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JOINT MEETING OF THE CHEMICALS COMMITTEE AND  
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY**

**GUIDANCE DOCUMENT ON HISTOPATHOLOGY TECHNIQUES AND EVALUATION (PART 1)  
FOR THE LARVAL AMPHIBIAN GROWTH AND DEVELOPMENT ASSAY (LAGDA)**

**Series on Testing & Assessment  
No. 228**

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**Series on Testing and Assessment**

**No. 228**

**Guidance Document on Histopathology Techniques and  
Evaluation (Part 1)**

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**Paris 2015**

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Environment, Health and Safety Division  
2 rue André-Pascal  
75775 Paris Cedex 16  
France**

**Fax: (33-1) 44 30 61 80**

**E-mail: [ehscont@oecd.org](mailto:ehscont@oecd.org)**

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## FOREWORD

The project to develop a Larval draft Amphibian Growth and Development Assay (LAGDA) was initiated by Japan and the United States and included in the work plan of Test Guidelines Programme in 2009.

The Integrated Summary Report and first draft TG were submitted to the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) in 2014, followed by subsequent commenting rounds in 2014. The draft guidance document on amphibian histopathology was prepared to accompany the draft Test Guideline and help users of the test become more proficient in applying tissue sampling and preparation techniques, evaluation techniques and in the interpretation of the slides.

The guidance document on amphibian histopathology techniques and evaluation was approved by the WNT at its 27<sup>th</sup> meeting in April 2015. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to the declassification of the guidance document on 10<sup>th</sup> July, 2015.

This document presents **Part 1** of the guidance document which in total consists of three parts.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

**GUIDANCE DOCUMENT ON HISTOPATHOLOGY TECHNIQUES AND EVALUATION  
(PART 1)**

**FOR THE LARVAL AMPHIBIAN GROWTH AND DEVELOPMENT ASSAY (LAGDA)**

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- (a) **Introduction.** This document is based on works undertaken in the United States and Japan between 2004 and 2014 in support of the validation of the Larval Amphibian Growth and Development Assay. The goal of this document is to serve as guidance for the collection, histological preparation, and pathological evaluation of thyroid glands, gonads and gonadal ducts, kidney, and liver specimens from African clawed frogs (*Xenopus laevis*) in support of the Larval Amphibian Growth and Development Assay (LAGDA) which is a test generating data on effects of test chemicals on important life-stage of amphibian organisms. The test is indicated at Level 4 of the OECD Conceptual Framework on Endocrine Disrupters Testing and Assessment, comprising in vivo assays providing data on adverse effects on endocrine relevant endpoints. For the histopathology endpoint, guidance is required to ensure that histological procedures and pathological evaluations are performed accurately and consistently.

This document is divided into three sections: I) Necropsy Procedures, II) Histology Procedures, and III) Pathology Evaluation. The Pathology Evaluation section includes written descriptions and illustrations of normal tissues and abnormal changes, with special emphasis on findings that are likely related to endocrine disruption, and specific examples of lesion severity grades as applicable. Additional guidance is provided on the topics of severity grading (in general), data recording, statistical analysis, data interpretation, and report formatting.

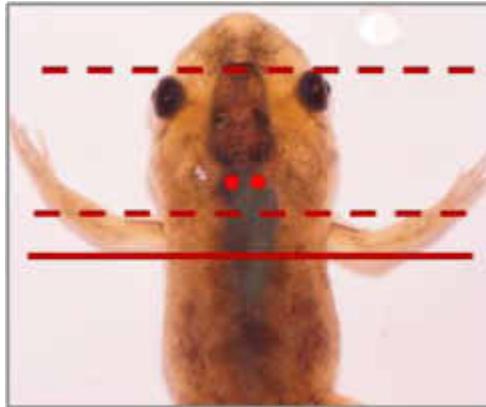
- (b) **Section I: Necropsy and Gross Trimming Procedures.**

At study termination, each frog selected for sampling is euthanized via injection of a 10% solution (w/v) of MS-222 dissolved in a physiological buffer (e.g., 0.1M phosphate buffered saline pH 7.4) at a dose of 0.2 mL per 50 g body weight into the dorsal lymph sac.

Three different types of samples are collected for histopathology: 1) larval head and neck region for evaluation of the thyroid glands, 2) juvenile liver, and 3) juvenile dorsal trunk region for evaluation of the gonads, gonadal ducts, and kidneys.

- (1) **Larval Thyroid Glands (Interim sub-sample)**

A transverse cut is made at the posterior margin of the arms prior to fixation (solid line; Figure 1). The head / neck samples are placed in appropriately sized cassettes and fixed in Davidson's solution for 48 hours, and are then rinsed thoroughly in tap water, after which they may be maintained in 10% neutral buffered formalin prior to shipment or processing. At any time following fixation, two transverse cuts are made through the head and neck region at the anterior margin of the eyes and anterior margin of the forelimbs, respectively (dashed lines; Figure 1). Care must be taken to ensure that the cut made anterior to the forelimbs is as square to the axial midline as possible and includes as much of the neck region as possible. This ensures that the thyroid glands will be included in the head / neck sample.



**Figure 1. Dorsal view of a larval (NF stage 62) *X. laevis* specimen to be evaluated for thyroid histopathology with solid red line indicating trimming plane prior to fixation, dashed red lines indicating trimming planes following fixation and red dots indicating *approximate* location of glands in relation to posterior trim plane.**

### **(2) Juvenile Liver (Test termination)**

The ventral abdominal wall is removed; the liver is carefully excised, weighed, transferred to an appropriately sized cassette that does not compress the tissue and fixed in Davidson's solution for 48 hours. The liver is then rinsed with tap water and maintained in 10% neutral buffered formalin prior to shipment or processing.

### **(3) Juvenile Gonads, Gonadal Ducts, and Kidneys**

Following removal of the liver, the gastrointestinal tract is excised and discarded. When manipulating the abdominal viscera, care must be taken not to disrupt the dorsally positioned gonads and fragile gonadal ducts. One transverse cut is made through the trunk at the anterior margin of the hips (posterior margin of the kidneys) (Figure 2). Another transverse cut is made through the middle of the spleen or several millimeters rostral from the anterior margin of the gonad. The resulting trunk segment contains the intact gonads, gonadal ducts and kidneys. The head, legs and other residual excised tissues are discarded. The trunk is transferred to an appropriately sized cassette or container that does not compress the tissue and fixed in Davidson's solution for 48 hours. The fixed tissues are then rinsed with tap water and maintained in 10% neutral buffered formalin prior to shipment or processing.

