# Amphibian Metamorphosis Assay
# OCSPP Guideline 890.1100

*Standard Evaluation Procedure (SEP)*

ENDOCRINE DISRUPTOR SCREENING PROGRAM
U.S. Environmental Protection Agency
Washington, DC 20460

30 September 2011

## Table of Contents

## Appendices

# I. Introduction

This document was developed by EPA to provide guidance to EPA staff who will be reviewing the data submitted in response to Tier 1 Orders issued under the Endocrine Disruptor Screening Program (EDSP). This document provides general guidance and is not binding on either EPA or any outside parties. The use of language such as "will," "is," "may," "can" or "should" in this document does not connote any requirement for either EPA or any outside parties. As such, EPA may depart from the guidance where circumstances warrant and without prior notice. The SEPs are intended to be used in conjunction with the EDSP Test Guideline Series 890 and the Corrections and Clarifications document available on the EDSP web page.

This Standard Evaluation Procedure (SEP) provides guidance on how EPA generally intends to review studies conducted using the OCSPP Guideline 890.1100 Amphibian Metamorphosis (Frog) Assay (AMA) that are submitted to support requirements imposed under the U.S. Environmental Protection Agency's Endocrine Disruptor Screening Program (EDSP). The objective of EDSP Tier 1 assays is to characterize the potential of a chemical to interact with the endocrine system.

The product of the review will be a Data Evaluation Record (DER) that reflects how well the study conforms to the Guideline, evaluates how well the study and analyses were performed, and provides the conclusions supported by the data. The DER will include, for example, a list of any significant deviations from the guideline and their potential impacts, a list of significant information missing from the study report, a description of how the statistical analyses were performed and whether they were performed according to the guideline, and any other information about the performance of the study that affects interpretation of the data within the context of the EDSP. The DER should record details on all endpoints required by the guideline. The DER is intended to contain enough information to provide EPA with the ability to determine whether the study is scientifically valid and provides the necessary information.

The guideline recommends the critical materials, methods, and analyses that lead to successful performance of the assay. If a particular material, method, or analysis is specified in the guideline, it is usually because other materials, methods, or analyses are either known to be inappropriate, or at least have not been validated and there is concern for their potential influence on results. The Agency has posted Corrections and Clarifications on Technical Aspects of the EDSP Tier 1 Assays (OCSPP Test Guideline Series 890) in the docket; the link to this document may be found by way of the EDSP web page (http://www.epa.gov/endo/). It is therefore important to note deviations from specific materials, methods, or analyses in the DER, and provide the Agency's opinion on whether the deviation/deficiency has an impact on the performance and results of the study or the acceptability of the study.

# II. The Amphibian Metamorphosis Assay

## A. **Purpose of the Assay**

The AMA is a screening assay intended to empirically identify substances which may interfere with the normal function of the Hypothalamus-Pituitary-Thyroid (HPT) axis (Fort *et al*., 2007). The AMA represents a generalized vertebrate model to the extent that it is based on the conserved structures and functions of the HPT axis. It is an important assay because amphibian metamorphosis provides a well-studied, thyroid-dependent process which responds to substances known to be active along the HPT axis, and it is the only assay in the EDSP Tier 1 battery that detects thyroid activity in an animal undergoing morphological development. It is intended to be included in a battery of *in vitro* and *in vivo* tests to identify substances with potential to interact with the endocrine system.

## B. **Study Design**

The general experimental design entails exposing Nieuwkoop-Faber (NF) stage 51 African clawed frog (*Xenopus laevis*) tadpoles, in separate treatment groups, to a minimum of three respective concentrations of a test chemical or a negative (clean water) control for 21 days. There are four replicates of each test treatment. Larval density at test initiation is 20 tadpoles per test tank (replicate) for all treatment groups (80 larvae/treatment). The observational endpoints are hind limb length (HLL), snout-to-vent length (SVL), developmental stage, body weight, thyroid histopathology, and daily observations of mortality and clinical signs. In addition, whole thyroid tissue and plasma samples may be collected for analysis of thyroxine (T4) (OECD 2009), although this is not specified in the OCSPP 890.1100 test guideline.

# III. Evaluation of Study Conduct

This section provides a summary description of the information that would generally be expected to be obtained from a study that had been conducted following the recommendations in the Test Guidelines. As described in this section, the DER reviewer is responsible for summarizing how the study was conducted, the extent to which that is consistent with the Guidelines, and how, if at all, that affected the validity of the study. This information will factor into the Agency's interpretations of the data contained in the study report. Specific points that are important for the DER to address are highlighted in the individual sections below, as appropriate.

The summary in this section is offered as a general outline to aid in preparation of the DER. The purpose of this section is not to serve as substitute for the Test Guidelines, nor to provide any guidance on how the study should be conducted. Rather, the summary is intended to provide context and examples illustrating to the individual preparing the DER what the DER would be expected to contain.

## A. Test Species

African clawed frog *(Xenopus laevis)* is the recommended species. The assay method was validated and OCSPP 890.1100 is explicit for this species. However, the Corrections and Clarifications document (EPA 2011) states that the method may also be applicable to *Silurana (Xenopus) tropicalis*, as demonstrated by Mitsui *et al.* (2006). If an alternate species is used, the DER should detail the relevant or significant deviations in the method in order to accommodate the alternate species (*e.g., S. tropicalis*). The DER should include a discussion of whatever evidence was used in the study report for the performance criteria that were used to support the reliability of the test.

## B. Equipment and Supplies

The list of equipment and supplies from the test guideline is provided only as a non-exhaustive recommendation of what is typically needed to conduct a successful test. If equipment and supplies are used that differ from those identified in the test guideline, it is recommended that the DER identify the differences and state whether and how they may have affected the performance or outcome of the study.

## C. Chemical Testability

The DER should summarize the results of any tests conducted to evaluate the extent to which concentration and stability of the test chemical in the exposure system were verified. The AMA is based upon an aqueous exposure protocol whereby the test chemical is introduced into the test chambers via a flow-through system. If a successful test is not possible for the chemical using a flow-through test system, a static renewal system is recommended. If neither system is capable of accommodating the test chemical even using approved co-solvents, then the default is to not test the chemical using this guideline.

## D. Exposure System

### 1. System Description

A flow-through diluter system is preferred, when possible, over a static renewal system (OECD 2009). The system components should be described in the DER as well as the extent to which they generally comport with the exposure system description in the guideline and are capable of maintaining the experimental conditions recommended in **Appendix 1** of the guideline.

### 2. Water Quality

It is recommended that a description of the source water and chemical analysis results be provided in the DER, in addition to evidence that the water can support normal growth and development of *X. laevis*.

### 3. Iodide Concentration in Test Water

Based on the available data from the validation studies, the assay has been demonstrated to work well when test water iodide ($I^-$) concentrations ranged between 0.5 and 10µg/L. This is in addition to dietary iodide exposure at levels present in the recommended feeding regime using Sera Micron® diet. Ideally, the minimum iodide concentration in the test water would be 0.5 µg/L. Iodide supplementation is recommended if the concentration is naturally below 0.5 µg/L in the test water; then, the it is recommended that iodide supplementation not exceed 2 µg/L. For example, if the test water is reconstituted from deionized water, it is recommended that iodide be added at a minimum concentration of 0.5µg/L. Any supplementation of the test water with iodide or other salts would be relevant information for inclusion in the DER. In addition, any measurements of iodide concentrations in the test water (and diet, if applicable) should be reported in the DER.

## E. Holding of Animals

### 1. Adult Care and Breeding

One option for adult care and breeding of *X. laevis* is to conduct these activities in accordance with existing guidance for the frog teratogenesis assay (ASTM 2004). A full description of any alternative animal care and breeding used would also be relevant information to be described in the DER, along with whether deviations from the recommended method may have had any significant impact on the study performance or interpretation, using performance criteria (Section IV-B of the SEP) as a guide.

### 2. Embryo Selection

The guideline recommends that 2-3 of the best individual spawns be retained to evaluate the quality of the spawns. From these 2-3 spawns, it is recommended that the test organisms originate from the best single spawn, based upon embryo viability/appearance and the presence of an adequate number ($\geq$ 1,500) of embryos. The guideline recommends against co-mixing spawns because this has been demonstrated to increase variability and decrease the statistical power of the test (OECD 2007a). It is useful for the DER to report how many spawns were evaluated, along with details of the evaluation method (*e.g.*, timing of evaluation and number of eggs evaluated per sample), and whether the organisms selected for the definitive test originated from the best single spawn.

### 3. Larval Culture and Feeding

The test guideline provides recommendations for handling the embryos and larvae (tadpoles) during the pre-exposure phase. If no tadpoles develop to NF stage 51 within

17 days after fertilization, then inappropriate environmental conditions, disease, or other stressors may be potential culprit(s).

The test guideline recommends tadpoles be fed Sera Micron® (Sera GmbH, Heinsberg, Germany), or other diet that has demonstrated to allow equal performance of the AMA (OECD 2009), throughout the pre-exposure period (after NF stage 45/46) and during the entire test period of 21 days. It is recommended that the feeding regime during both the pre-exposure period and the test follow the recommendations in the guideline; the recommended regime (and associated nutrient and dietary iodide availability) resulted in adequate performance (*i.e.*, growth and development of tadpoles) of the AMA during validation (OECD 2007a). The DER should document the feeding regime used.

### F. Analytical Chemistry

The guideline recommends test solutions from each replicate tank at each concentration be sampled for analytical chemistry analyses at test initiation (day 0), and weekly during the test for a minimum of four samples. It is also recommended that each test concentration be analyzed during system preparation, prior to test initiation, to verify system performance. In addition, it is recommended that stock solutions be analyzed when they are changed, especially if the volume of the stock solution does not provide adequate amounts of chemical to span the duration of routine sampling periods. The sampling schedule and analyses performed should be provided in the DER.

### G. Selection of Test Concentrations

#### 1. Establishing the High Test Concentration

The test guideline recommends that the high test concentration be set as the lowest of the following values: (1) 100 mg/L, (2) the solubility limit of the test substance, or (3) the highest test concentration of the chemical which results in less than 10% acute mortality. The justification for using the highest test concentration that results in less than 10% mortality (if this is the lowest value of the three options above) is that this demonstrates that the chemical is tested up to a level where overt toxicity is observed, and thus adequately challenges the organism, without compromising the validity of the test. The DER should describe any basis for selecting the highest test concentration included in the study.

#### 2. Test Concentration Range

The guideline-specified minimum number of test concentrations is three plus a negative (clean water) control, plus a solvent/vehicle control if necessary. The recommended minimum differential between the highest and lowest test concentrations is approximately one order of magnitude [lowest = 0.1X(highest)]. The minimum recommended separation between individual concentrations is a factor 0.33X and the

maximum is a factor of 0.1X (OECD, 2008). The DER should identify the range of test concentrations and include any justification provided in the study report, if the test employed a range different from the recommended guidance.

## H. Test Procedure

### 1. Day 0 (Test Initiation)

It is recommended that the DER identify the selection method for tadpoles used in the definitive test, along with the age (days post-fertilization), Nieuwkoop and Faber (1994) stage, and size range (if determined) of the tadpoles selected, as described in this section. The exposure is initiated when a sufficient number of tadpoles in the pre-exposure stock population, which are less than or equal to 17 days of age post-fertilization, have reached developmental stage 51 (**Appendix 1**) according to Nieuwkoop and Faber (1994). The test guideline includes additional guidance on optional size selection, based on total length (not snout-vent length), that complements the stage selection. The optional addition of size selection further reduces variability by ensuring that the NF stage 51 tadpoles fall within a specified size range.

For selection of test animals, healthy and normal looking tadpoles of the stock population are pooled in a single vessel containing an appropriate volume of dilution water. For developmental stage determination, the Guidelines recommend that the tadpoles be individually removed from the pooling tank using a small net or strainer and transferred to a transparent measurement chamber (*e.g*., 100 mm Petri dish) containing dilution water. The developmental stage of the animals is determined using a binocular dissection microscope. Total length, in millimeters (mm), may also be determined at this time.

To reduce variability associated with measurement (staging) error, it is important that staging be conducted as accurately as possible. It is preferred not to use anesthesia; however, it is possible to individually anesthetize the tadpoles using an appropriate method [*e.g.*, 100 mg/L tricaine methanesulfonate (MS-222), appropriately buffered with sodium bicarbonate (pH 7.0)], prior to initial staging. Animals are carefully handled during transfer in order to minimize handling stress and to avoid any injury. If anaesthesia is used, the DER should document the method.

While the complete Nieuwkoop and Faber (1994) guide may be consulted for comprehensive information on staging tadpoles, one can reliably determine NF stage using prominent morphological landmarks. For test initiation, the guidelines recommend the tadpoles selected should be at NF stage 51. The most prominent morphological staging landmark at NF stage 51 is hind limb morphology, where the hind limb bud is conical in shape and is 1.5X as long as it wide. Tadpoles that meet the stage criteria are held in a tank of clean culture water until the staging process is completed. Tadpoles exhibiting grossly visible malformations or injuries should be excluded from the assay. Once the staging is completed, the larvae are randomly distributed to exposure treatment

tanks until each tank contains 20 larvae. Each treatment tank is then inspected for animals with abnormal appearance (*e.g*., injuries, abnormal swimming behavior, *etc*.). Tadpoles that appear unhealthy are typically removed from the treatment tanks and replaced with larvae newly selected from the pooling tank. The DER should report any observations of abnormal appearance or behavior and whether tadpoles with the specified abnormality/ies were excluded from the assay.

### 2. Day 7

The guidelines recommend on day 7 of exposure, five randomly chosen tadpoles per replicate are removed from each test tank. The procedure used gives each test organism an equal probability of being selected; therefore, each tadpole is netted. Any randomization method is scientifically appropriate. Tadpoles not selected for day 7 measurements are returned to the tank of origin. The five tadpoles selected for measurements are humanely euthanized (*e.g.*, in 150 to 200 mg/L MS-222, buffered with bicarbonate to pH 7.0). The euthanized tadpoles are rinsed in water and blotted dry. Body weight (also referred to as wet weight or blotted dry weight) is recorded to the nearest milligram (mg). Hind-limb length (HLL) and snout-vent length (SVL) are recorded to the nearest millimeter (mm). NF developmental stage is determined using a binocular dissection microscope. This assay does not track individuals (*i.e.*, tadpoles are not marked) and thus it would not be scientifically appropriate to pair individual Day 7 observations with individual observations (*e.g.*, size) at test initiation. It is recommended that the DER identify which measurements and observations were recorded on Day 7 of the assay and briefly describe the method used to subsample tadpoles (*i.e.*, whether the selection method was consistent with the randomization procedure recommended in the guideline).

### 3. Day 21 (Test Termination)

The guideline recommends that at test termination (day 21), the remaining tadpoles are removed from the test tanks and humanely euthanized, as described above. Tadpoles are rinsed in water and blotted dry. Body weight (mg), HLL and SVL (mm), and NF developmental stage are recorded for each individual. The guideline recommends that all larvae be placed in Davidson's fixative for 48 to 72 hours (OECD 2007b), either as whole body samples or as trimmed head tissue samples containing the lower jaw. (Whole body samples may be preferable, since this preserves the association between individual observations of morphology and thyroid histopathology at test termination.) To maximize the viability of samples for potential future reference, it is recommended that all specimens be preserved using this method. However, the guideline recommends that a subset of only five tadpoles from each replicate tank be selected for thyroid histopathology in the AMA. The DER should identify which measurements and observations were recorded on Day 21 of the assay, along with the number of tadpoles selected for thyroid pathology and the methods of selection and preservation for these and any other archived specimens. More detailed information for the evaluation of study conduct with respect to biological endpoints is provided in section (III)(I) of this SEP.

Normal divergence in development is expected to result in different stage distributions within each replicate tank. Since follicular cell height is stage-dependent, the guideline recommends that individuals selected for histopathology (n=5 per replicate) be stage-matched to the median stage (pooled replicates) of the negative (clean water) control, when possible. Therefore, it is recommended that control specimens be processed first. After control specimens are selected for histopathology based on the median control developmental stage, all larvae in each remaining replicate (test item treatments and any solvent controls) are staged prior to selection of organisms from that replicate for histopathology.

If a replicate tank contains more than five larvae at the appropriate stage, then five larvae are randomly selected from the stage-matched individuals. If a replicate tank contains less than five larvae at the appropriate stage, then additional individuals are typically randomly selected from the next lower or upper developmental stage to reach a total sample size of five larvae per replicate. The decision to sample additional larvae from either the next lower or upper developmental stage is based on an overall evaluation of the stage distribution in the control and chemical treatments. That is, if the chemical treatment is associated with developmental delay based on NF stage, then it is recommended that the additional larvae be sampled from the next lower stage. In turn, if the chemical treatment is associated with accelerated development based on NF stage, the recommendation is that the additional larvae be sampled from the next higher stage. If there is no overlap of the stage distribution in the chemical treatment(s) with the median control developmental stage, as may occur with severe alterations of tadpole development due to treatment with a test chemical, the histopathology selection process may be modified by matching to a stage different from the control median stage. Furthermore, if NF stages are indeterminate (*i.e.*, asynchronous development), then five tadpoles from each replicate are randomly chosen for histological analysis. The DER should identify the histopathology selection method used and any justification provided in the study report for selection of any larvae not stage-matched to the median control developmental stage.

