticoids have a bimodal action on anuran metamorphosis (Hayes, 1997). When TH levels are low during early stages of metamorphosis, corticoids inhibit development but synergize with higher doses of TH later in development. The mechanism of the dual effects of corticoids on anuran metamorphosis is currently unknown. However, it is known that corticoids can directly act on metamorphosing tissues in the *in vitro* culture of epidermal and red blood cells, in addition to tail cultures (Galton, 1990; Nishikawa et al., 1992; Shimizu-Nishikawa and Miller, 1992; Schneider and Galton, 1995; Tata, 1997). It has also been hypothesized that corticoids exert negative feedback on the pituitary and hypothalamus in anurans (Denver and Licht, 1989; Gancedo, et al., 1992; Denver, 1993; Hayes, 1997).

Blocking the synthesis of endogenous corticoids can inhibit anuran metamorphosis (Kikuyama et al., 1982). When endogenous TH synthesis is blocked using thiourea, metamorphosis can be induced with exogenous T4 supplementation. The investigators tested the role of endocorticoids on anuran metamorphosis by simultaneously inhibiting endogenous corticoid synthesis using Amphenone B and determined that T4 administration was substantially less effective in inducing metamorphosis. However, addition of supplemental exogenous corticoids, in addition to the T4, induced tail resorption.

Although most of the effects of corticoids on metamorphosis are based on the resorption of the tail, similar synergies between corticoids and TH have been observed in other organs such as the limb and cell, including erythrocytes (Galton, 1990; Kikuyama et al., 1993; Hayes, 1997). At a molecular level, expression of adult keratin genes in the epidermis of *X. laevis* is induced synergistically by the combination of TH and corticoids. Although the molecular mechanism involved in this synergistic response is currently unknown, corticoids are presumed to act through its nuclear receptor, the glucocorticoid receptor (GR). The GR belongs to the same superfamily of receptors that includes TH receptors (Evans, 1988; Green and Chambon, 1988; Mangelsdorf et al., 1995). The corticoids' effects are, thus, most likely mediated through transcriptional regulation by the GR.

In summary, the synthesis and secretion of endogenous corticoids are under the direct or indirect control of TH, ACTH, and CRF. In essence, 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). Overall, physiological synthesis and secretion of corticoids plays an important role in metamorphosis.

3.4.2 Gonadal Steroids

Unlike corticoids, the role of gonadal steroids on metamorphosis is less obvious. Only one early study by Frieden and Naile (1955) in *Bufo bufo* indicated that estrone enhanced the

effect of T4 on metamorphosis. The majority of studies today, indicate that estradiol and testosterone antagonize the effects of T4 in *R. temporaria* (Roth, 1941; Roth, 1948) and inhibit larval development in *R. pipiens* and *X. laevis* (Richards and Nace, 1978; Gray and Janssens, 1990) *in vivo*, but not tail resorption in *in vitro* cultures. These results suggest that an inhibitory action of gonadal steroids most likely acts at the hypothalamic-pituitary-thyroid axis level by ultimately down-regulating circulating TH levels or by up-regulating prolactin levels (Gray and Janssens, 1990; Hayes, 1997).

3.4.3 Prolactin and Other Hormones

Prolactin, considered by many early investigators as a growth factor in amphibians, is capable of promoting growth prior to metamorphosis, but inhibits metamorphosis in anuran species (Etkin and Lehrer, 1960; Denver, 1996). Anuran prolactin was first purified and cloned from bullfrogs and was found to be highly homologous to its mammalian counterpart (60%) (Yamamoto and Kikuyama, 1981; Yasuda et al., 1991; Takahashi et al., 1990; Buckbinder and Brown, 1993). Prolactin in anuran species is produced in the distal lobe of the pituitary gland (Yamamoto et al., 1986; Tanaka et al., 1991). Once produced, prolactin is secreted and transported to target tissues though the plasma. Contrary to the suspicion that as an inhibitor of metamorphosis prolactin levels should be high during premetamorphosis and decrease with the onset of metamorphosis, plasma prolactin levels were found to be low during pre- and prometamorphosis, but rise to peak levels late in metamorphic climax (Clemons and Nicoll, 1977; Yamamoto and Kikuyama, 1982; Yamamoto et al., 1986). Prolactin secretion is under both stimulatory and inhibitory regulation by the hypothalamus (Kaltenbach, 1996). Although TRH does not stimulate production of TSH in the amphibian pituitary as in mammals, it is a primary prolactin-releasing neurofactor. Dopamine serves as the inhibitor of prolactin release.

Interestingly, the hypothalamic factors do not affect prolactin gene expression during metamorphosis. Upregulation of prolactin synthesis during metamorphic climax appears to be controlled by TH. Blockage of TH synthesis with the anti-thyroid drug methimazole prevents prolactin gene expression. However, treatment of tadpoles with T3 leads to precocious upregulation of prolactin (Buckbinder and Brown, 1993). The current hypothesis on the role of prolactin during metamorphosis is that prolactin counteracts high concentrations of TH present during metamorphic climax to coordinate sequential transformation of different tissues. This potential role is significant in tadpole-frog transformation, since different tissues/organ systems require differing TH levels at different times during metamorphosis. For prolactin to exert this effect, it must act directly at the tissue level and relatively early in the TH signal transduction process (Leloup and Buscaglia, 1977). Tata and coworkers recently demonstrated that prolactin 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 prolactin inhibits the function of the TH-TR complex.

Another hypothesis is that prolactin interacts with a membrane bound receptor that initiates a cascade of biochemical events that give rise to transcription factors known as Stats (signal transducers and activators of transcription). Interaction of Stats and TR leads to the inhibition of TR and thus, blocks TH-induced metamorphosis (Kanamori and Brown, 1992). Based on this model, the effects of prolactin on TH action are tissue-dependent, because receptor

and Stat levels likely differ in the different cell types. This may provide a method of coordinating systematic transformation of different tissues during metamorphosis. Overall, this discussion demonstrates the importance of other related hormonal systems in the control of metamorphosis.

Prolactin acts generally like a juvenile hormone in insects (Riddiford, 1996). However, more recent studies by Nieuwkoop and Faber (1956), Leloup and Buscaglia, (1977), and Tata (1997) suggest that the upregulation of prolactin during metamorphosis indicates there is an alternative function during metamorphosis. Unlike the other hormones and hormonal factors, the anti-metamorphic effect of prolactin appears to be exerted at the tissue level rather than in the brain. It is currently thought that prolactin inhibits TH activity at the thyroid hormone receptor (TR) level. Although this model needs further confirmation, it appears that prolactin gene products interfere with TH binding to TR, thus blocking the action of TH. Interestingly, this prolactin-TR interaction seems to be both tissue and stage specific indicating that prolactin may systematically coordinate transformation of specific tissues during metamorphosis (Boutin et al., 1988; Edery et al., 1989; Scott et al., 1992; Shirota et al., 1990; Ali et al., 1991; Tanaka et al., 1992; Clarke et al., 1993; Moore and Oka, 1993; Bignon et al., 1997; Schuler et al., 1997).

Several other hormones are capable of influencing anuran metamorphosis, including melatonin (pineal gland) and somatostatin. Both factors inhibit TSH secretion and are capable of retarding metamorphosis (Denver, 1996). Melatonin may inhibit metamorphosis though the induction of prolactin pathways (Rose and Rose, 1998). On the contrary, gonadotropin-releasing hormone (GnRH) elevates circulating TH levels in axolotls and frogs, thus accelerating metamorphosis. Limited information is available on these and other hormones and the understanding of their roles in metamorphosis remains unclear.

3.5 Morphological Changes During Metamorphosis

Three primary changes in tadpoles take place during metamorphosis in order to transform almost all of the tadpole organs to their adult form. The first change is the complete resorption of tadpole-specific organs, such as the tail. On the opposite spectrum, the second change is the *de novo* development of new tissues from newly proliferated and subsequently differentiated cell lines ultimately leading to tissue morphogenesis (i.e., hind limbs). The last major type of transformation is the remodeling of existing organ systems, such as the liver, lungs, and intestine into their adult forms. Many investigators have reviewed the morphological changes that occur during metamorphosis (Dodd and Dodd, 1976; Hourdry and Dauca, 1977; Gilbert and Frieden, 1981; Fox, 1983; Balls and Bownes, 1985; Yoshizato, 1989). For the sake of brevity, only morphological features, that are relevant to the development of amphibian metamorphosis assays will be discussed in this paper.

Of the organ systems resorbed during metamorphosis, two systems degenerate completely, the tail and the gills. Of these two organ systems, resorption of the tail has been the most widely studied (Dodd and Dodd, 1976; Houdry and Dauca, 1977; Gilbert and Frieden, 1981; Fox, 1983; Balls and Bownes, 1985; Yoshizato, 1989). All tissues that comprise the tadpole tail are resorbed during metamorphosis, including an epidermis, connective tissue, muscular tissue, blood vessels, and the notochord. Generally tail resorption begins at the onset

of metamorphic climax around stage 60 with the loss of cross-striations of the myofibrils and disintegration of the cristae in the mitochondria (Weber, 1964; Dodd and Dodd, 1976) and concludes around stage 65 to 66 with the complete disintegration of the tail fin.

Two primary processes appear to contribute to tail resorption, condensation and histolysis. The process of condensation contributes primarily to the reduction in tail length and is the result of water loss. Water loss, in turn, causes compaction of the cells and extracellular matrix (Frieden, 1961; Lapiere and Gross, 1963; and Yoshizato, 1989; Yoshizato, 1996). Although the mechanism of condensation is not completely clear, extensive studies have focused on histolysis (Kerr et al., 1974; Kinoshita, 1985; Yoshizato, 1989). The cells of the tail undergo programmed cell death or apoptosis, and the extracellular matrix is degraded by various enzymes. This process is consistent with apoptosis in other vertebrates. The tail is genetically pre-determined to resorb, requiring only sufficient levels of TH to initiate the process.

The classic example of *de novo* development is the limbs. Hind limb buds are first visible in *X. laevis* at stage 46 and continue to grossly develop until stage 54 without the assistance of TH. However, between stages 54 and 58, TH levels rise and induce the differentiation of the limb bud cells allowing morphogenesis to form the specific features of the hind limb and toes. The forelimb develops in a similar pattern with the undifferentiated limb bud developing without endogenous TH.

