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GUIDANCE DOCUMENT ON AMPHIBIAN THYROID HISTOLOGY

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No. 82

GUIDANCE DOCUMENT ON AMPHIBIAN THYROID HISTOLOGY

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This publication was produced within the framework of the Inter-Organisation Programme for the Sound Management of Chemicals (IOMC).

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The participating organisations are FAO, ILO, OECD, UNEP, UNIDO, UNITAR and WHO. The World Bank and UNDP are observers. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

This publication is available electronically, at no charge.

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FOREWORD

This document was prepared in addition to the validation of the amphibian metamorphosis assay for the development of an OECD Test Guideline, to assist those involved in the conduct of the amphibian metamorphosis assay for the detection of thyroid-active substances. The amphibian metamorphosis assay was included in the OECD conceptual framework for the testing and assessment of endocrine disrupting chemicals for the detection of thyroid active substances in aquatic vertebrates.

The guidance material is meant to support technicians and pathologists involved in the preparation and evaluation of biological samples. To keep the document of reasonable size for the download from the OECD website, the document is split in two parts:

Part 1: Technical guidance for morphologic sampling and histological preparation

Part 2: Approach to reading studies, diagnostic criteria, severity grading, and atlas

Scientists from the United States Environmental Protection Agency took the lead in the preparation of the document. A Consultation Meeting was organised in January 2006 in Washington to discuss the draft guidance. Several expert pathologists from various OECD countries contributed to the development of the document between 2005 and 2007, in parallel of the validation studies, which helped enrich the document based on experience.

The document was circulated for comments to the Task Force on Endocrine Disrupters Testing and Assessment and to the Working Group of National Coordinators of the Test Guidelines Programme (WNT) in 2006-2007, and it was approved by the 19th Meeting of the WNT in April 2007.

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

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Guidance Document on Amphibian Thyroid Histology Part 1: Technical guidance for morphologic sampling and histological preparation

Prepared by: Christiana Grim, OSCP/EPA, USA, May 16, 2007

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Overall Objectives:

The goals of this document are to provide guidance on the collection of morphologic data and preparation of thyroid tissues, from *Xenopus laevis*, for histopathological analysis to fulfill the needs of the Amphibian Metamorphosis Assay. This document draws from the guidance developed for the "Histopathology guidelines for Phase 1B of the OECD Fish Screening Assay for EDC's" and the guidance given in the general protocol for the Phase 2 Amphibian Metamorphosis Assay. The recommended procedures were derived from pathologists experienced with preparing and analyzing thyroid tissues of *Xenopus laevis*, from previous documents relating to histotechniques associated with preparing amphibian tissues, and from the scientific literature. The procedures are to guide the handling of animals and preparation of tissues for thyroid histology. These methods may not apply to studies that involve other amphibian species, at earlier or later life stages, under alternate treatment durations, or for different desired endpoints.

This initial guidance document is divided into two sections: 1. Humane sacrifice and necropsy procedures and 2. Tissue preparation and histotechniques. Please see "Guidance Document on Amphibian Thyroid Histology Part 2: Approach to reading studies, diagnostic criteria, severity grading, and atlas" for information regarding approach to reading studies, evaluation of histopathologic diagnostic criteria, terminology and data recording.

I. Humane Sacrifice Procedures

The purpose of this section is to outline steps and procedures involved in humane sacrifice of *Xenopus laevis* tadpoles, necropsy technique, and fixation of tissues.

Objectives:

1. Humanely euthanize tadpoles
2. Obtain body weights, developmental stage and evaluate gross morphology
3. Acquire photodocumentation to determine hind limb lengths, whole body lengths, and snout-vent lengths
4. Provide for adequate fixation of the thyroids and the carcass

This section include:

A. Humane euthanasia of *Xenopus laevis* tadpoles
B. Tissue fixation and decapitation procedures

Recommended Materials:

1. Tadpole transport container (~500 ml, containing dilution water from the system reservoir).
2. Small mesh dip net.
3. Euthanasia chamber (~500 ml vessel).
4. Euthanasia solution (200 mg/L tricaine methanesulfonate (MS-222) appropriately buffered with sodium bicarbonate) (See Appendix 1).
5. Razor blade
6. Electronic analytical balance (minimum display: ≤ 0.1mg) and tarred vessels.
7. Davidson's fixative (See Appendix 1)
8. 10% neutral buffered formalin (See Appendix 1)
9. Appropriately sized pre-labeled plastic tissue cassettes (one per tadpole).
10. Fixation containers (15-20 mL, one per tadpole).

A. Humane euthanasia using MS-222 of *Xenopus laevis* tadpoles

Procedures:

1. Tadpoles should be sacrificed within one to two minutes prior to fixation. Therefore, unless multiple prosectors are available, numerous tadpoles should not be sacrificed simultaneously.
2. Using the mesh dip net, a tadpole is removed from the experimental chamber and transported to the necropsy area in the transport container.
3. The tadpole is placed into the euthanasia solution. The tadpole is removed from the solution when it is unresponsive to external stimuli.
4. Place the tadpole in a Petri dish containing euthanasia solution.
5. Determine the developmental stage according to Nieuwkoop and Faber (1994). *A single person should conduct all developmental stage determinations in a study to avoid inter-individual differences.
6. At this time, digital documentation is to be performed and notes made on any gross morphologic abnormalities. (Please see Appendix 2 for photo documentation details)

7. After digital documentation, blot the tadpole dry, weigh to the nearest milligram and measure whole body length and snout-vent length according to the protocol.
8. At this time, tadpoles that will be used for histological analysis are to be fixed.

B. Tissue fixation and decapitation procedures

It is recommended that tadpoles be fixed prior to decapitation. The advantage of decapitating the tadpole post-fixation is that the tissue becomes more rigid, and the decapitation can be done cleanly to facilitate sectioning. In addition, it will save time during the final sacrifice because decapitation can be performed at a later date. Finally, preliminary evidence suggests that complete fixation is obtained when the tadpole is submerged into Davidson's solution intact.

Procedures:

1. Euthanized whole tadpoles that are to be preserved for histology (approximately 50/chemical) are placed into individually labelled containers (50 mL tubes) with Davidson's fixative. The volume of fixative in the container should be at least 10 times the approximated volume of the tissues.
2. All tissues remain in Davidson's fixative for at least 48 hours, but no longer than 96 hours at which time they are rinsed in tap water and stored in 10% NBF.
3. Once fixed, the tadpole is placed in ventral recumbency and decapitated in a plane perpendicular to the caudal-rostral axis. To ensure that the decapitated tissue contains the thyroid gland, transect the carcass just rostral to the heart using a razor blade.

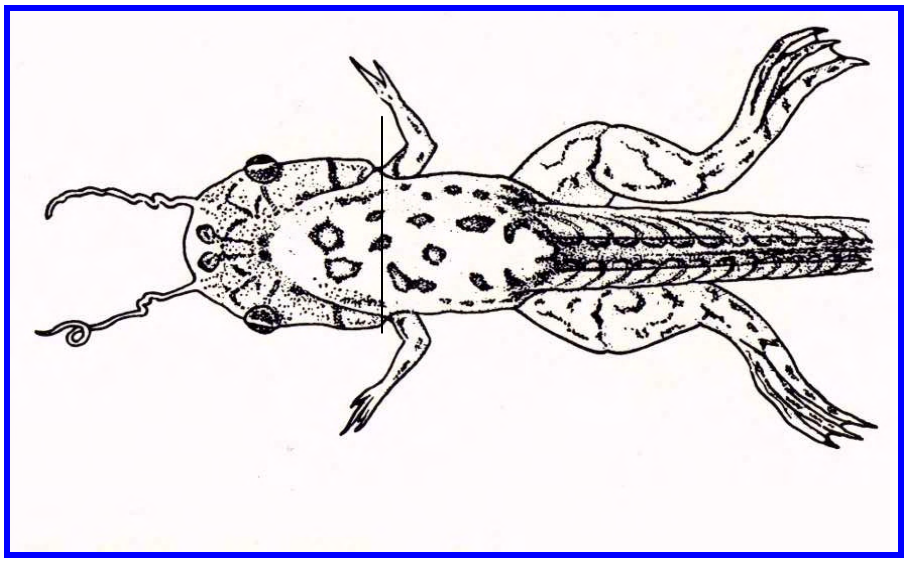


Figure 1. Site of decapitation.

4. Decapitated head tissue samples containing the lower jaw are placed in pre-labeled plastic cassettes and transferred to individual containers of 10% neutral buffered formalin [4% formaldehyde (NBF)]. Gently agitate the NBF for 5 seconds to dislodge any air bubbles that might adhere to the cassettes.

5. Place the remaining carcass into the container of NBF with the corresponding head tissue to facilitate tracking histological observations with developmental stage and hind limb data.

II. Histological preparation of tissues

The purpose of this section is to outline steps and procedures involved in histological preparation of tissues obtained from *Xenopus laevis* tadpoles.

Objectives:

1. Describe methods for trimming tissue
2. Describe tissue dehydration and embedding
3. Describe methods for microtomy

This section includes:

A. Tissue Trimming
B. Tissue Dehydration and Embedding
C. Microtomy
D. Staining, Coverslipping and Slide Labeling

A. Tissue Trimming

Either entire head sections, or mandibular sections with adjacent tissue, are acceptable tissues for embedding and microtomy. The tissues can be oriented ventral to dorsal on a horizontal plane in the mold to allow for sectioning of the ventral face first (frontal plane), or with the decapitation site oriented down, to accomplish transverse sectioning.

B. Tissue Dehydration and Embedding

Objectives:

1. Dehydrate tissue to provide for adequate penetration of paraffin.
2. Impregnate the tissue with paraffin to maintain tissue integrity and create a firm surface for microtomy.

Recommended Materials:

1. Tissue processor
2. Processing unit oven
3. Activated charcoal
4. Paraffin (Paraplast®, or equivalent)
5. 10% neutral buffered formalin. (See Appendix)
6. Ethyl alcohol (80%, 90%, 96%)
7. 100% isopropanol
8. Methyl benzoate
9. Embedding station (thermal, dispensing and cryo consoles)
10. Paraffin heating pots
11. Paraffin transfer pots

12. Thermometer
13. Embedding molds
14. Block drawers
15. Forceps
16. Scraper

Procedures:

1. A general dehydration, clearing and embedding procedure is listed below. Changes to this procedure that reflect differences in individual laboratory protocols are acceptable as long as laboratory protocols consistently provide high quality tissue sections. The tissue samples will be embedded in a manner that allows either the ventral surface of the tissues to be microtomed first (frontal sectioning), or the caudal portion of the head/mandibular tissue first (transverse sectioning). A routine *processor* protocol for dehydration and initial embedding is provided below. This protocol can be adapted for manual dehydration, clearing and embedding.

A.	80% EtOH	60 min	room temperature
B.	80% EtOH	60 min	room temperature
C.	90% EtOH	60 min	room temperature
D.	90% EtOH	60 min	room temperature
E.	96% EtOH	60 min	room temperature
F.	96% EtOH	60 min	room temperature
G.	100% isopropanol	60 min	room temperature
H.	100% isopropanol	60 min	room temperature
I.	methyl benzoate	60 min	room temperature
J.	methyl benzoate	12 hours	room temperature
K.	methyl benzoate	3 hours	room temperature
L.	paraffin I	3 hours	57°C (or 30 minutes at 65°C)
M.	paraffin II	12 hours	57°C (or 30 minutes at 65°C)

2. Labeled tissue cassettes are removed from formalin storage and are rinsed in tap water.
3. The cassettes are placed in the processing basket(s) in a single layer. The processing basket is loaded into the tissue processor and the dehydration, clearing and initial embedding processes are executed (A – M).
4. The cryo console of the embedding station is turned on. (Power to the dispensing console and thermal console should remain on at all times.)
5. The basket(s) of cassettes is/are removed from the processor and immersed in the paraffin-filled front chamber of the embedding station thermal console.
6. The first cassette to be embedded is removed from the front chamber of the thermal console. The cassette lid is removed and discarded, and the cassette label is checked against the animal records to resolve potential discrepancies prior to embedding.
7. An appropriately sized embedding mold is selected.
8. The mold is held under the spout of the dispensing console and filled with molten paraffin.
9. The tissues are removed from the base of the cassette and are placed in the molten paraffin in the mold. The tissues are oriented ventral to dorsal on a horizontal plane

in the mold to allow for sectioning of the ventral face first (place the ventral plane down such that it is on the leading edge of the block), or rostral to caudal to allow for transverse sectioning (placing the caudal portion of the head/mandibular tissue down so that it is on the leading edge of the block).

10. The base of the cassette is placed on top of the mold. Additional paraffin is added to cover the bottom of the base.
11. The mold with the cassette base is placed on the cooling plate of the cryo console.
12. After the paraffin has solidified, the block (i.e., the hardened paraffin containing the tissues and the cassette base) is removed from the mold.
13. Steps 3 through l0 are repeated for each cassette to be embedded.

C. Microtomy

Objective:

Create and mount histologic sections for staining.

Materials:

Microtome.
Disposable microtome knives.
Lipshaw Pike® oil (or equivalent lightweight, machine oil).
Temperature-controlled water bath.
Ice.
Microscope slides.
Staining racks.
Permanent slide marking pen.
Forceps.
Fine-tipped paint brush.
Temporary labels.
Slide warmer/oven.

Procedures:

1. The temperature in the water bath is allowed to stabilize so that ribbons cut from the tissue blocks will spread out uniformly on the surface without melting. This temperature assessment is a qualitative judgment made by the microtomist before and during microtomy. A good starting point is approximately 48-49°C.
2. If necessary, a new blade is mounted onto the microtome and the microtome is lubricated with oil.
3. The initial phase of microtomy is termed “facing” the block and is conducted as follows:
    a. The block is placed in the chuck of the microtome.
    b. The chuck is advanced by rotating the microtome wheel and thick sections are cut from the paraffin surface of the block until the knife reaches the embedded tissues. This process is referred to as “rough trimming” of the block.
    c. The section thickness on the microtome is set between 4 -10 microns. The chuck is advanced and multiple sections are cut from the block to remove any

artifacts created on the cut surface of the tissue during rough trimming. This process is termed “fast trimming” of the block.

d. The block is removed from the chuck and placed facedown on ice to soak the tissue (at the discretion of the microtomist).
e. Steps a through d are repeated until all blocks to be microtomed have been faced.
f. If it is determined during facing that any block is not of acceptable quality for microtomy (e.g. evidence of incomplete tissue infiltration), it is returned for re-embedding before proceeding with microtomy.
g. Any extraneous pieces of paraffin are removed from the microtome and workstation periodically during facing and before proceeding with the next phase of microtomy.

4. The next phase of microtomy is final sectioning and mounting of tissue sections on slides. These procedures are conducted as follows:
    h. Macroscopic lesions (if any) that are reported in the records are noted. Care is taken to include any macroscopic lesions in the sections collected during final sectioning.
    i. The block is removed from the ice and placed in the chuck of the microtome.
    j. With the section thickness on the microtome set to 5-7 microns, the chuck is advanced by rotating the microtome wheel. Initially, five step sections (30 microns apart) are taken from each block, and two serial sections of each step are placed on each of five slides. These slides are examined to assure that a sufficient amount of thyroid tissue is present bilaterally in at least two of the step sections. If necessary to meet this criterion, additional step sections can be cut from the block. Sections should ideally be acquired from central portions of the thyroid glands rather than peripheral areas to provide an accurate reflection of thyroid size. Serial sectioning rather than step sectioning may be necessary to acquire central sections of the thyroid glands. Sections are cut from the block until a “ribbon” containing at least two acceptable sections has been produced. As necessary during sectioning, the block may be removed from the chuck, placed on ice to soak the tissue, and replaced in the chuck.
    k. Each ribbon is floated flat on the surface of the water in the water bath. An attempt is made to obtain at least two sections with sufficient amount of thyroid tissue present bilaterally, from 30 microns apart in the ribbon, and which have no wrinkles and have no air bubbles trapped beneath them.
    l. A microscope slide is immersed beneath the best sections in the floating ribbon. The sections are lifted out of the water using the slide. This process is referred to as “mounting” the section on the slide.
5. With a slide-marking pen, the block number from which the slide was produced is recorded on the slide along with a sequential number indicating the order in which the sections were obtained.
6. The slide is placed in a staining rack.
7. The block is removed from the chuck and placed facedown for storage.
8. Steps a through h are repeated for all blocks to be microtomed.

Notes:

To ensure that the thyroid glands have been obtained while sectioning, it may be necessary to examine the sections under a microscope prior to staining. The landmarks that are generally used for transverse sections include the eyes bilaterally and hyoid cartilage in the dorsal third of the section for transverse sectioning, however landmarks for frontal sectioning have yet to be described.

D. Staining, Coverslipping, and Slide Labeling

Objectives:

1. Differential staining of intra- and inter-cellular components of the thyroid glandss to facilitate diagnostic examination by brightfield microscopy.
2. Permanently seal mounted and stained tissues.
3. Permanently identify stained sections in a manner that allows complete traceability.

Materials:

1. Automated slide stainer (optional)
2. Robot coverslipping machine (optional)
3. Orange terpene
4. 0.1% Eosin-Y
5. Hematoxylin
6. Xylene
7. Absolute ethyl alcohol (100% ETOH)
8. ETOH (96%, 90%, 80%, 70%)
9. 100% isopropanol
10. H_2O
11. Coverslipping mountant (Permount or equivalent)
12. Glass coverslips, No. 1, 24 x 50 (or 60) mm
13. Slide flats

Procedures:

1. Staining
    A. Slides are routinely air-dried overnight before staining.
    B. Harris' H&E Staining protocol

a.	orange terpene	3 x 5 min
b.	100% isopropanol	2 x 5 min
c.	96% EtOH	3 x 3 min
d.	90% EtOH	3 x 3 min
e.	80% EtOH	3 x 3 min
f.	70% EtOH	3 x 3 min
g.	H_2O	2 min
h.	hematoxylin	15 min
i.	running H_2O	10 min
j.	0.1% eosin Y	3 min
k.	H_2O	short
l.	70% EtOH	short
m.	80% EtOH	short

n.	90% EtOH	short
o.	96% EtOH	1 min
p.	100% isopropanol	2 x 5 min
q.	orange terpene	3 x 5 min

C. A similar schedule can be adapted for manual staining. Differences in procedures that are reflected in individual laboratory protocols are acceptable as long as laboratory protocols consistently provide well stained sections with adequate contrast.