## I. Determination of Biological Endpoints

Daily observation of test animals is recommended to record any mortality or other clinical signs, including behavioral effects. During the 21 days of exposure, determinations of developmental stage and measurements of hind limb length (HLL), snout-vent length (SVL), and body weight are performed on days 7 and 21. Thyroid gland histology is evaluated at test termination (Day 21). A summary of the recommended time points for observation is provided in **Table 1**.

**Table 1**. **Observation Time Points in the Amphibian Metamorphosis Assay.**

| Observation | Daily | Day 7 | Day 21 |
|---|---|---|---|
| Mortality | ● | | |
| Clinical Signs | ● | | |
| Developmental Stage | | ● | ● |
| Hind Limb Length (HLL) | | ● | ● |
| Snout-Vent Length (SVL) | | ● | ● |
| Body Weight | | ● | ● |
| Thyroid Gland Histology | | | ● |

### 1. Mortality and Clinical Signs

The DER should indicate whether mortality, abnormal behavior, and any other clinical signs were recorded on a daily basis.

### 2. Developmental Stage

Consistent with the staging method used for specimen selection at test initiation, the developmental stage at Day 7 and Day 21 (test termination) is determined, preferably using the staging criteria of Nieuwkoop and Faber (1994). Developmental stage data are used to determine if development is accelerated, asynchronous, delayed, or unaffected at either or both of these time points. Acceleration or delay of development is determined by making a comparison between the median stage achieved by the control and treated groups. Any asynchronous development should be reported in the DER when the NF stage cannot be determined because the tissues examined are not malformed or abnormal, but the relative timing of the morphogenesis or development of different tissues is disrupted within a single tadpole. For example, hind limb morphology may indicate a particular NF stage, whereas forelimb emergence and morphology of the same specimen may indicate a later NF stage. Therefore, analysis of developmental stage is potentially based upon two types of observations at Day 7 and Day 21: the median developmental stage of each group (treatments and controls), and the number of tadpoles (if any) in each group that cannot be staged due to asynchronous development.

### 3. Hind Limb Length (HLL)

Differentiation and growth of the hind limbs are under control of the thyroid hormones and are major developmental landmarks in the determination of developmental stage (through NF stage 57). In addition, hind limb length (HLL), normalized to snout-vent length (SVL), is a quantitative endpoint used to detect potential effects on the thyroid axis in the AMA (**Appendix 2**). For consistency, it is recommended that HLL be measured on the left hind limb. HLL is evaluated both at Day 7 and at Day 21 of the test.

Changes in the normalized HLL at Day 7, even if not evident at Day 21, are still considered indicative of potential thyroid activity in the AMA. For consistency and archival purposes, the Agency recommends that length measurements be acquired from digital photographs using image analysis software, as described in the OECD Guidance Document on Amphibian Thyroid Histopathology (2007b, 2007c). The DER should briefly describe the method of measurement.

### 4. Snout-Vent Length (SVL) and Body Weight

Determinations of SVL and body weight are included in the test guideline to assess possible effects of test substances on the growth rate of tadpoles in comparison to the control group and are useful in detecting generalized toxicity due to the test compound. Because the removal of adherent water for weight determinations can cause stressful conditions for tadpoles and may cause skin damage, weight and length measurements are performed on the day 7 sub-sampled tadpoles only and on all remaining tadpoles at day 21 (test termination). For consistency, it is recommended that the cranial aspect of the vent be used as the caudal limit of the measurement SVL. However, measurement based on termination of the abdomen has been identified as an acceptable alternative to SVL [see Corrections and Clarifications on Technical Aspects of the Test Guidelines for the Endocrine Disruptor Screening Program Tier 1 Assays (OCSPP Test Guideline Series 890); March 3, 2011]. The DER should describe which method was used.

### 5. Thyroid Gland Histopathology

Thyroid gland histopathology is recommended (1) when other endpoints in the AMA (developmental stage, HLL) show no significant effect of the test chemical, (2) when results are equivocal or conflicting, or (3) when there is evidence of developmental delay. Evidence of developmental delay cannot, by itself, be considered a diagnostic indicator of anti-thyroidal activity, and some changes related to effects on the HPT axis may be observable only by histopathological analysis. Thyroid gland histopathology may not be needed to confirm an HPT-related effect in the AMA when there is clear evidence of advanced development or asynchronous development based on other apical endpoints (developmental stage, HLL), because these effects in the AMA are only known to occur through thyroid-hormone related processes (see **Section D**).

Diagnostic criteria include thyroid gland hypertrophy/atrophy, follicular cell hypertrophy, follicular cell hyperplasia. Additional qualitative criteria include follicular lumen area, colloid quality, and follicular cell height and shape. Severity grading (4 grades; *e.g.*, 0=Not remarkable, 1=Mild, 2=Moderate, 3=Severe) should be reported in the DER. Please refer to "Amphibian Metamorphosis Assay: Part 1 – Technical guidance for morphologic sampling and histological preparation" (OECD, 2007b) and "Amphibian Metamorphosis Assay: Part 2 – Approach to reading studies, diagnostic criteria, severity grading and atlas" (OECD, 2007c) for information on obtaining and processing samples for histological analysis and for performing histology analyses on tissue samples. The

DER should identify which diagnostic criteria and additional characteristics, if applicable, were evaluated by the pathologist and the associated severity grading scale(s).

#### 6. Specimens Archival

It is recommended that any archival practices, *e.g.*, for histopathology slides, digital images, and tissue samples, be documented by the reviewer in the DER.

#### 7. Data Reporting and Completeness

The DER should document whether data were collected using electronic or manual systems which conform to good laboratory practices (GLP). The reviewer should confirm whether the study report contains the information identified for reporting in the test guideline, Section (g), including but not limited to the endpoints described in the foregoing sections of this SEP.

## IV. Study Interpretation

This section of the DER is intended to address the interpretation of the study results and any conclusions regarding the acceptability of the study. As part of this evaluation, the DER may include a discussion of how well the study conforms to specific validity and performance criteria identified in the test guideline; the analysis of validity and performance criteria would draw, in part, upon the evaluation of study conduct described in the previous section. This is intended to clarify the reviewer's conclusions regarding whether, to what extent, and how any deviation(s) affect the interpretation or acceptability of the study. Section (IV)(D) provides specific recommendations on interpreting the biological data obtained from the AMA and directions on how to report this information in the DER.

The following sections are based on a summary of the information generally expected to be obtained by a study that was conducted following the Test Guidelines, and that would generally be relevant to interpreting the results of the Amphibian Metamorphosis Assay. This summary is provided as a general outline to aid the reviewer in preparing a DER, and not as a substitute for the Test Guidelines, nor as guidance on how to conduct the assay.

### A. Test Validity Criteria

The DER should note whether the assay met the validity criteria identified in the OCSPP 890.1100 test guideline, based on the data and other information provided in the study report. The validity criteria are summarized below in **Table 2**. The validity criteria are based upon the guideline-recommended experimental design and the associated statistical power of the AMA.

When met, the validity criteria are used to support the scientific soundness of conclusions drawn from the test results. These validity criteria provide an indication of the general performance of the AMA under test conditions; *e.g.*, control mortality that exceeds the reference value may be an indication of underlying problems associated with husbandry, environmental conditions, or the particular strain of test organisms used in the study. Therefore, the extent to which the validity criteria are met would be relevant to any conclusions drawn from the study.

The DER should include a discussion of any rationale provided in the study report for any validity criteria that were not met. This includes identification of whether, to what extent, and in what way failure to meet the criteria has had an impact on the quality or acceptability of the study.

**Table 2: Validity Criteria for the Amphibian Metamorphosis Assay.**

| Criteria | Reference Value(s) |
|---|---|
| Mortality (each treatment, including controls) | ≤ 10% |
| Mortality (each replicate)<br>If positive for thyroid interaction<br>If negative for thyroid interaction | <br>≤ 2 tadpoles/replicate (≤10%)<br>≤ 3 tadpoles/replicate (≤15%) |
| Uncompromised treatment levels (all 4 replicates available for analysis) | ≥ 2, in addition to controls |
| Treatment levels without overt toxicity (mortality) | ≥ 2, in addition to controls |

## B. **Performance Criteria**

Performance criteria applicable to the guideline are summarized in **Table 3** below. Additional performance criteria are provided for tests under static-renewal conditions (**Table 4**). Finally, tests that utilize a solvent are recommended to include a separate solvent control, run concurrently with the negative (clean water) control and test item treatments, and to report all control results. The DER should include enough information to determine whether the assay met the relevant criteria.

Where deviations from the guideline are reported the performance criteria may be used to demonstrate that such deviations had a minor impact on study outcome. A study that fails to meet one or more performance criteria may still provide useful information and is not necessarily rejected. The DER includes a discussion of any rationale provided in the study report for any performance criteria that were not met. That discussion should also identify whether, to what extent, and in what way failure to meet the specified performance criteria had an impact on the quality or interpretation of the study. For additional guidance on the interpretation of performance criteria related to analytical chemistry and general water quality parameters, the reviewer may consult OCSPP Guideline 850.1000, special considerations for conducting aquatic laboratory studies.

**Table 3: General Performance Criteria for the Amphibian Metamorphosis Assay, when Conducted Under Flow-Through or Static-Renewal Conditions**

| Criteria | Reference Value(s) |
|---|---|
| Measured test concentrations | CV ≤ 20% over 21 days |
| Control mortality | ≤ 2 tadpoles/each replicate (≤10%) |
| Median NF stage in controls at test termination | ≥ 57 |
| Difference between $10^{th}$ and $90^{th}$ percentile of NF stage distribution in controls at test termination | ≤ 4 stages |
| Dissolved oxygen | ≥ 40% air saturation |
| pH | 6.5-8.5 (≤ 0.5 difference between replicates, treatments) |
| Water temperature | 22±1°C (≤0.5°C difference between replicates, treatments) |

**Table 4: Additional Performance Criteria for the Amphibian Metamorphosis Assay, when Conducted Under Static-Renewal Conditions**

| Criteria | Guideline Recommendation(s) |
|---|---|
| Measured test concentrations | Analysis immediately prior to and immediately following renewal |
| General water quality parameters, including ammonia | Analysis immediately prior to renewal |
| Renewal period | ≤ 72 hours (≤ 24 hours preferred) |
| Feeding schedule | Adjusted as necessary (*e.g.*, 50%) based on renewal scheme |

## C. General Analysis

### 1. Statistical Analyses

Where appropriate, formal statistical analysis is used to identify significant differences. Recommended statistical procedures for analyzing median developmental stage, normalized HLL, and continuous growth endpoints (*e.g.*, SVL and body weight) are summarized in **Appendix 4**. As recommended by the guideline, all statistically significant differences would be reported by endpoint and by concentration. The direction (*e.g.*, accelerated or delayed) and magnitude (*e.g.*, percent change as compared to control) are stated, as relevant. The level of significance (*e.g.*, $\alpha = 0.05$) would also typically be reported for each test when there is a statistical significance. Specific guidance for the statistical analysis of histopathology findings is not provided, but summary statistics for severity and incidence of observations should be reported in the DER.

#### a. Statistical Tests Employed

The DER should identify the extent to which the statistical methods used were consistent with those recommended in the test guideline. The DER should include the statistical tests (and software, as appropriate) used, along with any rationale provided for

the use of either parametric or nonparametric tests. Any data transformations would also be reported.

The extent and specific nature of statistical verification by the reviewer may vary. Typically, the reviewer confirms the accuracy of the statistical analyses by recalculating summary statistics and pertinent statistical tests for endpoints. This verification is facilitated if individual data for all results, including negative and solvent controls (if applicable), are submitted with the study report in electronic format (*e.g.*, spreadsheet files).

### b. Test Using Solvents

If a solvent was used, confirmation of comparisons between solvent controls and clean water controls is performed to determine potential solvent effects on the organisms. All control results that have been provided are included in the DER. Evidence of a solvent effect will be considered in determining the utility of a study. The reviewer should consult existing guidance on this topic (*e.g.*, OCSPP 850.1000, EPA 2008). In addition, one or both of the following analyses is performed:

- Clean water control versus treatment concentrations: Statistical comparison of responses in the test concentrations in relation to the dilution water control is recommended for all tests, and all control results are included in the DER. Unless stated in other policy or guidance documents this method is the preferred method for identifying treatment-related effects in the AMA and also for the reviewer to generally employ in generating the DER.

- Solvent control versus treatment concentrations: Statistical comparison of responses in the test concentrations in relation to the solvent control is another method of evaluation. All control results should be included in the data report.

### c. Outliers

The DER should also identify the method used to identify any outliers, if applicable. If outliers were excluded from the statistical analysis in the DER, it is recommended that the biological and/or statistical justification be described.

## 2. Trends

Reporting of all trends in the DER whether positive or negative is recommended. A discussion of the significance of the trends and implications for the interpretation of the test is also suggested.

### 3. Histological Findings

Reporting of histological findings by diagnostic criterion, severity grades, and opinion of the pathologist and reviewer is recommended. The severity grades recommended in the guideline are 0=Not remarkable, 1=Mild, 2=Moderate, 3=Severe. As indicated above, summary statistics are to be presented and verified by the reviewer.

## D. Endpoint Interpretation

Interpretation of the biological endpoints in the AMA is described in the following sections. The order in which possible effects are discussed is consistent with the proposed Decision Logic for endpoint interpretation, presented in **Appendix 3** (OCSPP 890.1100, OECD 2009).

### 1. Advanced Development (Developmental Stage and Normalized HLL)

Advanced development in the AMA is only known to occur through effects which are thyroid hormone related. These may be peripheral tissue effects such as direct interaction with the thyroid hormone receptor (such as with T4) or effects which alter circulating thyroid hormone levels. In either case, this may be considered sufficient evidence to indicate that the chemical has thyroid activity. The guideline recommends evaluating advanced development in one of two ways. First, the guidelines recommend evaluating the general developmental stage of tadpoles using the standardized approach detailed in Nieuwkoop and Faber (1994). Second, specific morphological features are quantified, such as HLL, at both days 7 and 21, which is positively associated with agonistic effects on the thyroid hormone receptor. If statistically significant advances in development or HLL occur, then the test indicates that the chemical may be thyroid active. As recommended in the test guideline, the evaluation of test animals for the presence of accelerated development relative to the control population will be based on results of statistical analyses performed for the following four endpoints:

- HLL (normalized by SVL) on study day 7,
- HLL (normalized by SVL) on study day 21,
- developmental stage on study day 7, or
- developmental stage on study day 21.

The recommended normalization of HLL is performed by calculating the ratio between individual HLL and snout-to-vent length (SVL) measurements. The means of the normalized values for each treatment level are then compared. Acceleration of development, for example, can be indicated by a significant increase of mean normalized HLL in a chemical treatment group, compared to the control group, on study day 7 and/or study day 21 (**Appendix 3**).

The recommended statistical analyses of developmental stage are performed based on determination of developmental stages according to the morphological criteria described by Nieuwkoop and Faber (1994). Acceleration of development is indicated when the multi-quantal analysis detects a significant increase of developmental stage values in a chemical treatment group compared to the control group on study day 7 and/or study day 21.

As recommended in the AMA test guideline, a significant effect on any of the four endpoints mentioned above may be regarded sufficient for a positive detection of accelerated development (**Appendix 3**). That is, significant effects on normalized HLL at a specific time point would not typically require corroboration by significant effects on normalized HLL at the alternative time point or by significant effects on developmental stage. In turn, significant effects on developmental stage at a specific time point generally would not require corroboration by significant effects at developmental stage on the alternative time point nor by significant effects on normalized HLL.

### 2. Asynchronous Development (Unable to Stage)

Asynchronous development is generally characterized by disruption of the relative timing of the morphogenesis or development of different tissues within a single tadpole. The inability to clearly establish the NF developmental stage of an organism using the suite of morphological endpoints considered typical of any given stage indicates that the tissues are developing asynchronously through metamorphosis. Asynchronous development in the AMA is an indicator of thyroid activity. The only known modes of action causing asynchronous development in the AMA are through effects of chemicals on peripheral thyroid hormone action and/or thyroid hormone metabolism in developing tissues, such as is observed with deiodinase inhibitors.

The recommended evaluation of test animals for the presence of asynchronous development relative to the control population is based on gross morphological assessment of test animals on study day 7 and study day 21. The description of normal development of *Xenopus laevis* by Nieuwkoop and Faber (1994) provides the framework for identifying a sequential order of normal tissue remodeling. The term “asynchronous development” refers specifically to those deviations in tadpole gross morphological development that disallow the definitive determination of a developmental stage according to the criteria of Nieuwkoop and Faber (1994) because key morphological landmarks show characteristics of different stages. As implied by the term “asynchronous development”, only cases showing deviations in the progress of remodeling of specific tissues relative to the progress of remodeling of other tissues should be considered. Some classical phenotypes include delay or absence of fore limb emergence despite normal or advanced development of hind limbs and tail tissues, or the precocious resorption of gills relative to the stage of hind limb morphogenesis and tail resorption. The test guideline recommends that an animal be evaluated as showing asynchronous development if it cannot be assigned to a stage because it fails to meet a majority of the landmark developmental criteria for a given NF stage, or if there is

extreme delay or acceleration of one or more key features (*i.e.* tail completely resorbed, but forelimbs not emerged).