The majority of organ systems are present in both the tadpole and the adult anuran; however, most require some form of modification in the adult. The liver undergoes extensive morphological and biochemical changes during metamorphosis. Fine structural changes in liver cells, including increases in the size of mitochondria, endoplasmic reticulum, and Golgi complexes during early and intermediate stages of metamorphosis; and increases in the number of nucleoli and heterochromatic nature of the nuclei during the latter stages of metamorphosis increase the biosynthetic capacity of the liver during metamorphosis. On a biochemical level, hepatocytes within the liver convert from ammonotelic to ureotelic (Atkinson, 1994; Atkinson et al., 1996; Chen et al., 1994). The nervous system is also reorganized during metamorphosis (Kollros, 1981; Fox, 1983; Gona et al., 1988; Tata, 1993). At the gross morphological level, changes in the shape of the diencephalon, medulla, and restructuring of neurons in the cerebellum occur during metamorphosis (Gona et al., 1988). At the neuronal level, one of the most dramatic changes includes the programmed death of the Mauthner cells. These cells represent a pair of giant neurons responsible for tail function in the tadpole. On the contrary, Purkinje cells acquire extensive dendritic trees, and lateral motor column neurons, and the dorsal root ganglia neurons differentiate during metamorphosis (Hoskins, 1990). The tadpole intestine is proportionately longer, but more simplistic than the adult anuran intestine, which resembles most vertebrate intestines in terms of structure and function. The tadpole intestine is comprised of a single layer of columnar epithelial tissue surrounded by thin layers of muscle and connective tissue (McAvoy and Dixon, 1977; Kordylewski, 1983; Ishizuya-Oka and Shimozawa 1987; Shi and Ishizuya-Oka, 1996). Remodeling of the intestine during metamorphosis creates a shorter, but substantially more sophisticated epithelium with numerous microvilli and increased luminal surface area.

The role of TH in primary and secondary sexual differentiation has only recently been investigated (Hayes, 1997). Administration of goitrogens, such as thiourea, which block TH production, resulted in skewed sex ratios (100% female) in *X. laevis* (Hayes, 1997; Hayes, 1998). TH has also been shown to directly induce the testosterone 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 estradiol induces female coloration in both male and female specimens (Hayes, 1997). However, when estradiol is administered concurrently with thiourea, the skewing toward female coloration does not occur. When thiourea is co-administered with testosterone, induction of gular pouch development does not occur. Gular pouch development is induced when testosterone is administered alone (Hayes, 1997).

3.6 Biochemical Changes During Metamorphosis

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 (discussed previously), 3) increase in serum protein levels, 4) changes in hemoglobin isoforms, 5) alterations in digestive enzymes, and 6) changes in the respiratory system. 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. The primary feature of apoptosis is the fragmentation of chromatin, which can be detected by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling) 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 stage 59 in *X. laevis*.

During the process of tail resorption, upregulation of a series of proteases, acid and alkaline hydrolases, and ribonucleases occurs. Upregulation of the degradative enzymes is regionally specific in the tail. For example, acid phosphatase levels in the dorsal fin region, which resorbs first in this process, are dramatically elevated in this region during this time. Similar lysosomal hydrolases are upregulated in the intestine during re-modeling. In each case, TH has been shown to control up and down-regulation of these degradative enzymes during metamorphosis (Tables 3-2 and 3-3).

Table 3-2. Thyroid Hormone Upregulated Genes - Early

Gene Name	Tissue Source	Homologous Genes	Possible Function
Tail 1/3	Tail[b], brain[c]	Zinc finger (BTEB)	Transcription factors[b,c,d,f,g,m]
Tail 7	Tail[b]	bZip (Fra-2)	
IU 16/33	Intestine[d]	NFI	
Tail 8/9	Tail[b], intestine[d]	TH/bZip (E4BP4)	
TR Beta	Tail[b], intestine[d]		
Tail 11	Tail[b]	Collagenase-3	Matrix metalloproteins[b,d,f,h]
Tail 14	Tail[b], intestine[d]	Stromelysin-3	
Tail 13	Tail[b]	FAPalpha	Proteases[f]
Tail D	Tail[b]	*N*-Aspatryl dipeptidase	
Tail C	Tail[b]	Fibronectin	ECM protein[f]
Tail 14	Tail[b]	Integrin alpha-1	ECM receptor[f]
Tail 15	Tail[b], brain[c]	Type III deiodinase	TH inactivation[b,c,l]
Xh20	Brain[c]	Protein disulfide isomerase	Protein isomerization[c]
Xh1	Brain[c]	Cytochrome c oxidase subunit	Oxidative phosphorylation[c]
IU22	Intestine[d]	Nonhepatic arginase	Proline biosynthesis, etc.[d,f]
IU24	Intestine[d]	Na^+/PO_{4-} cotransporter	PO_{4-} transport[d,k]
IU12	Intestine[d]	Transmembrane protein	Amino acid transport[l]
IU27	Intestine[d]	Sonic hedgehog	Morphogen[d,n]
P	Limb[e]	Rat clathrin B chain	Vesicular intracellular transport[c]
B	Limb[e]	Heat-shock protein	Rapid cell growth[e]
I	Limb[e]	Heat-shock protein	
M	Limb[e]	Heat-shock protein	
N	Limb[e]	Heat-shock protein	
H	Limb[e]	Yeast MCM3, mouse P1	
J	Limb[e]	Mouse Eif-4A	

[a] A direct response indicates that the regulation is resistant to protein synthesis inhibition; ND, not determinable.
[b] Wang and Brown (1993)
[e] Buckbinder and Brown (1992)
[h] Patterton et al. (1995)
[k] Ishikuya-Oka et al. (1997b)
[m] Puzianowska-Kunicka and Shi (1996)

Table 3-3. Thyroid Hormone Response Genes in *Xenopus laevis* and *Rana catesbeiana* - Late Response

Gene	Tissue	Frog Species	References
Carbamyl-phosphate synthase I	Liver	*R. catesbeiana*	Morris (1987); Helbing et al. (1992); Galton et al. (1991)
Arginosuccinate synthase	Liver	*R. catesbeiana*	Morris (1987)
Arginosuccinate lyase	Liver	*R. catesbeiana*	Iwase et al. (1995)
Arginase	Liver	*X. laevis*	Xu et al. (1993); Helbing and Atkinson (1994);
		R. catesbeiana	Atkinson et al. (1994); Iwase et al. (1995)
Ornithine carbamyltransferase	Liver	*R. catesbeiana*	Helbing et al. (1992)
N-CAM	Liver	*X. laevis*	Levi et al. (1990)
Albumin	Liver	*R. catesbeiana*	Schulz et al (1988); Moskaitis et al. (1989)
alpha1-microglobulin	Liver	*X. laevis*	Kawahara et al. (1997)
Myosin heavy chains	Limb	*X. laevis*	Buckbinder and Brown (1992); Dhanarajan et al. (1988);
		R. catesbeiana	Sachs et al. (1997b)
Tropomyosin	Limb	*R. catesbeiana*	Dhanarajan et al. (1988)
M-Line protein	Limb	*R. catesbeiana*	Dhanarajan and Atkinson (1981)
alpha-Actinin	Limb	*R. catesbeiana*	Dhanarajan and Atkinson (1981)
Keratins	Epidermis	*X. laevis*	Mathisen and Miller (1989)
			Ketola-Pirie and Atkinson (1988, 1990)
Magainin	Skin	*X. laevis*	Reilly et al. (1994)
Trypsin	Pancreas	*X. laevis*	Shi and Brown (1990)
Intestinal fatty acid-binding protein	Intestine	*X. laevis*	Shi and Hayes (1994)
Mdr (multidrug resistence)	Intestine	*X. laevis*	Zucker et al. (1997)
Gelatinase A	Intestine	*X. laevis*	Patterton et al. (1995)
Collagenase-4	Tail	*X. laevis*	Stolow et al. (1996)

In most anuran species, serum protein levels nearly double during metamorphosis. The ratio of serum albumin to globins is markedly elevated during TH-induced metamorphosis. Other plasma proteins that increase during metamorphosis include ceruloplasmin, transferrin, and carbonic anhydrase (Inaba and Frieden, 1967; Frieden and Just, 1970; Wise 1970). The increase in serum proteins is thought to play an adaptive role as the tadpole transforms into the frog. In the case of albumin, the biochemical properties of this critical protein not only fulfill osmotic requirements, but also provide circulatory transport needs associated with the terrestrial living habitat (Frieden, 1968; Weber, 1967; Broyles, 1981).

Anurans undergo changes in hemoglobin synthesis during development similar to mammals and birds. However, in anurans, only one primary change occurs as opposed to other animals, which typically undergo several changes in synthesis patterns. This change occurs during metamorphosis and involves a complete replacement of globulin chains (Weber, 1996). Hemoglobin in tadpoles is independent of pH and has a markedly greater affinity for oxygen than frog hemoglobin, which has lower oxygen binding affinity and is subject to the Bohr Effect (decreased affinity with decreasing pH) (McCutcheon, 1936; Riggs, 1951; Frieden, 1961). The greater affinity of tadpole hemoglobin for oxygen most likely allows for adaptation to low oxygen environments characteristic of the aquatic tadpole habitat. The switch in the form of hemoglobin occurs at metamorphic climax, although larval hemoglobin persists for some time in metamorphosed frogs in some cases (Just and Atkinson, 1972; Weber et al., 1991). Adult frogs require hemoglobins with lower oxygen affinity to facilitate terrestrial locomotion, which has more rapid and extensive oxygen requirements (Bennett and Frieden, 1962; Dodd and Dodd, 1976).

In addition to an increase in plasma proteins, the metabolic capacity of the liver markedly changes in the metamorphosing anuran. During metamorphosis, drastic increases in nucleic acid and protein synthesis result in large increases in several enzymes including: catalase, uricase, phosphatases, and the urea cycle enzymes. Of these enzymes, the urea cycle has been the most widely studied. Although each of the four primary enzymes associated with the urea cycle increase at least several fold, the mitochondrial enzyme carbamyl phosphate synthetase actually increases nearly 30-fold during metamorphic climax. However, the most widely studied enzyme of this excretory cycle is the cytosolic enzyme arginase, which catalyzes the conversion of arginine to urea and ornithine (Figure 3-2).