2. Coverslipping
   A. Coverslips can be applied manually or automatically.
   B. A slide is dipped in xylene, and the excess xylene is gently knocked off the slide.
   C. Approximately 0.1 ml of mounting medium is applied near the end of the slide opposite to the frosted end.
   D. A coverslip is tilted at a shallow angle as it is applied to the slide.
3. Labeling

   Each slide label should contain the following information:

Laboratory name
Species
Specimen No. / Slide No.
Chemical / Treatment group
Date (optional)

Appendix 1: Solutions

Euthanasia Solution:

200 mg/liter MS-222 appropriately buffered with sodium bicarbonate at 0.42 -1.05 g/liter. The goal is a pH of approximately 7.0. The characteristics of the water used to make this solution may impact the pH, therefore no exact recipe exists. However, begin using a recipe of one part MS-222 (150 mg/L of MS-222) plus two parts of sodium bicarbonate (300 mg/L of NaHCO3).

*Unbuffered MS-222 solution is irritating to frog skin and poorly absorbed resulting in a prolonged induction time.

Davidson's Fixative*:*

Formaldehyde (37-40%)	200 ml
Glycerol	100 ml
Glacial acetic acid	100 ml
Absolute alcohol	300 ml
Distilled water	300 ml

10% Neutral Buffered Formalin:

Formalin, full strength (37-40% formaldehyde)	100.0 ml
Sodium phosphate dibasic (anhydrous)	6.5 g
Sodium phosphate monobasic	4.0 g
Distilled water	900 ml

Appendix 2: Digital Documentation

Computer software analysis of digital images has been successfully applied in diverse biological research (Johnson et al., 2001; Machado-Silva et al., 2000; Merris et al., 2003), and is more accurate that the traditional caliper method. The following describes one procedure for acquiring digital photographs for measuring tadpole whole body length, snout-vent length, and hind limb length.

Procedure 1: Stereomicroscope

Note: Please refer to individual laboratory standard operating procedures for more detailed instructions.

Equipment:

1. Stereomicroscope equipped with a digital camera with micro function (e.g. Canon, EOS 10D, 4 mega pixels)
2. Lighting system (e.g. LEICA, CLS 150X)
3. Metric ruler
4. Image analysis software (e.g. Image Pro® Plus, Version 4.0, Media Cybernetics, U.S.A.) 1. Digital camera with micro function (e.g. Canon, EOS 10D, 4 mega pixels)

Procedure:

1. Place tadpole in a central position on the stage of the stereomicroscope in dorsal recumbency.
2. Place a calibration metric ruler and identifying number within the camera view next to the tadpole. Switch on lamps so that it illuminates the tadpole.
3. Two images of each tadpole should be acquired. One should be taken at a sufficiently low magnification to encompass the entire organism in order to document the developmental stage. The second should be taken at a higher magnification to be used in hindlimb measurements. When taking hindlimb photographs and measurements, it is suggested that one limb be analyzed consistently from specimen to specimen (e.g left hind limb only).
4. Adjust the focus prior to each photograph such that the hind limbs and metric scale are in full view.
5. Save the image files electronically. Each image file name should contain the identifying number.
6. Analyze digital images as described below.
7. If desired, lateral-view photographs of sampled tadpoles can also be taken to provide a means to electronically document length measurements. Calibration with a metric ruler as described above is needed for consistency.

Procedure 2: Glass board method

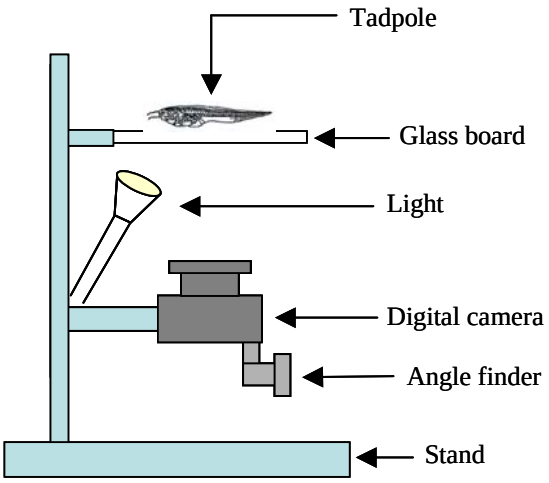
Equipment:

1. Digital camera with micro function (e.g. Canon, EOS 10D, 4 mega pixels)
2. Remote switch (e.g. Canon, RS-80N3)
3. Glass board (15 cm x 15 cm).
4. Lens (e.g. Canon, Macro Photo Lens MP-E 65 mm F2.8 1-5x)
5. Lighting system (e.g. LEICA, CLS 150X)
6. Image analysis software (e.g. Image Pro® Plus, Version 4.0, Media Cybernetics, U.S.A.)
7. Metric ruler

Procedure:

1. Attach the glass board to the stand.
2. Attach the camera to the stand such that the lens faces upward and under the glass board (see Figure 2).
3. Adjust the light under the glass board.
4. Attach the remote switch and the angle finder to the camera (Figure 1).
5. Wet the glass board slightly and place the tadpole carefully on the wet glass such that the ventral side faces the camera lens.
6. Place a calibration metric ruler and identifying number within the camera view next to the tadpole. This identifying number should be included in the image file name.
7. Switch on lamp so that it illuminates the tadpole from below.
8. Two images of each tadpole should be acquired. One should be taken at a sufficiently low magnification to encompass the entire organism in order to document the developmental stage. The second should be taken at a higher magnification to be used in hindlimb measurements.
9. Adjust the focus prior to each photograph such that the hind limbs and metric scale are in full view.
   *If measuring bent hind limbs is difficult, one can hyper-extend the hind limb as long as a deep anesthetic plane has been achieved.
10. Save each image electronically. Include the identifying number in the file name.

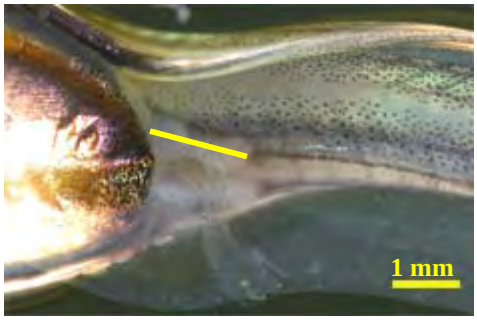
1. **Camera setup**



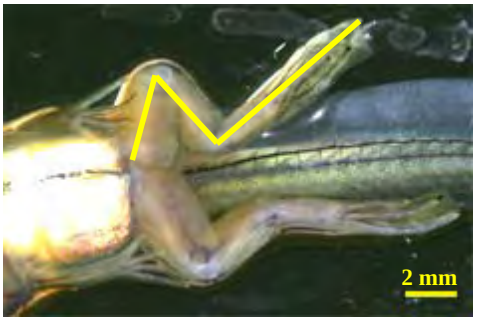
2. **System schematic**

Image analysis

1. Download the images from the camera to a computer. Use appropriate image-analyzing software for analysis of the digital images (e.g. Image-Pro® Plus).
2. Select the best images for measurements of the body length and the hind limb length, respectively.
3. For body length measurements, locate the tip of the snout, the end of the vent and the tip of the tail. Draw two lines, one from the tip of the snout to the end of the vent (snout-vent length) and another from the tip of the snout to the tip of the tail (whole body length).
4. For hind limb length measurements, locate the shining gold-colored part of the abdomen where the limb attaches to the abdomen, and draw a line right to the tip of the limb (Figure 3A).
5. If the limb is bent, draw the line from that part of the abdomen to the knee joint (Figure 3B). Click and create a node at the knee joint, and continue drawing the line all the way to the tip of the limb (Figure 3B).
6. Calculate the limb length accordingly.



3. **Hindlimb measurment – straight limb**



4. **Hindlinb measurement - bent limb**

References:

Johnson, I., Harman, M., Farrow, D. and Norris, M. (2001): An assessment of the feasibility of using image analysis in the oyster embryo-larval development test. Environ. Toxicol. 16, 68-77.

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Merris, M., Wadsworth, W.G., Khamrai, U., Bittman, R., Chtwood, D.J. and Lenard, J. (2003): Sterol effects and sites of sterol accumulation in *Caenorhabditis elegans*: developmental requirement for 4-methyl sterols. J. Lipid Res. 44, 172-181

Nieuwkoop, P.D. & Faber, J. 1994. Normal Table of *Xenopus laevis* (Daud.): A systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis. Garland Publishing, Oxford.

Guidance Document on Amphibian Thyroid Histology Part 2: Approach to reading studies, diagnostic criteria, severity grading, and atlas

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Objective:

The goal of this document is to facilitate the standardization of the histopathologic analysis of thyroid tissues from *Xenopus laevis* to fulfill the needs of the Amphibian Metamorphosis Assay. Standardization of reading practices, diagnostic criteria, severity grading and data reporting and compilation will be addressed in an effort to maximize comparability between pathologists and to reduce bias.

This document draws from the guidance provided for the "Histopathology guidelines for Phase 1B of the OECD Fish Screening Assay for EDC's" and the guidance given in the general protocol for the Phase 2 Amphibian Metamorphosis Assay, from pathologists experienced reading toxicologic pathology studies in amphibians, the scientific literature, and from the OECD Amphibian Metamorphosis Assay thyroid histopathology consultation held in Washington, DC 2006.

This guidance document is divided into four sections:

I. General guidelines for study reading practices

II. Diagnostic criteria and severity grading

III. Reference atlas of normal microanatomy of *X. laevis* thyroid glands and an atlas of core diagnostic criteria with examples of severity grades

IV. Data recording and compilation.

I. General guidelines for reading studies

The purpose of this section is to provide general guidance for the light microscopic evaluation of thyroid gland tissue sections to improve diagnostic consistency among pathologists.

IA. Pathologist qualifications

Studies are to be read by individuals experienced in reading toxicologic pathology studies, and who are familiar with normal *X. laevis* thyroid histology, with thyroid gland physiology, and with general responses of the thyroid gland to agonists or antagonists. 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 thyroid histology and toxicologic pathology. Technicians should not be used to conduct readings due to the subtle nature of some changes and the need for subjective judgments based on past experience.

It is recognized that there is a limited pool of pathologists with the necessary training and experience that are available to read the thyroid histopathology for the amphibian metamorphosis assay. If an individual has toxicological pathology experience and is familiar with thyroid histology in other species, he/she may be trained to read the

amphibian assay. If pathologists with little experience are used to conduct the histopathological analysis, informal peer review may be necessary.

IB. Approach to reading studies

Pathologists are to read the studies non-blinded (i.e. with knowledge of the treatment group status of individual tadpoles). However, it is expected that any potential compound-related findings will be re-evaluated by the pathologist in a blinded manner prior to reporting such findings, when appropriate. Certain diagnostic criteria, such as thyroid gland hypertrophy or atrophy, cannot be read in a blinded manner due to the diagnostic dependence on control thyroid glands. As a rule, treatment groups should be evaluated in the following order: Control, High-dose, Intermediate-dose, and Low-dose.

It is suggested that the pathologists be provided with all available information related to the study prior to conducting their readings. Information regarding developmental stage at sacrifice, 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 [1].

1C. Selection of Histologic Sections for Evaluation

Examination of two sections is sufficient for evaluation of the thyroid lesions that may present in the amphibian metamorphosis assay. These two sections should be selected from a minimum of five sections, they should be selected for maximum cross sectional amount of representative thyroid tissue present, the absence of artifacts in the tissue section, and they should be at least two step sections apart, if possible. If, for some reason, the five sections initially evaluated have prominent, though not consistent changes, up to three sections should be evaluated.

II. Diagnostic Criteria and Severity Grading

The purposes of this section are to provide common technical “language” and to describe an approach to severity grading of lesions.

IIA. Diagnostic Criteria

Histopathology is a descriptive and interpretive science, and therefore somewhat subjective. However, histopathologic evaluations of the same study by any qualified pathologist should identify the same treatment-related findings [1]. Therefore, we aim to define the diagnostic criteria that will likely be encountered during the histopathologic analysis of the amphibian metamorphosis assay.

A consolidated set of diagnostic criteria follow. These criteria are based on pathologists’ experience with the amphibian metamorphosis assay and known changes of the thyroid gland in response to chemical exposure, however novel findings that are

exposure-related shall also be reported. These criteria were not necessarily chosen based on biological significance and it is not implied that other lesions that may be found in the thyroid gland are biologically less significant.

The criteria below have been divided into two sections: 1. Core criteria, and 2. Additional criteria. The core criteria are severity graded on a numerical scale. The additional criteria are either graded on a numerical scale, or are qualitatively described. The additional criteria need only be addressed if they represent exposure-related findings.

Core Criteria:

1. **Thyroid gland hypertrophy/atrophy**: Increases (hypertrophy) or decreases (atrophy) in the overall size of the thyroid gland are consequent of changes in follicular cell size and number. The severity of either hypertrophic or atrophic observations is to be graded on an overall, general appearance of the thyroid gland. Because the diagnosis of hypertrophy or atrophy is dependant on a comparison to controls, it is necessary to establish the normal variability of thyroid gland sizes in control tadpoles prior to making determinations on thyroid gland size in dose groups.

2. **Follicular cell hypertrophy**: Hypertrophic follicular cells, defined as tall columnar cells, are to be graded based on the percentage of the cells exhibiting this feature. It is recognized that follicular cell hypertrophy may present as a generalized lesion and interpreted thus. Because normal amphibian thyroid glands show heterogeneity in follicular cell shape, ranging from squamous to tall columnar, severity is determined by the change in percentage of cells exhibiting tall columnar structure.

3. **Follicular cell hyperplasia**: Follicular cell hyperplasia is diagnosed when there is follicular cell crowding, stratification (multiple layers), or papillary infolding of single or multiple layers of follicular cells. The severity grading scheme for follicular cell hyperplasia is based on the percentage of follicles that exhibit hyperplasia, and/or the percentage of tissue that is affected.

Additional Qualitative Criteria:

1. **Follicular lumen area (previously colloid area)**: Lumenal area can be reduced or increased. Severity of effects on lumenal area is to be graded based on the generic grading scheme. Colloid quantity shall also be considered.

2. **Colloid quality**: Colloid quality is generally considered in association with colloid content decreases. However, changes in colloid quality can exist independent of changes in follicular lumen area. Typical descriptions include homogeneous, heterogeneous, lacy or granular. If present, these findings are to be reported in a narrative format.

3. **Follicular cell height/shape**: Follicular cell height/shape can range from squamous, to cuboidal, to low columnar to high columnar. Increased epithelial cell height (progression from cuboidal to columnar) can lead to glandular hypertrophy. A narrative description of the predominant cell shape (squamous, cuboidal, low columnar, tall columnar) can be provided if necessary to reflect a chemical-dependent change.

IIB. Severity Grading

Severity grading is a method by which a range of variation is assigned to ordinal classes, generally being listed as minimal, mild, moderate and severe [2] and is semi-quantitative. The purpose of severity grading is to provide an efficient, semi-objective mechanism for comparing changes (compound-related effects) among animals, treatment groups, and studies.

The current approach for grading severity incorporates a four score range, including: 0 – non-remarkable, 1-mild, 2-moderate, and 3-severe. This severity grading method has been evaluated and determined to be the best method for discriminating subtle pathologic changes in the thyroid gland.

For the frog metamorphosis studies, quantitative scoring may not be suitable for all criteria. The core criteria (i.e., atrophy/hypertrophy, follicular cell height/shape, and follicular hyperplasia) will most effectively be measured using the severity scoring system. In addition to the severity grade, pathologists can document qualitative changes associated with the lesions. The additional criteria will be documented using descriptive terms only, except for follicular lumen area, which will be documented using the generalized grading scheme. Follicular lumen area need only be documented if the finding is exposure-related.

For thyroid gland hypertrophy and glandular atrophy, the pathologist is to score compound-exposed animals relative to the control animals using the atlas provided and the descriptions below. For follicular cell hypertrophy and hyperplasia, the pathologist is to base severity grades on the percentage of cells affected, and/or the percentage of the tissue which is affected.

Severity grading shall employ the following system:

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

General severity grading scale:

- **Grade 0:** Non-remarkable to minimal. Ranging from inconspicuous to barely noticeable but so minor, small, or infrequent as to warrant no more than the least assignable grade. For multifocal or diffusely-distributed alterations, this grade is used for processes where less than 20% of the tissue in the section is involved.

- **Grade 1: Mild.** A noticeable feature of the tissue. For multifocal or diffusely-distributed alterations, this grade is used for processes where 30-50% of the tissue in the section is involved.

- **Grade 2: Moderate.** A dominant feature of the tissue. For multifocal or diffusely-distributed alterations, this grade is used for processes where 60-80% of the tissue in the section is involved.

- **Grade 3: Severe.** An overwhelming feature of the tissue. For multifocal or diffusely-distributed alterations, this grade is used for processes where greater than 80% of the tissue in the section is involved.

Because the grading system to be used for the amphibian metamorphosis assay is to promote consistency in the histological analysis, proposed descriptions for scoring the various criteria are presented below. These descriptions are to support the photomicrographic examples of each criterion with severity grades, which follow. In addition examples of normal histomorphology of the amphibian thyroid gland during metamorphosis, and examples of common artifacts are presented.

Core Criteria:

Table 1. Severity grading scheme for thyroid gland hypertrophy.

Grade	Descriptor	Criteria
0	Non-remarkable	Less than 20% enlargement of glands in comparison to controls.
1	Mild	Diffuse enlargement of glands that exceeds control glands size by 30-50%.
3	Moderate	Diffuse enlargement of glands that exceeds control glands size by 60-80%.
4	Severe	Diffuse enlargement of glands that exceeds control glands size by over 80%. There is contact of both glands at the midline and they exceed normal boundaries into surrounding tissue space.

Table 2. Severity grading scheme for thyroid gland atrophy.

Grade	Descriptor	Criteria
0	Non-remarkable	Less than a 20% reduction in size in comparison to controls.
1	Mild	Gland size is 30-50% reduced from the size of control glands.
2	Moderate	Gland size is 60-80% reduced from the size of control glands.
3	Severe	Gland size is over 80% reduced from the size of control glands.

Table 3. Severity grading scheme for follicular cell hypertrophy.

Grade	Descriptor	Criteria
0	Non-remarkable	Fewer than 20% of the cells exhibit hypertrophy.
1	Mild	30-50% of follicular cells exhibit hypertrophy.
2	Moderate	60-80% of follicular cells exhibit hypertrophy.
3	Severe	Over 80% of follicular cells exhibit hypertrophy.

Table 4. Severity grading scheme for follicular cell hyperplasia.

Grade	Descriptor	Criteria
0	Non-remarkable	Focal or diffuse crowding of follicular cells affecting less than 20% of the tissue.
1	Mild	Focal or diffuse crowding of follicular cells affecting 30-50% of the tissue, and/or single or multiple papillary infoldings of follicular cell layer.
2	Moderate	60-80% of the follicles exhibit focal hyperplasia characterized by pseudostratified or stratified follicular epithelium – papillary infolding may be present.
3	Severe	Over 80% of follicles exhibit extensive hyperplasia with stratification 2-3 cell layers thick – papillary infolding may be present.