The recommended assessment is performed qualitatively and should examine the full suite of landmark features listed by Nieuwkoop and Faber (1994). Under this analysis, animals recorded as showing asynchronous development are not assigned to a Nieuwkoop and Faber (1994) development stage. Thus, it is recommended that a central criterion for designating cases of abnormal morphological development as "asynchronous development" be that the relative timing of tissue remodeling and tissue morphogenesis is disrupted, whereas the morphology of affected tissues is not overtly abnormal. One example to illustrate this interpretation of gross morphological abnormalities is that retarded hind limb morphogenesis relative to development of other tissues could fulfill the criterion of "asynchronous development," whereas cases showing missing hind limbs, abnormal digits (*e.g.*, ectrodactyly, polydactyly), or other overt limb malformations should not typically be considered as "asynchronous development." Depending on the mode of chemical action, different gross morphological phenotypes can occur. Some classical phenotypes include delay or absence of fore limb emergence in spite of normal or advanced development of hind limbs and tail tissues, precocious gill resorption relative to hind limb, and tail remodeling. The DER should identify whether asynchronous development was observed in tadpoles and the associated treatment concentration(s) (*e.g.*, negative control, solvent control, or specific test item concentration), along with the number of tadpoles (if any) affected in each treatment.

### 3. Histopathology

If the chemical does not cause overt toxicity (.*e.g.*, significant mortality at a given treatment level) and does not accelerate development or cause asynchronous development, an evaluation of the histopathology of the thyroid glands is recommended, with consideration of the appropriate guidance document (OECD, 2007b; 2007c). Developmental delay, in the absence of toxicity, can be a strong indicator of anti-thyroid activity in the AMA, but the developmental stage analysis is less sensitive and less diagnostic than the histopathological analysis of the thyroid gland. In addition, effects on thyroid gland histology have been demonstrated in the absence of developmental effects. The thyroid gland is under the influence of thyroid-stimulating hormone (TSH); any chemical which alters circulating thyroid hormone sufficiently to alter TSH secretion will result in histopathological changes in the thyroid gland. Therefore, conducting histopathological analyses of the thyroid gland would typically be appropriate when other endpoints indicate developmental delay or no discernible effect.

Changes in thyroid histopathology provide evidence that the chemical is thyroid active in the AMA. If thyroid histopathology is performed and no developmental delays or histological lesions are observed in the thyroid gland, then the chemical may be considered to be thyroid inactive in the AMA, provided that there are no observations of advanced or asynchronous development (based on developmental stage and normalized HLL analyses). The determination of an effect associated with exposure to a chemical

may be heavily weighted by the expert opinion of a qualified pathologist, as presented in the histopathology description of the study report. Summary statistics should be presented in the DER.

#### 4. Developmental Delay (Developmental Stage and Normalized HLL)

Statistically significant developmental delays, in the absence of other signs of overt toxicity, indicate that the chemical is thyroid active (antagonistic) in the AMA. In the absence of strong statistical responses, it may be appropriate to augment the outcome with results from thyroid histopathology.

Delayed development can also occur through anti-thyroidal mechanisms and through indirect toxicity. Mild developmental delays coupled with overt signs of toxicity (*e.g.*, mortality, reduction in body weight) may indicate a non-specific toxic effect. Excessive mortality is an obvious indication that other toxic mechanisms are occurring. Other clinical signs to be considered in determining overt toxicity include (but are not necessarily limited to) edema, hemorrhagic lesions, lethargy, reduced food consumption, erratic/altered swimming behavior, *etc*. The guideline recommends that $\geq$ 2 treatment levels, in addition to controls, be uncompromised by overt toxicity and be available for analysis.

#### 5. Growth (SVL and Body Weight)

Apparent increases in growth (SVL and/or body weight), in conjunction with observed effects on developmental endpoints (developmental stage, normalized HLL, and/or histopathology) may support the conclusion of a thyroid active substance, as apparent increases in growth are commonly observed with compounds that negatively affect normal development. However, growth endpoints alone should not be the basis of determining whether or not a substance is thyroid-active.

Reductions in growth (SVL and/or body weight), possibly in conjunction with mildly delayed development (*i.e.*, not statistically significant or corroborated by histopathology findings), may suggest non-thyroidal toxicity.

### E. Special Data Analysis Considerations

#### 1. Use of Compromised Treatment Levels

Several factors are typically considered when determining whether a replicate or entire treatment demonstrates overt toxicity and should be removed from the analysis. Overt toxicity is generally defined in the AMA test guideline as greater than two mortalities in any replicate, which can only be explained by toxicity and not technical error. Other signs of overt toxicity include hemorrhage, abnormal behaviors, abnormal swimming patterns, anorexia, and any other clinical signs of disease. For sub-lethal signs

of toxicity, qualitative evaluations may be necessary and would be made in reference to the clean water control group.

### 2. Solvent Controls

The use of a solvent is typically only considered as a last resort, after all other chemical delivery options have been determined to be inappropriate, because it has the potential to interfere with the test results. If a solvent is used, then a clean water control is typically run in concert. At the termination of the test, an evaluation of the potential effects of the solvent is performed. This is typically done through a statistical comparison of the solvent control group and the clean water control group. If significant differences are detected between the clean water control and solvent control groups, the guideline recommends that best professional judgment be used to determine if the validity of the test has been compromised. If there are no significant differences between the clean water control and solvent control for any of the measured response variables, it is recommended that the study endpoints be determined based on comparison to the clean water control unless other guidance or policy recommends otherwise.

### 3. Treatment Groups Achieving NF Developmental Stage 60 and Above

After NF stage 60, tadpoles show a reduction in size and weight due to tissue resorption and reduction of absolute water content. Thus, measurements of body weight and SVL cannot appropriately be used in statistical analyses for differences in growth rates. Therefore, body weight and length data from organisms > NF stage 60 are censored and not used in analyses of replicate means or replicate medians. Further discussion is provided in **Appendix 4**.

### 4. Histological Analyses and Developmental Stage

Since the test is based on a fixed exposure termination point, organisms in the various treatment groups progress to different developmental stages. This increases difficulty of evaluating histological preparation of the thyroid gland. Analysis of thyroid gland histology may be done either in the context of what is normal for the relevant NF stage or based upon comparison to histology of the negative control specimens (OECD 2007c). These approaches are only substantively different if observations of the negative controls are uncharacteristic of what is normal for their NF developmental stage. The method of comparison should be clearly described in the DER.

## V. Data Evaluation Record

It is recommended that the submitted study be reviewed according to the principles in the previous sections of this SEP. The review is then documented in the Data Evaluation Record (DER). A template that provides additional guidance to the reviewer for preparation of the DER is available. Generally, the DER will include a

cover sheet, executive summary and results synopsis, quality assurance elements, description of the material and methods used, summary of the study author's reported results and analysis, and the reviewer's interpretation of results and discussion. Consistent with the recommendations in the test guideline and this SEP, the DER should typically identify deviations from the guideline-recommended methods and validity and performance criteria, along with a discussion of their significance. The DER should generally identify the effective concentrations associated with each endpoint in the AMA. Finally, the DER will include a conclusion as to whether the test item was potentially active in the AMA thyroid axis in the submitted study (see **Appendix 3**), and whether the study satisfied the Test Order for an Amphibian Metamorphosis Assay using OCSPP Guideline 850.1100.

## VI. References

Additional background information is available from the sources in the Expanded Bibliography (**Appendix 5**).

ASTM International (2004). E 1439: Standard Guide for Conducting the Frog Embryo Teratogenesis Assay – *Xenopus*, West Conshohocken, PA.

Fort, D.J., Degitz, S., Tietge, J., and Touart, L.W. (2007). The hypothalamic-pituitary-thyroid (HPT) axis in frogs and its role in frog development and reproduction. *Critic Rev Toxicol* 37:117-161.

Nieuwkoop, P.D., and Faber, J. (1994). Normal Table of *Xenopus laevis*. Garland Publishing, New York and London.

OECD (2004). Detailed Review Paper (DRP) on Amphibian Metamorphosis Assay for the Detection of Thyroid Active Substances, No. 46. Organisation for Economic Co-operation and Development. Washington Center (www.oecdwash.org).

OECD (2006). Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application. *Environ Health Safety Public* No. 54. Series on Testing and Assessment. Paris.

OECD (2007a). Validation of the Amphibian Metamorphosis Assay as a Screen for Thyroid-Active Chemicals: Integrated Summary Report. Organisation for Economic Co-operation and Development. Washington Center (www.oecdwash.org).

OECD (2007b). Amphibian Metamorphosis Assay Histopathology Part 1 – Technical guidance for morphologic sampling and histological preparation. Organisation for Economic Co-operation and Development. Washington Center (www.oecdwash.org).

OECD (2007c). Amphibian Metamorphosis Assay Histopathology Part 2 – Approach to reading studies, diagnostic criteria, severity grading and atlas. Organisation for Economic Co-operation and Development: Washington Center (www.oecdwash.org).

OECD (2009). Guideline for the Testing of Chemicals: The Amphibian Metamorphosis Assay. Test Guideline 231, Adopted 7 September 2009. Organisation for Economic Co-operation and Development: Washington Center (www.oecdwash.org).

U.S. Environmental Protection Agency (EPA). (2011). Corrections and Clarifications on Technical Aspects of the Test Guidelines for the Endocrine Disruptor Screening Program Tier 1 Assays (OCSPP Test Guideline Series 890). March 3, 2011. Office of Chemical Safety and Pollution Prevention (OCSPP), Washington, D.C. (http://www.epa.gov/endo/pubs/assayvalidation/clarificationdoc.pdf).

U.S. Environmental Protection Agency (EPA). (2008). Guidance for the use of dilution-water (negative) and solvent controls in statistical data analysis for guideline aquatic toxicology studies. September 25, 2008. Memo from Statistics Workgroup and Aquatic Biology Technical Team to Donald Brady, Director, Environmental Fate and Effects Division. Office of Pesticide Programs, Office of Chemical Safety and Pollution Prevention (OCSPP), Washington, D.C.

## Appendix 1: Staging

The most prominent morphological staging landmark for NF Stage 51 tadpoles of *X. laevis* is hind limb morphology, which is illustrated in the figure below (OECD 2009).

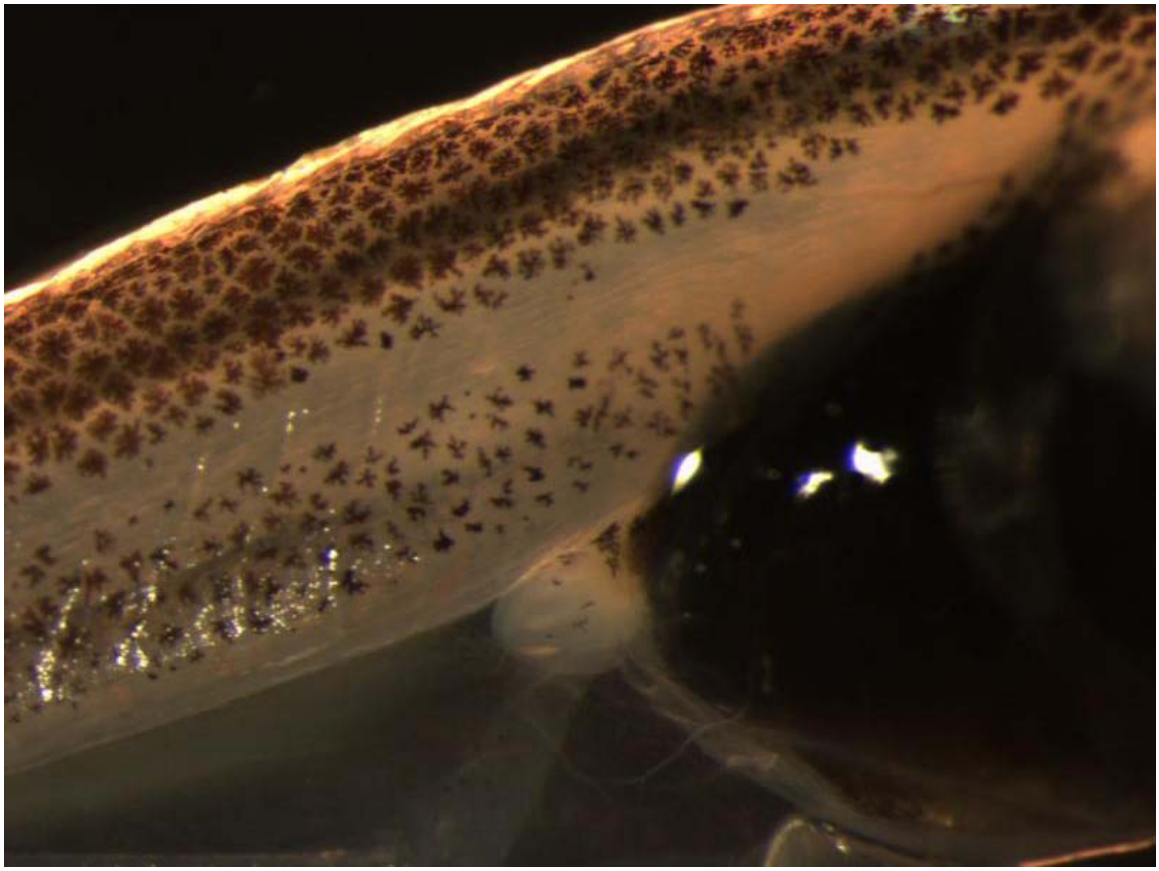


**Figure 1. Hind limb morphology of a stage 51 *X. laevis* tadpole.**

## Appendix 2: Length Measurement

Length in *X. laevis* is measured in accordance with the diagrams provided below (OECD, 2008).

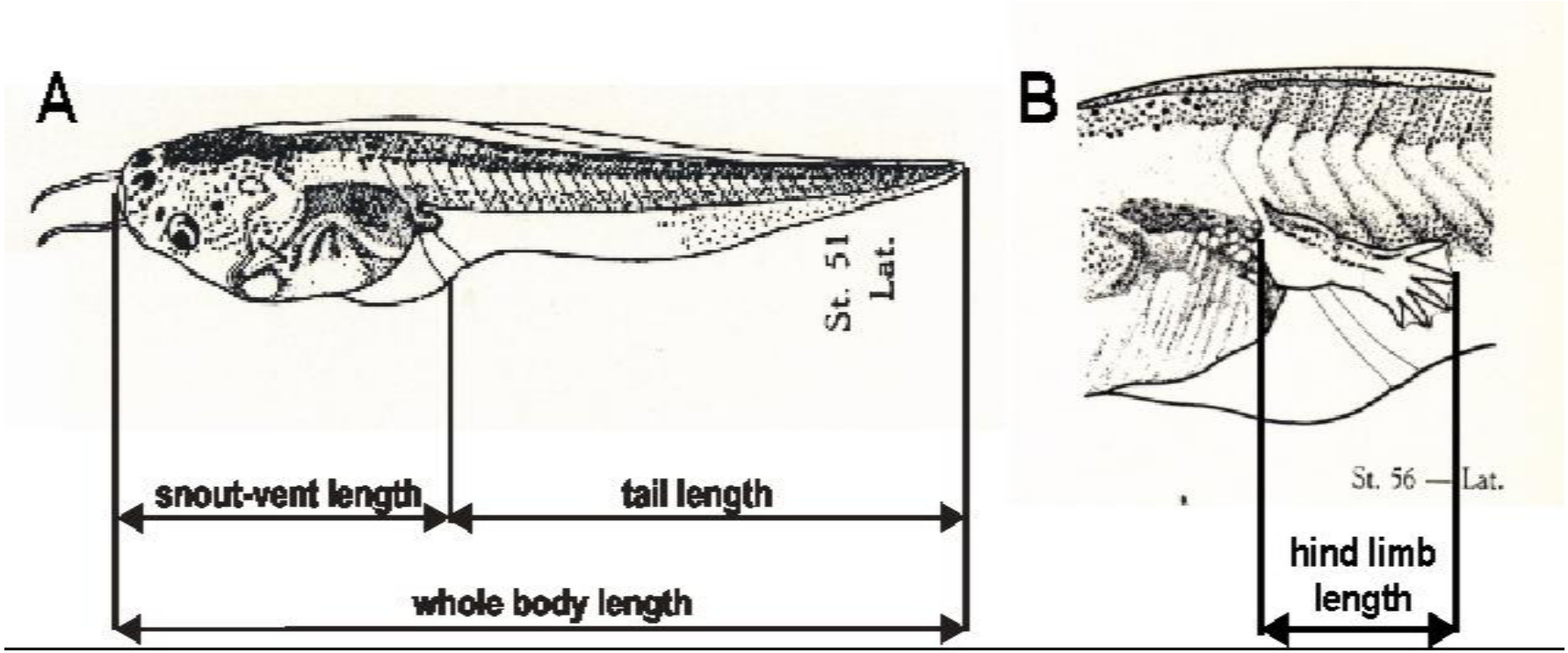



**Figure 2. (A) Types of body length measurements and (B) Hind limb length measurements for *X. laevis* tadpoles**

## Appendix 3: Decision Logic for the Conduct and Interpretation of the AMA

A recommended decision logic is presented in the AMA guideline (OCSPP 890.1100, OECD 2009) that provides logical assistance in the conduct of the assay and interpretation of the results. The flow chart below illustrates outcomes in the AMA, such as advanced development, asynchronous development, and effects on thyroid histopathology, which tend to carry weight in determinations of whether a substance is thyroid-active in the AMA. The flow chart does not include other outcomes considered in the decision logic, such as delayed development (based on developmental stage and/or HLL), effects on growth (snout-vent length and wet body weight), and effects on other parameters that can potentially be affected by general toxicity; these outcomes may be weighed less heavily in the determination of whether a substance is thyroid-active in the AMA because they are less specific to potential interference with the HPT axis. Nonetheless, as indicated in the SEP (**Section D**), statistically significant developmental delays (in the absence of other signs of overt toxicity) can indicate that the chemical is thyroid-active (antagonistic) in the AMA. In the absence of strong responses, the interpretation may be augmented with results from thyroid histopathology.