3.7 Production of Thyroid Hormone and Mechanism of Thyroid Hormone Action

Both of the natural occurring THs, T4 and 3,3', 5–triiodothyronine (T3) are produced in the thyroid gland, although T4 can be converted to T3 in other tissues (Fox, 1983; Dodd and Dodd, 1976). Biosynthesis of TH requires activation of the thyroglobulin gene in the thyroid gland, which in turn produces thyroglobulin. Thyroglobulin then undergoes a series of post-translational modifications, including iodination and condensation of the tyrosine residue to produce T4 (Figure 3-3). T4 can either be secreted into the plasma, or directly converted to T3 in the thyroid by 5'-deiodinase. Both T4 and T3 can be inactivated by 5-deiodinases by converting either to T2 or reverse T3, respectively. This allows different tissues to possess different ratios of T3 to T4 depending on their specific requirements. Two different 5-deiodinases have been isolated (St. Germain and Galton, 1997) that have different enzymatic

properties and tissue distributions in mammals. A 5'-deiodinase and a 5-deiodinase in *R. catesbeiana* (Davey et al., 1995; Becker et al., 1995) and a 5-deiodinase in *X. laevis* (St. Germain, 1994) have been cloned and found to have distinctly different regulation patterns in different tissues.

FIGURE 3-2
UREA CYCLE IN METAMORPHOSING TADPOLE

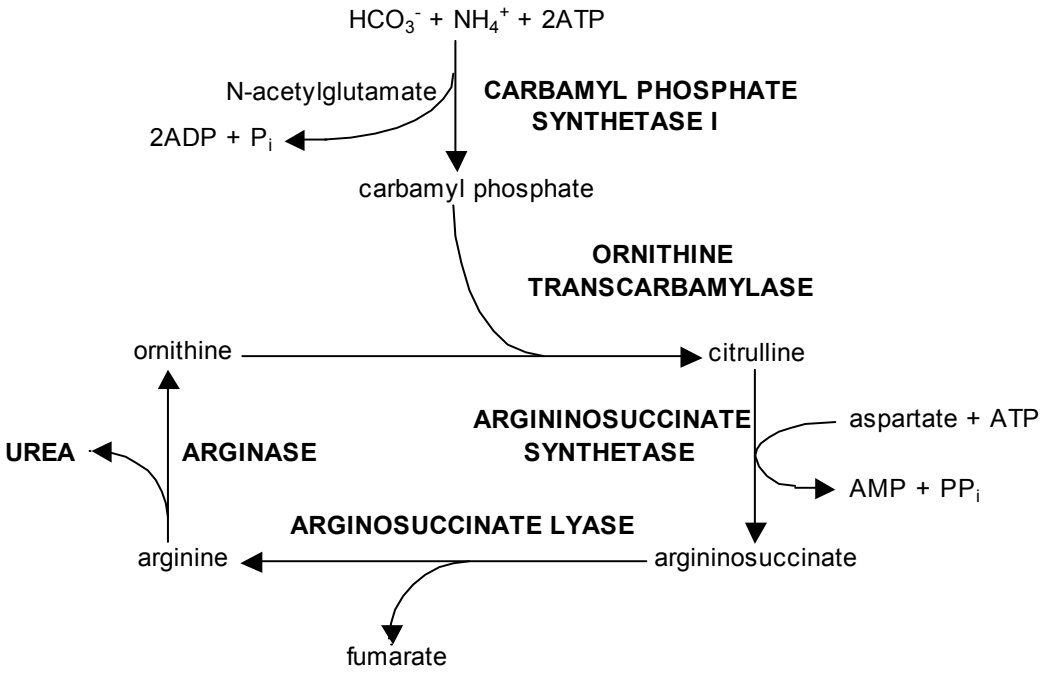
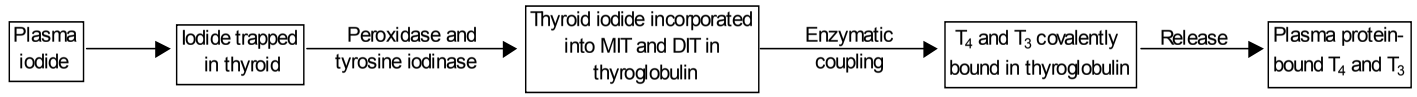
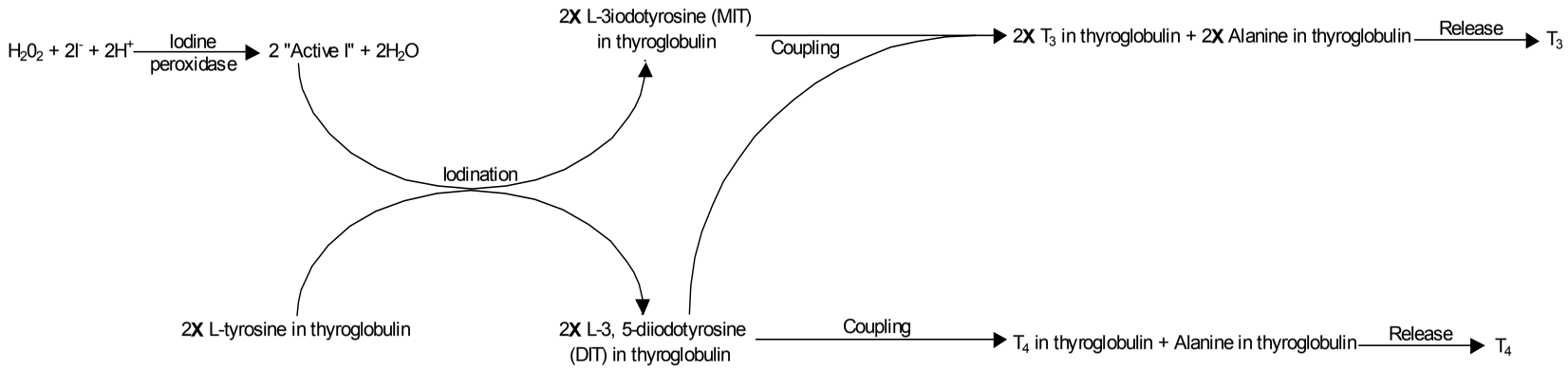


FIGURE 3-3
BIOSYNTHETIC PATHWAY OF T_3 AND T_4



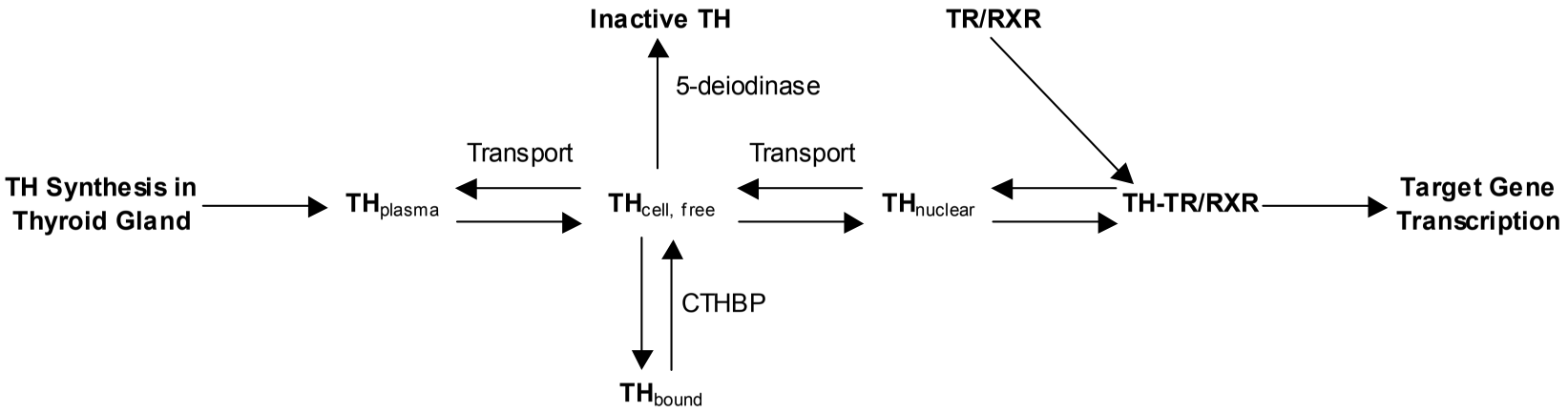


The action of TH during development is regulated at many different levels, due in part to the presence of numerous TH binding proteins in the plasma, cytosol, and nucleus. Since many of the proteins with which TH interacts are cytosolic and many of the effects occur at a non-genomic level, it was originally 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 know to transport TH, although several are more significant factors. T4 can also be converted to T3 in various target tissues by 5'deiodinase (St. Germain, 1994; St. Germain and Galton, 1997).

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. Both T3 and T4 are highly hydrophobic at physiological pH. 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 encounters another 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.

Higher vertebrate animals possess two TR genes (TR alpha and TR beta) (Lazar, 1993). *Xenopus* possesses 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 *Xenopus* (Brooks et al., 1989; Yaoita et al., 1990). In general, TRs belong to the superfamily of nuclear hormone receptors, including glucocorticoid, estrogen, retinoic acid, and vitamin D (Evans, 1988; Tsai and O'Malley, 1994; Yen and Chin, 1994). The TR contains five different binding domains, A/B, C, D, E, F (amino to carboxy terminus), which are reasonably consistent within this class of nuclear receptors. In the TR, the A/B domain has no known function. DNA binding occurs in domain C. Domain D is the variable hinge region which contains a nuclear localization signal and influences both DNA binding and transactivation (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 conceptual model for transcriptional regulation by TR is illustrated in Figure 3-4. TR is presumed to form a heterodimer with the retinoic acid X receptor (RXR). The heterodimer binds to the TH response element in a target gene. In the absence of TH, the heterodimer represses gene transcription, most likely through the activation of a co-repressor (Horlein et al., 1995; Chen and Evans, 1995).