Additional Criteria:

Follicular lumenal area increase or decrease: Use the generic grading scheme presented above to document exposure related changes.

Colloid Quality: Document changes in colloid quality using narrative descriptions. Examples include homogeneous, heterogeneous, lacy or granular. Pathologists may also comment on the tinctorial quality of the colloid.

III. Reference Atlases

Below are photographic examples of normal thyroid histomorphology of *X. laevis* during metamorphosis, examples of the core criteria and assigned severity grades for each lesion, examples of colloid quality changes, some miscellaneous changes that might be encountered while performing histological analyses on the test subjects, and common artifacts that might contribute to interpretive difficulty. Several caveats to these images are as follows:

1. The examples are not all-inclusive, nor complete, and may not be the most representative example of each lesion.
2. Sections are either transverse or frontal, however it is expected that the thyroid changes presented can be accurately applied to multiple sectioning planes.
3. Examples have been obtained from tadpoles in different developmental stages.
4. Some photographs contain several different lesions that are to be scored independently.

IIIA. General normal appearance of *X. laevis* thyroid microanatomy

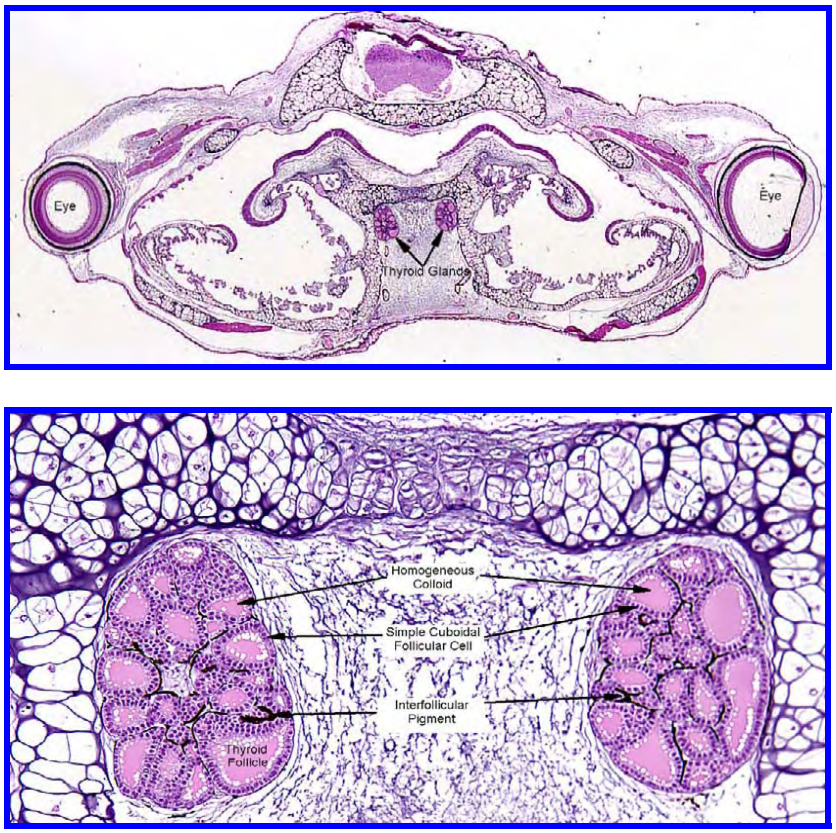


Figure 1. Low (1.25x) and high (10x) magnification photomicrographs of transversally sectioned thyroid glands. The colloid is homogeneous and light eosinophilic, and the follicles are lined by simple cuboidal epithelium.

IIIB. Developmental series of normal *X. laevis* thyroid microanatomy – H&E

Figure 2. 48 hours post-stage 54

Figure 3. 96 hours post-stage 54

Figure 4. 144 hours post-stage 54.

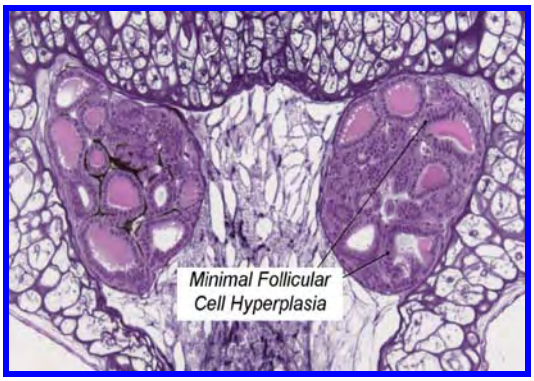


Figure 5. 192 hours post-stage 54

In an effort to establish histologic references for *X. laevis* thyroid glands, a time course experiment was executed. Beginning at stage 54, tadpoles were sequentially sacrificed at 48-, 96-, 144-, and 192-hours post stage 54, as represented above. These images were obtained to demonstrate the normal changes of the amphibian thyroid gland during metamorphosis.

IIIC. Reference atlas of diagnostic criteria and severity grades

IIIC1. Thyroid gland hypertrophy – general range of changes

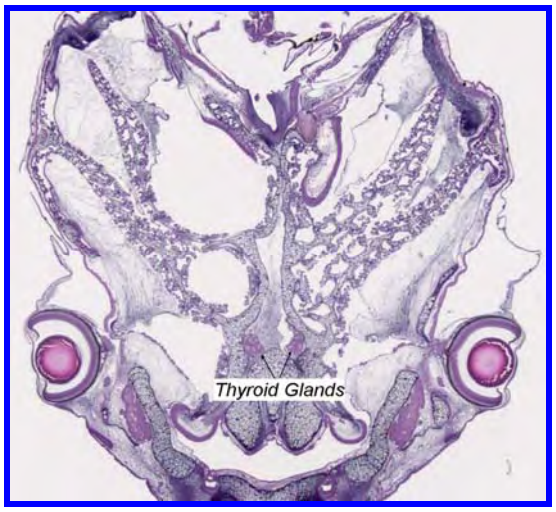


Figure 6. Thyroid hypertrophy - Grade 0

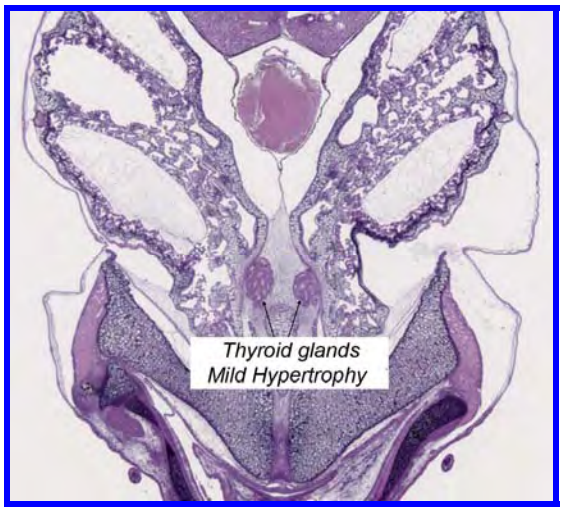


Figure 7. Thyroid hypertrophy – Grade 1

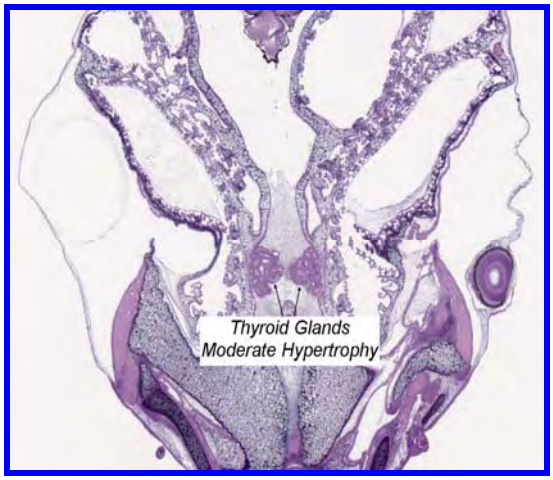


Figure 8. Thyroid hypertrophy – Grade 2

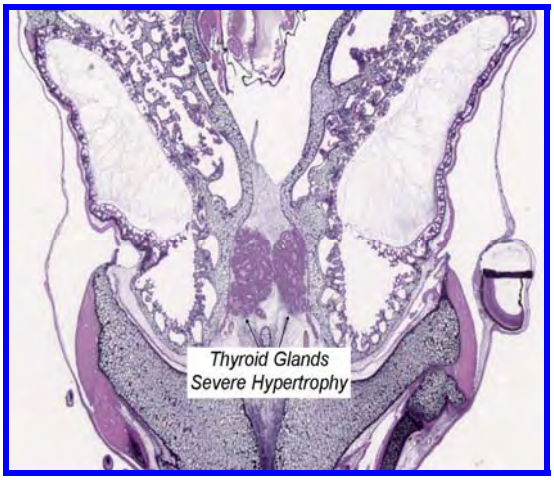


Figure 9. Thyroid hypertrophy – Grade 3

Grade 0 – Thyroid gland hypertrophy:

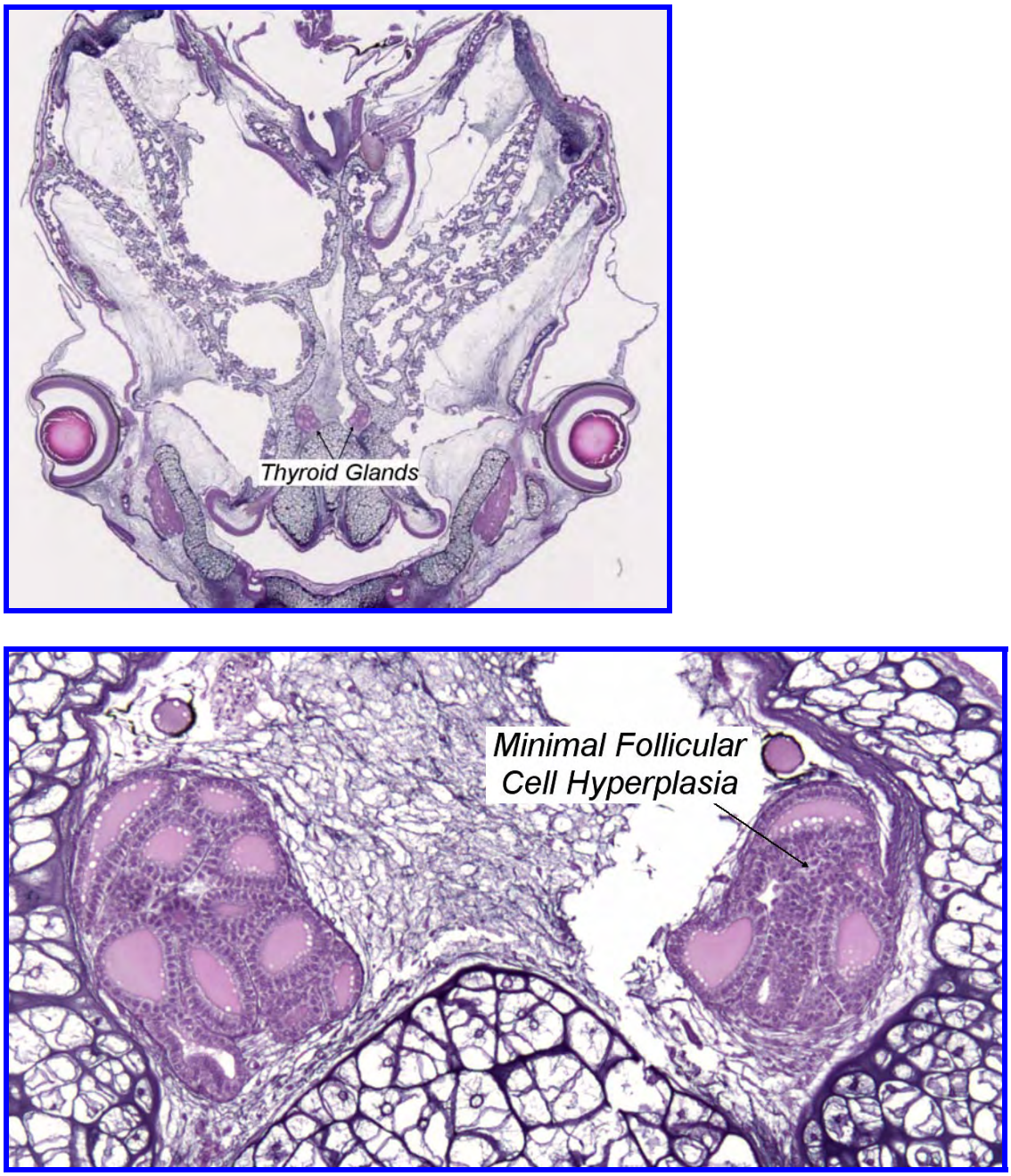
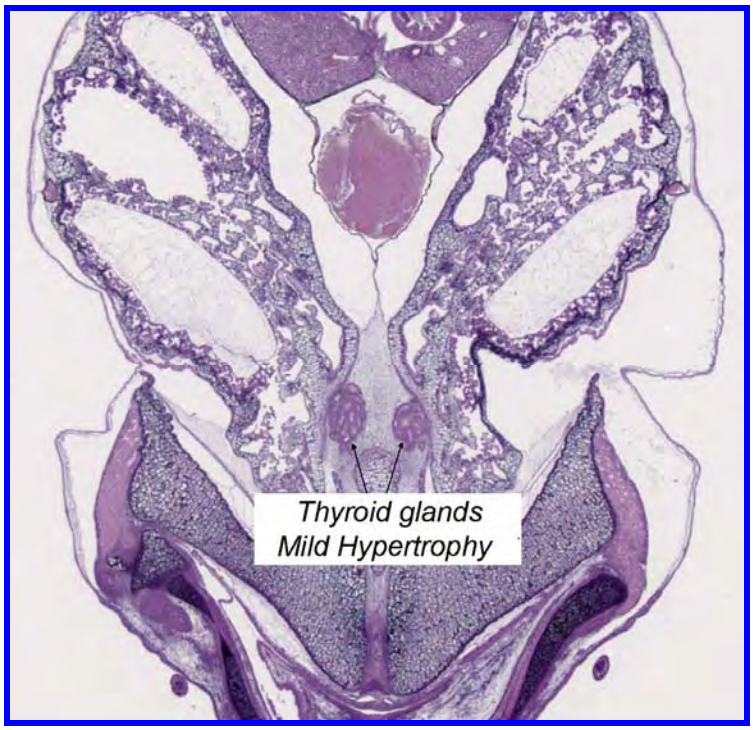


Figure 10. Low (1.25x) and medium (10x) magnification photomicrographs of thyroid glands from a *X. laevis* that was a control animal. The thyroid follicles are colloid-filled and lined by simple cuboidal follicular cells. There is minimal follicular cell hyperplasia.

Grade 1 (Mild) – Thyroid gland hypertrophy:



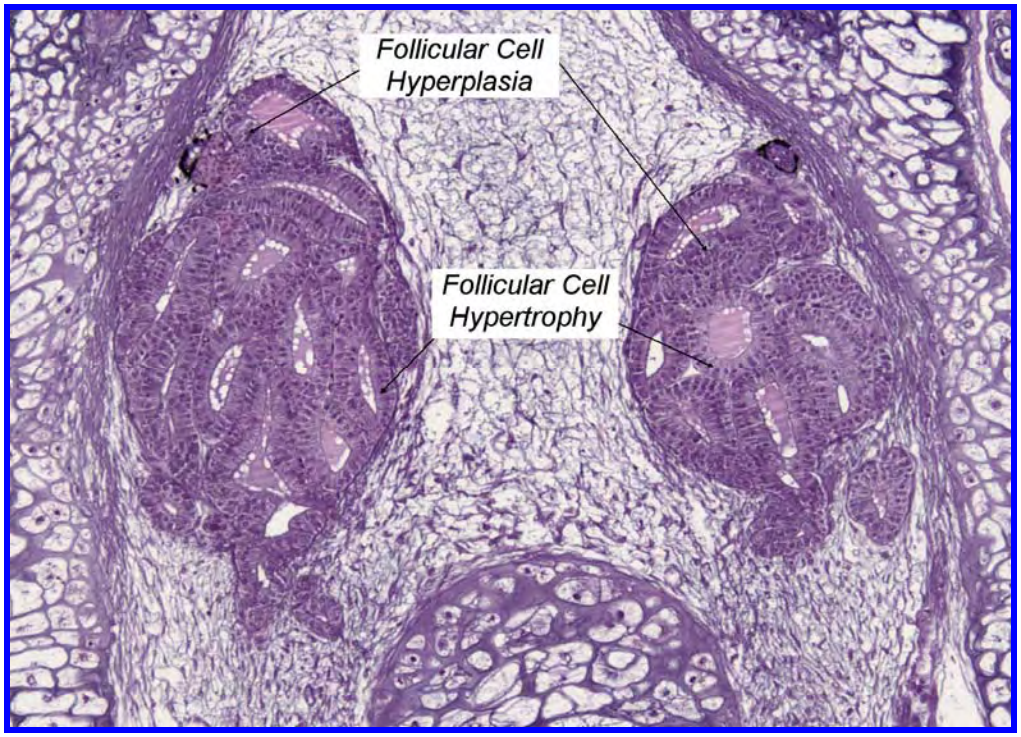


Figure 11. Low (1.25x) and medium (10x) magnification photomicrographs of a *X. laevis* exposed to 62.5 µg/L perchlorate for 21 days. There is moderate hypertrophy of the follicular cells and slight/mild follicular cell hyperplasia. There is mild glandular hypertrophy and there is slight/mild decreased colloid in the thyroid follicles.