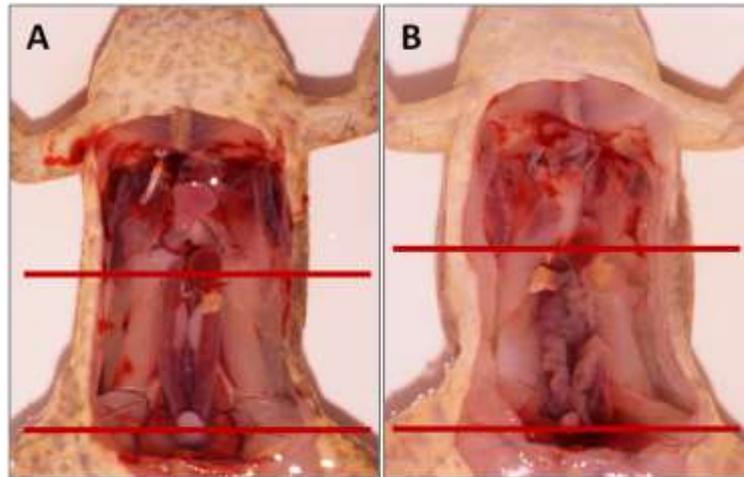


Figure 2. Ventral view of a: A) male; and B) female; juvenile (10 weeks post-NF62) *X. laevis* specimens with lines approximately indicating the tissue trimming to be performed before fixation, processing and evaluation for gonad and duct histopathology.

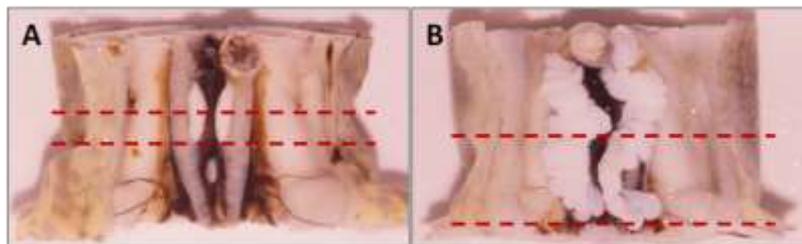


Figure 3. Dorsal trunk segments of A) male; and B) female juvenile (10 week post-NF62) *X. laevis* specimens which have been trimmed for evaluation of gonad and gonadal duct histopathology. The anterior margins of the gonads are positioned toward the tops of the images. The dashed lines represent the cuts used to bisect the specimens into anterior and posterior segments and to provide a sectioning plane for the posterior margin of the gonad.

Prior to processing and embedment, the dorsal trunk segment is transected (bisected) at the axial midpoint of the gonads to create one anterior and one posterior segment. The trunk is also transected at the posterior margin of the gonad which will be the plane microtomed for the posterior sections (dashed lines in Fig. 3).

(c) **Section II: Histologic Procedures.**

(1) **Larval Thyroid Glands**

- i. **Decalcification.** The acetic acid in modified Davidson's solution will soften bone and cartilage; therefore, decalcification prior to processing may or may not be required. The study tissues can be tested by processing and microtoming a few control frogs prior to the other study animals. If decalcification is found to be necessary, commercial preparations that contain a mixture of formic acid and EDTA are preferred, because they

provide the optimum combination of gentleness and speed. Decalcification times can often be 24 hours or less.

- ii. **Processing and Embedding.** Each head / neck specimen is processed in an automated tissue processor and infiltrated with paraffin according to routine methods. Samples are embedded in the paraffin block so that the cut surface of the posterior margin (the neck side) is sectioned first.
- iii. **Microtomy.** Each block is faced (excess paraffin trimmed away) until at least one of the thyroid glands has been reached (approximately 500 microns into the tissue). Step sections (4-5 microns thickness) are then taken at 50-micron intervals until the maximum diameter of at least one gland has been reached. Two additional step sections are then cut at an interval of 50 microns, and these two sections are placed on a single glass slide. Following microtomy, each paraffin block is sealed with paraffin.
- iv. **Staining and Coverslipping.** The thyroid gland sections are stained with hematoxylin and eosin (H&E) and are mounted with a glass coverslip using an appropriate permanent mounting medium.
- v. **Labeling.** Slides are labeled with at least the following information:
  - (i) Study number
  - (ii) Exposure Laboratory
  - (iii) Name of the test chemical
  - (iv) Individual animal identification number
- vi. **Quality Control.** The bilateral thyroid glands in the two step sections should contain the maximum number of follicles. If inadequate numbers of follicles are represented, and it is apparent that further thyroid tissue remains in the block, additional step sections are cut at 50 micron intervals until the appropriate sections are captured or until it is determined that additional recuts will not yield the required tissue. The pathologist ultimately selects the sections to be evaluated. Following microtomy, each paraffin block is re-sealed with paraffin

## (2) Juvenile Liver

- i. **Processing and Embedding.** Each liver specimen is processed in an automated tissue processor and infiltrated with paraffin according to routine methods. Samples are embedded in the paraffin block so that the area of liver to be examined is maximized.
- ii. **Microtomy.** Each block is faced (excess paraffin trimmed away) until the liver surface is reached. Step sections (4-5 microns thickness) are then taken at 50-micron intervals until the maximum diameter of the liver samples is attained. Two additional step sections are then microtomed at an interval of 50 microns, and these two sections are placed on a single glass slide. Following microtomy, each paraffin block is sealed with paraffin.

iii. **Staining and Coverslipping.** The liver sections are stained with hematoxylin and eosin (H&E) and are mounted with a glass coverslip using an appropriate permanent mounting medium.

iv. **Labeling.** Slides are labeled with at least the following information:

- (i) Study number
- (ii) Exposure Laboratory
- (iii) Name of the test chemical
- (iv) Individual animal identification number

### (3) Juvenile Gonads, Gonadal Ducts, and Kidneys

i. **Decalcification.** The acetic acid in modified Davidson's solution will soften bone and cartilage; therefore, decalcification prior to processing may or may not be required. The study tissues can be tested by processing and microtoming a few control frogs prior to the other study animals. If decalcification is found to be necessary, commercial preparations that contain a mixture of formic acid and EDTA are preferred, because they provide the optimum combination of gentleness and speed. Decalcification times can often be 24 hours or less.

ii. **Processing and Embedding.** The trunk segments are processed in an automated tissue processor and infiltrated with paraffin according to routine methods. The anterior and posterior trunk segments are embedded in separate blocks. Blocks should be labeled as anterior (a) or posterior (p). Each sample is embedded in the paraffin block so that the middle gonad surface of the anterior segment and the posterior gonad surface of the posterior segment are microtomed first.