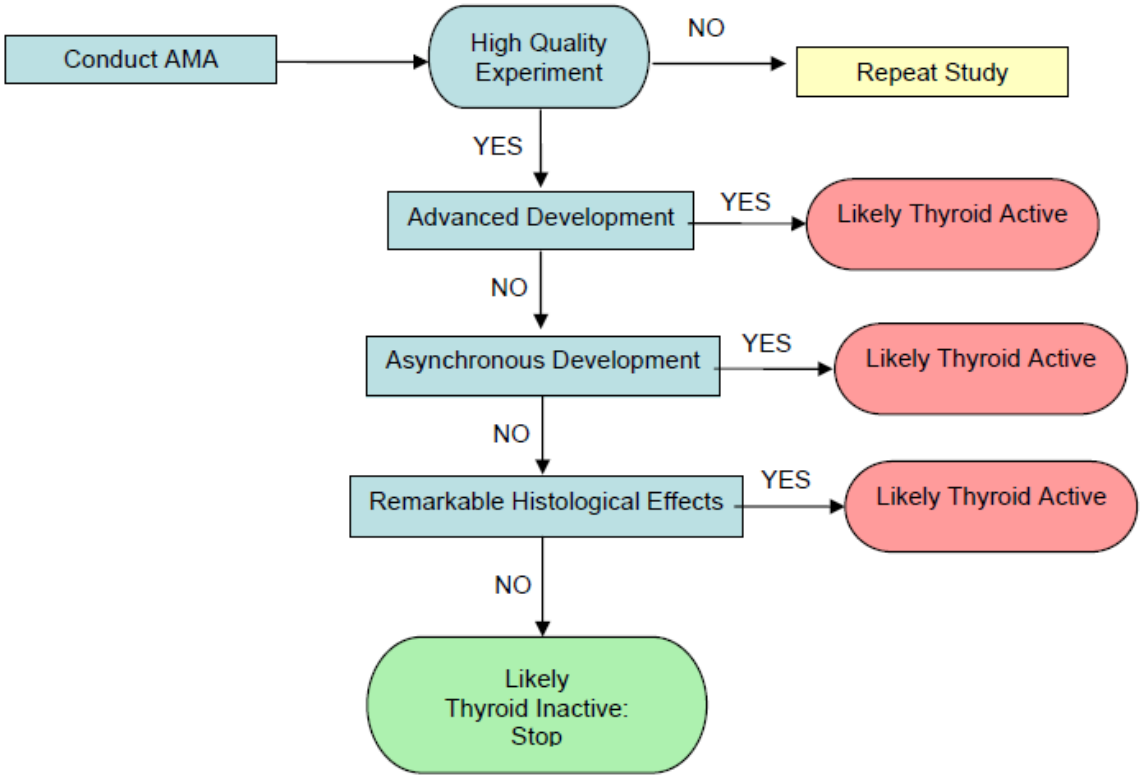
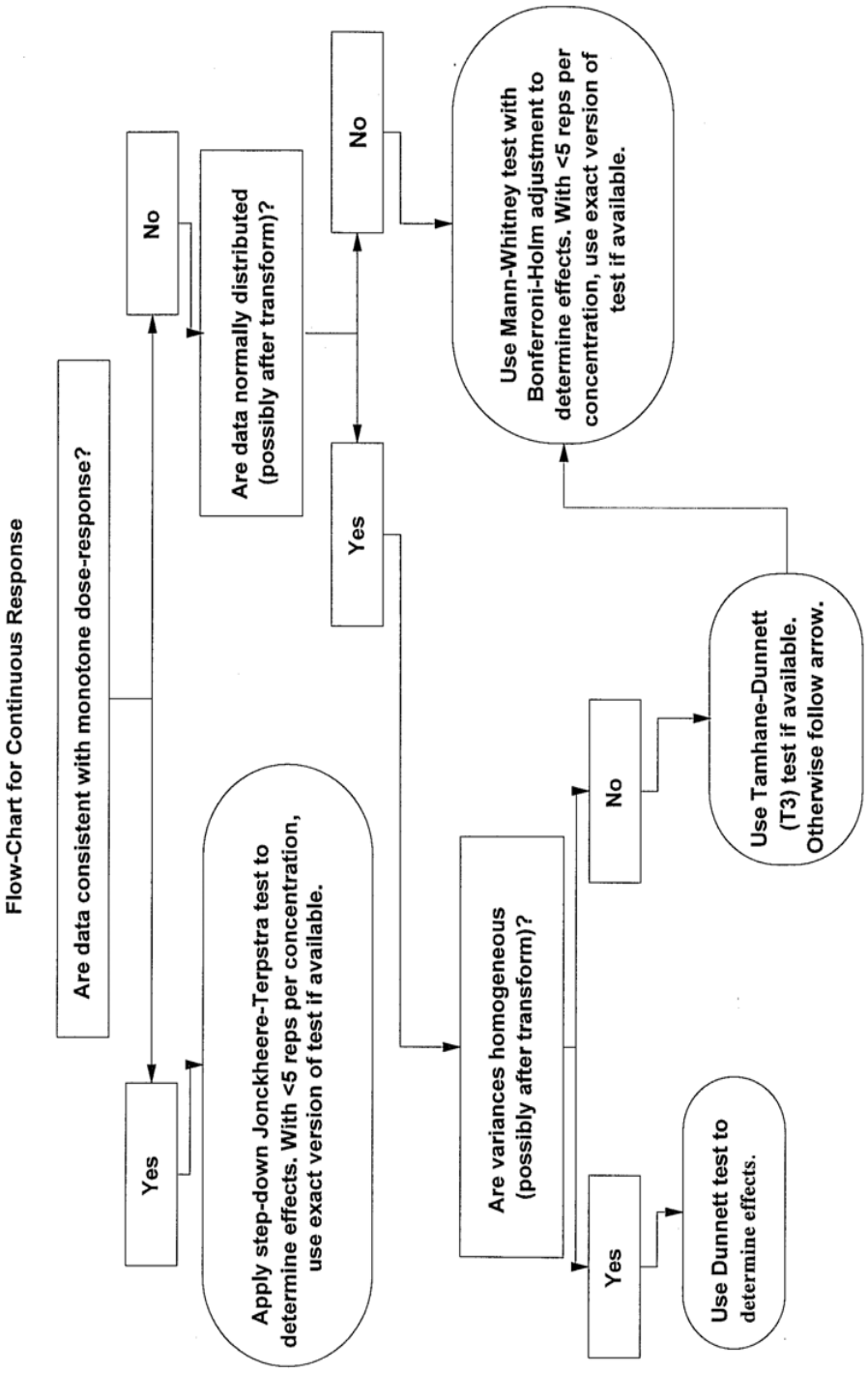



**Figure 3. Suggested decision logic for certain outcomes in the AMA.**

## Appendix 4: Recommendations for Statistical Analyses Based on OECD 2006

The statistical analyses recommended in the AMA test guideline (OCSPP 890.1100, OECD 2009) are described in more detail in the document, Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application (OECD 2006). For all continuous quantitative endpoints (HLL, SVL, body weight) consistent with a monotonic dose-response, it is recommended that the Jonckheere-Terpstra test be applied in step-down manner to establish a significant treatment effect. For continuous endpoints that are not consistent with a monotone dose-response, it is recommended that the data be assessed for normality (preferably using the Shapiro-Wilk or Anderson-Darling test) and variance homogeneity (preferably using the Levene test). Both tests are performed on the residuals from an ANOVA. Expert judgment can be used in lieu of these formal tests for normality and variance homogeneity, though formal tests are preferred. Where non-normality or variance heterogeneity is found, it is recommended that a normalizing, variance stabilizing transformation be sought. If the data (perhaps after a transformation) are normally distributed with homogeneous variance, EPA recommends determining a significant treatment effect using Dunnett's test. If the data (perhaps after a transformation) are normally distributed with heterogeneous variance, it is recommended that a significant treatment effect be determined using the Tamhane-Dunnett or T3 test or from the Mann-Whitney-Wilcoxon U test. Where no normalizing transformation can be found, it is recommended that a significant treatment effect be determined from the Mann-Whitney-Wilcoxon U test using a Bonferroni-Holm adjustment to the p-values. The Dunnett's test is applied independently of any ANOVA F-test and the Mann-Whitney test is applied independently of any overall Kruskal-Wallis test.

Significant mortality is not expected, but if it occurs, EPA recommends assessing mortality from the step-down Cochran-Armitage test where the data are consistent with dose-response monotonicity, and otherwise from Fisher's Exact test with a Bonferroni-Holm adjustment. The Guideline recommends that a significant treatment effect for developmental stage be determined from the step-down application of the Jonckheere-Terpstra test applied to the replicate medians. Alternatively, and preferably, the multi-quantal Jonckheere test from the 20th to the 80th percentile could be used for effect determination, as it takes into account changes to the distribution profile. The appropriate unit of analysis is the replicate so the data consist of replicate medians if the Jonckheere-Terpstra or Mann-Whitney U test is used, or the replicate means if Dunnett's test is used. Dose-response monotonicity can be assessed visually from the replicate and treatment means or medians or from formal tests. With fewer than five replicates per treatment or control, the exact permutation versions of the Jonckheere-Terpstra and Mann-Whitney tests is recommended, if available. It is typically recommended that the statistical significance of the tests described be judged at the 0.05 significance level.


Flow-Chart for Continuous Response
Are data consistent with monotone dose-response?
Yes
No
Apply step-down Jonckheere-Terpstra test to determine effects. With <5 reps per concentration, use exact version of test if available.
Are data normally distributed (possibly after transform)?
Yes
No
Use Mann-Whitney test with Bonferroni-Holm adjustment to determine effects. With <5 reps per concentration, use exact version of test if available.
Are variances homogeneous (possibly after transform)?
Yes
No
Use Dunnett test to determine effects.
Use Tamhane-Dunnett (T3) test if available. Otherwise follow arrow.

The test guideline recommends that body weight and length data from organisms > NF stage 60 be censored and generally not be used in analyses of replicate means or replicate medians because late stage effects on growth may skew the distribution. Two different approaches could be used to analyze these growth-related parameters. One approach is to consider only tadpoles with developmental stages lower or equal to stage 60 for the statistical analyses of body weight and/or SVL. This approach is believed to provide sufficiently robust information about the severity of possible growth effects as long as only a small proportion of test animals are removed from the analyses ($\leq$ ( 20%). If an increased number of tadpoles show development beyond stage 60 ($\geq$ 20%) in one or more nominal concentration(s), then it is recommended that a two-factor ANOVA with a nested variance structure be undertaken on all tadpoles to assess growth effects due to chemical treatments while taking into account the effect of late stage development on growth. Guidance on the two-factor ANOVA analysis of weight and length is provided in the following paragraph.

An alternative analysis of weight data may be appropriate if an increased number of tadpoles show development beyond stage 60 ($\geq$ ( 20%) in one or more nominal concentration(s). A two-factor ANOVA with a nested variance structure is recommended on all tadpoles to assess growth effects due to chemical treatments while taking into account the effect of late stage development on growth. The approach is to use all data, but take into account the effect of late stage development. This can be done with a two-factor ANOVA with a nested variance structure. Then a two-factor ANOVA with concentration and stage, and their interaction can be used. Replicate concentrations and tadpoles per replicate serve as random factors. This process still considers the replicate as the unit of analysis and gives essentially the same results as a weighted analysis, weighted by the number of animals per mean. If the data violate the normality or variance homogeneity requirements of ANOVA, a normalized rank-order transform can be done to correct the violation. In addition to the standard ANOVA F-tests for the effects of concentration, advanced stage, and their interactions, the interaction F-test can be divided into two additional ANOVA F-tests. One F-test applies to the mean responses across concentrations for advanced stage, and another on the mean responses across concentrations for advanced stage. Further comparisons of treatment means against control are done within each level of advanced stage. A trend analysis using appropriate contrasts or simple pair-wise comparisons may be performed if there is evidence of non-monotonic dose-response within a level of the advanced stage variable. A Bonferroni-Holm adjustment to the p-values is made only if the corresponding F-slice is not significant.

## Appendix 5: Expanded Bibliography

The following sources, in addition to the references cited in **Section VI** above, provide useful background information related to the background, conduct, and interpretation of the Amphibian Metamorphosis Assay.

Abe, T., Kakyo, M., Sakagami, H., Tokui, T., Nishio, T., Tanemoto, M., Nomura, H., Hebert, S.C., Matsumo, S., Kondo, H., and Yawo, H. (1998). Molecular Characterization and Tissue Distribution of a New Organic Anion Transporter Subtype (oatp3) that Transports Thyroid Hormones and Taurocholate and Comparison with oatp2. *J Biol Chem* 273 (35):22395-22401.

Achen, M.G., Harms, P.J., Thomas, T., Richardson, S.J., Wettenhall, R.E., and Schreiber, G. (1992). Protein synthesis at the blood-brain barrier: The major protein secreted by amphibian choroids plexus is a lipocalin. *J Biol Chem* 267 (32): 23170-23174.

Adkins, E. (1975). Hormonal basis of sexual differentiation in Japanese quail. *J Comp Physiol Psychol* 89: 61-71.

Allen, B. M. (1916). The results of extirpation of the anterior lobe of the hypophysis and of the thyroid of *Rana pipiens* larvae. *Science* 44: 755-758.

Allen, B. M. (1929). The influence of the thyroid gland and hypophysis upon growth and development of amphibian larvae. *Quarterly Rev Biol* 4: 325-352.

Allran, J. W., and Karasov, W.H. (2000). Effects of atrazine and nitrate on Northern leopard frog (*Rana pipiens*) larvae exposed in the laboratory from posthatch through metamorphosis. *Envir Toxicol and Chem* 19: 3850-2542.

Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W., and Yuan, J. (1996). Human ICE/CED-3 protease nomenclature. *Cell* 87: 171.

Ankley, G., Mihaich, E., Stahl, Tillitt, D., and Colborn, T. (1998). Overview of a workshop on screening methods for detecting potential (anti-) estrogenic/androgenic chemicals in wildlife. *Envir Toxicol and Chem* 7: 68-87.

Ashley, H., and Frieden, E. (1972). Metabolism and distribution of triiodothyronine and thyroxine in the bullfrog tadpole. *Gen Comp Endocrinol* 18: 22-31.

Atkinson, B.G., and Just, J.J. (1975). Biochemical and histological changes in the respiratory system of *Rana catesbeiana* larvae during normal and induced metamorphosis. *Develop. Biol.* 45: 151-165.

Atkinson, B.G. (1981). Biological basis of tissue regression and synthesis. In: Metamorphosis: A problem in Developmental Biology, Second Edition, L.I.Gilbert, and E.Frieden, eds., pp. 397-444. Plenum Press, NY.

Atkinson, B.G. (1994). Metamorphosis: Model systems for studying gene expression in postembryonic development. *Developmental Genetics* 15: 313-319.

Baiser, W.V., Hertoghe, J., and Eeckhaut, W. (2000). Thyroid Insufficiency: Is TSH measurement the only diagnostic tool? *J Nutr and Envir Med* 10: 105-113.

Baker, B.S., and Tata, J.R. (1992). Prolactin prevents the autoinduction of thyroid hormone receptor mRNAs during amphibian metamorphosis. *Developmental Biol* 149: 463-467.

Balinsky, J.B., Cragg, M.M., and Baldwin, E. (1961). The adaptation of amphibian waste nitrogen excretion to dehydration. *Comprehensive Biochem and Physiol* 3: 236-244.