Grade 2 (Moderate) – Thyroid gland hypertrophy:

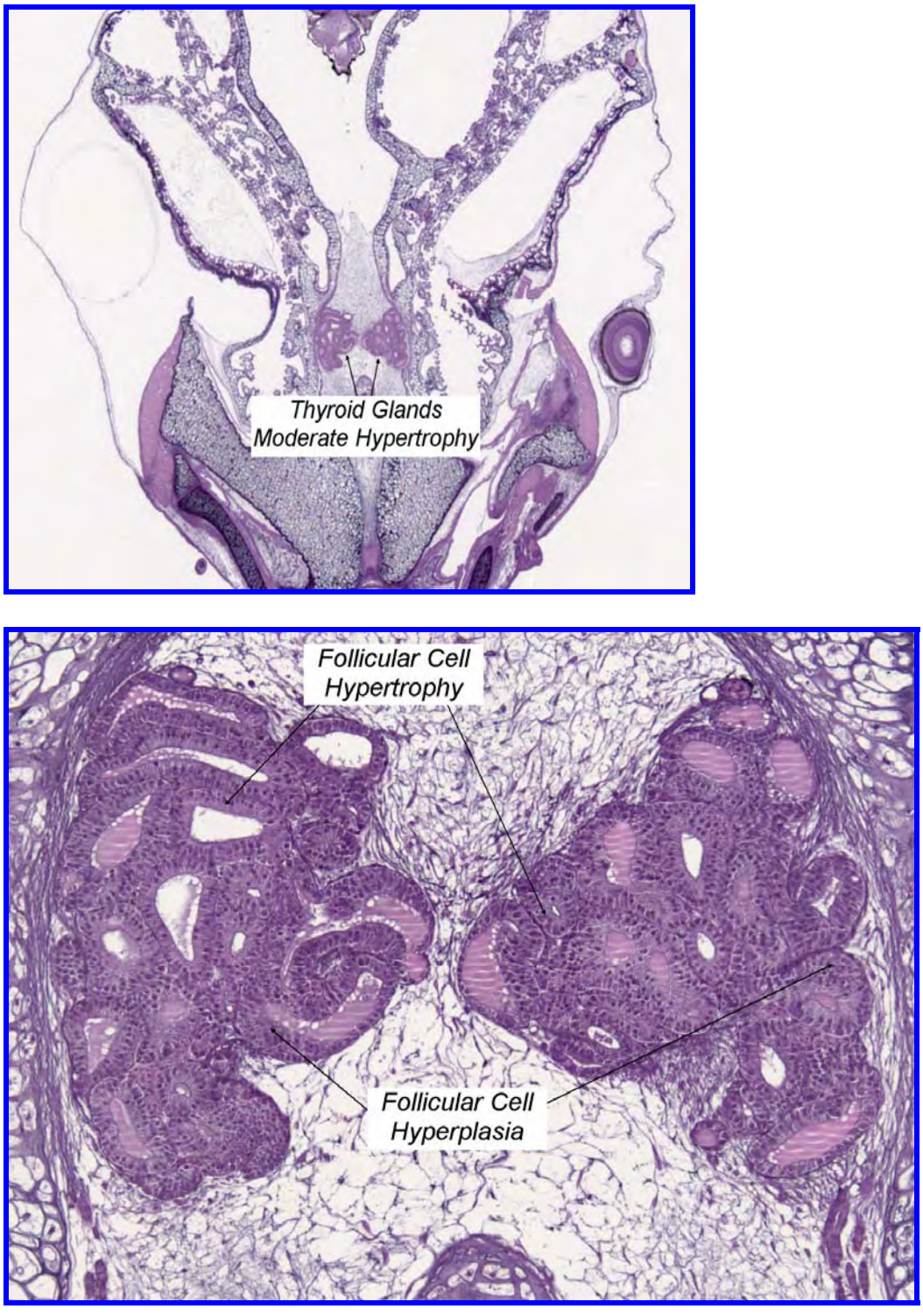


Figure 12. Low (1.25x) and medium (10x) magnification photomicrographs of a *X. laevis* exposed to 62.5 µg/L perchlorate for 21 days. Moderate follicular cell hypertrophy and moderate follicular cell hyperplasia are present. The thyroid glands are moderately enlarged overall and there is slight/mild decreased colloid in the thyroid follicles.

Grade 3 (Severe) – Thyroid gland hypertrophy:

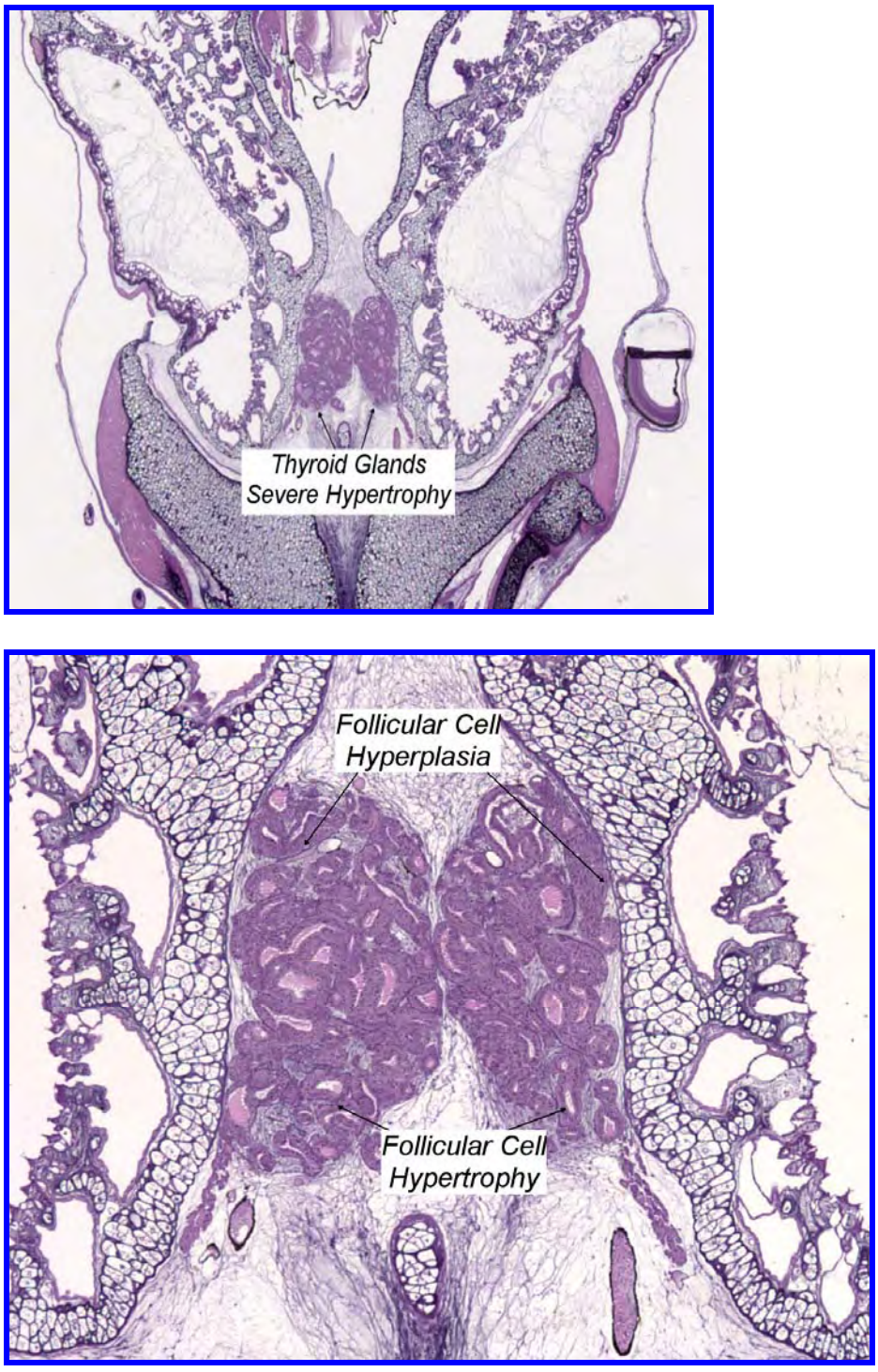


Figure 13. Low (1.25x) and medium (4x) magnification photomicrographs of a *X. laevis* exposed to 250 µg/L perchlorate for 21 days. There is severe hypertrophy of the thyroid glands with contact at the midline. There is moderate follicular cell hypertrophy and there is moderately severe follicular cell hyperplasia. There is slight/mild decreased colloid in the thyroid follicles.

IIIC2. Thyroid gland atrophy - general range of changes

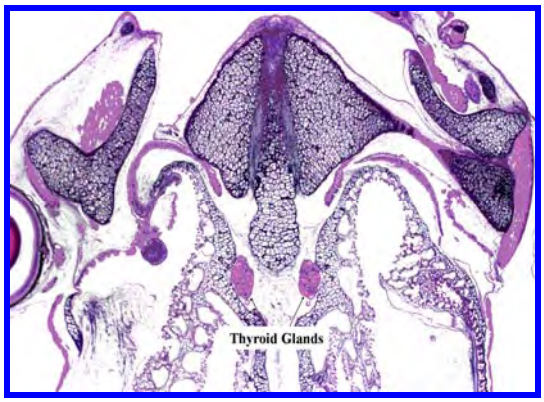


Figure 14. Thyroid atrophy – Grade 0

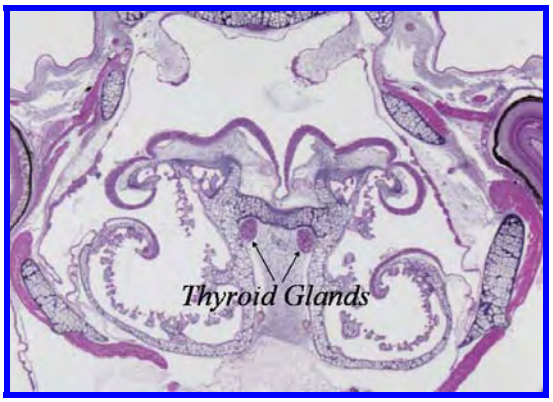


Figure 15. Thyroid atrophy – Grade 1

Figure 16. Thyroid atrophy - Grade 2

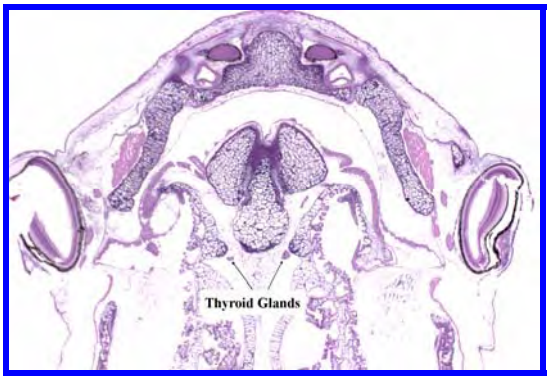


Figure 17. Thyroid atrophy - Grade 3

Grade 0 – Thyroid gland atrophy

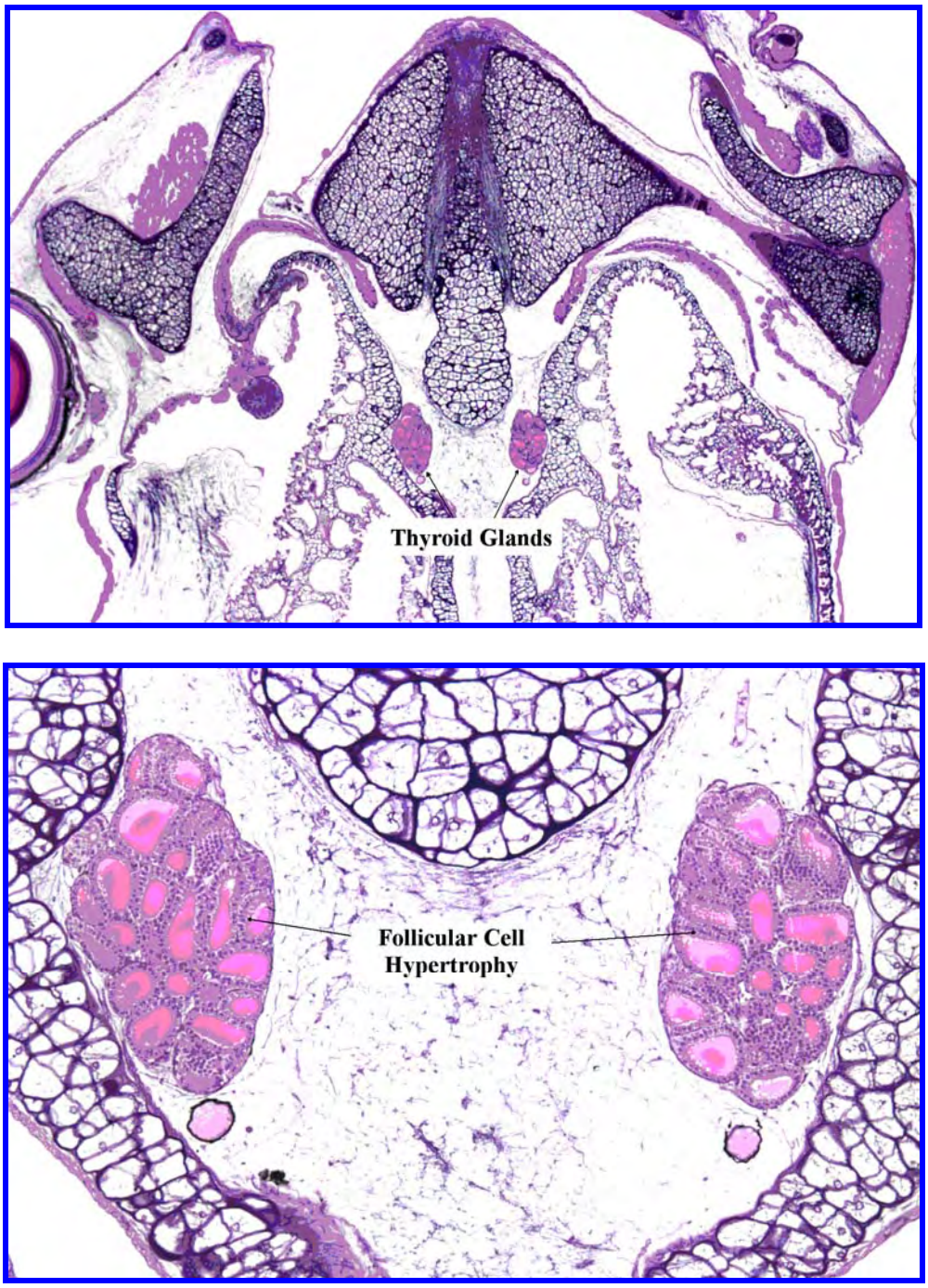


Figure 18. Low (1.25x) and medium (10x) magnification photomicrographs of thyroid glands from *X. laevis* that was a control animal. The thyroid follicles contain eosinophilic homogeneous colloid and are lined by simple cuboidal follicular cells. There is minimal follicular cell hyperplasia and hypertrophy which may be present in normal thyroid glands. The thyroid follicles are variable in size.

Grade 1 – Thyroid gland atrophy:

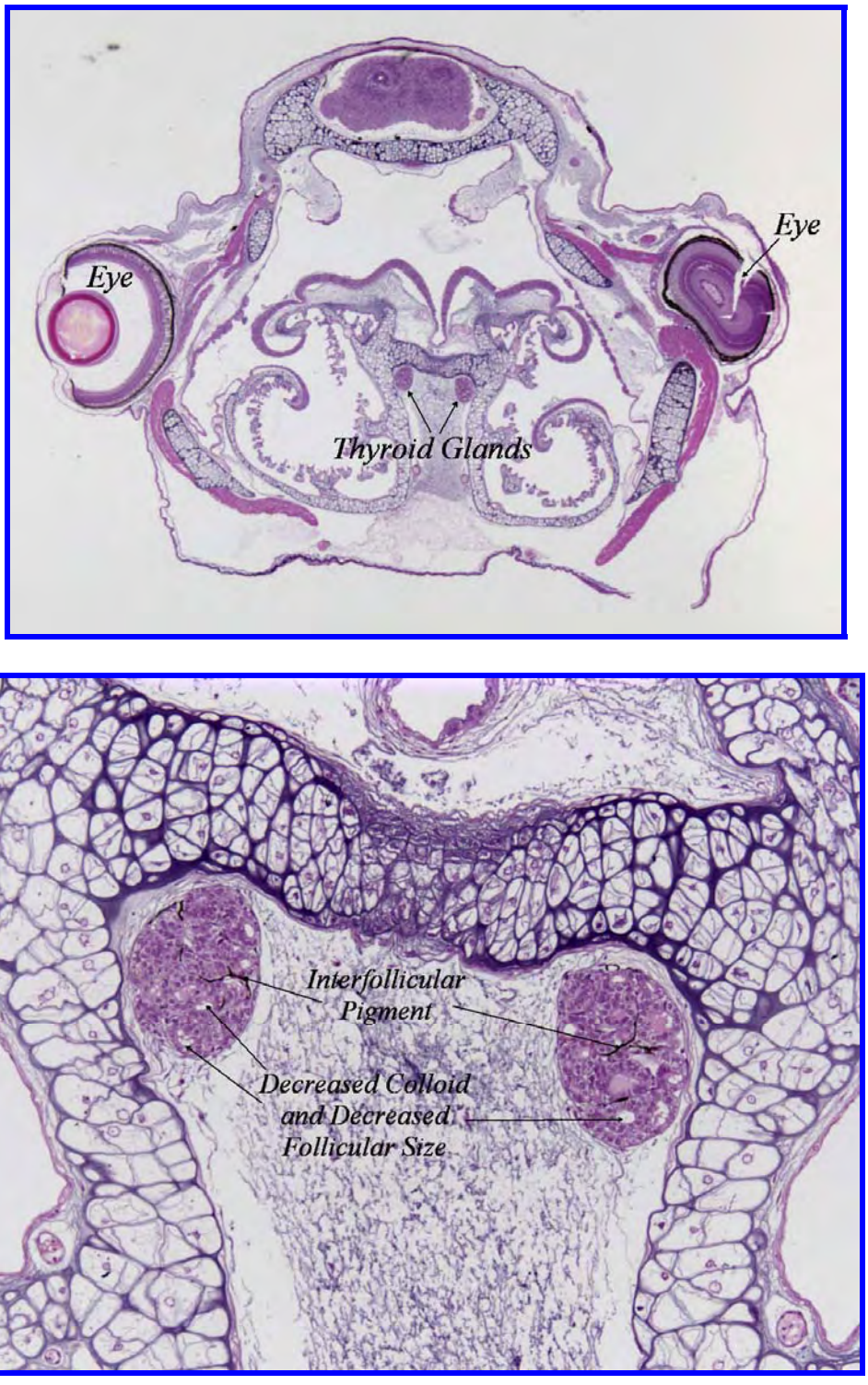


Figure 19. Low (1.25x) and medium (10x) magnification photomicrographs of thyroid glands from *X. laevis* exposed to 1 µg/L thyroxine. There is minimal decreased quantity of colloid and a minimal decrease in the size of thyroid follicles as compared to control frogs. There is mild atrophy of the thyroid glands.