iii. **Microtomy.** For blocks containing anterior segments, two serial sections (each 5 microns thick) are acquired as soon as the block is faced (excess paraffin trimmed away), and placed on a single slide; these will be the middle sections. Each anterior segment block is then microtomed 500 microns for males, or 1000 microns for females, and then a second pair of serial sections is taken and placed on a single slide; these will be the anterior sections. Finally each posterior section block is faced and then microtomed 1000 microns for males and females, and two serial sections are taken which are placed on a single slide; these will be the posterior sections. If the gonads are much larger or smaller than anticipated, adjustments may be made in the depths at which anterior and posterior sections are acquired. Such adjustments should be documented in the methods section of the pathology report. Following microtomy, each paraffin block is sealed with paraffin.

iv. **Staining and Coverslipping.** The gonad/kidney/duct sections are stained with hematoxylin and eosin (H&E) and are mounted with a glass coverslip using an appropriate permanent mounting medium.

v. **Labeling.** Slides are labeled with at least the following information:

- (i) Study number
- (ii) Exposure Laboratory
  - (iii) Name of the test chemical
- (iv) Individual animal identification number
  - (v) Lower case letters are used to indicate the anatomic locations of the sections as follows: a = anterior; m = middle; p = posterior to indicate the gonad region for the slide (e.g. 141a = slide 141 anterior gonad sections).

(d) **Section III: Pathologic Evaluation.**

- (1) **General Approach to Pathologic Evaluations.** Slides are to be read by individuals experienced in reading toxicologic pathology slides, and who are familiar with normal, amphibian thyroid and gonad histology, physiology, and general responses of these organs to toxicologic insult. Pathologists may be board certified (e.g., American College of Veterinary Pathologists, The European Centre of Toxicologic Pathology, or other certifying organizations); however, certification is not a requirement as long as the pathologist has obtained sufficient experience with, and knowledge of, amphibian histology and toxicologic pathology.

It is recognized that there is a limited pool of pathologists with the necessary training and experience that are available to evaluate the histopathology endpoint for the LAGDA assay. If an individual has toxicologic pathology experience and is familiar with thyroid, liver, and gonadal histology in amphibian species, he/she may be trained to evaluate the LAGDA assay. If pathologists with little experience are used to conduct the histopathological analysis, informal peer review may be necessary.

Pathologists are to read these studies unblinded (i.e., with knowledge of the treatment group status of individual frogs). This is because endocrinological effects on histomorphology tend to be incremental, and subtle differences between exposed and unexposed animals may not be recognizable unless tissue sections from high dose animals can be knowingly compared to those from controls. Thus the aim of the initial evaluation is to ensure that diagnoses are not missed (i.e., to avoid false-negative results). On the other hand, it is expected that ***all potential treatment-related findings will be re-evaluated by the pathologist in a blinded manner***, in order to prevent the reporting of false-positive results. As a rule, treatment groups should be evaluated in the following order: Control, High-dose, Mid-dose, and Low-dose.

Pathologists should specifically evaluate the target tissues identified in the guidelines; however, changes observed in other tissue types may also be recorded. This especially pertains to findings suspected to be treatment-related, or findings that might otherwise impact the study results (e.g., systemic inflammation or neoplasia).

It is suggested that the pathologist be provided with all available information related to the study. Information regarding gross morphologic abnormalities, mortality rates, and general test population performance and health are useful for pathologists to provide comprehensive reports and to aid in the interpretation of findings. For a more comprehensive discussion of standard reading approaches

for toxicologic pathology studies, please refer to the Society of Toxicologic Pathology Best Practices for reading toxicologic histopathology studies (Crissman JW et al. 126-31).

- (2) **Severity Grading.** In toxicologic pathology, it is recognized that compounds may exert subtle effects on tissues that are not adequately represented by simple binary (positive or negative) responses. Severity grading involves a semi-quantitative estimation of the degree to which a particular histomorphologic change is present in a tissue section (Shackelford et al., 2002). The purpose of severity grading is to provide an efficient, semi-objective mechanism for comparing changes (including potential compound-related effects) among animals, treatment groups, and studies.

Severity grading should usually use the following system:

- 0 (not remarkable)
- Grade 1 (minimal)
- Grade 2 (mild)
- Grade 3 (moderate)
- Grade 4 (severe)

Findings that are not present are not graded and assigned a zero (0) to represent that the tissue section is not remarkable. This is not to mean “Grade 0.” This practice provides continuity with subsequent statistical analyses.

[It is recognized that this severity grading scheme differs from that of Grim et al., 2009, which advocated the following scale for the assessment of thyroid gland changes in *X. laevis*: Grade 1 = mild, Grade 2 = moderate, and Grade 3 = severe. However, for consistency, all Tier 2 tests should use the Grade 1-Grade 4 system].

A grading system needs to be flexible enough to encompass a variety of different tissue changes. In theory, there are three broad categories of changes based on the intuitive manner in which people tend to quantify observations in tissue sections:

1. **Discrete:** These are changes that could be readily counted. Examples include atretic follicles, oocytes in the testis, and clusters of apoptotic cells.
2. **Spatial:** These are changes that could be quantified by area measurements. Includes lesions that are typically classified as focal, multifocal, coalescing, or diffuse. Specific examples include granulomatous inflammation and tissue necrosis.
3. **Global:** These are generalized changes that would usually require more sophisticated measurement techniques for quantification. Examples include increased hepatocyte basophilia, thyroid follicular cell hypertrophy, or quantitative alterations in cell populations.

Listed below are general guidelines for the use of a severity grading system, with examples of how the system could be applied to each of the above categories. Please understand that the terms Discrete, Spatial, and Global are used for illustrative purposes only; it is not intended that these terms be incorporated into any diagnosis or grade. It should be stressed that the examples below should be modified as needed for each particular type of change (diagnosis).

**Grade 1:**

Discrete change example: 0 to 2 occurrences per microscopic field, or 1 to 2 occurrences per tissue section.

Spatial change example: The change occupies a miniscule area of either a specific tissue type or the entire tissue section.

Global change example: The least perceptible alteration relative to control animals or prior experience.

**Grade 2:**

Discrete change example: 3 to 5 occurrences per microscopic field or tissue section.

Spatial change example: The change occupies a larger area than Grade 1, but still less than or equal to 25% of either a specific tissue type or the tissue section.

Global change example: The alteration is easily appreciated, but still not dramatic.

**Grade 3:**

Discrete change example: 6 to 8 occurrences per microscopic field or tissue section.