FIGURE 3-4
PROPOSED PATHWAY FOR THYROID HORMONE
REGULATION OF CELLULAR GENE TRANSCRIPTION



3.8 Anticipated Sites of EDC Impact on the Thyroid Axis

Based on the previous discussion, EDCs could potentially affect the thyroid axis at three levels, 1) CNS (including pituitary and hypothalamus), 2) thyroid, and 3) TR. The effect at the pituitary level is even more complex since it may involve thyrotropes (TSH), corticotropes (ACTH), and lactotropes (prolactins). In addition to the thyroid gland, the interrenal gland may also be a site of EDC action, which could potentially impact metamorphosis. Thus, the impact of potential EDCs on metamorphosis may occur at multiple different levels. In addition other physical environmental factors such as temperature and water level (densities) may also alter metamorphosis. Biochemical factors outside the thyroid axis, such as the corticotropes, may also affect metamorphosis. The liver should not be overlooked since it plays a role in T4 and T3 homeostasis. The complexity of metamorphosis and control by the neuroendocrine system must be strongly considered in the design of appropriate test methods. Since the objective of the test method is to screen for thyroid axis disruption, a rapid, high throughput biochemical measurement test or molecular test would likely be more advantageous. However, alone, biochemical measurements and molecular tests might not provide adequate information on the morphological effect of the EDC on metamorphosis at the whole organism level. Incorporation of a high throughput biochemical or molecular assay within a short-term morphological method would be advantageous. It is crucial that the methodology used demonstrate diagnostic power by distinguishing between non-thyroid and thyroid-related delays in developmental progress. Morphological components of the assay must address this issue.

4.0 CULTURE AND HANDLING OF TEST SPECIES

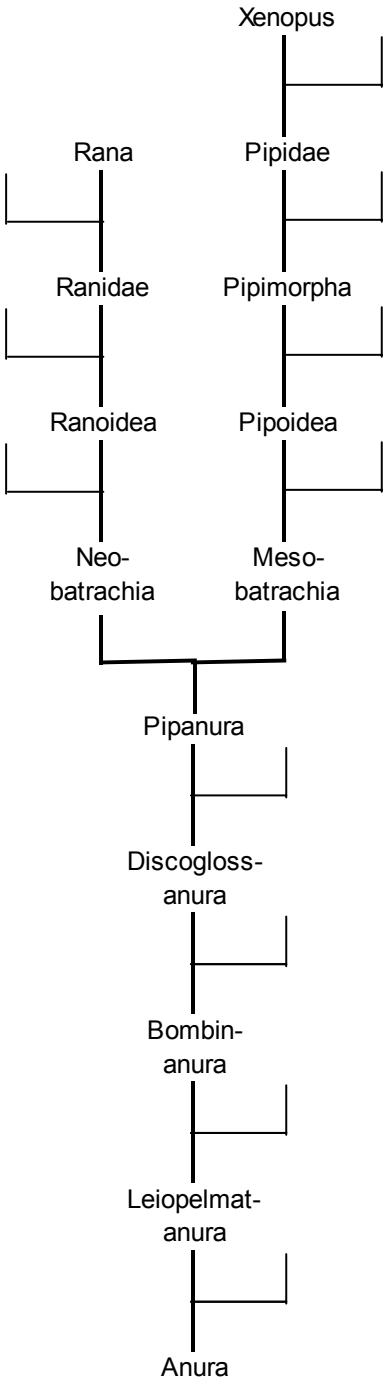
4.1 Anurans

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*



Phylogenetic relationships between *Xenopus* and R*ana*. 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), and salmon diet (Xenopus I, Dexter, MI). Although beef liver is the classic diet for *Xenopus*, the salmon diet has 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. 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). Adults may be treated with a subcutaneous injection of ivermectin or tetracycline to treat these diseases.

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 E1439-98; 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. After the embryos have reached stage 47 (ca. 4.5 d), feeding is required. Strained baby food green beans or peas, or a well-mixed slurry of salmon food is used to feed the developing tadpoles. Generally, 100 mg food per tadpole every 3 to 5 days is provided. As tadpoles reach metamorphosis, the rate of feeding is increased. Only partial replacement of culture water is recommended, especially during metamorphosis, as the larvae become sensitive to more drastic environmental changes. 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 to 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. (Silurana) 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 lifecycle. The lifecycle for *X. tropicalis* is roughly 4 to 5 months, whereas, the lifecycle in *X. laevis* can run from 1.5 to 2 years. *X. tropicalis* is capable of producing 1,500 to3,000 fertilized embryos per breeding. Because of the closeness in the family relationship between these two *Xenopus* species, many techniques of animal husbandry, breeding, and tadpole rearing are similar. Further, many of the molecular probes developed historically for *X. laevis* can be also used with *X. tropicalis*. 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 25°C. Prolonged exposure to water temperatures below 22°C 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 moderately buffered solution (MBS). 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. One-half strength FETAX Solution is also effective in culturing *X. tropicalis* embryos and tadpoles.

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, Rana Pipiens , 2002). The only regions within the U.S. where *R. pipiens* is not located are in the south-eastern, 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, and sexual maturity is achieved in roughly 1-2 years.

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.* In most amphibians, juvenile frogs resemble the adults with respect to dorsal coloration and patterning. The reed frog (*H. viridiflavus* and *H. argus*), native to the forests of Kenya, represents one of a very few anurans, that undergo ontogenic color change. Several species of the genus *Hyperolius*, undergo ontogenic changes in coloration and patterning resulting in sexual dichromatitism (Hayes, 1997; Hayes and Menendez, 1999). In the case of *H. argus*, both males and females metamorphose with a bright green dorsum. Several months after metamorphosis, females develop a reddish brown dorsal color with white spots (Hayes, 1997), and males develop gular pouches.

Hyperolius can be maintained in captivity, although they must be obtained originally from Africa. Currently, 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. 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. Interestingly, these animals breed spontaneously about every 10 days (Hayes and Menendez, 1999). Larvae are raised in 10% Holtfreter's solution (Holtfreter, 1931), at a density of 30 tadpoles per 5 L of solution. The tadpoles are fed boiled spinach, and the solutions changed daily following feeding. The water temperature is maintained at 27˚ C, and a 12-hour light cycle is used.

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 3 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 2 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 urodeles species 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 to 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 Amphibian Metamorphosis Assays. 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 capacity and ease of developing transgenic lines (see section 4.1.1.2) and the rate of development. Since the objective of the assay is to screen for chemical thyroid axis disruptors, the origin of the species is not a primary factor in selection. Next to *Xenopus sp*., there is some literature available on the native American *Rana sp*. metamorphic 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 metamorphosis is for *R. catesbeiana* (bull frog). However, use of *R. catesbeiana* in the laboratory for metamorphosis studies is impractical due to the long length of development, thus warranting the consideration of *R. pipiens*. The use of *Hyperolius sp*. as described by Hayes (1997), 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 metamorphic processes to the other anurans. Further, the procedures reviewed do not represent a direct test of thyroid axis activity.

The only potential advantage to the inclusion of the urodeles sp. to the list of potential candidate species for the Amphibian Metamorphosis Assays, 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 4-1.

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

Species	Strengths	Weaknesses
South African clawed frog (*Xenopus laevis*)	- Wealth of literature base on all aspects of development, reproduction, metamorphosis, including molecular models - Ease of culture in lab - husbandry - Many laboratories are familiar with culture and testing - Breeds naturally and repeatedly with hormone stimulus - High productivity from breeding and rapid development - Mapped genome	- Relatively long life cycle with respect to sexual maturity - Nonnative species - Oligotetraploid genome
South African clawed frog (*Xenopus tropicalis*) [compared to *X. laevis* only]	- Relatively short life cycle - Rapid development - Diploid genome - Good transgenic capacity - Capacity to develop inbred lines - Mapped genome	- More challenging animal husbandry - Less information currently available, data base - Disease susceptibility - Availability
Northern Leopard Frog (*Rana pipiens*)	- Native species - Reasonable database - Relatively short metamorphic period for native species - Terrestrial and aquatic life phase	- More difficult animal husbandry and breeding - Limited testing experience
Urodeles *sp.*	- Represent different Order (non-frog amphibian) - Native species - Terrestrial and aquatic life phase	- Limited testing experience - More difficult animal husbandry and breeding - Limited database
Hyperolius sp.	- External endpoints - Straight-forward end points - Suitable animal husbandry - Connection to sexual development (gonadotrophs)	- Limited database - Non-native genus - Availability - Does not directly measure thyroid dysfunction

5.0 EXPERIMENTAL DESIGN CONSIDERATIONS FOR AMPHIBIAN METAMORPHOSIS ASSAYS

5.1 Exposure Period

In a general sense, an increase in exposure time often results in increased sensitivity of an organism to a given toxicant. The developmental period during which exposure occurs can also influence the results of a study. In the case of metamorphosis, premetamorphosis, prometamorphosis, and metamorphic climax are three distinct periods. Premetamorphosis is characterized as a phase of embryogenesis and early tadpole growth, including development of the thyroid gland. During prometamorphosis, amphibians acquire TH synthesis. This phase of development is characterized by concentration of endogenous TH. Metamorphic climax is the period in which endogenous TH is at its peak levels and when rapid and drastic morphological changes (i.e., tail resorption) occur. However, the toxicity of a given chemical or chemical mixture is also dependent upon the mechanism of action. In this case, the aim is to identify the ability of a test substance to interfere with normal thyroid function. If the mechanism involves a developmental period not included in the experimental design of the test, the testing method will not be predictive of that chemical's action. For example, compounds that inhibit TH synthesis such as thiourea, would have little effect prior to prometamorphosis. However, since the mechanism of action of most chemicals is unknown, test approaches covering representative developmental periods have the potential to ensure that the appropriate periods are covered. The only rationale for extending the exposure period to earlier stages of development is when the physicochemical nature of the test substance is such that it has extremely low water solubility and increased exposure time is required to reach effective tissue concentrations during prometamorphosis.

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 Amphibian Metamorphosis Assays, several options are available including: 1) exposure from late premetamorphosis through metamorphic climax, 2) exposure from prometamorphosis through metamorphic climax, 3) exposure during prometamorphosis only, and 4) exposure during metamorphic climax. As noted in the preceding paragraph, exposure prior to late premetamorphosis would most likely be counter productive since no thyroid activity (other than thyroid development) occurs prior to premetamorphosis. The first scenario is most inclusive since it covers most of the developmental phase in which the thyroid is active. However, the length of an assay could easily be in the order of 35 d in *X. Laevis*. Exposure from prometamorphosis to metamorphic climax provides a similar advantage in that it covers most of the significant events occurring during metamorphosis, but still requires approximately 28 d in *X. laevis*. An assay that includes only the prometamorphic period could be performed in roughly 14 d in *X. laevis*. The use of a prometamorphic exposure protocol provides an interesting scenario in that it covers a

developmental period in which the thyroid is acquiring activity and morphological changes are occurring as the result of endogenous TH-induced metamorphosis. Although it can be completed more quickly, it does not account for exposure immediately prior to the onset of thyroid activity, which could reduce its sensitivity. The metamorphic climax assay can effectively be performed over a 16 d period in *X. laevis*. The assay is relatively quick compared to the other exposure scenarios, however, it is only capable of evaluating only the final processes of metamorphosis. Since many morphological metamorphic programs are already underway during metamorphic climax and endogenous TH is at its greatest level, exposure during metamorphic climax may not provide a sensitive or realistic estimation of the effect of a given test substance on the thyroid axis. The capacity of an exposure period to provide adequate developmental time to address delays in development and differentiate between thyroidal and non-thyroidal mechanisms will be extremely important.