Grade 2 – Thyroid gland atrophy:

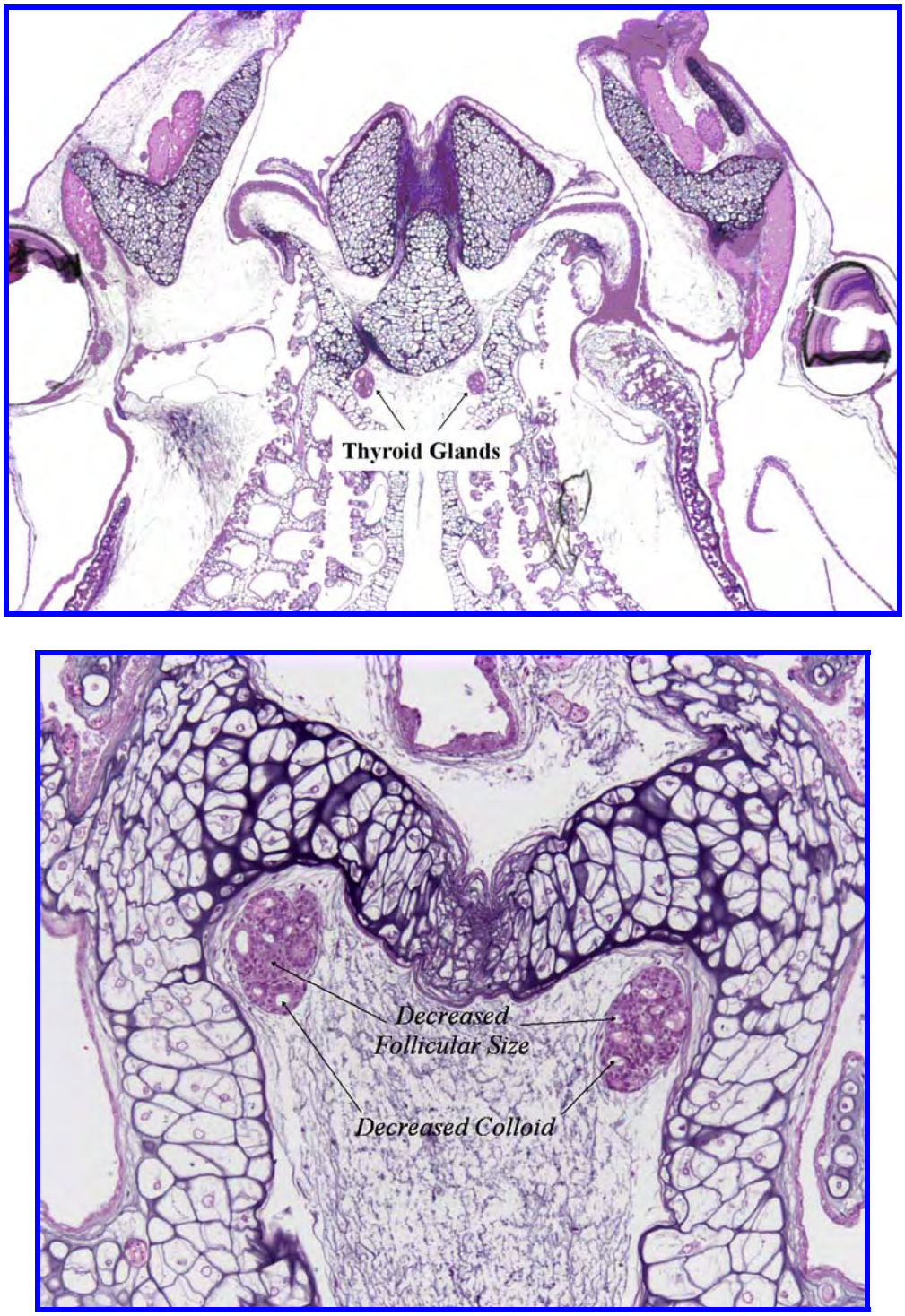


Figure 20. Low (1.25x) and medium (10x) magnification photomicrographs of thyroid glands from thyroid glands of *X. laevis*. There is a moderate decrease in the size of the thyroid follicles and moderately decreased colloid. The glands are moderately atrophied.

Grade 3 – Thyroid gland atrophy:

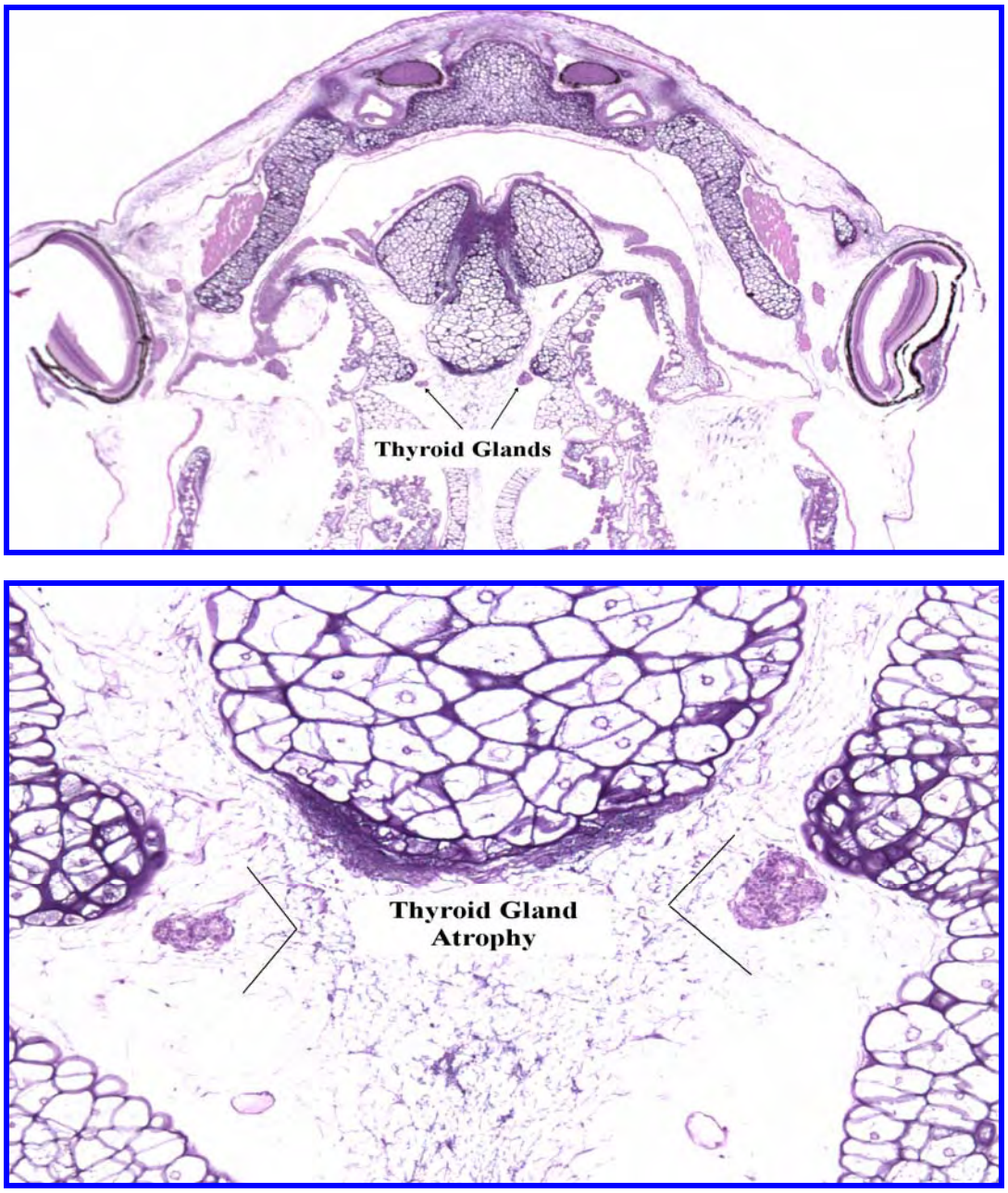


Figure 21. Low (2x) and medium (10x) magnification photomicrographs of thyroid glands from *X. laevis*. There is severe thyroid gland atrophy and the thyroid follicles are uniformly small. There is a moderately severe decease in amount of colloid, but what little colloid is present is eosinophilic and homogeneous.

IIIC3. Follicular cell hypertrophy – general range of changes

Figure 22. Follicular cell hypertrophy - Grade 0

Figure 23. Follicular cell hypertrophy – Grade 1

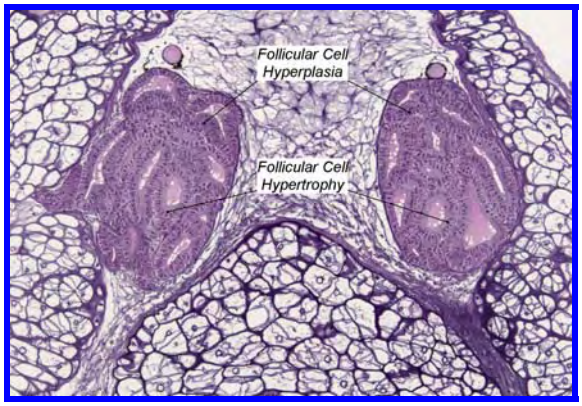


Figure 24. Follicular cell hypertrophy – Grade 2

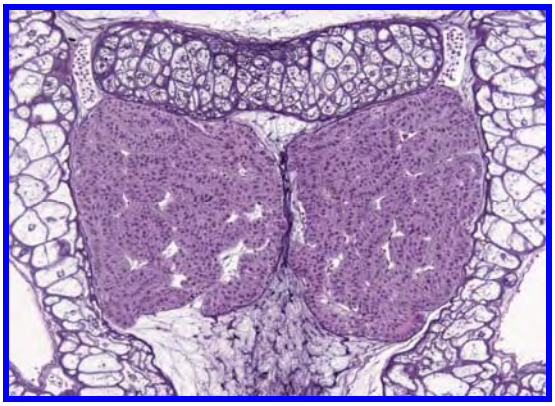
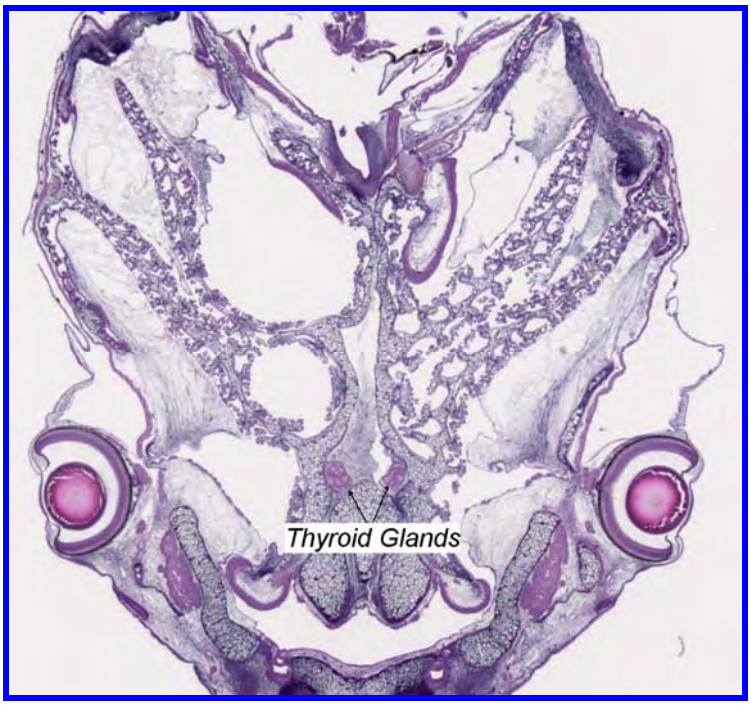


Figure 25. Follicular cell hypertrophy – Grade 3

Grade 0 - Follicular cell hypertrophy:



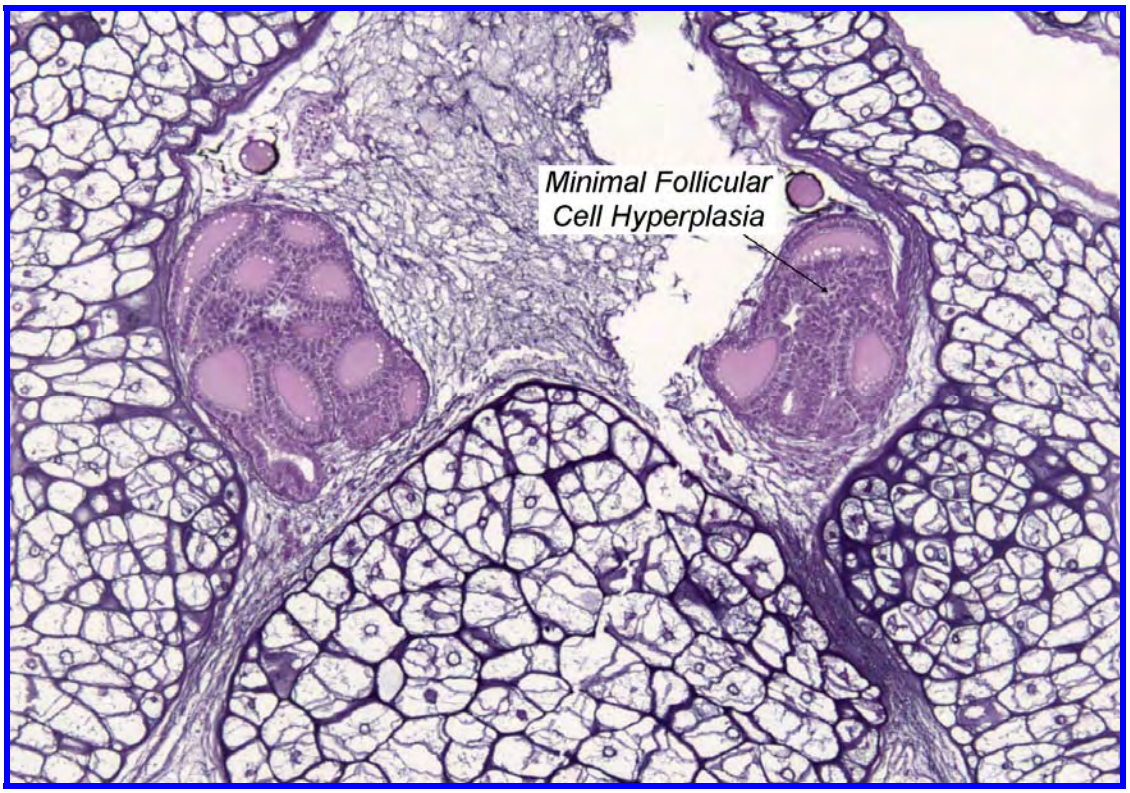


Figure 26. Low (1.25x) and medium (10x) magnification photomicrographs of thyroid glands from *X. laevis* that was a control animal. The thyroid follicles are colloid-filled and lined by simple cuboidal follicular cells. There is minimal follicular cell hyperplasia.

Grade 1 - Follicular cell hypertrophy:

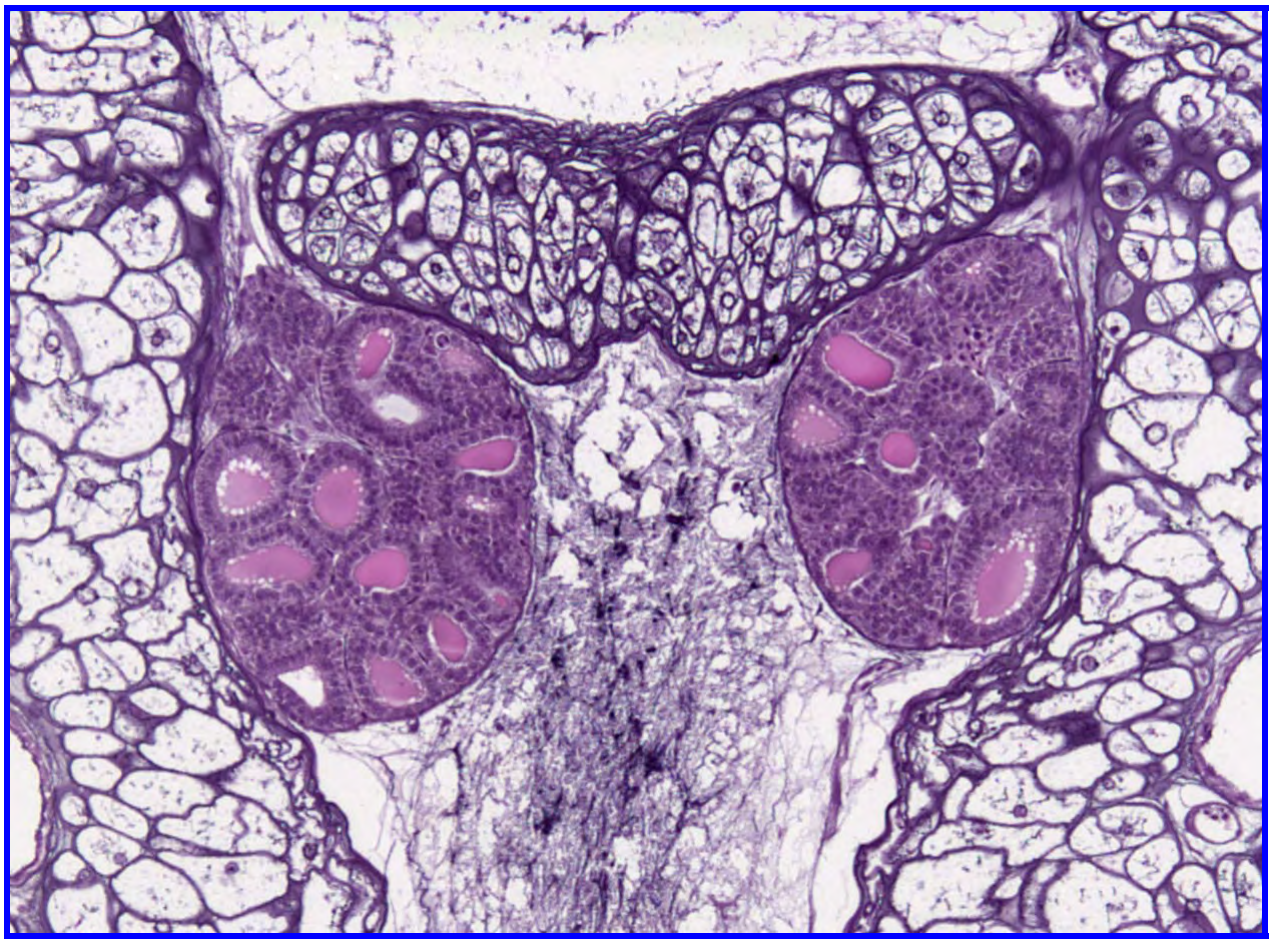
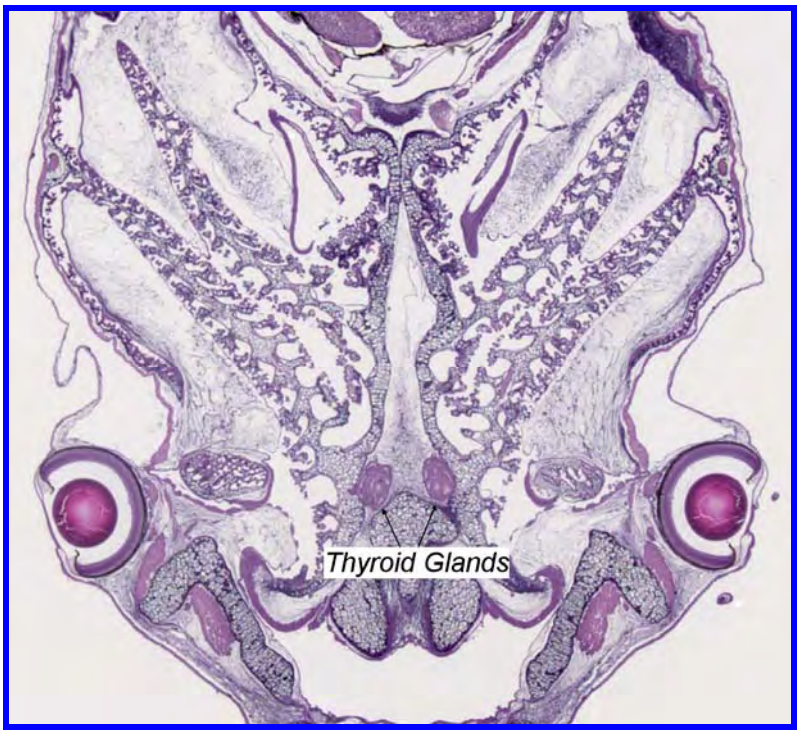


Figure 27. Thyroid glands from a Stage 54 *X. laevis* exposed to methimazole for 96 hours. There is mild follicular cell hypertrophy characterized by cells that are plump and columnar as compared to the usual cuboidal cells. There is also mild follicular cell hyperplasia. A lower magnification image is not available.