Spatial change example: The change occupies more than 25% but less than or equal to 50% of either a specific tissue type or the entire tissue section.

Global change example: The alteration is dramatic, but a more pronounced alteration can be envisioned.

**Grade 4:**

Discrete change example: 9 or more occurrences per microscopic field or tissue section.

Spatial change example: The change occupies more than 50% of either a specific tissue type or the entire tissue section.

Global change example: Essentially, the most pronounced imaginable alteration.

At least some of the histomorphologic changes that have been associated with EDCs in wildlife are considered to be exacerbations of “normal”, physiologic

findings. Whenever possible, the severity of a given change should be scored relative to the severity of the same change in concurrent control animals. For each important (i.e., treatment-associated) finding, the severity scoring criteria should be stated in the Materials and Methods section of the pathology narrative report. By convention, it is recommended that severity grading should not be influenced by the estimated physiologic importance of the change. For example, the presence of two oocytes in the testis should not be graded as “severe”, even if the pathologist considers this finding to be highly significant in terms of endocrine modulation. The reason is that estimating the physiologic importance adds a further layer of subjectivity to the findings that complicates inter-laboratory results comparisons.

- (3) **Data Recording.** The pathologist records the results on a spreadsheet template provided by EPA. For each frog, the pathologist records the presence of a diagnosis by indicating the severity grade. In rare instances (e.g., tumor diagnoses), severity grading may not be applicable. If there are no findings for a particular frog, this should be recorded specifically. It is also important to record a notation if the target tissue is missing or if the amount of tissue present is insufficient to make a diagnosis. Adding modifiers to a diagnosis may help to further describe or categorize a finding in terms of chronicity, spatial distribution, color, etc. In many instances, modifiers are superfluous or redundant (e.g., fibrosis is always chronic); therefore, the use of modifiers should be kept to a minimum. An occasionally important modifier for evaluating paired organs is unilateral; unless specified in this manner, all diagnoses for paired organs are assumed to be bilateral. Other modifiers can be created sparingly as needed by the pathologist.
- (4) **Statistical Analysis.** Histopathology data are analyzed using a recently described method, the Rao-Scott Cochran-Armitage by Slices, or RSCABS (Green et al., 2013). Advantages of using RSCABS as a statistical method for analyzing histopathology data include the ability to account for: 1) experimental designs with multiple replicates, 2) lesion severity scores of individual animals in addition to group-wise lesion prevalence, and 3) dose-response relationships. Additionally, the RSCABS test is easy to perform and interpret.
- (5) **Data Interpretation.** Once the microscopic examinations have been completed and statistical analyses have been performed on the resulting data, the pathologist interprets the histopathologic findings. The initial task is to determine which, if any, of the recorded findings are related to administration of the test article, and which are not. The goal is to classify each type of recorded finding (i.e., diagnosis) into one of three categories: 1) **Treatment-related**, 2) **Potentially treatment-related**, and 3) **Non-treatment-related**. Criteria for these determinations are listed below.
  - (i) **Determining Relationship to Treatment.** A weight-of-evidence (WOE) approach is used to determine if a particular finding should be considered treatment-related. Such evidence may include any or all of the following as available:
    - a. Differences between groups of control and treated animals in terms of lesion prevalence and severity, utilizing

statistical analytical results to test for significance as warranted.

- b. Ancillary data from the current study, involving information such as behavioral observations, liver and body weights, genotypic sex, time-to-metamorphosis, and age at sacrifice.
- c. Results from other submitted or pending agency studies.
- d. The at-large scientific literature, giving greater weight to studies in which the quality of the research can be established and is considered superior.
- e. Overall biological, physiological, and toxicological plausibility.

Findings that are considered potentially treatment-related may be those that have borderline statistical significance, or those in which the relationship to treatment is considered equivocal for other reasons (e.g., lack of corroborating evidence from other sacrifices or other studies, biological or toxicological implausibility, or commonality of the diagnosis as a background finding).

There are several points to be made regarding the determination of treatment-relatedness. First, it is possible for a finding to be treatment-related but not be caused by the test chemical. This can include situations in which group-wise differences may be associated with an uncontrolled (and possibly unrecognized) variable involving conduct of the in-life assay, specimen preparation, or some other non-systemic bias. Second, not all statistically significant differences are real, as a p-value significance level of 0.05 allows for the probability that in 5% of cases the result occurred by chance. Third, a finding may be statistically significant and not necessarily biologically or toxicologically important. Fourth, in some instances, treatment-related findings may not be statistically significant. For example, this can occur when treatment induces a low frequency of a lesion type that rarely occurs spontaneously.

- (ii) **Determining Relationship to Endocrine Disruption.** A similar weight-of-evidence (WOE) approach can be used to determine if a particular finding is likely to be endocrine-related; however, in this case the WOE will more heavily depend on ancillary data, results of other assays, and the published literature, including mechanistic studies where available.

(6) **Report Format.** The pathologist is responsible for deliverables that include: 1) Pathology Narrative Report, 2) Spreadsheet with recorded data, and 3) TIFF image files of figures.

- (i) **Pathology Narrative.** Each histopathology narrative report should contain at least the first five of the following sections: Introduction, Materials and Methods, Results, Discussion, Summary / Conclusions, References, Tables, and Figures. The ***Introduction*** section briefly outlines the

experimental design. The **Materials and Methods** section briefly describes procedures used during the slide preparation and examination phases of the study. If specific severity grading criteria were created for a particular finding, they should also be listed in this section. The **Results** section should report findings that are: 1) treatment-related; 2) potentially treatment-related; and 3) non-treatment-related findings that are novel or unusual. Detailed histomorphologic descriptions need only be included for findings that differ substantially from diagnoses presented the Histopathology Atlas. It is intended that the Results section should be as objective as possible (i.e., opinions and hypotheses should be reserved for the Discussion section). The **Discussion** section, which contains subjective information, should address relevant findings that were reported in the Results section. Opinions and hypotheses can be included in this section, preferably backed by references from peer-reviewed sources, but unsupported speculation should be avoided. The **Summary / Conclusions** section should encapsulate the most important information from the Results and Discussion sections. The **References** section should include only material that is cited specifically in the narrative report. A separate **Tables** section may not be necessary if tables are embedded in the Results section. The **Figures** section should include photomicrographic examples of treatment-related findings, plus unusual or noteworthy lesions. The Figures section should include normal tissues for comparison, and digital images should be taken at magnifications that clearly illustrate the salient features of the findings. Figures embedded in the narrative should be in a universally readable compressed file format such as JPEG.