6.0 EXPOSURE PROTOCOLS FOR AMPHIBIAN METAMORPHOSIS ASSAYS

6.1 Route of Administration

6.1.1 Water

Water exposure is the most common route to expose larval and metamorphic 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-E1439-98, 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). 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.

6.1.2 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., 2001). 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., 2001). In a large-scale screening program like EDSP, oral dosing is probably not as practicable as aqueous exposure, unless required due to limitation in aqueous solubility.

6.1.3 Parenteral

Intramuscular or intravenous administration of a toxicant in a premetamorphic tadpole (stage 47-48) is possible. However, the technique is technically difficult and the environmental relevance is questionable since it is difficult to determine or estimate effective environmental concentrations. Thus, as with oral dosing, parenteral routes of administration are not practicable for high volume screening programs.

6.2 Dose Selection

Dose levels for Amphibian Metamorphosis Assays 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 less 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 Amphibian Metamorphosis Assay study should be conducted with at least five treatment levels, and the treatment levels should be separated by approximately a factor of two. At least one of the treatment levels should be below the no-observed adverse effect concentration (NOAEC). Concentrations should be selected to produce an adequate concentration-response curve for the endpoints measured during the study.

6.3 Stages of Exposure

As previously indicated, two primary developmental periods need to be considered in the exposure regime used in the Amphibian Metamorphosis Assays, prometamorphosis (stages 54 to 57) and metamorphic climax (stages 58 to 66) (see Table 3.1). A toxicant may or may not induce an effect during these periods depending on the mechanism of action. Most compounds that adversely affect the thyroid axis would show activity at least during prometamorphosis. Some of these thyroid disruptors might also demonstrate effects during metamorphic climax. Thus, since a short-term test is desired, an exposure protocol incorporating either prometamorphosis or metamorphic climax would appear to be most effective. It is likely though that late premetamorphosis to early prometamorphosis (stages 51 to 54+) will be the most

sensitive period. However, it is remotely possible, that a toxic insult could result in abnormal development of the thyroid during the premetamorphic stage.

6.4 Statistical Considerations

The objective of the Amphibian Metamorphosis Assays 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. Power can be increased by increasing the number of replicates. The choice of the test species and endpoints with the least inherent variability, by default, requires the least replication for a given level of power and, thus, are more cost effective.

6.4.1 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 specimen). 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 metamorphosis will require larger sample sizes than used for short-term partial life-cycle amphibian tests. As a useful guide, 100 fish 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 less specimens will be required (i.e., 5 replicates of 20 specimen per concentration). However, before sample size and replicate requirements can be determined for the Amphibian Metamorphosis Assays, formal statistical power analysis will be required.

6.4.2 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 Amphibian Metamorphosis Assay 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 thyroid axis function.

7.0 DESCRIPTION OF THE TEST BATTERIES AND ASSAY ENDPOINTS REFLECTIVE OF THYROID DYSFUNCTION

7.1 Whole Organism Tests

7.1.1 Morphological Measures

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.

7.1.1.1 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-d) "*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-d, the assay will require a 50-d 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), 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, this endpoint is one which could be considered. Issues regarding exposure design, including the use of flow-through systems, need to be addressed.

7.1.1.2 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 et al. (1996) 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.

7.1.1.3 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. Immunohistochemical 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.

7.2 Biochemical Measures

7.2.1 Corticotropin Releasing Factor and Thyroid Stimulating Hormone

As previously discussed, CRF, produced and secreted by the hypothalamus, stimulated the production and secretion of both ACTH and TSH from the pituitary. Although the structure of CRF is known (41 amino acid peptide), there is currently no diagnostic test, such as a RIA or ELISA that has been readily used. The development of such a test for CRF is possible. In humans, TSH is measured by high affinity RIA. As with CRF, an amphibian RIA (or ELISA) could be developed considering the similarities in structure between mammals and amphibians. However, considering the context of use of the Amphibian Metamorphosis Assays, measurement of more downstream hormones and factors would appear to be more appropriate in a screening assay battery.

7.2.2 Thyroid Hormones

Measurement of TH, specifically T4 and T3, produced by the thyroid provides a valuable measure of thyroid status during metamorphosis. Both T4 and T3 can be measured using conventional serum or tissue RIAs. Both serum and tissue (whole brains, thyroid, or carcass following cardiac puncture to obtain serum) should be considered. Three different methodologies of TH analysis are currently being evaluated, 1) high sensitivity RIA, 2) ELISA, and 3) liquid chromatography/gas chromatography with mass selective detection (LC/GC-MS). The former two techniques are reasonably well established in mammals (Ekins, 1999; Baiser et al., 2000), and to a lesser extent in amphibians (Galton et al., 1991). The chromatographic technique is not as well established (Moller et al., 1983; De Brabandere et al., 1998), but has significant promise, because it may be able to simultaneously analyze monoiodotyrosine (MIT), diiodotyrosine (DIT), reverse T3, T3, and T4. In some cases it will be important to measure the concentration of free T4 and T3 in relation to the transport protein-bound TH, since the majority of TH is protein bound (Baiser et al., 2000). Overall, TH analysis will be an important component of the Amphibian Metamorphosis Assays. However, the most beneficial use of TH analysis will be in combination with the histological, morphological, and molecular test methods

used. It is possible, but unlikely, that TH analysis alone will provide sufficient information to be a stand-alone measure of thyroid dysfunction.

7.2.3 Iodothyronine Deiodinase

Two deiodinase isoforms are present in most anurans. One isoform, type II, catalyzes the conversion of T4 to T3 in the thyroid and various target tissues, whereas the other isoform, type III, selectively inactivates T3 and T4 by converting them to T2 and reverse T3 (Huang et al., 1999). It is thought that type III deiodinase in anurans (*X. laevis*) is responsible for protecting the tissues from circulating TH. Koopdonk-Kool et al. (1993) developed a method for measuring deiodinase activity by measuring the conversion of [^{125}I]T3 to T2. In most cases deiodinase activity is not considered in evaluating thyroid function; however differences in tissue levels of T4 and T3 can in some cases be explained by differing deiodinase activities. Further work will be required to fully determine the usefulness of deiodinase measurement in evaluating thyroid dysfunction.

7.2.4 Thyroid Hormone Transport Proteins and Thyroid Hormone Receptors

TH synthesis and secretion, TH transport by carrier proteins, and TH binding to TR constitutes the primary thyroid axis pathway. Thus, measurement of TH transport proteins and TR provide a measure of TH activity and responsiveness (Tata, 1999). The most practicable methods of evaluating these processes appear to be quantifying changes in protein levels during metamorphosis. Conventional ELISA is suitable for the analysis of specific transport proteins, such as transthyretin (Yamauchi et al., 1993). Evaluating TR gene expression also represents a suitable method for evaluating the up and down regulation of TR. Immunohistochemical analysis of TR in the whole organism can be used to regionally quantify the presence of TR in a metamorphosing tadpole.

7.2.5 Clinical Tests of Thyroid Function

Aside from analytically measuring pituitary and TH levels, associated regulatory enzymes of the thyroid axis, and TH transport proteins; several classical methods of measuring thyroid function in higher animals, including humans, could be considered. These classical assays include, radioactive I (^{125}I) uptake by the thyroid and T3 resin tests, which measure thyroglobulin binding protein among a host of others (Thomson, 1974). None of these tests have been specifically adapted for use in amphibians due to the advent of more sophisticated molecular techniques. Therefore, it is unlikely that the classical methods of measuring thyroid dysfunction in humans will be more effective in measuring thyroid disruption in amphibians, than the methods already described or the approaches discussed in the following section.

7.2.6 Thyroid Pathology

In most cases, thyroid dysfunction, such as goiter or myxedema, manifests changes in the morphometry of the thyroid. For example, tadpoles exposed to the goitrogen methimizole develop enlarged thyroid glands that are visible under low magnification in the transparent *Xenopus* tadpole (Fort et al., 1996). In this case, the thyroid gland can be digitally photographed and the size quantified by photodigitization. Some EDCs may induce myxedema, or a shrinking

of the thyroid gland (Wollman, 1980). Again, myxedema can also be quantified using the methods discussed for goiter. Distinct histopathology is also associated with goiter, including a thickened capsule wall, shrinking of capillaries, fusion of the follicles, increased follicular size, and infiltration of connective tissue (partitions) entering the lobe from the capsule (Wollman, 1980). The histological techniques are relatively simple, using thin sections of the thyroid gland and standard light microscopy of hemotoxylin/eosin stained tissue. Use of thyroid morphometry and pathology, particularly in *Xenopus*, where the thyroid gland is fully visible in the intact specimen, should be included in the analysis of thyroid function.

7.3 Molecular Biomarkers

7.3.1 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 metamorphosis. 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. Although differential display analysis is a reasonably well developed technique for measuring multiple gene activity, the method is somewhat out-of-date and is plagued by difficulty in interpretation of the results.