Balls, M., Clothier, R.H., Rowles, J.M., Kiteley, N.A., and Bennett, G.W. (1985a). TRH distribution levels, and significance during the development of *Xenopus laevi*s. In: Metamorphosis: The Eighth Symposium of the British Society for Developmental Biology, B.Balls, and M.Bownes (eds.), pp. 260-272. Clarendon Press, Oxford, England.

Balls, M., and Bownes, M. (1985b). Metamorphosis, M. Balls and M. Bownes, eds. Clarendon Press, Oxford, UK.

Banker, D., Bigler, J., and Eisenman, R. (1991). The thyroid hormone receptor gene (c-erb Aα) is expressed in advance of thyroid hormone naturation during the early embryonic development of *Xenopus laevis. Mol Cell Biol* 11: 5079-5089.

Barsano, C.P., and De Groot, L. (1983). Nuclear cytoplasmic interrelationships in *Molecular basis of thyroid hormone action*, pp, 139-177. Oppenheimer, J., and Samuels, H. (eds.). Academic Press: New York.

Battelle (2005). Frog Metamorphosis Assay Demonstration Study, OECD – Phase I. USEPA Contract No. 68-W-01-023.

Becker, K.B., Schneider, M.J., Davey, J.C., and Galton, V.A. (1995). The type III 5-deiodinase in *Rana catesbeiana* tadpoles is encoded by a thyroid hormone-responsive gene. *Endocrinol* 136: 4424-4431.

Bennett, T.P., and Frieden, E. (1962). Metamorphosis and biochemical adaptation in amphibians. In: Comparative Biochemistry, Florkin, M., and Mason, H.S., (eds.), 4: 483-556. Academic Press, New York.

Benvenga, S., and Robbins, J. (1993). Lipoprotein-thyroid hormone interactions. *Trends in Endocrinol and Metab* 4: 194-198.

Blondeau, J-P., Osty, J., and Francon, J. (1988). Characterization of the thyroid hormone transport system of isolated hepatocytes. *J Biol and Chem* 263: 2685-2692.

Bray, T., and Sicard, R.E. (1982). Correlation among the changes in the levels of thyroid hormones, thyrotropin and thyrotropin-releasing hormone during the development of *Xenopus laevis*. *Experim Cell Biol* 50: 101-107.

Bridges, C.M., (2000). Long-term effects of pesticide exposure at various life stages of the southern leopard frog (*Rana sphenocephala*). *Arch Env Contam and Toxicol* 39: 91-96.

Britson, C.A., and Threlkeld, S.T. (1998). Abundance, metamorphosis, developmental, and behavioral abnormalities in *Hyla chrysoscelis* tadpoles following exposure to three agrichemicals and methyl mercury in outdoor mesocosms. *Bull Env Contam and Toxicol* 61: 154-161.

Brooks, A.R., Sweeney, G., and Old, R.W. (1989). Structure and functional expression of a cloned Xenopus thyroid hormone receptor. *Nucleic Acid Res* 17: 9395-9405.

Brown, D.D., Wang, Z., Kanamori, A., Eliceiri, B., Furlow, J.D., and Schwartzman, R. (1995). Amphibian metamorphosis: A complex program of gene expression changes controlled by the thyroid hormone. *Recent Prog Hormone Res* 50: 309-315.

Brown, D.D., Wang, Z., Furlow, J.D., Kanamori, A., Schwartzman, R.A., Remo, B.F., and Pinder, A. (1996). The thyroid hormone-induced tail resorption program during *Xenopus laevis* metamorphosis. *Proc Natl Acad Sci USA* 93: 1924-1929.

Brown, G.W. Jr., and Cohen, P.P. (1958). Biosynthesis of urea in metamorphosing tadpoles. In: The Chemical Basis of Development, W. McElroy and B. Glass (eds.), pp. 495-513. Johns Hopkins Press, Baltimore.

Brown, G.W. Jr., Brown, W.R., and Cohen, P.P. (1959). Comparative biochemistry of urea synthesis. *J Biol Chem* 234: 1775-1780.

Broyles, R.H. (1981). Changes in the blood during amphibian metamorphosis. In: Metamorphosis: A Problem in Developmental Biology, Second Edition, L.I.Gilbert, and E. Frieden, eds., pp. 461-490. Plenum Press, New York.

Brucker-Davis, F. (1998). Effects of environmental synthetic chemicals on thyroid function. *Thyroid* 8, 827-856.

Buchholz, D.R., Hsia, S-CV., Liezhen, F., and Shi, Y-B. (2003). A dominant-negative thyroid hormone receptor blocks amphibian metamorphosis by retaining corepressors at target genes. *Mol and Cell Biol* 23: 6750-6758.

Buckbinder, L., and Brown, D.D. (1992). Thyroid hormone-induced gene expression changes in the developing frog limb. *J Biol Chem* 267: 25786-25791.

Buckbinder, L., and Brown, D.D. (1993). Expression of the *Xenopus laevis* prolactin and thyrotropin genes during metamorphosis. *Proc Natl Acad Sci USA* 90: 3820-3824.

Carr, J.A., and Norris, D.O. (1990). Immunohistochemical localization of corticotropin releasing factor- and arginine vasotocin-like immunoreactivities in the brain and pituitary of the American bullfrog (*Rana catesbeiana*) during development and metamorphosis. *Gen Comp Endocrinol* 87: 180-188.

Cartensen, H., Burgers, A.C.J., and Li, C.H. (1961). Demonstration of aldosterone and corticosterone as the principal steroids formed in incubates of adrenals of the American bullfrog (*Rana catesbeiana*) and stimulation of their production by mammalian adrenocorticotropin. *Gen Comp Endocrinol* 1: 37-50.

Ceusters, R., Darras, W.M., and Kuhn, E.R. (1978). Difference in thyroid function between male and female frogs (*Rana temporia L.*) with increasing temperature. *Gen Comp Endocrinol* 36 (4): 598-603.

Chang, L., Munro, S.L., Richardson, S.J., and Schreiber, G. (1999). Evolution of thyroid hormone binding by transthyretins in birds and mammals. *Eur J Biochem* 259: 534-542.

Cheek, A.O., Ide, C.F., Bollinger, J.E., Rider, C.V., and McLachlan, J.A. (1999). Alteration of leopard frog (*Rana pipiens*) metamorphosis by the herbicide acetochlor. *Arch of Environ Contam and Toxicol* 37: 70-77.

Chen, J.D., and Evans, R.M. (1995). A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature* 377: 454-457.

Chen, Y., Hu, H., and Atkinson, G. (1994). Characterization and expression of C/EBP-like genes in the liver of *Rana catesbeiana* tadpoles during spontaneous and thyroid hormone-induced metamorphosis. *Devel Gen* 15: 366-377.

Cheng, S-Y. (1991). Thyroid Hormone Metabolism — Regulation and Clinical Implications, S.Y. Wu, ed., pp. 145-166. Blackwell Scientific Publications, Boston.

Clemons, G.K., and Nicoll, C.S. (1977). Effects of antisera to bullfrog prolactin and growth hormone on metamorphosis of *Rana catesbeiana* tadpoles. *Gen Comp Endocrinol* 31: 495-497.

Cohen, M.A., and Kelley, D.B. (1996). Androgen-induced proliferation in the developing larynx of *Xenopus laevis* is regulated by thyroid hormone. *Devel Biol* 178: 113-123.

Cohen, P.P. (1970). Biochemical differentiation during amphibian metamorphosis. *Science* 168: 533-543.

Cohen, R.N., Brzostek, S., Kim, B., Chorev, M., Wondisford, F.E., and Hollenberg, A.N. (2001). The specificity of interactions between nuclear hormone receptors and coreceptors is mediated by distinct amino acid sequences within the interacting domains. *Mol Endocrin* 15: 1049-1061.

Colborn, T., and Clement, C., eds. (1992). Chemically-induced alterations in sexual and functional development: The wildlife/human connection. Princeton Scientific Publishing Co, Inc., Princeton, NJ.

Cole, K.D., and Little, G.H. (1983). Bile pigments and bilirubin UDP-glucuronyl transferase during metamorphosis of *Rana catesbeiana* tadpoles. *Comp Biochem and Physio* B76: 503-506.

Collingwood, T.N., Urnov, F.D., Krishna, V., Chatterjee, K., and Wolfee, A.P. (2001). Chromatin remodeling by the thyroid hormone receptor in regulation of the thyroid-stimulating hormone α-subunit promoter. *J Biol Chem* 276: 34227-34234.

Cryns, V., and Yuan, J. (1998). Proteases to die for. *Genes Development* 12: 1551-1470.

Davey, J.C., Becker, K.B., Schneider, M.J., St. Germain, D.L., and Galton, W.A. (1995). Cloning of a cDNA for the type II iodothyronine deiodinase. *J Biol Chem* 270: 26786-26789.

Davis, P.J., and Davis, F.B. (1996). Nongenomic actions of thyroid hormone. *Thyroid* 6: 497-504.

de Brabandere, V.I., Hou, P., Stockl, D., Thienpont, L.M., and De Leenheer, A.P. (1998). Isotope dilution-liquid chromatography/electrospray ionization-tandem mass spectrophotometry for the determination of serum thyroxine as a potential reference method. *Rapid Communications in Mass Spectrophotometry* 16: 1099-1103.

de Luze, A., Sachs, L., and Denebeux, B. (1993). Thyroid hormone-dependent transcriptional regulation of exogenous genes transferred into *Xenopus* muscle in vivo. *Proc Natl Acad Sci* 90:7322-7326.

Debouck, C., and Goodfellow, P.N. (1999). DNA microarrays in drug discovery and development. *Nat Genet* 21: 48-50.

Degitz, S.J., Holcombe, G.W., Flynn, K.M., Kosian, P.A., Korte, J.J., and Tietge, J.E. (2005). Progress towards development of an amphibian-based thyroid screening assay using *Xenopus laevis*. Organismal and thyroidal responses to the model compounds 6-propylthiouracil, methimazole, and thyroxine. *Toxicol Sci* 87 (2): 353-64.

Dent, J.N. (1968), Survey of amphibian metamorphosis. In: Metamorphosis: A Problem in Developmental Biology, W. Etkin, and I.L. Gilbert, eds., pp. 271-311. Appleton-Crofts, New York.

Denver, R.J. (1988). Several hypothalamic peptides stimulate in vitro thyrotropin secretion by pituitaries of anuran amphibians. *Gen Comp Endocrinol* 72: 383-393.

Denver, R.J., and Licht, P. (1989). Neuropeptide stimulation of thyrotropin secretion in the larval bullfrog: Evidence for a common neuroregulator of thyroid and interregnal activity during metamorphosis. *J Exper Zool* 252: 101-104.

Denver, R.J. (1993). Acceleration of anuran amphibian metamorphosis by corticotropin releasing hormone-like peptides. *Gen Comp Endocrinol* 91: 38-51.

Denver, R.J. (1996). Neuroendocrine control of amphibian metamorphosis. In: Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells, I.L. Gilbert, J.R. Tata, and B.G.Atkinson, eds., pp. 433-464. Academic Press, New York.

Denver, R.J. (1997a). Environmental stress as a developmental cue: Corticotropin-releasing hormone is a proximate mediator of adaptive phenotypic plasticity in amphibian metamorphosis. *Hormones and Behavior* 31: 169-179.

Denver, R.J. (1997b). Proximate mechanisms of phenotypic plasticity in amphibian metamorphosis. *Am Zool* 37: 172-184.

Denver, R.J. (1998). Hormonal correlates of environmentally induced metamorphosis in the western spadefoot toad *Scaphiopus hammondii*. *Gen Comp Endocrinol* 119: 326-336.

Denver, R.J., Glennemeier, K.A., and Boorse, G.C. (2002). Endocrinology of Complex Lifecycles: Amphibians. In: Hormones, Brain and Behavior, A.A. Pfaff, A. Etgen, S. Fahrbach, R. Moss, and R. Rubin, eds., pp. 469-513. Academic Press, San Diego.

Divino, C.M., and Schussler, G.C. (1990). Receptor-mediated uptake and internalization of transthyretin. *J Biol Chem* 265 (3): 1425-9.

Dodd, M.H.I., and Dodd, J.M. (1976). The biology of metamorphosis. In: Physiology of Amphibia, B. Lofts, ed., pp. 467-599. Academic Press, New York.

Duggan, D. J., Bittner, M., Chen, Y., Meltzer, P., and Trent, J.M. (1999). Expression profiling using cDNA microarrays. *Nature Genetics* 21:10-14.

Dumont, J.N. (1972). Oogenesis in *Xenopus laevis*. I. Stages of oocyte development in laboratory maintained animals. *J Morphol* 136: 153-180.

Eddy, L., and Lipner, H. (1976). Amphibian metamorphsis: The role of thyrotropin-like hormone. *Gen Comp Endocrinol* 29: 333-336.

EDSTAC (Endocrine Disruptor Screening and Testing Advisory Committee) (1998). Final Report from the Endocrine Disruptor Screening and Testing Advisory Committee, August, 1998. *http://www.epa.gov/oppintr/opptendo/finalrpt.htm*.

Ekins, R.P. (1999). Immunoassay, DNA analysis, and other ligand binding assay techniques: From electropherograms to multiplexed, ultrasensitive microarrays on a chip. *J Chem Ed* 76: 769-780.

Eliceiri, B., and Brown, D.D. (1994). Quantitation of endogenous thryroid hormone receptors α and β during embryogenesis and metamorphosis in *Xenopus laevis*. *J Biol Chem* 269: 24459-24465.

Ellis, H.M., and Horovitz, H.R. (1986). Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* 44: 817-829.

Etkin, W., and Lehrer, R. (1960). Excess growth in tadpoles after transplantation of adenohypophysis. *Endocrinol* 67: 457-466.

Etkin, W. (1964). Metamorphosis. In: Physiology of the amphibian, J. Moore, ed., pp. 427-468. Academic Press, New York.

Etkin, W. (1968). Hormonal control of amphibian metamorphosis. In: Metamorphosis: A Problem in Developmental Biology, pp. 313-348. Appleton, New York.

Evans, R.M. (1988). The steroid and thyroid hormone receptor superfamily. *Science* 240: 889-895.

Favre-Young, H., Dif, F., Roussille, F., Demeneix, B.A., and Kelly, P.A. (2000). Cross-talk between signal transducer and activator of transcription (Stat5) and thyroid hormone receptor -β 1 (TR β 1) signaling pathways. *Mol Endocrinol* 14:1411-1424.

Fort, D.J., and Stover, E.L. (1996). Effect of low-level copper and pentachlorophenol exposure on various early life stages of Xenopus laevis. In: Environmental Toxicology and Risk Assessment: Biomarkers and Risk Assessment, D.A. Bengtson and D.S. Henshel, eds. Philadelphia, PA, American Society for Testing and Materials, v. 5 ASTM STP 1306:188-203.

Fort, D.J., and Stover, E.L. (1997). Development of short-term, whole embryo assays to evaluate detrimental effects on amphibian limb development and metamorphosis using *X. laevis*. In: Environmental Toxicology and Risk Assessment: Modeling and Risk Assessment, F.J. Dwyer, T.R. Doane, and M.L. Hinman, eds. Philadelphia, PA: American Society for Testing and Materials, v. 6 ASTM STP 1317:376-390.

Fort, D.J., Rogers, R.L., Morgan, L.A., Miller, M.F., Clark, P.A., White, J.A., Paul, R.R., and Stover, E.L. (2000). Preliminary validation of a short-term morphological assay to evaluate adverse effects on amphibian metamorphosis and thyroid function using Xenopus laevis. *J Appl Toxicol* 20:419-425

Fort, D.J., Stover, E.L., Bantle, J.A., Dumont, J.N., and Finch, R.A. (2001a). Evaluation of a reproductive toxicity assay using Xenopus laevis: Boric acid, cadmium, and ethylene glycol monomethyl ether. *J Appl Toxicol* 21:41-52.

Fort, D.J., Rogers, R.L., Paul, R.R., Miller, M.F., Clark, P., Stover, E.L., Yoshioko, J., Rolland, R., Quimby, F., Sower, S.A., Reed, K.L., and Babbitt, K.J. (2001b). Effects of pond water, sediment, and sediment extract samples from New Hampshire USA, on early Xenopus development and metamorphosis: Comparison to native species. *J Appl Toxicol* 21:199-209.

Fort, D.J., Rogers, R.L., Thomas, J.H., Buzzard, B.O., Noll, A.M., and Spaulding, C.D. (2004). The comparative sensitivity of *Xenopus tropicalis* and *Xenopus laevis* as test species for the FETAX model. *J Appl Toxicol* 24(6):443-457.

Fox, H. (1983). Amphibian morphogenesis. Clifton, NJ: Humana Press.

Frieden, E., and Naile, B. (1955), Biochemistry of amphibian metamorphosis: I Enhancement of induced metamorphosis by glucocorticoids. *Science* 121:37-39.

Frieden, E. (1961). Biochemical adaptation and anuran metamorphosis. *Am Zool* 1:115-149.