- (ii) **Spreadsheet.** In addition to the recorded histopathology findings, the completed spreadsheet should indicate the animals from which figure images were photographed, and the number of images obtained per photographed organism.
  - (iii) **Figures.** A complete set of unembedded and unannotated photomicrographic figures should be submitted electronically on portable media as uncompressed TIFF files.
- (7) **Pathology Peer Review.** Following the initial slide evaluation and creation of a draft report by the study pathologist (SP), it is encouraged that at least a subset of the original histologic sections be assessed by a second reviewing pathologist (RP). Known as pathology peer review, the purpose of this exercise is to increase confidence in the histopathology data by ensuring diagnostic accuracy and consistency. Commonly, this procedure involves the targeted examination of one or more tissue types in which treatment-related findings were initially detected (this helps to guard against false positive results), plus all tissues from a randomly selected percentage (e.g., 10-20%) of animals from the control and high-dose groups (this helps guard against false negatives). The RP is tasked with determining the accuracy and consistency of diagnostic criteria, diagnostic terminology, severity grading, and the interpretation of findings. The peer review can be performed in-house or (preferably) by an external pathologist, and frequently the reviewing pathologist has at least equal or greater expertise than the SP. Following the peer review, the SP and RP typically meet to resolve

diagnostic differences. In unusual cases in which such differences cannot be resolved, a panel of experts (Pathology Working Group) may be convened to determine the final diagnoses. In addition to enhancing confidence in the histopathology results, benefits of peer review may include decreased inter-laboratory variability, and cross-training of pathologists (i.e., the initial study pathologist may not always need to be an avian expert). Recommended procedures for conducting pathology peer reviews have been described elsewhere (Morton et al., 2010; The Society of Toxicologic Pathologists, 1991; The Society of Toxicologic Pathologists, 1997).

### (8) Summary of Diagnostic Criteria for Selected Histopathologic Findings.

Table 1 lists criteria for selected findings according to tissue type.

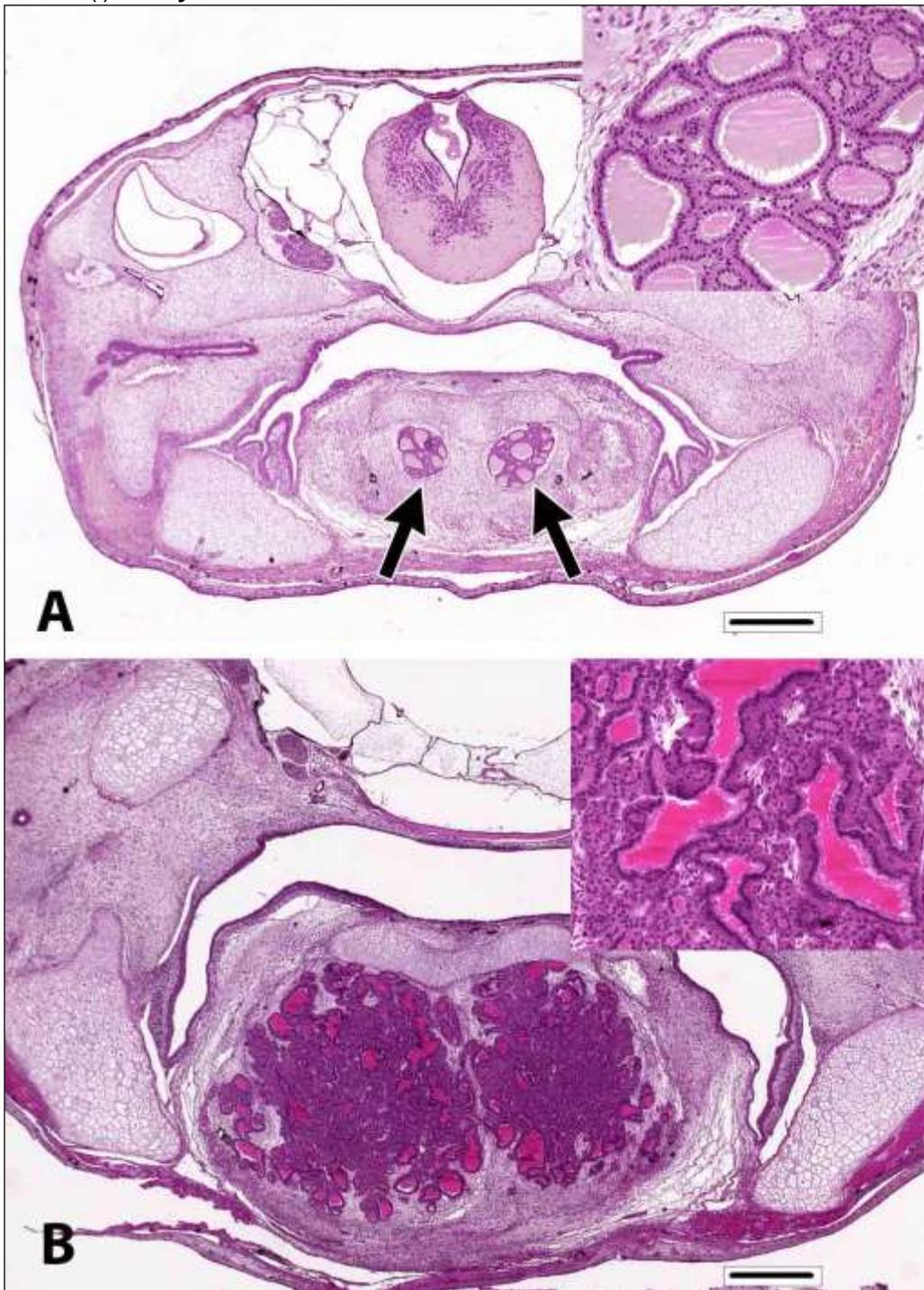
Table 1. Diagnostic Criteria for Potential Histopathological Findings in LAGDA Studies		
Thyroid	Criteria for thyroid gland assessment are available in Grim et al., 2009.	
Liver	Decreased (Glycogen) Vacuolation	1 Hepatocellular vacuolation is decreased slightly relative to controls, but at least 75% of the cytoplasm is vacuolated.
		2 50-75% of the cytoplasm is vacuolated.
		3 25-50% of the cytoplasm is vacuolated.
		4 Less than 25% of the cytoplasm is vacuolated.
Gonad	Testis Stage	1 Undifferentiated gonad
		2 Individual primary spermatogonia and undifferentiated somatic cells populate the medullary region
		3 Seminiferous tubules with primary spermatogonia and cysts of secondary spermatogonia
		4 Primary spermatocytes with rete testis formation; may have occasional spermatocysts that contain round or elongated spermatids
		5 All stages of spermatogenesis evident
	Tubule Development Score	1 Less than 50% of seminiferous tubules have visible lumens
		2 50% or greater seminiferous tubules have visible lumens
	Ovary Stage	1 Undifferentiated gonad
		2 Gonad identifiable as an ovary based on the presence of a discontinuously open lumen lined with epithelial cells; germ cells within the cortex consist of primary oogonia, cysts of primary mitotic oogonia, secondary oogonia, and very early meiotic oocytes
		3 First appearance of diplotene oocytes in cortex; the most prevalent germ cell types at this stage are cysts of secondary oogonia and cysts of leptotene-pachytene primary meiocytes
		4 Pre-vitellogenic (Dumont Stage I) diplotene oocytes are the most prevalent germ cell type observed by area and absolute cell counts; the central lumen is proportionately smaller while the whole ovary grows greatly in size and volume due to the growth of the oocytes; cysts in earlier stages of oogenesis become fewer in number and are located along the periphery of ovary
		5 Ovary consists almost entirely of vitellogenic oocytes (Dumont Stage IV); pre-vitellogenic diplotene oocytes can be found along the periphery of the ovary and germ patches of primary and secondary oogonia are difficult to locate
	Gonad Phenotype Score	0 Gonad phenotype matches genotype
		1 Gonad phenotype primarily matches genotype, with a relatively smaller portion of opposite sex gonad tissue
		2 Gonad is approximately equal parts testis and ovary
		3 Gonad phenotype primarily matches opposite genotype, with a relatively smaller portion of genotypic sex gonad tissue
	Germ Cell Degeneration	4 Gonad phenotype matches opposite genotype (sex reversed)
		1 A single degenerating germ cell to less than three clusters of degenerating germ cells per gonad
		2 Three or more clusters of degenerating germ cells, but less than 25% of gonad affected
		3 Gonad consists of greater than 25% but less than 50% of degenerating germ cells
Mononuclear Cell Infiltrates	4 Gonad consists of 50% or more of degenerating germ cells	
	1 Small, focal infiltrates in one gonad	
	2 Small focal infiltrates in both gonads, or large area of infiltrates in one gonad	
	3 Large areas of infiltrates in both gonads	
Oviduct	Oviduct Stage	4 Gonads contain greater than 50% of infiltrates by area
		1 The oviduct is either a fibrous connective tissue tag, or is essentially non-existent
		2 The oviduct has an epithelial cell lining (1-3 cell layers), and is comparable in size to the Wolffian duct
		3 The oviduct has convoluted, multilayered epithelial cell lining, and is substantially larger (1.5x or greater) than the size of the Wolffian duct
		4 The oviduct dwarfs the size of the Wolffian duct. Epithelial cells have formed large, basophilic glandular structures