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). TH-mediated gene expression patterns have been identified in the rat (Witzel et al.,2001) and *X. laevis* (Denver et al., 1997) using cDNA expression arrays and a slightly different technique, PCR-based subtractive hybridization. Recent studies using this approach have identified sets of T3-responsive genes in various tadpole tissues, including the tail (Wang and Brown, 1993; Brown et al., 1996), hind limb (Buckbinder and Brown, 1992), brain (Denver et al., 1997), and intestine (Shi and Brown, 1993). These analyses have demonstrated that some early TH-response genes are common to all tadpole tissues, whereas others are tissue specific. Genes identified by Denver et al. (1997) to be up-regulated during prometamorphosis and metamorphic climax include *xh*4, *xh*7, *xh*15, *xh*6, *xh*1, TR beta, and bZIP. Gene array constructs using TH-responsive genes and TREs could be developed to evaluate the effect of potential EDCs on multiple TH-dependent gene expression. Although the gene array technology offers advantages over differential display, it is not yet clear how interpretable the gene array analysis approach will be

in a screening test 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 metamorphosis. For example, genes that code for TR beta or the urea cycle enzyme, arginase, could be selectively monitored for activity during metamorphosis. However, the most intriguing TH responsive genes that could be evaluated during prometamorphosis are TR beta or stromelysin-3 (ST3). ST3 is expressed in frogs, mice, and humans during development, in tissues were selective cell death takes place (Bassett et al., 1990; Lefebvre et al., 1992). Results from these investigators suggest that ST3 is involved in both apoptosis and cell migration, both of which are involved in limb definition, intestinal remodeling, and tail resorption. Further, ST3 is ubiquitous in *Xenopus* tadpoles, and exogenous TH induces the precocious activation of ST3 (Wang and Brown, 1993; Shi and Brown, 1993; Patterton et al., 1995). *In vivo*, the developmental expression of ST3 mRNA correlates strongly with organ-specific metamorphosis. For example, in the tail ST3 mRNA is low until stage 62, when expression increases and apoptosis is detected by TUNEL (Kerr et al., 1974). ST3 mRNA levels are appreciably lower in the elongating hind limb until stage 54 to 56 when the interdigital cells degenerate to form the toes. Because ST3 is TH responsive, it is ubiquitous among tadpole tissues, and correlates well with morphological events associated with metamorphosis. 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 metamorphic events, both approaches show substantial promise for use with the Amphibian Metamorphosis Assays.

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 unhybridzed, 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 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 vitellogenin genes in *Xenopus* as the result of exposure to the weakly estrogenic compound bisphenol A (Kloas et al., 1999). 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.

7.3.2 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 metamorphosis (see Tables 3-3 and 3-4) in combination with tissue-specific promoters, will eventually allow construction of a transgenic line that models the expression of a series of genes important for successful metamorphosis. Adaptation to *X. tropicalis*, a diploid organism with a shorter lifecycle, further increases the feasibility of these studies. A transgenic line exploiting TH/TR response elements could be developed. Activation of these response elements 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.

7.3.3 Organ and Cell Culture

Consistent TH-induced tail resorption is relatively easy to monitor in organ cultures (Weber, 1967; Tata et al., 1991). Whole tail cultures could be used to evaluate TH agonists and antagonists downstream from the thyroid gland. More specifically, tail culture assays could potentially evaluate TH interaction with the TR, TR activation of the TREs, and the cascade of molecular events associated with selective cell death in the tail. Similar transfected cell culture lines could also be established to measure the influence of EDCs on TREs and associated gene products (Denver et al., 2002).

7.3.4 Receptor and Protein Binding Assays

Since EDC binding to the TR and plasma TH transport proteins represents an additional process, which may be affected by EDCs, a receptor or transport protein binding assay could also be considered. Currently, TR binding assays, which measure the relative binding affinities of potential EDCs to the TR, are available and are being evaluated (Burkhart et al., personal communication). In addition, a transthyretin binding assay has also been developed for the study of TH transport (Denver et al., personal communication). However, this assay technique has not been evaluated for use in evaluating EDC activity on the thyroid axis.

8.0 RESPONSE TO THYROID AGONISTS AND ANTAGONISTS

8.1 Endpoint Sensitivity to Thyroid Stimulation and Inhibition

The sensitivity of the whole organism 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)≥ 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 diagonistic 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.

8.2 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 prolactin 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, 1997). Therefore, gender differences should not be a major variable of TH disruption unless precocious sexual development is simultaneously induced.

8.3 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.

9.0 RESPONSE TO OTHER HORMONAL DISTURBANCES

The effect of other hormones on metamorphosis is specifically described in section 3.0. In summary, gonadotrophs (estradiol and testosterone), melatonin, somatostatin, and prolactin have an inhibitory action on metamorphosis in anurans (Kaltenbach, 1996; Denver, 1996). Conversely, GnRH stimulates the production of TH. Due to the complex nature of amphibian endocrinology, undiscovered hormonal influences or disturbances may also affect the process of metamorphosis.

10.0 CANDIDATE PROTOCOLS

10.1 Whole Organism Tests

10.1.1 16-day Metamorphic Climax Assay

The 16-d Metamorphic Climax Assay is conducted during the final period of metamorphosis, which is most prominently marked by the resorption of the tail and the development of the forelimb. As originally proposed by Fort et al. (2000), this assay primarily quantitatively evaluated the rate of tail resorption in *X. laevis*, although maturation of the skin and forelimb development were noted anecdotally. Due to higher than acceptable levels of variability in the rate of tail resorption, the assay has been modified somewhat to increase robustness. However, this variability is primarily due to the over-whelming influence of the endogenous TH peak that occurs during this developmental window. Thus, it is anticipated that

this stage would be relatively insensitive to TR agonists since the system is fully stimulated by the TH cascade. Conversely, this stage may also be insensitive to TR antagonists as the endogenous TH surge initiates a cascade of gene activity that is insensitive to most synthesis inhibitors (Brown et al., 1996).

In terms of the original assay design, larvae are cultured in FETAX solution (ASTM E1439-98, 1998), a reconstituted water medium suitable for the culture of *X. laevis* embryos and larvae. FETAX Solution consists of 625 mg NaCl, 96 mg $NaHCO_3$, 75 mg $MgSO_4$, 60 mg $CaSO_4 \cdot H_2O$, 30 mg KCl, and 15 mg $CaCl_2$ per L of solution. Larvae are fed Salmon Starter® fish food (*Xenopus* 1®, Dexter, MI) starting after day 4 at a rate of approximately 250 mg/larvae/day. One-half strength FETAX Solution should be used for the culture of *X. tropicalis*. Twenty stage 58 larvae are placed in each of four 10-L vessels containing varying constant concentrations of the toxicants. Stock solutions were prepared in FETAX Solution. Dilutions are also prepared in FETAX Solution. Five test concentrations are tested in quadruplicate, using a flow-through delivery/diluter system. A solid phase concentrator may be used when necessary to maximize the water solubility of highly hydrophobic test materials. Four separate vessels containing 20 larvae each are exposed to FETAX solution alone. Treatment and control dishes contain a total of 8-L of solution. The pH of the test solutions is maintained between 7.0 to 8.0.

If a static renewal design is used, renewal must be performed daily unless degradation of the test substance is minimal over a long period of time. Waste, excess food material, and dead embryos and larvae are removed daily regardless of the exposure design and numbers recorded. Generally, the use of a flow-through exposure design is preferred. Staging is performed during the renewal process (Nieuwkoop and Faber, 1975). Tests are terminated once the larvae reach stage 66 for the evaluation of tail resorption. At the completion of the exposure, larvae are fixed in 3% (w/v) formalin, pH = 7.0, and the gross effects on limb development and skin maturation noted. Limb defect assessment is aided by the use of a dissecting microscope.

Digital images are captured using a high-resolution color digital video camera. A computer with image processing software is used to digitize the tail length at developmental stages 58 to 66. A ruler videotaped with the larvae is used to monitor image distortion and calibrate the length-measuring program to ensure accurate measurements of the larvae. Tail lengths are measured using digitizing software. The mean tail lengths at day 14 for each concentration of each test material evaluated are corrected for the starting tail length at d 0. Statistical comparisons of the control and exposure treatments and determination of NOAEC values are performed using ANOVA. Isotonic regression of monotonic data is performed to determine median inhibitory (IC50) or median stimulatory (SC50) data.

As originally described, the primary drawback to this approach, aside from questionable sensitivity, is that it relied merely on gross morphological endpoints. As previously discussed, in order to distinguish between thyroidal- and non-thyroidal-based changes in developmental rates, other biochemical and molecular endpoints need to be incorporated into the test protocol that can establish mechanistic links to the observed morphological effects. Based on this information, a modified morphological test based on the original metamorphic climax assay that incorporates a more sensitive stage of the metamorphic period, such as early prometamorphosis, that is capable

of utilizing biochemical and molecular endpoints might be more advantageous.

10.1.2 Full Metamorphosis Assay

The same methodology addressed above for the metamorphic climax assay generally applies to the German full metamorphosis assay. As with the metamorphic climax assay, this specific procedure is designed for *Xenopus sp*. Culturing practices and data collection are identical to those described with the following exceptions. First, the test is conducted from stage 48 to stage 66 (OECD, 2001). Second, additional morphological endpoints are included in the full metamorphosis exposure and include hind limb differentiation, forelimb development, thyroid anatomy and pathology (goiter and myxedema), skin maturation, and tail resorption. The rates and normalcy of each process are considered. As described originally by OECD (2001), this assay is designated as a 28-d assay. The length of time generally required for *X. laevis* to develop from stage 48 to 66 is at least 50 d under ideal laboratory conditions (Nieuwkoop and Faber, 1975), which is exceedingly long for a thyroid disruption screening test. Further, the assay incorporates a significant portion of premetamorphosis at the start of the test in which no significant thyroidal activity is occurring, making it counterproductive.

10.1.3 Prometamorphosis Assay in *Xenopus*

An approximately 14-d prometamorphosis assay protocol exposing *X. laevis* from late premetamorphosis stage 52 to the completion of the prometamorphic phase (stage 57) could potentially be the most advantageous whole organism exposure design. In this scenario, the rate and normalcy of hind limb differentiation is measured along with biochemical measures of TH and possibly a molecular screen of TH-inducible gene expression (i.e., TR beta). Previous work by Fort and Bantle (unpublished data) indicated that earlier developmental stages, starting around stage 52 to 54, were capable of responding to thyroid agonists and antagonists. Although tail resorption was used as a morphological marker of metamorphosis in this study, it is not unreasonable that hind limb development would follow the same pattern, only with increased sensitivity. A late premetamorphosis (stage 51) to early prometamorphosis (stage 54) exposure scenario which also is completed in ca. 14d could also be considered. Culturing and measurement methodology cited in Section 10.1.1 would apply to this assay design.

10.1.4 *Hyperolius argus* Endocrine Screening (HAES) Assay

No further discussion of this assay is provided in the DRP since, although it may be a useful model, it does not meet the objectives of developing a thyroid axis disruption screening assay.

10.2 Biochemical Measurements

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; Mellstrom 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.