Grade 2 - Follicular cell hypertrophy:



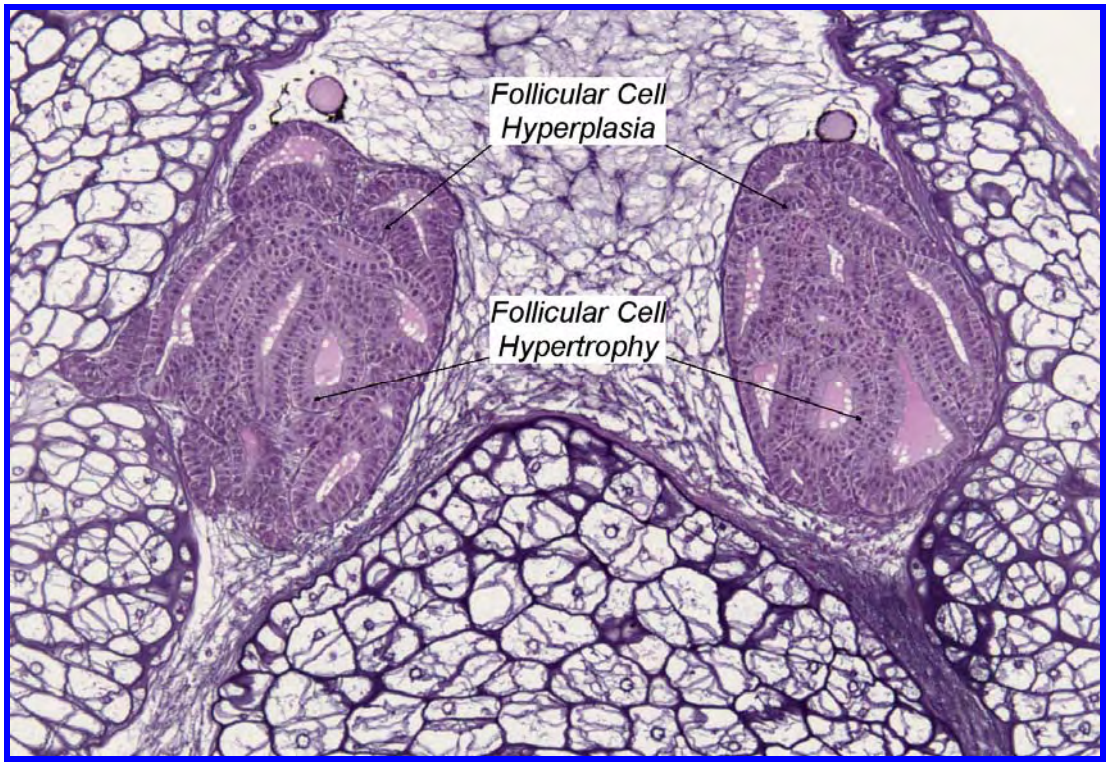


Figure 28. Low (1.25x) and medium (10x) magnification photomicrographs of a Stage 51 *X. laevis* exposed to 125 µg/L perchlorate for 21 days. There is slight/mild follicular cell hyperplasia and there is moderate follicular cell hypertrophy. There is slight/mild decreased colloid in the thyroid follicles.

Grade 3 - Follicular cell hypertrophy:

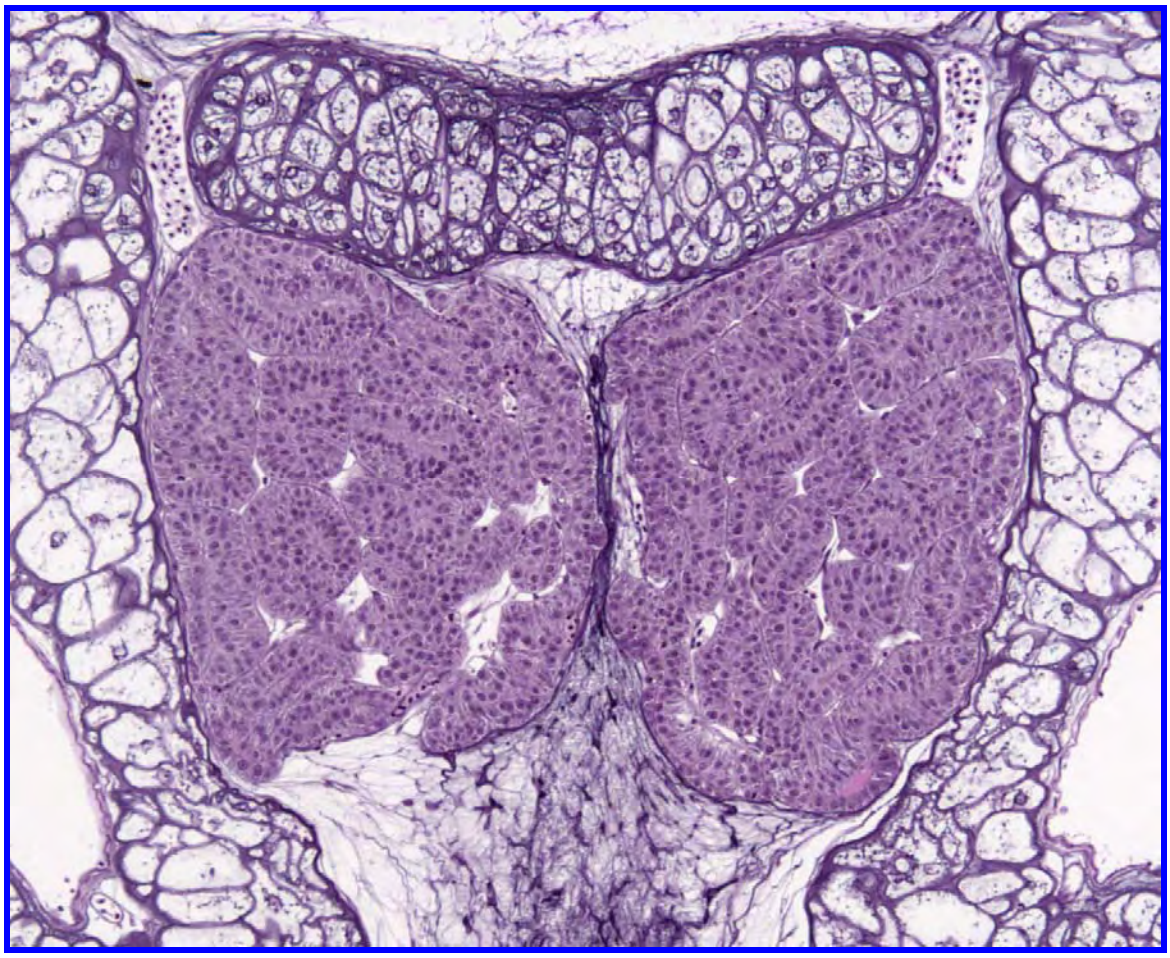


Figure 29. Thyroid glands from a Stage 54 X. laevis exposed to methimazole for 192 hours. There is moderately severe hypertrophy of the thyroid glands. The amount of colloid in the follicles is severely reduced and there is severe follicular cell hypertrophy and moderately severe hyperplasia. A lower magnification image is not available.

IIIC4. Follicular cell hyperplasia

Grade 0 – Follicular cell hyperplasia:

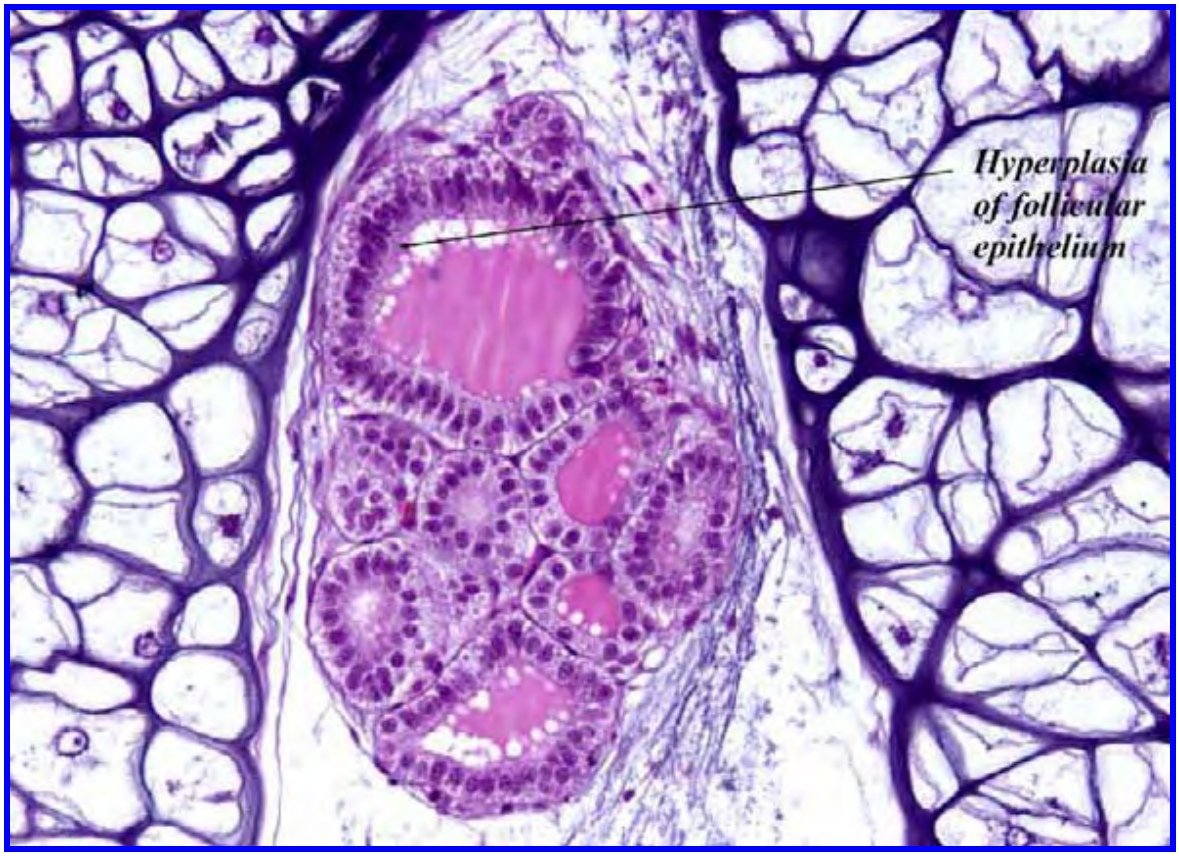


Figure 30. High (20x) magnification photomicrograph of thyroid gland from *X. laevis*. Minimal thyroid follicular cell hyperplasia is characterized by crowding of columnar cells. This follicular cell hyperplasia is representative of the upper limit of grade 0 because it is focal and affects less than 20% of the tissue.

Grade 1 – Follicular cell hyperplasia:

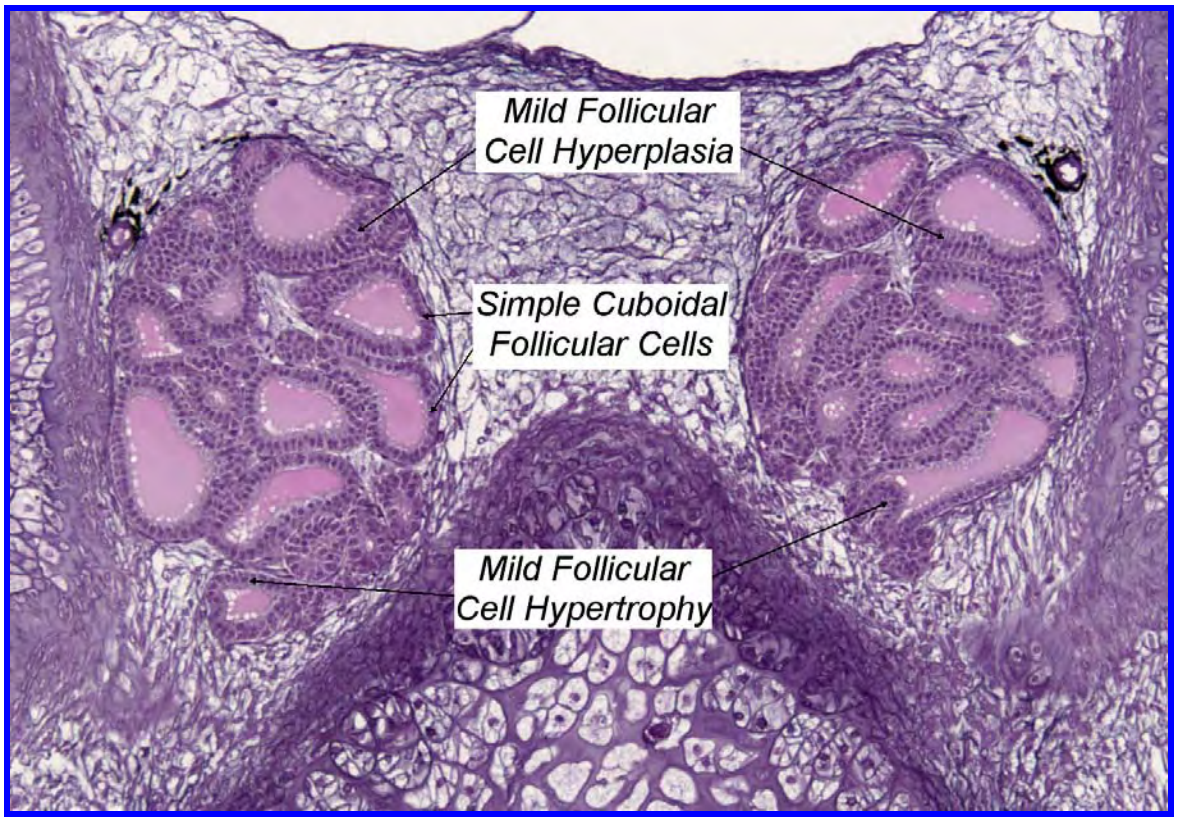


Figure 31. Medium (10x) magnification photomicrograph of thyroid glands from *X. laevis* that was a control animal. The thyroid follicles are colloid-filled and most are lined by simple cuboidal follicular cells. Slight/mild follicular cell hypertrophy and mild follicular cell hyperplasia are present demonstrated by papillary infolding and crowding of follicular cells.

Grade 2 – Follicular cell hyperplasia:

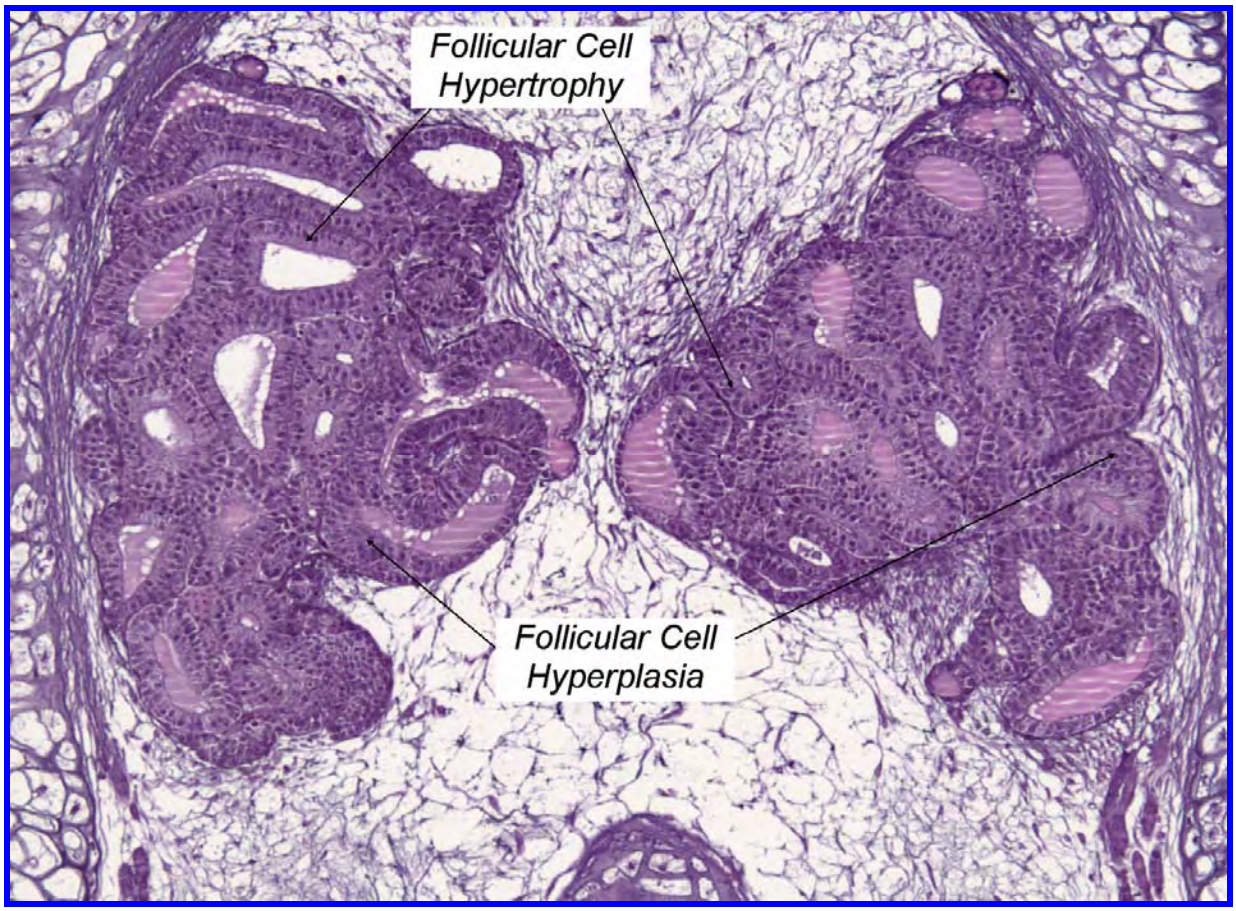


Figure 32. Medium (10x) magnification photomicrographs of a Stage 51 *X. laevis* exposed to 62.5 µg/L perchlorate for 21 days. Moderate follicular cell hypertrophy and moderate follicular cell hyperplasia are present. The thyroid glands are moderately enlarged overall and there is slight/mild decreased colloid in the thyroid follicles. The hyperplasia affects 30-50% of the tissue.