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Wolffian Duct	Wolffian Duct Stage	1	Epithelial lining of Wolffian duct is completely or focally less than two cell layers thick
		2	Epithelial lining of Wolffian duct is two cell layers thick or greater, but clear apical portion of cell (if present) is less than half the cell height
		3	Epithelial lining of Wolffian duct is two cell layers thick or greater, and clear apical portion of the columnar cells is at least half the cell height but less than 2/3 of the cell height
		4	The clear apical portion of the columnar cells represents 2/3 or greater of the total cell height
Kidney	Mineralization	1	Less than three small foci of mineralization per kidney
		2	Three or more small foci of mineralization per kidney, or less than three large foci
		3	Three or more large foci of mineralization per kidney
		4	Mineralized deposits in 50% or more tubules
	Tubule Dilatation	1	Minimal to mild dilatation of less than three tubule clusters
		2	Mild to moderate dilatation of three or more tubule clusters
		3	Moderate dilatation affecting 50% or more tubules
		4	Massive dilatation of one or more tubules

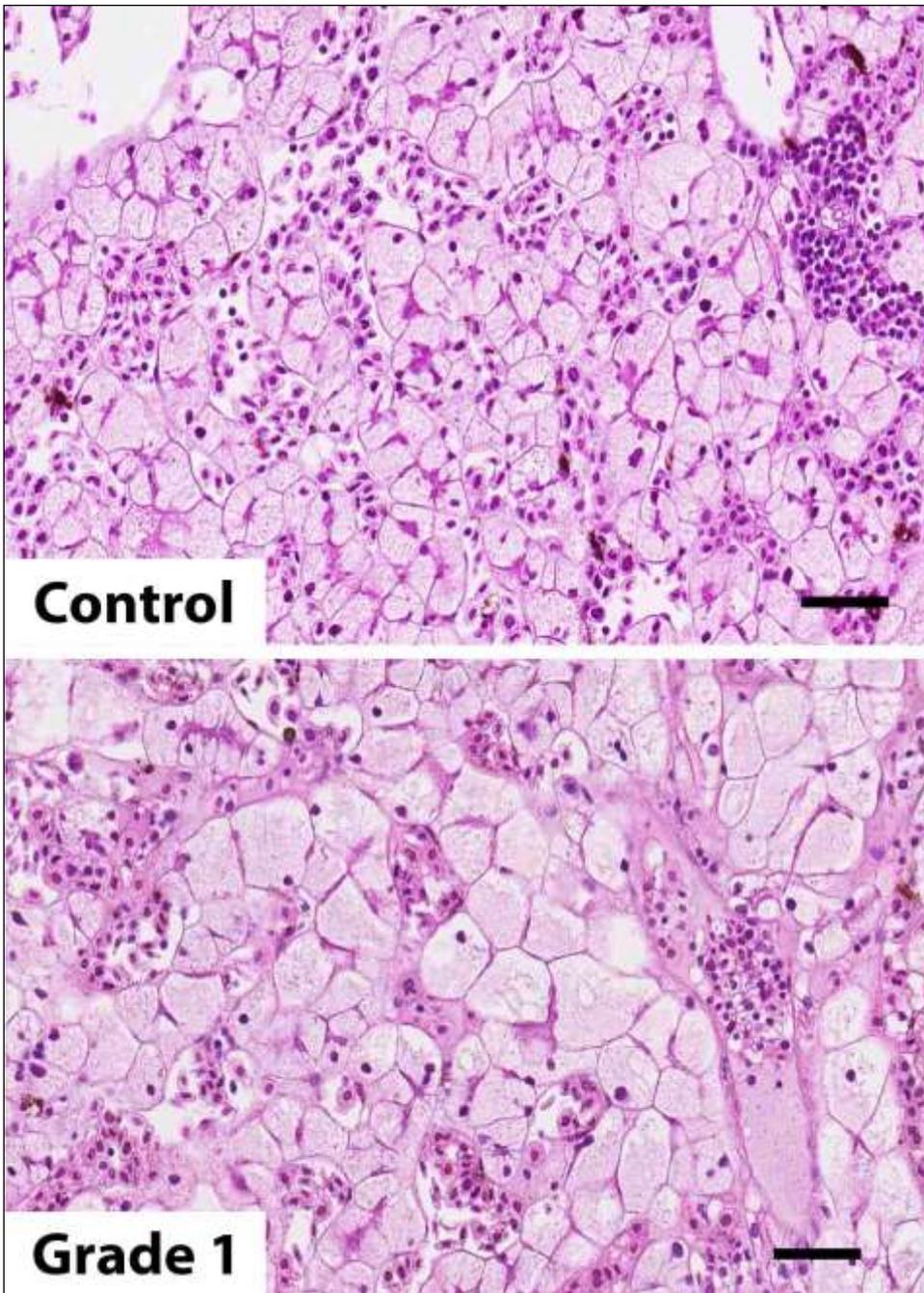
- 9) **Atlas of Histopathologic Findings.** The purposes of this section are: 1) to provide a common technical “language” and 2) to create a reference atlas of both normal microanatomical structures and potential pathological findings. Listed first by organ type and then alphabetically are a number of terms followed by working definitions or descriptions, and representative photomicrographic examples. The information in this section is derived from a number of sources including scientific articles, conference proceedings, related guidelines, toxicologic pathology textbooks, medical dictionaries, and the personal experience of various pathologists.