10.3 Molecular Tests

Three molecular approaches for measuring TH-induced metamorphosis are currently being evaluated: transgenesis, multiple gene expression assays, and single gene expression assays. The multiple gene expression assays reviewed include differential display and gene array techniques. The single gene expression assays reviewed included RT-PCR and RPA technology. Development of transgenic lines expressing novel TH-inducible gene sets (i.e.,TR beta and related TREs) are possible. However, the complexity and time required to create a transgenic line makes it somewhat less attractive than the gene expression assays. The potentially most useful molecular assays are the gene array and RT-PCR approaches. Differential display is plagued by difficulty in interpretation and quantification of the results of simultaneous multiple gene expression, and will thus not be considered further. Although the RPA technology could potentially be used to monitor single gene expression, the newer RT-PCR techniques are most efficient and sensitive. Based on this information, measurement of TH-inducible gene arrays (TR beta, TR alpha, ST3, and other related genes), including the respective TREs, could be used to measure changes in multiple gene activity as the result of EDC exposure. A more simplistic method is to use RT-PCR to measure changes in single gene activity. In this case, changes in TR beta, ST3, arginase, or other relevant TH-induced genes could be quantitatively measured for changes as the result of EDC exposure. Furthermore, both RT-PCR and gene arrays could be used as an endpoint in the short-term morphological tests, along with biochemical measurements, to determine if the responses are the result of thyroidal or non-thyroidal processes. The primary advantage of the gene array technology over the RT-PCR approach is that it is capable of monitoring multiple gene activity. Since TH-induced metamorphosis is the result of multiple genes acting within a highly complex program, it is more realistic and potentially useful.

11.0 RECOMMENDED PROTOCOL AND ADDITIONAL DATA NEEDS

11.1 Preferred Test Species

11.1.1 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, and to 2) ultimately develop a longer term Tier II assay that includes advanced developmental and reproductive endpoints, 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 thyroid axis-related genes and some knowledge of the genetic programs associated with TH dependent processes;

7. Biochemical information on the endocrine axis, particularly of the hypothalamus-pituitary-thyroid (HPT) axis; and

8. Metabolism information, especially as it relates to TH homeostasis.

11.1.2 Test Species

The only anuran species which meets the minimal criteria established above is *X. laevis*. 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. laevis* is relatively rapid compared to the two Ranid species commonly used in biological and toxicological research, *R. pipiens* and *R. catesbeiana*. In terms of thyroid dependent post-embryonic development, more is known about *X. laevis* than any other anuran species. In addition to meeting the minimal requirements stated above, the genetic information regarding *X. laevis* is more extensive than other anurans, especially in the area of the thyroid axis, where numerous publications have detailed the genes and the genetic program involved in the process of metamorphosis. Finally, the information on the biochemical and metabolic control of TH in this species is well-developed, and includes information on all of the typical hypothalamus-pituitary-thyroid (HPT) modulators as well as peripheral tissue enzymes, such as the deiodinases, which ultimately control the local and downstream effects of TH.

The only alternative species that should be considered is *X. tropicalis*. This species is similar in terms of ease of culture and reproduction. The primary advantages of this species are:

1) relatively rapid developmental rate that could shorten test protocols, especially those that include reproductive endpoints (not specifically discussed in this DRP), and 2) the genome of this species is diploid which will eventually facilitate the development and use of molecular endpoints. However, at this time, there is too little experience in the broader scientific community to support the selection of this species as the primary species for a Tier I screen. In addition, disease susceptibility may be greater in *X. tropicalis* than in *X. laevis*, making them more difficult to rear successfully in the laboratory. Eventually, as the genetic information is developed for this species (major initiatives have been proposed to sequence the genome of *X. tropicalis*) and as more laboratories develop biological and toxicological information to support the use of *X. tropicalis*, this species may replace *X. laevis*.

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.

11.2 Description of Method

11.2.1 Rationale

The utility of any biological protocol is necessarily constrained by inherent limitations of the model, costs of conducting the protocol, variability inherent in the endpoint responses, specificity of the response with respect to the hypothesis, and sensitivity of the endpoints using a practical and reproducible approach. Given these general considerations, it is important to focus the development of a Tier I screen for thyroid disruption on a specific hypothesis. This hypothesis is, "*Exposure to a test chemical causes changes in the homeostasis and action of thyroid hormone* or the thyroid axis."

Although simple, this hypothesis is useful as it limits the scope of a Tier I protocol to exclude other considerations, such as development of the thyroid gland and the effect of thyroid perturbation on reproduction. However, by establishing these limits, a more cost-effective protocol can be developed that will be more applicable to meet the needs of the EDSP.

In addition to the basic hypothesis, it should be the goal of this approach to develop a protocol which serves as a generalized vertebrate model that can be used to help evaluate the risk concerns among vertebrates. In order to accomplish this goal, it is necessary to understand the mechanisms involved in thyroid perturbation. Extrapolation to other species is dependent on defining the similarities and dissimilarities at the mechanistic level. There are many conserved aspects of the thyroid pathway among vertebrates, especially in the HPT axis. For example, substantial homology exists for many of the genes associated with TH homeostasis. And, in practice, commonly used inhibitors of thyroid synthesis behave similarly in anurans as they do in

mammals. In combination, these facts support the plausibility that results obtained from a *X. laevis* model could be extrapolated to other vertebrates.

11.2.2 Specific Protocol Recommendations

11.2.2.1 Developmental Stage. Assuming that *X. laevis* is the species of choice and assuming that testing the above hypothesis is the objective, establishing which developmental stage(s) should be included in the protocol is paramount. As noted earlier, the process of metamorphosis in *X. laevis* (and other anurans) can be divided into three phases: premetamorphosis, prometamorphosis, and climax. Premetamorphosis is the interval of development that proceeds from hatch to stage 54. This stage is characterized primarily by growth in the absence of a functional thyroid gland and consequently this development is considered TH independent. This is supported by the fact that inhibition of TH synthesis during premetamorphosis permits development up to stage 54, but not beyond. In fact, continuous exposure to TH synthesis inhibitors during premetamorphosis will maintain the organism at stage 54 indefinitely. At stage 54, prometamorphosis begins, which is characterized by the onset of thyroid gland function, rising levels of TH, and the process of TH-dependent morphogenesis. Prometamorphosis continues through approximately stage 60, and many tissues reach their respective metamorphic fate during this phase (e.g., limbs). At stage 60, metamorphic climax begins which is characterized by an abrupt rise in TH synthesis and the dramatic morphogenetic events, including tail resorption, which complete the transition from the larval to the juvenile phase at stage 66.

Given this background and the hypothesis to be considered, it seems obvious that exposures that cover the premetamorphosis period would be ineffective at detecting inhibitors of the thyroid pathway. Since inhibition of the thyroid pathway is the most likely mode of action of environmental chemicals, an assay that includes a substantial period of premetamorphosis is unwarranted.

At the other end of the developmental sequence, metamorphic climax, TH-dependent morphogenesis requires very high TH levels. This suggests that climax may be a relatively insensitive period as the level of inhibition would have to be relatively greater than for prometamorphosis in order to observe an effect. Stage 60 larvae are, in fact, less sensitive to inhibitors than prometamorphic larvae. In some cases, inhibition at stage 60 was ineffective, consistent with the observations of other laboratories (Brown, 1996; Fort and Bantle, unpublished data) that once the genetic program is initiated by rising TH levels, the cascade of the gene expression program continues even in the face of inhibition. Furthermore, in terms of stimulation, it is likely that further stimulation of the TH pathway during climax will be inconsequential to the developmental outcome because the overwhelming stimulation via the endogenous rise in TH overshadows any presumptive additional stimulation. Furthermore, it has also been demonstrated in the literature that larvae are competent to respond to stimulation as early as stage 48. And, the earlier developing tissues, particularly hind limb during prometamorphosis, are more sensitive than tissues that complete remodeling during climax. This is consistent with the natural sequence of TH-dependent morphogenesis when compared to the endogenous TH levels. That is, the more sensitive tissues remodel during prometamorphosis

when TH levels are low and that the endogenous TH elevation at climax is necessary for the late developing tissues.

Consequently, the general developmental period upon which an assay should be developed is prometamorphosis (stages 54 through 60). The rationale for this is that 1) the thyroid system is fully functional, 2) tissues are fully competent to respond, and 3) prometamorphosis is more sensitive to inhibition and stimulation than climax. This period, according to Nieuwkoop and Faber (1975), lasts for approximately 20 days, depending on culture conditions. But, since effects can be determined prior to the completion of prometamorphosis, it is unlikely that it will be necessary to conduct the exposure for the complete interval, making the ultimate protocol shorter. An additional argument that has merit is that the transition period from pre- to prometamorphosis would be particularly sensitive to perturbation of the TH pathway because the exposures would be initiated with organisms that are initially naive to TH, but that TH synthesis would be developmentally acquired during the protocol. Effects of inhibition that precede endogenous production of TH could be enhanced because there is no TH pool to promote TH-dependent development in the absence of nascent synthesis. Conversely, effects of stimulation could be enhanced since there is no TH at the initial stages of the study. To address the issues of necessary length of exposure and optimal developmental period, Tietge et al. (personal communication) have examined the effects of methimazole, perchlorate, and propylthiouracil on development of stage 51 and 54 organisms for periods of up to 14 d. Only marginal improvements are seen with stage 51 organisms and 14 day exposures to stage 54 are sufficient to observe developmental delay based on apical morphological endpoints.

11.3 Endpoints

Apical morphological endpoints are useful in that they demonstrate an organism-level effect that integrates all aspects of the toxicological process. While there is value in apical morphological endpoints, they are limited because: 1) they are not necessarily diagnostic of a particular mode of action, 2) they are generally less sensitive than sub-organismal endpoints, 3) they are the slowest of the endpoints to manifest effects, and 4) they may be difficult to use in terms of inter-species extrapolation. For example, Tietge et al. (personal communication) have demonstrated that histological analysis of the thyroid gland of organisms that have been exposed to several TH inhibitors can detect substantial thyroidal hypertrophy and hyperplasia in 8 days at concentrations where there is no effect on the apical morphological endpoints. These observations suggest that, based on apical endpoints, one would conclude that the thyroid pathway is unaffected, when, in fact, there is clear evidence of a thyroid-specific effect at a histological level. The interpretation of this pattern of responses is that the thyroid axis is inhibited, but the mechanisms that underlie homeostasis are able to compensate at a dose which results in incomplete inhibition. More specifically, partial inhibition of TH synthesis results in depressed TH levels, which is sensed by the CNS and results in TSH release which, in turn, stimulates thyroidal hyperplasia and hypertrophy. This response compensates for low TH levels by up-regulated synthetic pathways.