Frieden, E. (1968). Biochemistry of amphibian metamorphosis. In: Metamorphosis: A Problem in Developmental Biology, W. Etkin and L.I. Gilbert, eds., pp. 349-398. New York: Appleton.

Frieden, E., and Just, J.J. (1970), Hormonal responses in amphibian metamorphosis. In: Action of Hormones on Molecular Processes, G. Litwack, ed., 1:1-53. New York: Academic Press.

Friesema, E.C.H., Docter, R., Moerings, E.P.C.M., Stieger, B., Hagenbuch, B., Meier, P.J., Krenning, E.P., Hennemann, G., Visser, T.J. (1999). Identification of thyroid hormone transporters. *Biochem Biophys Res Commun* 254:497-501.

Friesema, E.C.H., Docter, R. Krenning, E.P., Everts, M.E., Hennemann, G., and Visser, T.J. (1998). Rapid sulfation of 3,3´,5´-triiodothyronine in native Xenopus laevis oocytes. *Endocrinol* 139:596-600.

Furlow, J.D., and Brown, D.D. (1999), In vitro and in vivo analysis of the regulation of a trascription factor gene by thyroid hormone during Xenopus laevis metamorphosis. *Mol Endocrinol* 13(12):2076-2089.

Galton, V.A. (1980). Binding of thyroid hormones in serum and liver cytosol of Rana catesbeiana tadpoles. *Endocrinol* 107:61-69.

Galton, V.A., and Munck, K. (1981), Metabolism of thyroxine in Rana catesbeiana tadpoles during metamorphic climax: *Endocrinol* 109(4):1127-1131.

Galton, V.A. (1983). Thyroid hormone action in amphibian metamorphosis. In: Molecular Basis of Thyroid Hormone Action, J. H. Oppenheimer and H. H. Samuels, eds., pp. 445-483. New York: Academic Press.

Galton, V.A., St. German, D.L., and Whittemore, S. (1986). Cellular uptake of 3,5,3'-triiodothyronine and thyroxine by red blood and thymus cells: *Endocrinol* 118:1918-1923.

Galton, V.A. (1989). The role of 3,5,3´-triiodothyronine in the physiological action of thryroxine in the premetamorphic tadpole. *Endocrinol* 124:2427-2433.

Galton, V.A. (1990). Mechanisms underlying the acceleration of thyroid hormone-induced tadpole metamorphosis by corticosterone. *Endocrinol (Baltimore)* 127: 2997-3002.

Galton, V. A., Morganelli, C. M., Schneider, M. J., and Yee, K. (1991). The role of thyroid hormone in the regulation of hepatic carbamyl phosphate synthetase activity in Rana catesbeiana. Endocrinology 129:2298-2304.

Gancedo, B., Corpas, I., Alonso-Gomez, A.L., Delgado, M.J., Morreale de Escobar, G., and Alonso-Bedate, M. (1992). Corticotropin-releasing factor stimulates metamorphosis and increases thyroid hormone concentration in prometamorphic Rana catesbeiana. *Endocrinol* 87:6-13.

Gao, X., Kalkhoven, E., Peterson-Maduro, J., van der Birg, B., and Destree, O.H.J. (1994). Expression of the glucocorticoid receptor gene is regulated during early embryogenesis of Xenopus Laevis. *Biochim Biophys Acta* 1218(2):194-198.

Gavrieli, Y., Sherman, Y., and Ben-Sasson, S. A. (1992). *J. Cell Biol.* 119:493−501.

Giguere, V., Hollenberg, S.M., Rosenfeld, M.G., and Evans, R.M. (1986). Functional domains of human glucocorticoid receptor. *Cell* 46:645-652.

Gilbert, L.I., and Frieden, E. (1981). Metamorphosis: A Problem in Developmental Biology, Second Edition. New York: Plenum Press.

Godowski, P. J., Picard, D., and Yamamoto, K.R. (1988). Signal transduction and transcriptional regulation by glucocorticoid receptor-LexA fusion proteins. *Science* 241:812-816.

Goleman, W.L., Urquidi, L.J., Anderson, T.A., Smith, E.E., Kendall, R.J., and Carr, J.A. (2002). Environmentally relevant concentrations of ammonium perchlorate inhibit development and metamorphosis in Xenopus laevis. *Environ Toxicol Chem* 21:424-430.

Gona, A.G., Uray, N.J., and Hauser, K.F. (1988). Neurogenesis of the frog cerebellum. In: Developmental Neurobiology of the Frog, E. D. Pollack and H. D. Bibb, eds., pp. 255-276. New York: Alan R. Liss.

Gonzalez, G.C., and Lederis, K. (1988). Sauvagine-like and corticotropin-releasing factor-like imunoreactivity in the brain of the bullfrog (Rana catesbeiana). *Cell Tissue Research* 253:29-37.

Goos, H.J., Zwanenbeek, H.C., and van Oordt, P.G. (1968). Hypothalamic neurosecretion and metamorphosis in Xenopus laevis. II. The effect of thryroxine following treatment with propylthiouracil. *Arch d Anatomie, d Histol et d Embryol* 51:267-274.

Gosner, K.L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetol* 16:183-190.

Gracia-Navarro, F., Lamacz, M., Tonon, M., and Vaudry, H. (1992). Pituitary adenylate cyclase-activating polypeptide stimulates calcium mobilization in amphibian pituitary cells. *Endocrinol* 131:1069-1074.

Gray, K.M., and Janssens, P.A. (1990). Gonadal hormones inhibit the induction of metamorphosis by thyroid hormones in Xenopus laevis tadpoles in vivo, but not in vitro. *Gen Comp Endocrinol* 77:202-211.

Green, S., and Chambon, P. (1988). Nuclear receptors enhance our understanding of transcription regulation. *Trends in Genetics* 4:309-313.

Greenspan, F.S. (1997). In: Basic and Clinical Endocrinology, F. S. Greenspan and G. J. Strewler, eds., pp. 192-262. Stamford, CT: Appelton and Lange.

Gudernatsch, J.F. (1912). Feeding experiments on tadpoles. I. Influence of specific organs given as food on growth and differentiation: a contribution to the knowledge of organs with internal secretion. *Arch Entwicklungsmech Org* 35:457-483.

Guiochon-Mantel, A., Loosfelt, H., Lescop, P. Sar, Atger, S.M., Perrot-Applanat, M., and Milgrom, E. (1989). Mechanism of nuclear localization of the progesterone receptor: Evidence for interaction between monomers. *Cell* 57:1147-1154.

Gutleb, Ac., Appelman, J. Bronkhorst, M., van den Berg, J. H. J., and Miurk, A. J. (2000). Effects of oral exposure to polychlorinated byphenyls (PCBs) on the development and metamorphosis of two amphibian species (Xenopus laevis and Rana temporaria). *Science of the Total Envir* 262:147-157.

Han, Y., Watling, D., Rogers, N.C., and Stark, G.R. (1997). JAK2 and STAT5, but not JAK1 and STAT1, are required for prolactin-induced beta-lactoglobulin transcription. *Mol Endocrinol* 11:1180-1188.

Hanaoka, Y. (1967). Effect of phenylthiourea upon posterior hypothalactomized tadpoles of Rana pipiens. *Gen Comp Endocrinol* 9:24-30.

Hayes, T.B., Chan, R., and Licht, P. (1993). Interaction of temperature and steroids on larval growth, development and metamorphosis in a toad (Bufo boreas). *J Exper Zool* 266:206-215.

Hayes, T.B. (1995a). An histological examination of the effects of corticosterone in larvae of the western toad, Bufo boreas (Anura: Bufonidae), and the Oriental fire bellied toad, Bombina orientalis (Anura: Discoglossidae). *J Morphol* 226:297-307.

Hayes, T.B. (1995b). Interdependence of corticosterone and thyroid hormones in larval growth and development in the western toad (Bufo boreas). I. Thyroid hormone dependent and independent effects of corticosterone on growth and development. *J Exper Zool* 271:95-102.

Hayes, T.B., and Wu, T.H. (1995c). Interdependence of corticosterone and thyroid hormones in larval growth and development in the western toad (Bufo boreas). II. Regulation of corticosterone and thyroid hormones. *J Exper Zool* 271:103-111.

Hayes, T.B., and Wu, T.H. (1995d). The role of corticosterone in anuran metamorphosis and its potential role in stress-induced metamorphosis. Proceedings of the 17th Conference of the European Society of Comparative Endocrinology. *Netherlands J Zool* 45:107-109.

Hayes, T.B. (1997a). Steroid as potential modulators of thyroid hormone activity in anuran metamorphosis. *Am Zool* 37:185-194.

Hayes, T.B. (1997b). Hormonal mechanisms as potential constraints on evolution: Examples from the Anura. *Am Zool* 37:482-490.

Hayes, T.B. (1998). Endocrine disruptors in amphibians: potential impacts and the usefulness of amphibian screens for detecting endocrine disrupting compounds. *Sci J* 68:557-568.

Hayes, T.B. (2000). Endocrine Disruption in Amphibians. In: Ecotoxicology of Amphibians and Reptiles, D. W. Sparling, G. Linder, and C. A. Bishop, eds., pp. 573-593. Pensacola, FL, SETAC Press.

Heery, D.M., Kalkoven, E. Hoare, S., and Parker, M.G. (1997). A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature* 387:733-736.

Hollenberg, S.M., and Evans, R.M. (1988). Multiple and cooperative transactivation domains of human glucocorticoid receptor. *Cell* 55:899-906.

Horlein, A.J., Naar, A.M., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C.K., and Rosenfeld, M.G. (1995). Ligand-independent

repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature* 377:305-404.

Hoskins, S.G. (1990). Metamorphosis of the amphibian eye. *J Neurobiol* 21:970-989.

Hourdry, J., and Dauca, M. (1977). Cytological and cytochemical changes in the intestinal epithelium during anuran metamorphosis. *Internat Rev Cytol* 5:337-385.

Huang, H., and Brown, D.D. (2000). Prolactin is not a juvenile hormone in Xenopus laevis metamorphosis. *Proc Natl Acad Sci USA* 97:195-199.

Huang, H., Cai, L.Q., Remo, B.F., and Brown, D.D. (2001). Timing of metamorphosis and the onset of the negative feedback loop between the thyroid gland and the pituitary is controlled by type II iodothyronine deiodinase in *Xenopus laevis*. *Proc Natl Acad Sci USA* 98:7348-7353.

Huang, M., Marsh-Armstrong, N., and Brown, D.D. (1999). Metamorphosis is inhibited in transgenic *Xenopus laevis* tadpoles that overexpress type III deiodinase. *Proc Natl Acad Sci USA* 96:962-967.

Hughes, A.F.W. (1957). The development of the primary sensory system in Xenopus laevis (Daudin). *J Anat* 91:323-338.

Inaba, T., and Frieden, E. (1967). Changes in ceruloplasmin during anuran metamorphosis. *J Biol Chem* 242:4789-4796.

Ishizuya-Oka, A., and Shimozawa, A. (1987). Development of connective tissue in the digestive tract of the larval and metamorphosing *Xenopus laevis*. *Ant Anz* 164:81-93.

Jackson, I.M.D., and Reichlin, S. (1977). Thyrotropin-releasing hormone: Abundance in the skin of the frog, *Rana pipiens*. *Science* 198:414-415.

Jacobs, G.F., Goyvaertws, M.P., Vandorpe, G., Quaghebeur, A.M., and Kuhn, E.R. (1988). Luteinizing hormone-releasing hormone as a potent stimulator of the thyroidal axis in ranid frogs. *Gen Comp Endocrinol* 70:274-283.

Jacobs, G.F.M., and Kuhn, E.R. (1992). Thyroid hormone feedback regulation of the secretion of bioactive thyrotropin in the frog. *Gen Comp Endocrinol* 88:415-423.

Jaffe, R.C. (1981). Plasma concentration of corticocosterone during *Rana catesbeiana* tadpole metamorphosis. *Gen Comp Endocrinol* 44:314-318.

Jolivet-Jaudet, G., and Leloup-Hatey, J. (1984). Variations in aldosterone and corticosterone plasma levels during metamorphosis in *Xenopus laevis* tadpoles. *Gen Comp Endocrinol* 56:59-65.

Jorgensen, E.C. (1978). Thyroid hormones and analogs. II. Structure-activity relationships. In: Hormonal Proteins and Peptides, C.H. Li, ed., pp. 107-199. Academic Press, New York.

Jung, R.E., and Walker, M.K. (1997). Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on development of anuran amphibians. *Envir Toxicol Chem* 16:230-240.

Just, J.J., and Atkinson, B.G. (1972). Hemoglobin transition in the bullfrog *Rana catesbeiana* during spontaneous and induced metamorphosis. *J Exper Zool* 182:271-280.

Just, J.J., Kraust-Just, J., and Check, D.A. (1981). Survey of chordate metamorphosis. In: Metamorphosis: A Problem in Developmental Biology, L.I. Gilbert, and E. Frieden, eds., pp. 265-326. Plenum Press, New York.

Kaltenbach, J.C. (1985). Amphibian metamorphosis: Influence of thyroid and steroid hormones. In: Current Trends in Comparative Endocrinology, B. Lofts, and W.N. Holmes, eds., pp. 533-534. Hong Kong University Press, Hong Kong.

Kaltenbach, J.C. (1996). Endocrinology of amphibian metamorphosis In: Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells, L.I. Gilbert, J.R. Tata, and B.G. Atkinson, eds., pp. 403-431. Academic Press, New York.

Kaltenbach, J.C., Faszewski, E.E., Nytch, K.E., Potter, C.H., Shanthakumar, N., and Fakin, A. (2004). Glycoconjugate localization in larval and adult skin of the bullfrog, *Rana catesbeiana*: a lectin histochemical study. *J Morphol* 261(2):184-95.

Kanamori, A., and Brown, D.D. (1992). The regulation of thyroid hormone receptor β genes by thyroid hormone in *Xenopus laevis*. *J Biol Chem* 267:739-745.

Kang, L., Marin, M., and Kelley, D. (1994). Developmental and regional expression of thyroid hormone receptor genes during *Xenopus* metamorphosis. *Development* 112:933-943.

Kavlock, R.J., Daston, G.P., DeRosa, C., Fenner-Crisp, P., Gray, L.E., Kaattari, S., Lucier, G., Luster, M., Mac, M.J., Maczka, C., Miller, R., Moore, J., Rolland, R., Scott, G., Sheehan, D.M., Sinks, T., and Tilson, H.A. (1996). Research needs for the risk assessment of health and environmental effect of endocrine disruptors: A report of the USEPA-sponsered workshop. *Envir Health Persp* 104:15740.

Kawahara, A, Kohara, S., Sugimoto, Y., and Amano, M. (1987). A change in the hepatocyte population is responsible for the progressive increase of vitellogenin synthetic capacity at and after metamorphosis of *Xenopus laevis*. *Dev Biol* 122:139-145.

Kawahara, A., Baker, B.S., and Tata, J.R. (1991). Developmental and regional expression of thyroid hormone receptor genes during Xenopus metamorphosis. *Development* 112:933-943.

Kelley, D.B., and Dennison, J. (1990). The vocal motor neurons of *Xenopus laevis*: Development of sex differences in axon number. *J Comp Neurol* 164:63-78.

Kelley, D.B. (1996). Sexual differentiation in *Xenopus laevis.* In: The Biology of Xenopus, R.C. Tinsley and H.R. Kobel, eds., pp. 143-193. Clarendon Press, Oxford, UK.

Kerr, J.F.R., Harmon, B., and Searle, J. (1974). An electron-microscope study of cell eletion in the anuran tadpole tail during spontaneous metamorphosis with special reference apoptosis of striated muscle fibres. *J Cell Sci* 14:571-585.

Khan, J., Saal, L.H., Bittner, M.L., Chen, Y., Trent, J.M., and Meltzer, P.S. (1999). Expression profiling in cancer using cDNA microarrays. *Electrophoresis* 20:223-229.

Kikuyama, S., Niki, K., Mayumi, M., and Kawamura, K. (1982). Retardation of thyroxine-induced metamorphosis by Amphenone B in toad tadpole. *Journal of Endocrinology* 29:659-662.

Kikuyama, S., Niki, K., Mayumi, M., Shibayama, R., Nishikawa, M., and Shitake, N. (1983). Studies on corticoid action on the toad tadpoles in vitro. *Gen Comp Endocrinol* 52:395-399.

Kikuyama, S., Suzuki, M.R., and Iwamuro, S. (1986). Elevation of plasma aldosterone levels of tadpoles at metamorphic climax. *Gen Comp Endocrinol* 63:186-190.

Kikuyama, S., Kawamura, K., Tanaka, S., and Yamamoto, K. (1993). Aspects of amphibian metamorphosis: Hormonal control. *International Review of Cytol* 145:105-148.

King, J.A., and Miller, R.P. (1981). TRH, GH-RIH, and LH-RH in metamorphosing *Xenopus laevis*. *Gen Comp Endocrinol* 44:20-27.

Kinoshita, T., Sasaki, F., and Watanabe, K. (1985). Autolysis and heterolysis of the epidermal cells in anuran tadpole tail regression. *J Morphol* 185:269-275.