Grade 3 – Follicular cell hyperplasia:

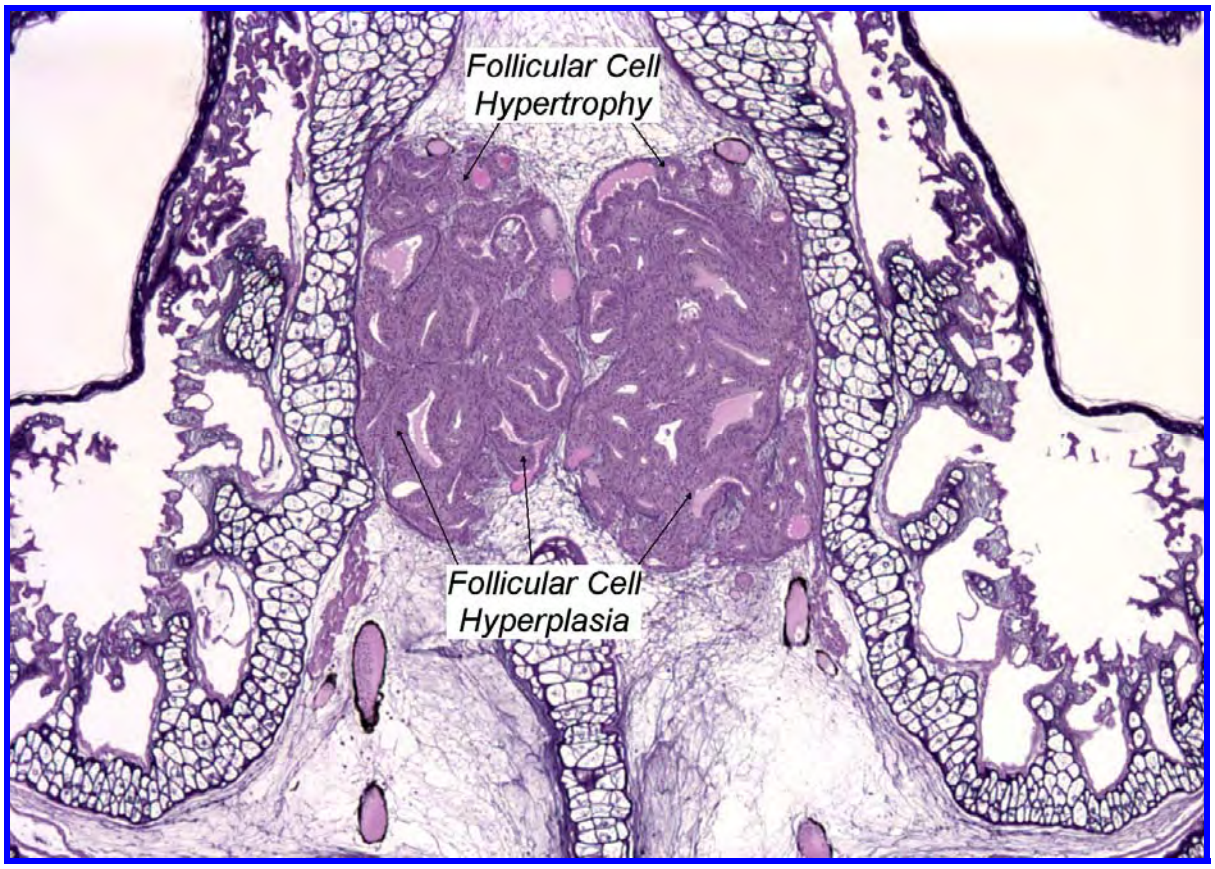


Figure 33. Medium (4x) magnification photomicrographs of a Stage 51 *X. laevis* exposed to 500 µg/L perchlorate for 21 days. There is severe hypertrophy of the thyroid glands and the colloid is slightly/mildly decreased in the thyroid follicles. There is severe follicular cell hyperplasia, affecting over 80% of the tissue, and there is moderate follicular cell hypertrophy.

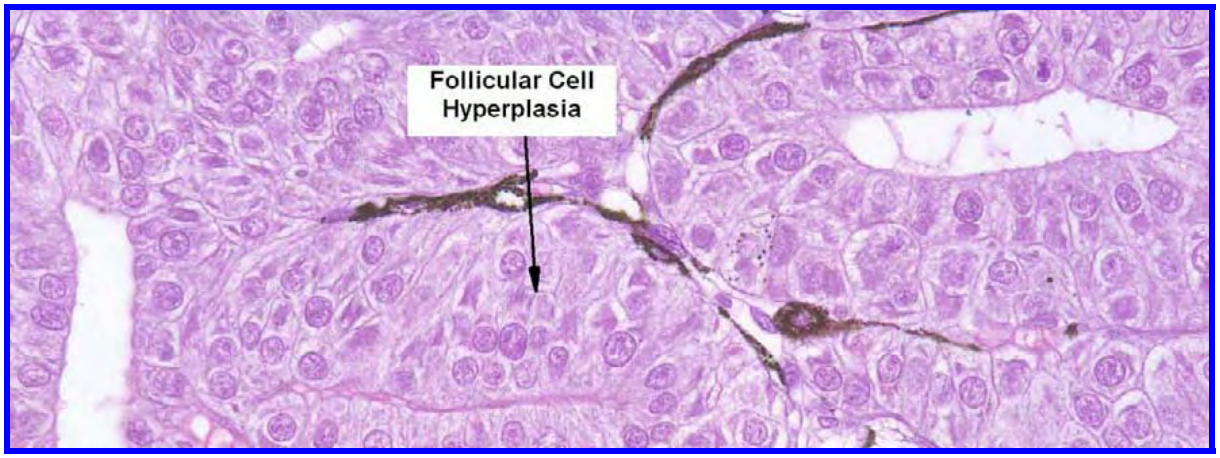


Figure 34. High (40x) magnification photomicrograph of thyroid gland from *X. laevis*. There is severe follicular cell hyperplasia demonstrated by the stratification of the follicular cells.

IIIC5. Colloid quality

Homogenous:

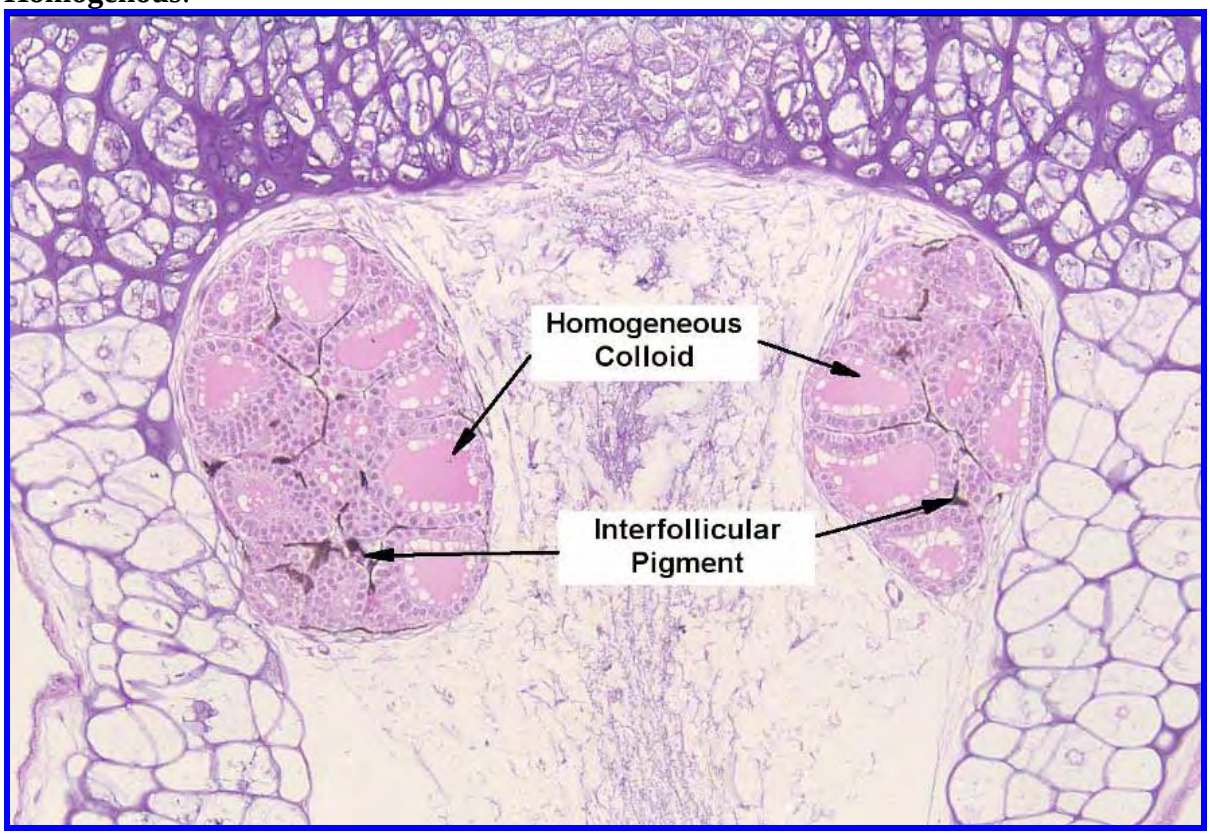


Figure 35. Medium (10x) magnification photomicrographs of thyroid glands from *X. laevis*. The colloid is homogeneous and light eosinophilic and the follicles are lined by simple cuboidal epithelium.

Foamy appearance:

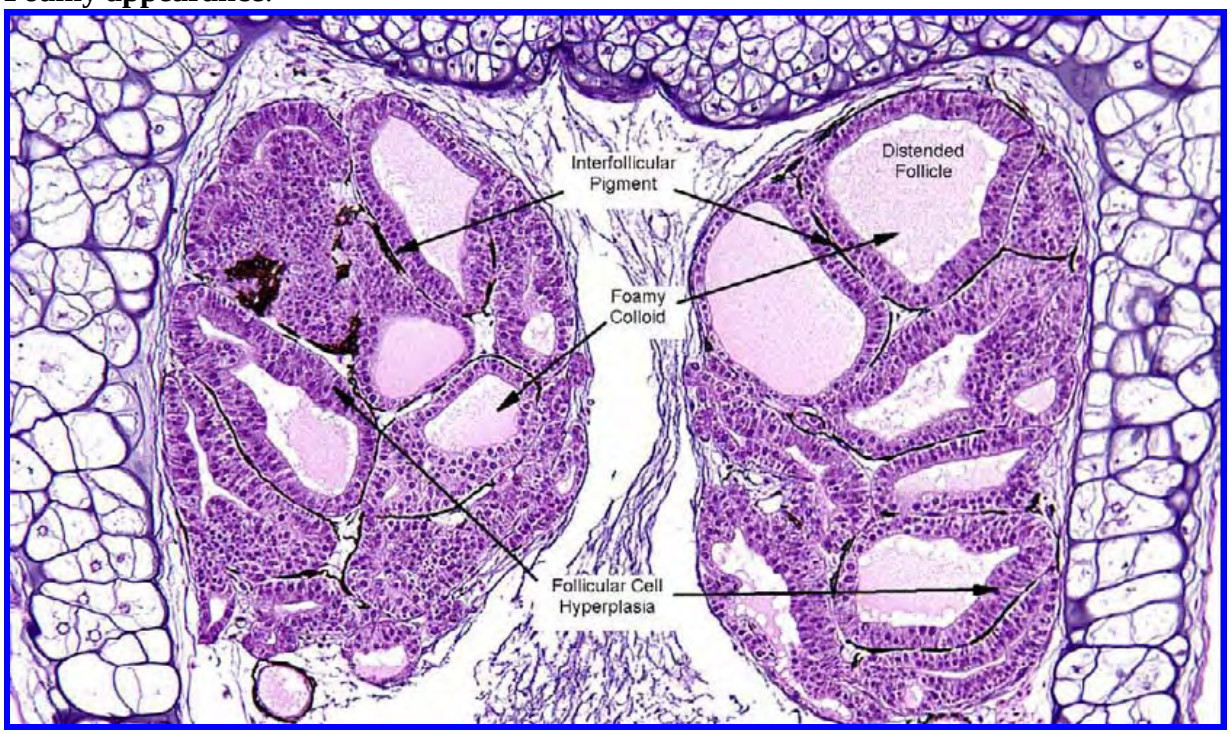


Figure 36. High (10x) magnification photomicrographs of thyroid glands from *X. laevis*. The colloid has a foamy appearance.

Granular colloid:

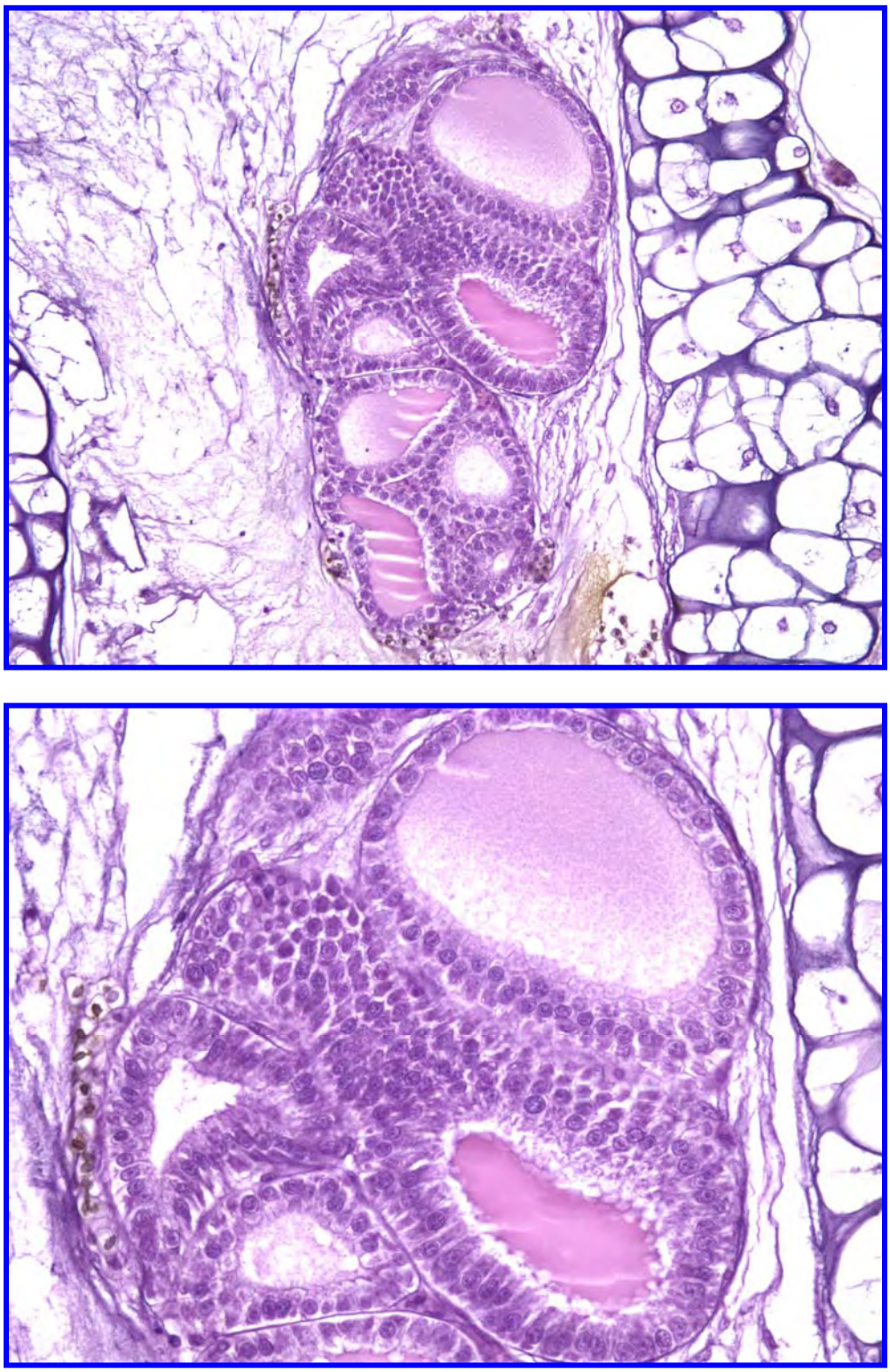


Figure 37. Medium (20x) and high (40x) magnification of an *X. laevis* thyroid gland. The colloid in the upper-right follicle is granular.