Another problem with apical morphological endpoints is that they tend to be divergent and do not lend themselves to inter-species extrapolation. Endpoints which address more central

and conserved processes are more desirable because they do lend themselves to inter-species extrapolation. For example, several chemicals are known to inhibit TH synthesis via different mechanisms. The same mechanisms are apparently affected similarly in both mammals and amphibians (e.g., inhibition of iodide uptake by perchlorate, inhibition of thyroid peroxidase by methimazole). The manifestation of these inhibitory mechanisms in an anuran at the apical level would be inhibition of metamorphosis based on a morphological response, such as tail resorption. It would be difficult to use this response to predict effects in mammals, where tail resorption does not occur. Therefore, if the subject protocol is to be used as a generalized vertebrate model, then apical endpoints are insufficient and endpoints with more diagnostic power are clearly necessary.

There are three additional classes of endpoints that should be considered for this assay: 1) molecular endpoints, 2) biochemical endpoints, and 3) histological endpoints. Classical toxicology tests focus primarily upon tissue and organism-level effects, which are often insufficient for discriminating the modes/mechanisms of action (MOA). A fundamental understanding of MOA, however, is critical to the ability to extrapolate toxicological effects among species and chemicals, and across biological levels of organization. This necessitates the development of tools capable of linking tissue and organism-level effects to MOA. Monitoring gene expression is one approach commonly used to link whole organism changes to specific MOA. However, traditional methods in molecular biology have generally utilized a "one gene in one experiment" approach and, as a consequence, the through-put is very limited and resource-intensive when more than a few genes are monitored. In the past several years, a new technology, called DNA array analysis, has been developed to simultaneously monitor a large number of genes simultaneously (see Nuwaysir et al., 1999; Khan et al., 1999; Debouck and Goodfellow, 1999). Application of this tool to the study of thyroid toxicology could allow investigators to easily and simultaneously monitor the expression of many thyroid related genes at multiple points within an organism. This approach would provide insight as to where in the organism or tissue toxicity is occurring, and which genes might serve as indicators of exposure and effects. Ultimately the combination of DNA arrays and whole organism testing would enhance our ability to link functional changes in the organism to specific MOAs.

Biochemical endpoints, like molecular endpoints, provide useful information on MOA and are, thus, important in developing a protocol that provides data that are diagnostic of a thyroid-specific effect and that can be extrapolated to other vertebrates. The most commonly used method to assess T3 and T4 status is RIA. RIA determinations on organisms exposed to a chemical represent the minimal data necessary to evaluate inhibition of T4 synthesis and metabolic conversion of T4 to T3 via deiodinase activity. Other methods should be considered that permit the analysis of synthetic precursors of T4, as well as metabolic products that are the result of elimination pathways (e.g., deiodination, glucuronidation, sulfation). Several HPLC-based methods have been utilized to achieve separation of such compounds, but they often rely on the use of radiolabeled iodine for detection. Alternative detection methods should be further investigated and possibly included as a routine analytical method to augment the utility of the Tier I protocol. Finally, histological analysis of the thyroid must be included as an endpoint at this time because it confers diagnostic specificity to the assay that is unattainable using apical morphological endpoints alone.

In summary, endpoints which confer diagnostic specificity are needed in order to accurately determine whether or not the thyroid pathway is the target of a test chemical. This is needed, not only to properly characterize the chemical for potential regulatory action, but to provide a basis for interspecies and interchemical extrapolation. Although the molecular and biochemical endpoints suggested herein require additional research and development, their successful implementation will reduce the time and cost associated with conducting an assay, and will improve the quality and utility of the data. Ultimately, this will help reduce testing needs in the future.

11.4 Exposure Protocol

Based on the information provided in the present DRP, the recommended exposure protocol will involve exposure of late premetamorphic *X. laevis* larvae from stage 51 to stage 54, resulting in a late premetamorphic to early prometamorphic test. In general, *X. laevis* larvae will be cultured as described in Section 4, during which time histological effects data will be collected on the thyroid, including hypertrophy, hypotrophy, hyperplasia, and hypoplasia. Since histological effects on the thyroid may occur well before apical morphological effects are manifested, exposure through stage 54 may not be required for EDCs that strongly affect the thyroid axis. Thus, collection of data prior to the ca.14-d prometamorphosis assay period could potentially shorten the test. Collection of control (positive and negative) and exposed specimens for histological evaluation of the thyroid gland should be performed on at least days 8 and 14. At this time, samples should also be collected for biochemical analysis of TH (T4 and T3) via RIA. Samples should also be collected for analysis of gene expression. Using tissue punch samples described by Veldhoen and Helbing (2001), molecular analysis could be performed without sacrificing specimens and allow for tracking of simultaneous histological effects and gene activity changes on the same individual. At this point, it is not completely clear which molecular assay will be best served for this exposure protocol. The two best options are RT-PCR analysis for measuring single gene activity and gene array analysis for measuring multiple genes activity. Of the single genes to be strongly considered, TR beta (or TR alpha) and ST3 are potential candidates for analysis. However, gene array analysis now provides a means of measuring multiple gene activity simultaneously, which is a tremendously powerful tool and potentially more useful in this assay. Finally, apical morphological endpoints should not be excluded from the assay. During this period of development, evaluation of hind limb differentiation should be monitored.

In summary, the recommended exposure protocol will involve a ca. 14-d into pre-/prometamorphosis assay with *X. laevis* initiated at stage 51 and concluded at stage 54. Static-renewal or flow-through exposure, with adequate test substance analysis based on the physicochemical properties of the test substance, is recommended. On, at least, days 8 and 14, specimens should be digitally photographed to document morphological development and stage. Hind limb development can be measured using a scanning digitizer. Specimens should be randomly selected for histological examination of the thyroid and samples should be collected for biochemical analysis and analysis of TH-induced gene expression via constructed DNA arrays. It should be noted that if one of the endpoints demonstrates particular sensitivity, reliability, and speed, it may be chosen as a single endpoint for the proposed assay. However, the use of multiple endpoints provides additional confirmation of the response and will help

distinguish between thyroidal and non-thyroidal responses. More research will be required to survey this issue and will be addressed in the Data Gaps Section (Section 11.6).

11.5 Interpretation of Results

Results from the ca. 14-d late premetamorphic/early prometamorphic assay (stages 51-54) will be classified as: 1) histological, 2) biochemical, 3) molecular, and 4) morphological. Changes in thyroid histology, including hyper- and hypoplasia, and hyper- and hypotrophy will be reviewed. Since histological changes can be somewhat subjective, additional confirmation may be required. Criteria for establishing what constitutes a significant change in thyroid histology will also be required. Chemicals which disrupt TH synthesis (goitrogens) cause hypertrophy of the thyroid due to increased stimulation from greater quantities of TSH induced by TH feedback loops. This results in a hyperactive thyroid that produces similar levels of TH. In this case, although distinct histopathology of the thyroid exists, no marked changes in TH may be observed. These scenarios confound interpretation of the results. Changes in TH levels directly indicate changes in thyroid activity. However, measurement of biochemical parameters alone will not demonstrate a specific histological or morphological effect. Thus, biochemical analyses of TH levels will be an important complement to the other endpoints. The molecular tests (gene arrays) will be most straightforward in terms of interpretation. Changes in differential gene expression for arrays under control of a TRE suggest that the test substance is capable of altering TH-inducible gene activity. For example, in the measure of TH, acetochlor (Veldhoen and Helbing, 2001) synerigistically induces TR beta genes. Changes in downstream gene activity could potentially affect thyroid histology or TH levels, although more work will be required to specifically evaluate these relationships. Apical morphological evaluation can not be used alone to determine if a substance alters thyroid homeostasis. Thus, if used, morphological endpoints will require other biochemical and/or molecular endpoints to distinguish between thyroid- and non-thyroid-mediated responses. In summary, because of the complexity in evaluating and interpreting results from these studies, use of each endpoint that has been sufficiently developed should be included until a sufficient database is developed that aids in interpretation of the data and the selection of specific endpoints to be used in the future.

11.6 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 thyroid agonists and antagonists are currently known? The effects of thyroid agonists and antagonists on apical morphological changes during anuran metamorphosis are reasonably well understood. However, the relationship between changes in thyroid axis homeostasis and apical morphological changes are not as obvious. Before the effects of unknown chemicals on thyroid function can be assessed, the response of known thyroid 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 thyroid-based mechanisms? At this point, it is understood that disruption of apical morphological changes during metamorphosis may or may not be the result of alteration

of thyroid function. More work will be required to assess confidence in the histology, biochemical, and molecular endpoints in terms of predictability of thyroid 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.

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 thyroid perturbation? To understand with confidence at what point molecular changes are an indicator of thyroid disruption, the results must somehow be shown to be related to an upstream or downstream response within the thyroid axis. If molecular changes, such as inhibition of TR beta mRNA synthesis, can be linked to a histological, biochemical, or possibly even an apical morphological change in metamorphic processes, this relationship can be addressed. Work will be required to determine the threshold of molecular change that results in a physiological change in thyroid 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 thyroid axis homeostasis and its relationship to gross morphological, molecular, biochemical, and histological changes? The degree to which thyroid homeostasis can be changed without adversely affecting the organism needs to be determined. In addition, the relationship between the sensitivity of thyroid 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.

12.0 IMPLEMENTATION CONSIDERATIONS

12.1 Animal Welfare Considerations

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.

12.2 Recommended Equipment/Capabilities

12.2.1 Laboratory Capabilities

Laboratories generally qualified to perform research and conduct testing associated with the prevalidation and validation of the amphibian metamorphosis assays 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.

12.2.1.1 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 seervices 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.

12.2.1.2 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, pulsed electrochemical 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 and fluorescence and light microscopes).

12.2.2 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.

12.2.3 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 judgement 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.

12.2.4 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.

12.2.5 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.

12.2.6 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).

12.2.7 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.

12.3 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. laevis* prometamorphosis assay protocol. The most significant work needs to be performed in the final development of the molecular endpoints. Use of TR beta single gene expression from tissue biopsy samples using RT-PCR analysis is reasonably well-developed. However, TH-inducible gene arrays need to be constructed for evaluating multiple gene activity. The latter approach appears to be the most powerful at this time.

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 thyroid agonists (e.g., thyroxin) and antagonists (i.e., perchlorate, propylthiouracil, and amiodarone) should be tested. 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 thyroid disruption capacity and perhaps one that has no information (fourth phase) regarding thyroid axis 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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