Kloas, W., Lutz, I., and Einspanier, R. (1999). Amphibians as models to study endocrine disruptors: II. Estrogenic activity of environmental chemical in vitro and in vivo. *Science of the Total Environment* 225:59-68.

Kobayashi, H. (1958). Effect of deoxycorticosterone acetate on metamorphosis induced bythyroxine in anuran tadpoles. *Endocrinol* 62:371-377.

Kollros, J.J. (1981). Transitions in the nervous systems during amphibian metamorphosis. In: Metamorphosis: A Problem in Developmental Biology, L.I. Gilbert and E. Frieden, eds., pp. 445-459. Plenum Press, New York.

Koopdonk-Kool, J.M., van Lopik-Peterse, M.C., Veenboer, G.J., Visser, T.J., Schoenmakers, C.H., and de Vijlder, J.J. (1993). Quantification of type III iodothyronine deiodinase activity using thin-layer chromatography and phosphor screen autoradiography. *Analyt Biochem* 214:329-331.

Kordylewski, L. (1983). Light and electron microscopic observation of the development of intestinal musculature in Xenopus. *Anat Forsch* 97:19-734.

Krain, L.P., and Denver, R.J. (2004). Developmental expression and hormonal regulation of glucocorticoid and thyroid hormone receptors during metamorphosis in *Xenopus laevis*. *J Endocrinol* 181(1):91-104.

Kroll, K.L., and Gehart, J.C. (1994). Transgenic *X. laevis* embryos from eggs transplanted with nuclei of transfected cultures cells. *Science* 266:650-653.

Kroll, K.L., and Amaya, E. (1996). Transgenic *X. laevis* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* 122:3173-3183.

Krug, E.C., Honn, K.V., Battista, J., and Nicoll, C.S. (1983). Corticosteroids in serum of *Rana catesbeiana* during development and metamorphosis. *Gen Comp Endocrinol* 52:232-241.

Kühn, E.R., Kikuyama, A., Yamamoto, K., and Darras, V.M. (1985). In vivo release of prolactin in *Rana ridibunda* following an intravenous injection of thyrotropin-releasing hormone. *Gen Comp Endocrinol* 60(1):86-89.

Langlois, M.F., Zanger, K., Monden, T., Safer, J.D., Hollenberg, A.N., and Wondisford, F.E. (1997). A unique role of the beta-2 thyroid hormone receptor isoform in negative regulation by thyroid hormone. Mapping of a novel amino-terminal domain important for ligand-independent activation. *J Biol Chem* 272:24927-24933.

Lapiere, C.M., and Gross, J. (1963). Animal collagenase and collage metabolism. In: Mechanisms of Hard Tissue Destruction, R.F. Sognnaes, ed., pp. 663-694. American Association for the Advancement of Science, Washington D.C.

Lazar, M.A. (1993). Thyroid hormone receptors: Multiple forms, multiple possibilities. *Endocr Rev* 14:184-193.

Leatherland, J.F. (1985). Effects of 17b-estradiol and methyl testosterone on the activity of the thyroid gland in rainbow trout, *Salmo gairdneri Richardson*. *Gen Comp Endocrinol* 60:343-352.

Lee, Y., and Mahdavi, V. (1993). The D domain of the thyroid hormone receptor $\alpha$ 1 specifies positive and negative transcriptional regulation functions. *J Biol Chem* 268:2021-2028.

Leloup, J., and Buscagalia, M. (1977). La triiodothyronine: Hormone de la metamorphose des amphibiens. *CR Acad Sci* 284:2261-2263.

Leloup-Hatey, J., Buscagalia, M., Jolivet-Jaudet, G., and Leloup, J. (1990). Interenal function during metamorphosis in anuran larvae. In: Biology and Physiology of Amphibians, W. Hanke, ed., pp. 139-154. Gustav Fischer Verlag, New York.

Levy, O., Riedel, G.D.C, Ginter, C.S., Paul, E.M., Lebowitz, A.N., and Carrasco, N. (1997). Characterization of the thryroid $Na^+/I^-$ symporter with an anti-COOH terminus antibody. *Proc Natl Acad Sci USA* 94:5568-5573.

Liang, V.C., Sedgwick, T., and Shi, Y-B. (1997). Characterization of the *Xenopus* homolog of an immediate early gene associated with cell activation: Sequence analysis and regulation of its expression by thyroid hormone during amphibian metamorphosis. *Cell Res* 7(2):179-93.

Lister, A., and Van Der Kraak, G. (2002). Modulation of goldfish testicular testosterone production in vitro by tumor necrosis factor alpha, interleukin-1beta, and macrophage conditioned media. *J Exper Zool* 292:477-486.

Macchi, I.A., and Phillips, J.G. (1966). In vitro effect of adrenocorticotropin on corticoid secretion in the turtle, snake, and bullfrog. *Gen Comp Endocrinol* 6:170-182.

MacKenzie, D.S., Licht, P., and Papkoff, H. (1978). Thyrotropin from amphibian (*Rana catesbeiana*) pituitaries and evidence of heterothyrotropic activity of bullfrog luteinizing hormone in reptiles. *Gen Comp Endocrinol* 36:566-574.

Manzon, R.G., and Denver, R.J. (2004). Regulation of pituitary thyrotropin gene expression during *Xenopus* metamorphosis: Negative feedback is functional throughout metamorphosis. *J Endocrinol* 182(2):273-285.

May, F.E., and Knowland, J. (1980). The role of thyroxine in the transition of vitellogenin synthesis from noninducibility to inducibility during metamorphosis in Xenopus laevis. *J Dev Biol* 77(2):419-30.

McAvoy, J.W., and Dixon, K.E. (1977). Cell proliferation and renewal in the small intestinal epithelium of metamorphosing and adult Xenopus laevis. *J Exp Zool* 202:129-138.

McCutcheon, F.H., (1936). Hemoglobin function during the life history of the bullfrog. *J Cell Comp Physiol* 8:63-81.

McMaster, M.E., Jardine, J.J., Ankley, G.T., Benson, W.H., Greeley, Jr., M.S., Gross, T.S., Guillette Jr., L.J., MacLatchy, D.L., Orlando, E.F., Van Der Kraak, G.J., and Muntkittrick, K.R. (2001). An interlaboratory study on the use of steroid hormones in examining endocrine disruption. *Environ Toxicol Chem* 20:2081-2087.

Mellstrom, B., Naranjo, J.R., Santos, A., Gonzalez, A.M., and Bernal, J. (1991). Independent expression of the alpha and beta c-erbA genes in developing rat brain. *Mol Endocrinol* 5:1339-1350.

Millar, R.P., Nicolson, S., King, J.A., and Louw, G.N. (1983). Functional significance of TRH in metamorphosing and adult anurans. In: Thyrotropin-Releasing Hormone, E.C. Griffiths and G.W. Bennett, eds., pp. 217-227. Raven, New York.

Mimnagh, K.M., Bolaffi, J.L., Montgomery, N.M., and Kaltenbach, J.C. (1987). Thyrotropin-releasing hormone (RRH): Immunohistochemical distribution in tadpole and frog brain. *Gen Comp Endocrinol* 66:394-404.

Miranda, L.A., Paz, D.A., Dezi, R.E., and Pisanó, A. (1995). Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. *Gen Comp Endocrinol* 98:166-176.

Moller, B., Bjorkhem, I., Falk, O., Lantto, O., and Larsson, A. (1983). Identification of thyroxine in human breast milk by gas chromatography-mass spectrometry. *J Endocrinol Metab* 56:30-34.

Moulton, J.M., Jurand, A., and Fox, H. (1968). A cytological study of Mauthner's cells in Xenopus laevis and Rana temporaria during metamorphosis. *J Embryol Exp Morphol* 19:415-431.

Munro, A.F. (1953). The ammonia and urea excretion of different species of amphibian development. *J Biochem* 54:29-36.

Niki, K., Yoshizato, K., and Kikuyama, S. (1981). Augmentation of nuclear binding capacity of triiodothyronine be aldosterone in tadpole tail. *Proc Japan Acad* 57:271-275.

Nishikawa, A., Shimizu-Nishikawa, K., and Miller, L. (1992). Spatial, temporal and hormonal regulation of epidermal keratin expression during development of the frog, Xenopus laevis. *Devel Biol* 151:145-153.

Norris, D.O., Duvall, D., Greendale, K., and Gern, W.A. (1977). Thyroid function in pre- and postspawning neotenic tiger salamanders (Ambystoma tigrinum). *Gen Comp Endocrin* 33(4):512-517.

Norris, D.O. (1996). Vertebrate Endocrinology, Third Edition, p. 634. Academic Press, San Diego.

Nuwaysir, E. F., Bittner, M., Trent, J., Barrett, J. C., and Afshari, C. A. (1999). Microarrays and toxicology: The advent of toxicogenomics. *Mol Carcinog* 24:153-159.

Oberste-Berghaus, C., Zanger, K., Hashimoto, K., Cohen, R.N., Hollenberg, A.N., and Wondisford, F.E. (2000). Thyroid hormone-independent interaction between the thyroid hormone 2 amino terminus and coactivators.receptor *J Biol Chem* 275:1787-1792.

OECD (2000). Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. *Environ Health Safety Public* No. 23. Series on Testing and Assessment. Paris.

OECD (2003). Compilation of Comments on Draft DRP on Amphibian Metamorphosis Assay, p. 52. Organisation for Economic Co-operation and Development: Washington Center (**www.oecdwash.org**).

Okada, R., Iwata, T., Kato, T., Kikuchi, M, Yamamoto, K., and Kikuyama, S. (2000). Cloning of bullfrog thyroid-stimulating hormone (TSH) β subunit cDNA: Expression of TSHβ mRNA during metamorphosis. *Gen Comp Endocrinol* 119(2):224-231.

Okada, R., Yamamoto, K., Koda, A., Ito, Y., Hayashi, H., Tanaka, S., Hanaoka, Y., Kikuyama, S. (2004). Development of radioimmunoassay for bullfrog thyroid-stimulating hormone (TSH): effects of hypothalamic releasing hormones on the release of TSH from the piuitary *in vitro*. *Gen Comp Endocrinol* 135(1):42-50.

Olivereau, M., Vandesande, F., Boucique, E., Ollevier, F., and Oliveau, J.M. (1987). Immunocytochemical localization and spatial relation to the adenohypophysis of a somatostatin-like and a corticotropin-releasing factor-like peptide in the brain of the four amphibian species. *Cell Tissue Research* 274:317-324.

Oofusa, K., Tooi, O., Kashiwagi, A., Kashiwagi, K., Kondo, Y., Watanabe, Y., Sawada, T., Fujikawa, K., and Yoshizato, K. (2001). Expression of thyroid hormone receptor beta a gene assayed by transgenic *Xenopus laevis* carrying its promoter sequences. *Mol Cellular Endocrinol* 18:97-110.

Opitz, R., Bögi, C., Levy, G., Lutz, I., and Kloas, W. (2002a). Bioassay development for detection of chemical affecting thyroid system in *Xenopus laevis* tadpoles. Proceedings of the Second Status Seminar Endocrine Disrupters, Berlin, Germany 2001:217-219.

Opitz, R., Levy, G., Lutz, I., and Kloas, W. (2002b). Development of molecular biomarkers to detect thyroid-disrupting activities of environmental chemicals in *Xenopus laevis* tadpoles. Proceedings of the 21st Conference of European Comparative Endocrinologiests (German), R. Keller, H. Dircksen, D. Sedlmeier, and H. Vaudry, eds., pp. 99-102. Monduzzi Editore S.P.A, Bonn.

Opitz, R., Braunbeck, T., Bogi, C., Pickford, D.B., Nentwig, G., Oehlmann, J., Tooi, O., Lutz, I., and Kloas, W. (2005). Description and initial evaluation of a *Xenopus metamorophosis* assay for detection of thyroid system-disrupting activities of environmental compounds. *Environ Toxicol. Chem* 24(3):653-664.

Oppenheimer, J. H., Schwartz, H. L., Mariash, C. N., Kinlaw, W. B., Wong, N. C. W., and Freake, H. C. (1987). Advances in our understanding of thyroid hormone action at the cellular level. *Endocrinol Rev* 8:288-308.

Oppenheimer, J.H. (1979). Thyroid hormone action at the cellular level. *Science* 203:971-979.

Packard, G.C., and Packard, M.J. (1975). The influence of acclimation temperature on the metabolic response of frog tissue to thyroxine administered in vivo. *Gen Comp Endocrin* 27(2):162-168.

Paik, W., and Cohen, P.P. (1960). Biochemical studies on amphibian metamorphosis. I. The effect of thyroxine on protein synthesis in the tadpole. *J Gen Physiol* 43:683-696.

Parmentier M., Libert F., Maenhaut C., Lefort A., Gerard C., Perret J., Van Sande J., Dumont J.E., and Vassart, G. (1989). Molecular cloning of the thyrotropin receptor. *Science* 246:1620-2.

Picard, D., and Yamamoto, K.R. (1987). Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *J EMBO* 6:3333-3340.

Pierce, J.G., and Parsons, T.F. (1981). Glycoprotein hormones: Structure and function. *Annual Rev Physiol* 50:465-495.

Prapunpoj, P., Yamauchi, K., Nishiyama, N., Richardson, S.J., and Schreiber, G. (2000). Evolution of structure, ontogeny of gene expression, and function of *Xenopus laevis* transthyretin. *Am J Physiol Regulatory Integrative Comp Physiol* 279:R2026-R2041.

Puzianowska-Kuznicka, M., Damjanovski, and S., Shi, Y-B. (1997). Both thyroid hormone and 9-cis retinoic acid receptors are required to efficiently mediate the effects of thyroid

hormone on embryonic development and specific gene regulation in *Xenopus laevis*. *Mol Cell Biol* 17:4738-4749.

Rabelo, E.M.L., and Tata, J.R. (1993). Thyroid hormone potentiates estrogen activation of vitellogenin genes and autoinduction of estrogen receptor in adult Xenopus hepatocytes. *Mol Cell Endocrinol* 96:37-44.

Rabelo, E.M.L., Baker, B.S., and Tata, J.R. (1994). Interplay between thyroid hormone and estrogen in modulating expression of their receptor and vitellogenin genes during Xenopus metamorphosis. *Mech Dev* 45:49-57.

Regard, E. (1978). Cytophysiology of the amphibian thyroid gland through larval development and metamorphosis. *Int Rev Cytol* 52:81-118.

Ribiero, R.C.J., Cavelieri, R.R., Lomri, N., Rahmaoui, C.M., Baxter, J.D., and Scharschmidt, B.F. (1996). Thyroid hormone export regulates cellular hormone content and response. *J Biol Chem* 271:17147-17151.

Richards, C.M., and Nace, G.W. (1978). Gynogenetic and hormonal sex reversal used in tests of the XX-XY hypothesis of sex determination in *Rana pipiens*. *Growth* 42:319-331.

Richardson, S.J., Bradley, A.J., Duan, W., Wettenhall, R.E., Harms, P.J., Babon, J.J., Southwell, B.R., Nicol, S., Donnellan, S.C., and Schreiber, G. (1994). Evolution of marsupial and other vertebrate thyroxine-binding plasma proteins. *Am J Physiol* 266(4 pt 2): R1359-1370.

Riddiford, L.M. (1996). Molecular aspects of juvenile hormone action in insect metamorphosis. In: Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells, L.I. Gilbert, J.R. Tata, and B.G. Atkinson, eds., pp. 223-251. Academic Press, New York.

Riggs, A. (1951). The metamorphosis of hemoglobin in the bullfrog. *J Gen Physiol* 35:23-40.

Ritchie, J.W.A., Peter, G.J., Shi, Y-B., and Taylor, P.M. (1999). Thyroid hormone transport by 4F2hc-IU12 heterodimers expressed in Xenopus oocytes. *J Endocrinology* 163:R5-R9.

Ritchie, J.W., Shi, Y.B., Hayashi, Y., Baird, F.E., Muchekehu, R.W., Christie, G.R., and Taylor, P.M. (2003). A role for thyroid hormone transporters in transcriptional regulation by thyroid hormone receptors. *Mol Endocrinol* 17:653-661.

Rivier, J., Rivier, C., and Vale, W. (1984). Synthetic competitive antagonists of corticotropin-releasing factor: Effect on ACTH secretion in the rat. *Science* 224:889-891.

Robbins, J. (1992). Thyroxine transport and the free hormone hypothesis. *Endocrinol* 131:546-547.

Robertson, J.C., Watson, J., and Kelley, D.B. (1991). Laryngeal nerve axon number in premetamorphic *Xenopus laevis* is androgen dependent: Abstract. *Society for Neuroscience* 18:1320.

Robertson, J.C., and Kelley, D. (1992). Gonadal and laryngeal development in hypothyroid tadpoles. *Amer Zool* 32:86A.

Robertson, J.C., and Kelley, D.B. (1996). Thyroid hormone controls the onset of androgen sensitivity in the developing larynx of *Xenopus laevis*. *Dev Biol* 176:108-123.