(i) **Thyroid Glands**

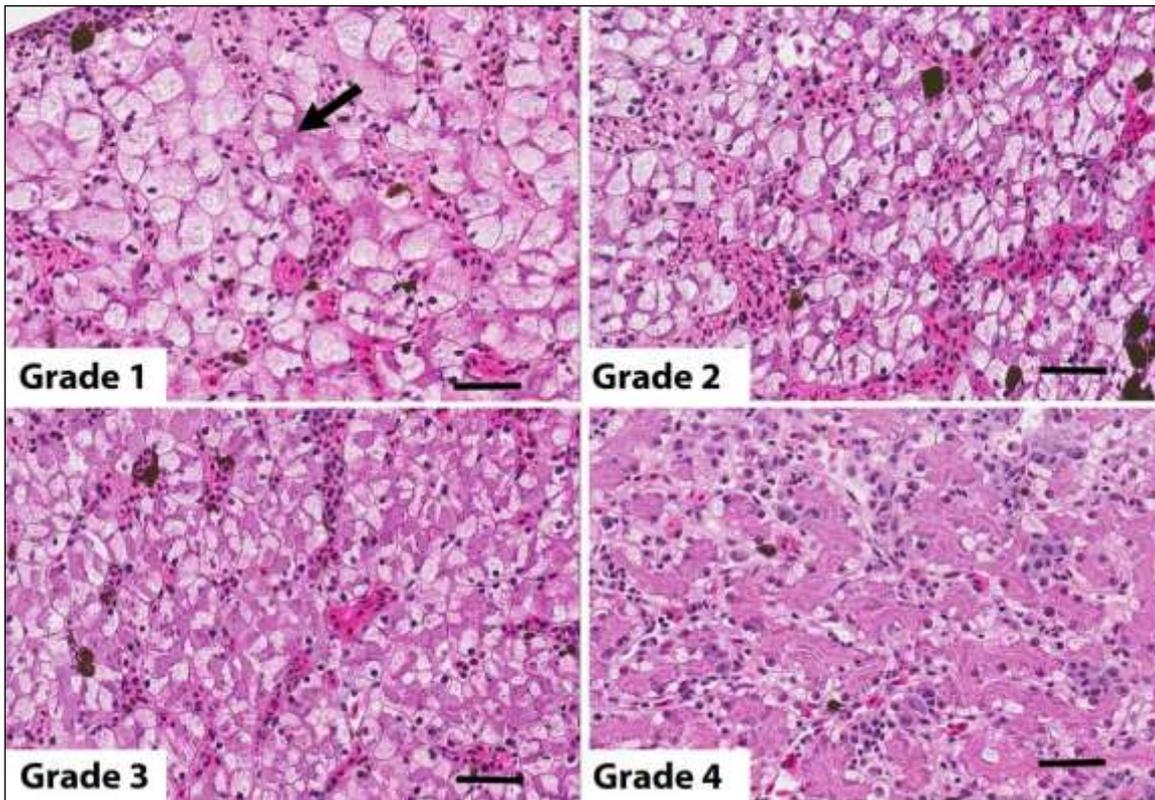


**Normal versus Hypertrophic Thyroid Glands.** A. Transverse section through the neck region to demonstrate thyroid glands (arrows and inset) in control frog. B. Severely hypertrophic thyroid glands. Morphologic criteria for the histopathologic evaluation of the thyroid glands are described and illustrated in Grim et al., 2009. Bar = 500 microns.

## (ii) Liver

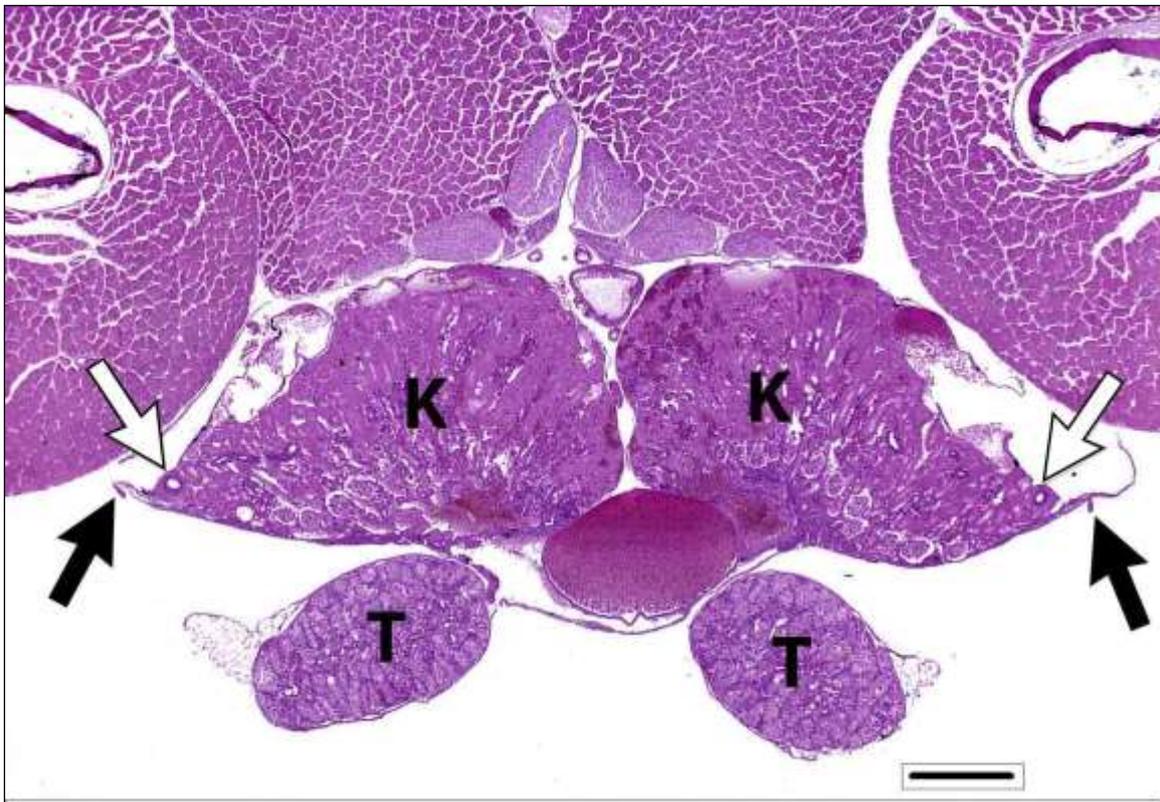


**Hepatocellular Vacuolation, Increased.** Relative to the control liver, affected hepatocytes in the treated frog liver contain greater amounts of pale granular cytoplasm, the contents of which are morphologically consistent with glycogen. Alterations in cell size can only be made by careful comparison of the treated frog livers to those of the concurrent controls. Bar = 50 microns.

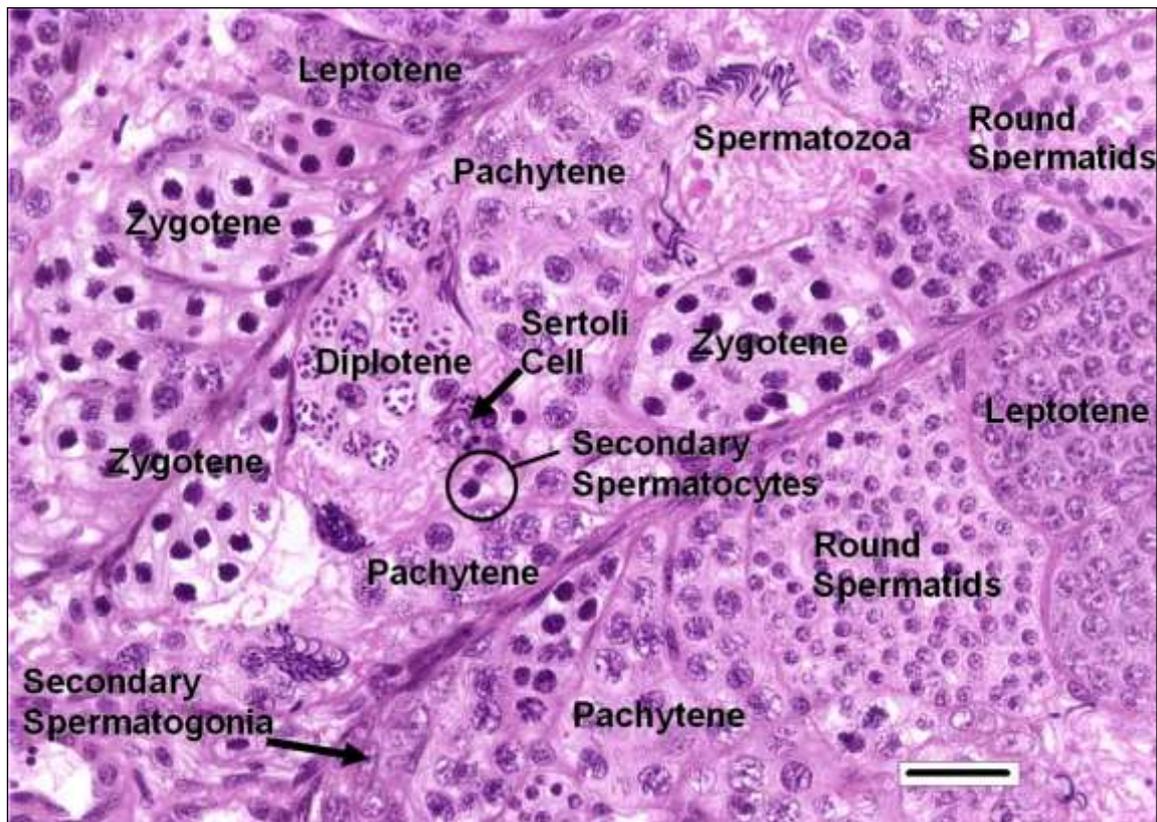


**Hepatocellular Vacuolation, Decreased.** Relative to the livers of control frogs (see previous figure), affected hepatocytes contain lesser amounts of cytoplasmic glycogen. This loss of glycogen vacuolation tends to occur preferentially in the basal (avascular) region of the cells, in the area adjacent to the biliary canals of Hering (arrow). As vacuolation decreases, the liver progressively acquires a more basophilic overall appearance. Bar = 50 microns.

## (iii) Gonads



**Normal Testes, Oviducts and Wolffian Ducts.** The testes (T) are attached to the peritoneal surface of the kidneys (K) by suspensory ligaments. The black arrows indicate the oviduct (Müllerian duct) remnants (Stage 1 oviducts in this case), whereas the white arrows indicate the Wolffian ducts. Bar = 500 microns.



**Normal Testis, Spermatogenic Stages.** Germ cell maturation occurs as follows: primary spermatogonia → secondary spermatogonia → primary spermatocytes → secondary spermatocytes → round spermatids → elongated spermatids → spermatozoa. The majority of visible spermatocytes, which are primary spermatocytes, represent only prophase I of meiosis. These include leptotene → zygotene → pachytene → diplotene phases. Secondary spermatocytes, which are haploid representatives of meiosis II, are comparatively rare. Sertoli cells are sparse, and those that are visible are usually surrounded by elongating spermatids. Interstitial areas contain low numbers of Leydig cells (not indicated). Bar = 25 microns.