Rollins-Smith, L.A., Barker, K.S., and Davis, A.T. (1997a). Involvement of glucocorticoids in the reorganization of the amphibian immune system at metamorphosis. *Dev Immunol* 5:145-152.

Rollins-Smith, L.A., Flajnik, M.F., Blair, P.J., Davis, A.T., and Green, W.F. (1997b). Involvement of thyroid hormones in the expression of MHC class antigens during ontogeny in Xenopus. *Dev Immunol* 5:133-144.

Rollins-Smith, L.A. (1998). Metamorphosis and the amphibian immune system. *Immunol Review* 166:221-230.

Rose, M.F., and Rose, S.R. (1998). Melatonin accelerates metamorphosis in *Xenopus laevis*. *J Pineal Research* 24:90-95.

Rosenkilde, P. (1979). The thyroid hormones in amphibia in Hormones and Evolution, E. J. W. Barrington, ed., pp. 437-491. Academic Press, New York.

Rosenkilde, P. (1982). The role of thyroid hormones in adult amphibians. In: Gunma Symposium on Endocrinology, Phylogenetic Aspects of Thyroid Hormone Actions, pp. 91-106. Institute of Endocrinology, Gunma University, Tokyo: Center for Academic Publications.

Rossi, A. (1959). Tavole cronologiche dello svilluppo embryionale e larvale del “Bufo bufo.” *Mont Zool Ital* 66:133.

Roth, P. (1941). Action antogoniste du propionate de testosterone dans la metamorphose experimentale des batraciens provoquee par thyroxine. *Bull Mus Natl Hist Nat* (Paris) 13:500-502.

Roth, P. (1948). Sur l’action antagoniste des substances oestrogenes dans la metamorphose experimentale des amphibiens (3e note). *Bull Mus Natl Hist Natl* (Paris) 20:408-415.

Rowe, I., Coen, L., Le Blay, K., Le Mevel, S., and Demeneix, B.A. (2002). Autonomous regulation of muscle fibre fate during metamorphosis in *Xenopus tropicalis*. *Dev Dynamics* 224:381-390.

Ruben, L.N., Clothier, R.H., Murphy, G.L., Marshall, J.D., Lee, R., Pham, T., Nol, C., and Shiigi, S. (1989). Thyroid function and immune reactivity during metamorphosis *Xenopus laevis*, the South African clawed toad. *Gen Comp Endocrinol* 76:128-138.

Sakai, M., Hanoaka, Y., Tanaka, S., Hayashi, H., and Kikuyama, S. (1991). Thyrotropic activity of various adenohypophysis hormones of the bullfrog. *Zool Sci* 8:929-934.

Schneider, M. J., and Galton, V.A. (1995). Effect of glucocorticoids on thyroid hormone action in cultured red blood cells from *Rana catesbeiana* tadpoles. *Endocrinol* 136:1435-1440.

Schneider, M.J., Fiering, S.N., Pallud, S.E., Parlow, A.F., St. Germain, D.L., and Galton, V.A. (2001). Targeted disruption of the type 2 selenodeiodinase gene (DIO2) results in a phenotype of pituitary resistance to T4. *Mol Endocrinol* 15:2137-2148.

Schreiber, G., and Richardson, S.J. (1997). The evolution of gene expression, structure, and function of transthyretin. *Comp Biochem Physiol B Biochem Mol Biol* 116(2):137-160.

Schussler, G.C. (2000). The thyroxine-binding proteins. *Thyroid* 10:141-149.

Shi, Y.F., Sahai, B.M., and Green, D.R. (1989). Cyclosporin A inhibits activation-induced cell death in T-cell hybridomas and thymocytes. *Nature* 339:625-626.

Shi, Y-B., and Brown, D.D. (1993). The earliest changes in gene expression in tadpole intestine induced by thyroid hormone. *J Biol Chem* 268:20312-20317.

Shi, Y-B., and Ishizuya-Oka, A. (1996). Biphasic intestinal development in amphibians: Embryogenesis and remodeling during metamorphosis. *Current Topics in Dev Biol* 32:205-235.

Shi, Y-B. (2000). Amphibian metamorphosis from morphology to molecular biology. Wiley-Liss Press, New York.

Shimizu-Nishikawa, K., and Miller, L. (1992). Hormonal regulation of adult type keratin gene expression in larval epidermal cells of the frog *Xenopus laevis*. *Differentiation* 49:77-83.

Shintani, N., Nohira, T., Hikosaka, A., and Kawahara, A. (2002). Tissue-specific regulation of type III iodothyronine 5-deiodinase gene expression mediates the effects of prolactin and growth hormone in Xenopus metamorphosis. *Develop Growth Differ* 44:327-335.

Simon, R, Tietge, J.E., Michalke, B., Degitz, S., and Schramm, K.W. (2002). Iodine species and the endocrine system: Thyroid hormone levels adult *Danio rerio* and developing *Xenopus laevis*. *Analy Bioannal Chem* 372:481-485.

St. Germain, D.L. (1994). Iodothyronine deiodinases. *Trends Endocrinol Metab* 5:36-42.

St. Germain, D.L., and Galton, V.A. (1997). The deiodinase family of selenoproteins. *Thyroid* 7:655-668.

Stearns, S. (1989). The evolutionary significance of phenotypic plasticity. *Bioscience* 39:436-445.

Stebbins, R.C., and Cohen, N.W. (1995). A Natural History of Amphibians. Princeton University Press, Princeton, NJ.

Stenzel-Poore, M.P., Heldwein, K.A., Stenzel, P., Lee, S., and Vale, W.W. (1992). Characterization of the genomic corticotropin-releasing factor (CRF) gene from *Xenopus laevis*: Two members of the CRF family exist in amphibians. *Mol Endocrinol* 6:1716-1724.

Su, Y., Shi, Y., Stolow, M., and Shi, Y-B. (1997). Thyroid hormone induces apoptosis in primary cell cultures of tadpole intestine: Cell type specificity and effects of extracellular matrix. *J Cell Biol* 139:1533-1543.

Suzuki, M.R., and Kikuyama, S. (1983). Corticoides augement nuclear binding capacity for triiodothyronine in bullfrog tadpole tail fins. *Gen Comp Endocrinol* 52:272-278.

Suzuki, S., and Fujikura, K. (1994). Circulating thyroglobulin in tadpoles and adult frogs of *Rana catesbeiana*. *Gen Comp Endocrinol* 94:72-77.

Takahashi, N., Yoshihama, K., Kikuyama, S., Yamamoto, K., Wakabayashi, K., and Kato, Y. (1990). Molecular cloning and nucleotide sequence analysis of complementary DNA for bullfrog prolactin. *J Mol Endocrinol* 5:281-287.

Tanaka, S., Sakai, M., Park, M.K., and Kurosumi, K. (1991). Differential appearance of the subunits of glycoprotein hormones (LH, FSH, and TSH) in the pituitary of bullfrog (*Rana catesbeiana*) larvae during metamorphosis. *Gen Comp Endocrinol* 84:318-327.

Tata, J.R., and Widnell, C.C. (1966). Ribonucleic acid synthesis during the early action of thyroid hormones. *J Biochem* 88:604.

Tata, J.R. (1967). The formation and distribution of ribosomes during hormone induced growth and development. *J Biochem* 104:1.

Tata, J.R. (1968). Early metamorphic competence of Xenopus larvae. *Dev Biol* 18:415-440.

Tata, J.R. (1972). Hormonal regulation of metamorphosis. *Symp Soc Exp Biol* 25:163-181.

Tata, J.R., Kawhara, A., and Baker, B.S. (1991). Prolactin inhibits both thyroid hormone-induced morphogenesis and cell death in cultured amphibian larval tissues. *Dev Biol* 146:72-80.

Tata, J.R. (1993). Gene expression during metamorphosis: An ideal model for post-embryonic development. *BioEssays* 15:239-248.

Tata, J.R. (1994). Gene expression during post-embryonic development: Metamorphosis as a model. Proceedings of the Indian National Science Academy, Part B. *Biol Sci* 60:287-301.

Tata, J.R. (1997). How hormones regulate programmed cell death during amphibian-metamorphosis. In: Programmed Cell Death, Y-B. Shi, Y. Shi, Y. Xu, and D.W. Scott, eds., pp. 1-11. Plenum Press, New York.

Tata, J.R. (1998). Amphibian metamorphosis as a model for studying the developmental actions of thyroid hormone. *Cell Res* 8:259-272.

Tata, J.R. (1999). Amphibian metamorphosis as a model for studying the developmental actions of thyroid hormone. *Biochem* 81:359-366.

Tata, J.R. (2000). Autoinduction of nuclear hormone receptors during metamorphosis and its significance. *Insect Biochem Mol Biol* 30:645-651.

Taylor, A.C., and Kollros, J.J. (1946). Stages in the normal development of *Rana pipiens* larvae. *Anat Rec* 93:7-23.

Thomson, J.A. (1974). Clinical Tests of Thyroid Function. Technomic Publishing Company, Inc., Westport, Connecticut.

Tonon, M-C., Cuet, P., Lamacz, M., Jegou, S., Cote, J., Gouteux, L., Ling, N, Pelletier, G., and Vaudry, H. (1986). Comparative effects of corticotropin-releasing factor, arginine vasopressin, and related -MSH by frog anterior pituitaryneuropeptides on the secretion of ACTH and  cells and neurointermediate lobes in vitro. *Gen Comp Endocrinol* 61:438-445.

Tsai, M-J., and O'Malley, B.W. (1994). Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annual Rev Biochem* 63:451-486.

Ulisse, S., and Tata, J.R. (1994). Thyroid hormone and glucocorticoid independently regulate the expression of estrogen receptor in male Xenopus liver cells. *Mol Cell Endocrinol* 105:45-53.

Ulisse, S., Esslemont, G., Baker, B.S., Chatterjee, V.K.K., and Tata, J.R. (1996). Dominant-negative mutant thyroid hormone receptors prevent transcription from  gene promoter in response to thyroid hormonęXenopus thyroid hormone receptor  in Xenopus tadpoles in vivo. *Proc Natl Acad Sci USA* 93:1205-1209.

Underhay, E.E., and Baldwin, W. (1955). Nitrogen excretion in the tadpoles of *Xenopus laevis Daudin*. *J Biochem* 61:544-547.

Uppaluri, R., and Towle, H.C. (1995). Genetic dissection of thyroid hormone receptor: Identification of mutations that separate hormone binding and transcriptional activation. *Mol Cell Biol* 15:1499-1512.

Valamparampil, T.T., and Oommen, O.V. (1997). Triiodothyronine ($T_3$) and thyroxine ($T_4$) levels in *Rana curtipes* during development and metamorphosis. *Indian J Exp Biol* 35:1375-1377.

Vandorpe, G., and Kuhn, E.R. (1989). Estradiol-17 beta silastic implants in female *Rana ridibunda* depress thyroid hormone concentrations in plasma and the in vitro 5'-monodeiodination activity of kidney homogenates. *Gen Comp Endocrinol* 76(3):341-345.

Veldhoen, N., and Helbing, C. (2001). Detection of environmental endocrine-disruptor effects on gene expression in live *Rana catesbeiana* tadpoles using a tail fin biopsy technique. *Environ Toxicol Chem* 20(12):2704-2708.

Verhaert, P., Marivoet, S., Vandesande, F., and De Loof, A. (1984). Localization of CRF immunoreactivity in the central nervous system of three vertebrate and one insect species. *Cell Tiss Res* 238:49-53.

Wakao, H., Gouilleux, F., and Groner, B. (1994). Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the PRL response. *EMBO J* 13:2182-2191.

Wang, Z., and Brown, D.D. (1993). Thyroid hormone-induced gene expression program for amphibian tail resorption. *J Biol Chem* 268:16270-16278.

Watanabe, M., Ohshima, M., Morohashi, M., Maeno, M., and Izutsu, Y. (2003). Ontogenic emergence and localization of larval skin antigen molecule recognized by adult T cells of *Xenopus laevis*: Regulation by thyroid hormone during metamorphosis. *Dev Growth Differ* 45(1):77-84.

Weber, R. (1964). Ultrastructural changes in regressing tail muscles of *Xenopus* larvae at metamorphosis. *J Cell Biol* 22:481-7.

Weber, R. (1967). Biochemistry of amphibian metamorphosis. In: The Biochemistry of Animal Development, R. Weber, ed., pp. 227-301. Academic Press, New York.

Weber, R., Blum, B., and Muller, P.R. (1991). The switch from larval to adult globin gene expression in *Xenopus laevis* is mediate by erythroid cells from distinct compartments. *Development* 112:1021-1029.

Weber, R. (1996). Switching of global genes during anuran metamorphosis. In: Metamorphosis: Postembryonic of Gene Expression in Amphibian and Insect Cells, L. I. Gilbert, J. R. Tata, and B. G. Atkinson, eds., pp. 567-597. Academic Press, New York.

White, B.A., and Nicoll, C.S. (1981). Hormonal control of amphibian metamorphosis. In: Metamorphosis: A Problem in Developmental Biology, L. I. Gilbert and E. Frieden, eds., pp. 363-396. Plenum Press, New York.

Wise, R.W. (1970). An immunochemical comparison of tadpole and frog hemoglobins. *Comp Biochem Physiol* 32:89-95.

Witschi, E. (1971). Mechanisms of sexual differentiation. In: Hormones in Development, M. Hamburg and E. Barrington, eds., pp. 601-618. Appleton Century Crofts, New York.

Wollman, S.H. (1980). Structure of the thyroid gland. In: The Thyroid Gland, M. De Vissher, ed., pp. 1-19. Raven Press, New York.

Woody, C.J., and Jaffe, R.C. (1984). Binding of dexamethasone by hepatic, intestine and tail fin cytosol in *Rana catesbeiana* tadpoles during spontaneous and induced metamorphosis. *Gen Comp Endocrinol* 54:194-202.

Yamamoto, K., and Kikuyama, S. (1981). Purification and properties of bullfrog prolactin. *Endocrinol Jpn* 28:59-64.

Yamamoto, K., and Kikuyama, S. (1982). Radioimmunoassay of prolactin in plasma of bullfrog tadpoles. *Endocrinol Jpn* 29:159-167.

Yamamoto, K., Niinuma, K., and Kikuyama, S. (1986). Synthesis and storage of prolactin in the pituitary gland of bullfrog tadpoles during metamorphosis. *Gen Comp Endocrinol* 62:247-253.

Yamamoto, K., and Kikuyama, S. (1993). Binding of aldosterone by epidermal cytosol in the tail of bullfrog larvae. *Gen Comp Endocrinol* 89:283-290.

Yamauchi, K., Kasahara, T., Hayashi, H., and Horiuchi, R. (1993). Purification and characterization of a 3, 5, 3'-L-triiodothyroninie-specific binding proteins from bullfrog tadpole plasma: A homolog of mammalian transthyretin. *Endocrinol* 132:2254-2261.

Yamauchi, K., Takeuchi, H-A., Overall, M., Dziadek, M., Munro, S.L.A., and Schreiber, G. (1998). Structural characteristics of bullfrog (*Rana catesbeiana*) transthyretin and its cDNA. *Eur J Biochem* 256:287-296.

Yang, Z, and Privalsky, M.L. (2001). Isoform-specific transcriptional regulation by thyroid hormone receptors: Hormone-independent activation operates through a steroid receptor mode of coactivator interaction. *Mol Endocrinol* 15:1170-1185.

Yaoita, Y., Shi, Y-B., and Brown, D.D. (1990a). *Xenopuș laevis* and thyroid hormone receptors. *Proc Natl Acad Sci USA* 87:7090-7094.

Yaoita, Y., and Brown, D.D. (1990b). A correlation of thyroid hormone receptor gene expression with amphibian metamorphosis. *Genes and Dev* 4:1917-1924.

Yasuda, A., Yamaguchi, K., Kobayashi, T., Yamamoto, K., Kikuyama, S., and Kawauchi, H. (1991). The complete amino acid sequence of prolactin from the bullfrog, *Rana catesbeiana*. *Gen Comp Endocrinol* 83:218-226.

Yen, P.M., and Chin, W.W. (1994). New advances in understanding the molecular-mechanisms of thyroid hormone action. *Trends Endocrinol Metab* 5:65-72.

Yoshizato, K., (1989). Biochemistry and cell biology of amphibian metamorphosis with a special emphasis on the mechanism of removal of larval organs. *Intl Rev Cytol* 119:97-149.

Yoshizato, K. (1996). Cell death and histolysis in amphibian tail during metamorphosis. In: Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells, L. I. Gilbert, J. R. Tata, and B. G. Atkinson, eds., pp. 647-671. Academic Press, New York.

Yuan, J., Shaham, S., Ledoux, S., Eliss, H.M., and Horvitz, H.R. (1993). The *C. elegans* cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell* 75:641-652.

Zechel, C., Shen, X-Q. Chen, J-Y., Chen, Z-P., Chambon, P., and Groenmeyer, H. (1994). The dimerization interfaces formed between the DNA binding domains of RXR, RARA and TR determine the binding specificity and polarity of the full-length receptors to direct repeats. *EMBO J* 13:1425-1433.