Tinctorial quality of colloid:

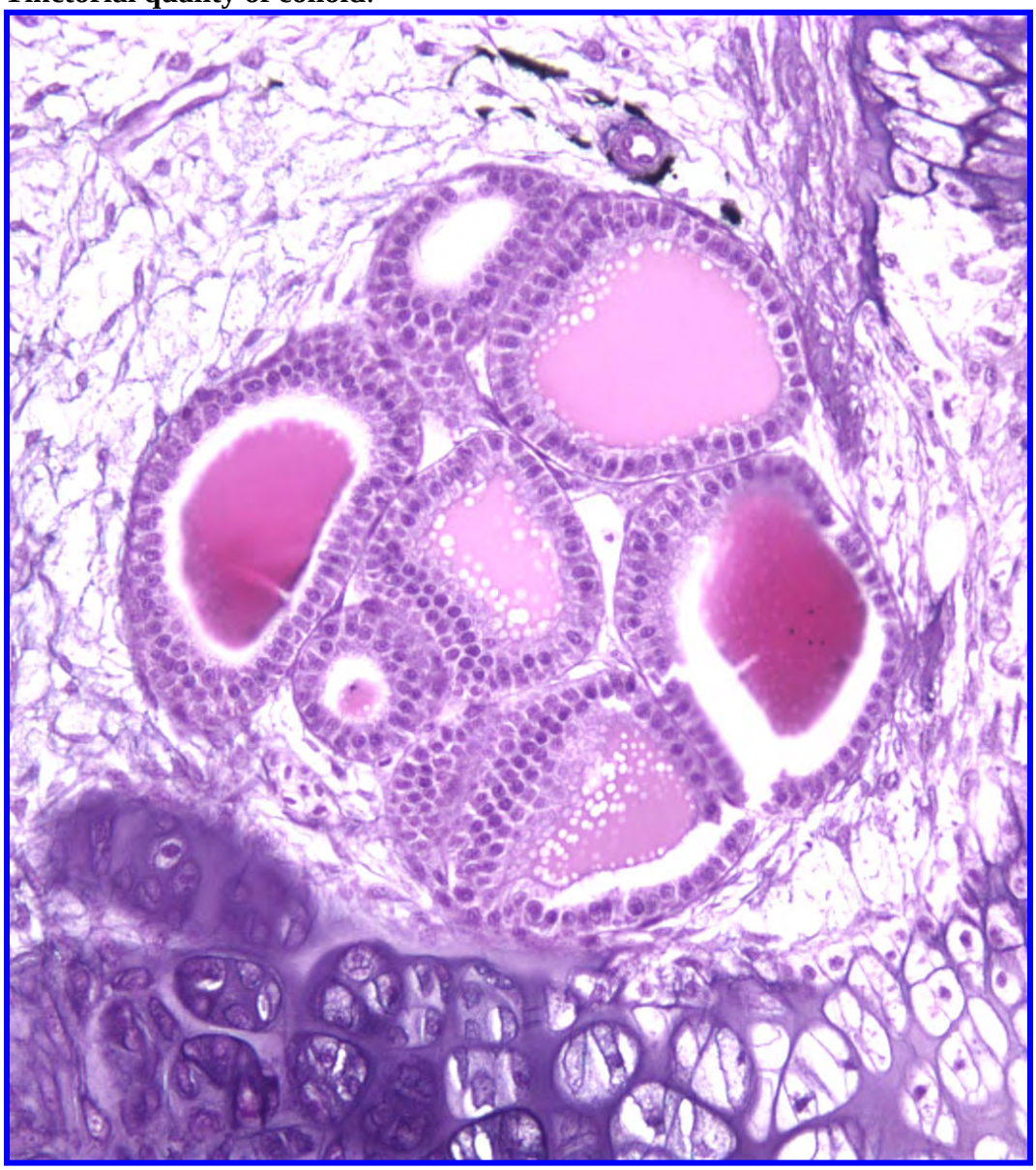


Figure 38. Thyroid gland of a stage 61 *X. laevis* tadpole. The tinctorial quality of the colloid is heterogenous in this example. Tinctorial quality may be affected by the thickness of the section, the batch of stain used, and variation of staining within the same gland.

IIIC6. Miscellaneous findings

Eosinophilic droplets:

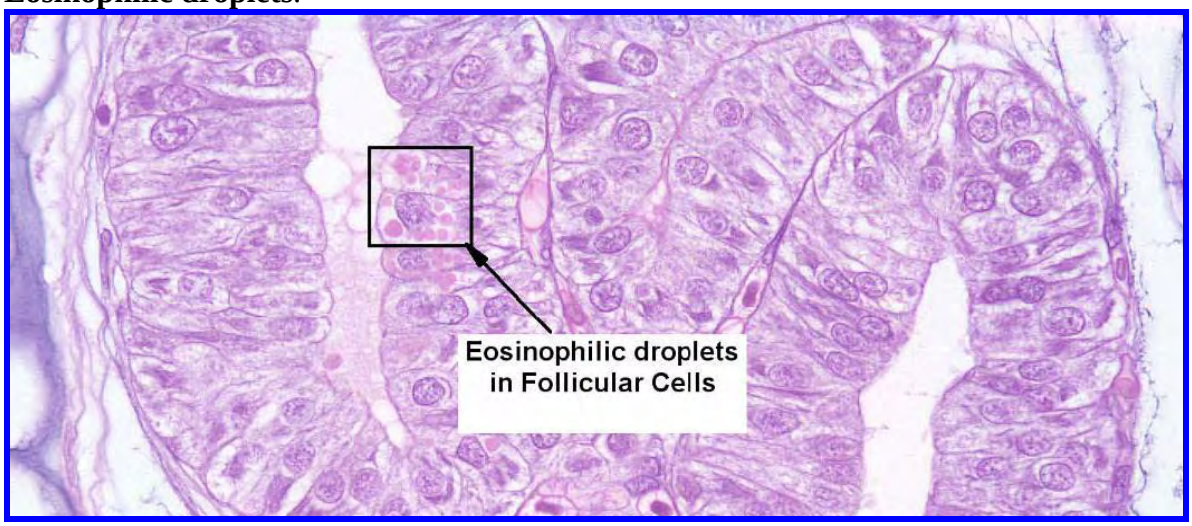


Figure 39. High (40x) magnification photomicrograph of thyroid gland from *X. laevis*. Note the eosinophilic droplets in follicular cells.

Karyomegaly:

Figure 40. High (20x) magnification photomicrographs of thyroid glands from *X. laevis*. Minimal karyomegaly is characterized by occasional enlarged nuclei.

Midline thyroid follicles:

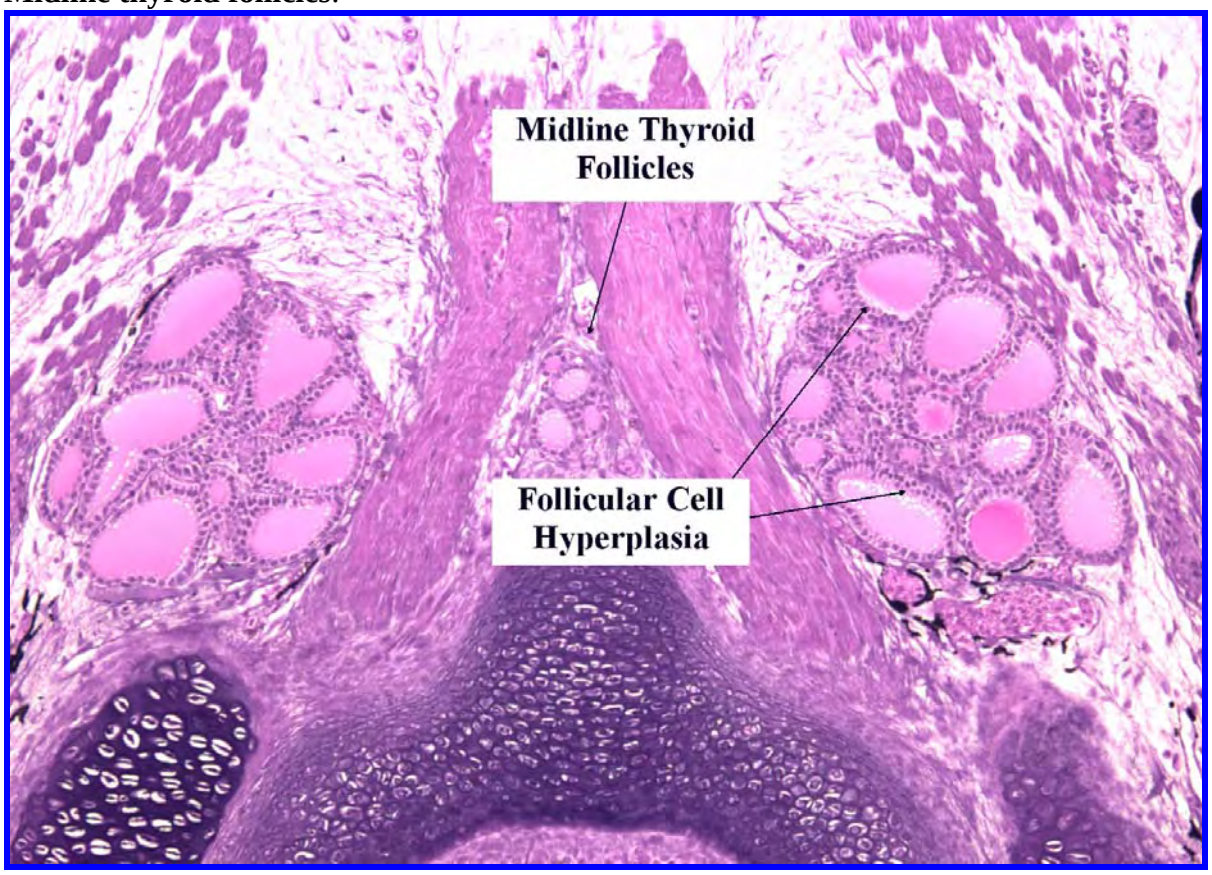


Figure 41. Medium (10x) magnification photomicrographs of thyroid glands from *X. laevis* (frontal section). The thyroid follicles contain eosinophilic homogeneous colloid and are lined by simple cuboidal follicular cells. There is minimal follicular cell hyperplasia. Thyroid follicles are present also on the midline in this frog.

Interfollicular pigment:

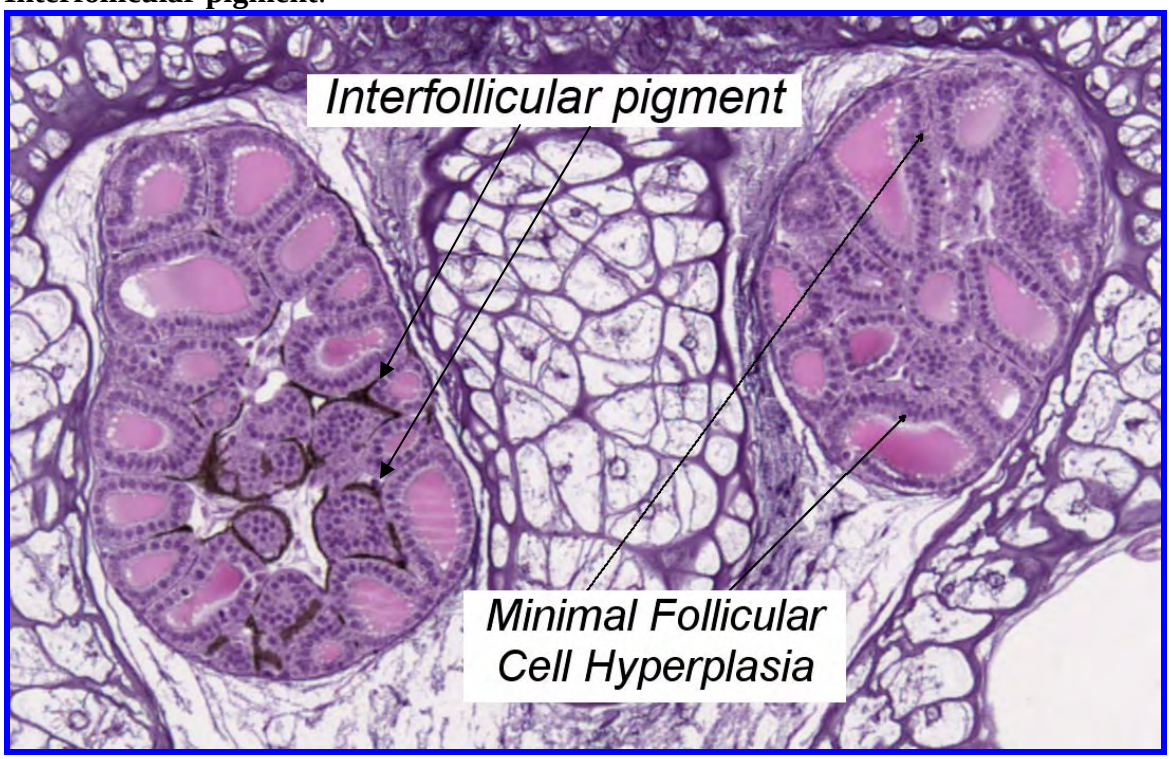


Figure 42. Medium (10x) magnification photomicrographs of thyroid glands from *X. laevis* (frontal section). Pigment surrounding follicles is present in the gland on the left. This pigment is inconsistently present in normal thyroid glands. It may occur unilaterally or bilaterally in the glands.

IIIC7. Artifacts

Colloid fall-out:

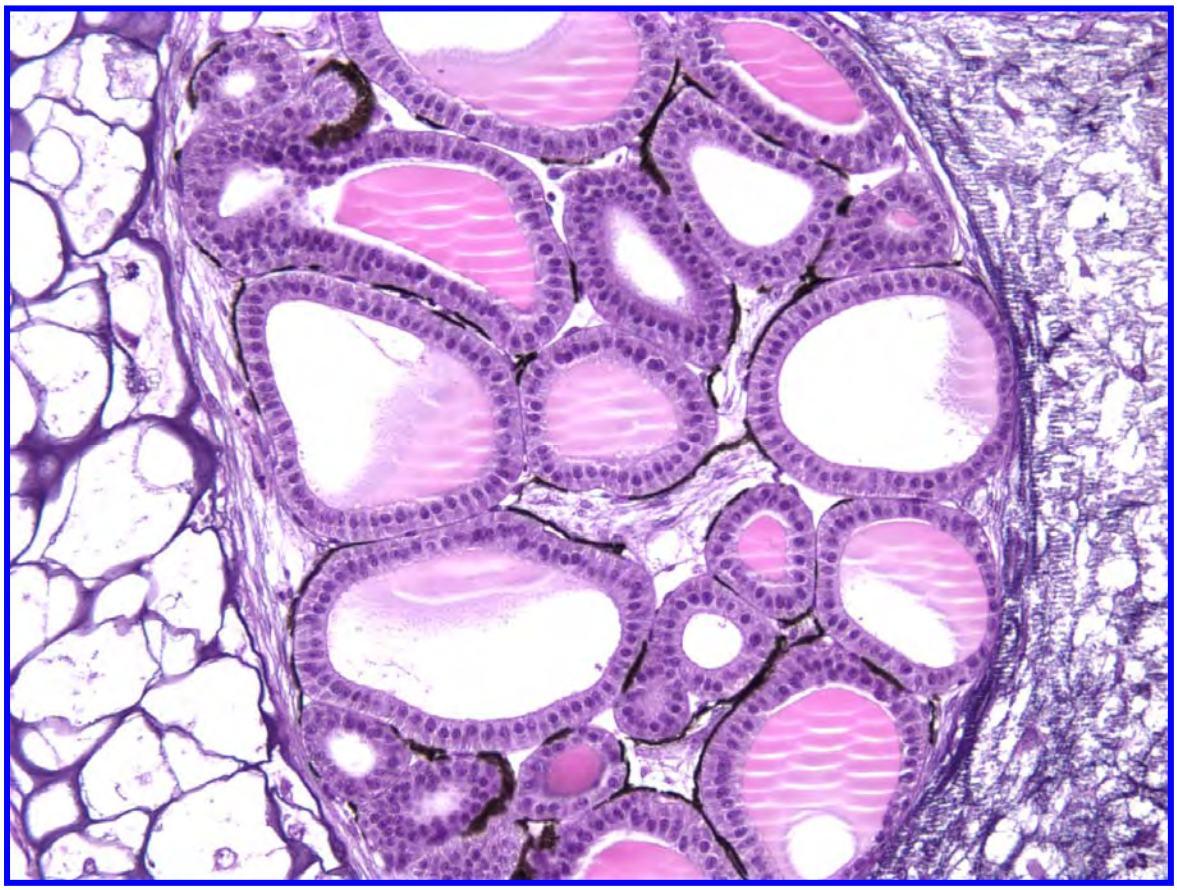


Figure 43. Thyroid gland from a stage 60 *X. laevis* tadpole. Some follicles are completely or partially devoid of colloid. This is an artifact that sometimes occurs during sectioning and is not to be mistaken with decreased colloid content. The equally distributed lines within the colloid are also a result of the sectioning process.

IV. Data Reporting

An Excel worksheet form has been created to facilitate histopathology data collection (Appendix A) and which will be provided electronically. In this worksheet, each data entry cell represents an individual tadpole. Space is also provided for comments. For each tadpole, the pathologist records the severity grade in the corresponding cell to the diagnostic criterion. If there is no reasonably appropriate diagnostic criterion for a particular finding, the pathologist can create a term that can be assigned to an unused portion of the table. If there are no findings for a tadpole, this should be recorded as ”0” (not remarkable). If insufficient tissue is available for diagnosis, this should be recorded as **IT** (insufficient tissue).

Adding a modifier 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 thyroid glands is *unilateral* (**UNI**); unless specified in this manner, all thyroid diagnoses are assumed to be bilateral. Other modifier codes can be created as needed by the pathologist.

Reference List

1. Crissman,J. *et al.* Best Practices Guideline: Toxicologic Pathology. *Toxicologic Pathology* **32**, 126-131 (2004). http://www.toxpath.org/ToxHistopath.pdf

2. Holland,T. A survey of discriminant methods used in toxicological histopathology. *Toxicol Pathol* **29**, 269-273 (2001).

Appendix A: Histopathology Reporting Tables

Core Criteria:

Date: Chemical: Pathologist:

		Thyroid gland hypertrophy	Thyroid gland atrophy	Follicular cell hypertrophy	Follicular cell hyperplasia
Control Animal ID - replicate 1					
Control Animal ID - replicate 2					
Total:					

		Thyroid gland hypertrophy	Thyroid gland atrophy	Follicular cell hypertrophy	Follicular cell hyperplasia
Dose ____ Animal ID - replicate 1					
Dose ____ Animal ID - replicate 2					
Total:					

		Thyroid gland hypertrophy	Thyroid gland atrophy	Follicular cell hypertrophy	Follicular cell hyperplasia
Dose ____ Animal ID - replicate 1					
Dose ____ Animal ID - replicate 2					
Total:					

		Thyroid gland hypertrophy	Thyroid gland atrophy	Follicular cell hypertrophy	Follicular cell hyperplasia
Dose ____ Animal ID - replicate 1					
Dose ____ Animal ID - replicate 2					
Total:					

Additional Criteria:

Date: Chemical: Pathologist:

		Follicular lumen area increase	Follicular lumen area decrease
Control Animal ID - replicate 1			
Control Animal ID - replicate 2			
Total:			

		Follicular lumen area increase	Follicular lumen area decrease
_____ Dose Animal ID - replicate 1			
_____ Dose Animal ID - replicate 2			
Total:			

		Follicular lumen area increase	Follicular lumen area decrease
_____ Dose Animal ID - replicate 1			
_____ Dose Animal ID - replicate 2			
Total:			

		Follicular lumen area increase	Follicular lumen area decrease
_____ Dose Animal ID - replicate 1			
_____ Dose Animal ID - replicate 2			
Total:			

Narrative Descriptions:

Date:
Chemical:
Pathologist:

		Narrative description
Control Animal ID - replicate 1		
Control Animal ID - replicate 2		
Dose ____ Animal ID - replicate 1		
Dose ____ Animal ID - replicate 2		
Dose ____ Animal ID - replicate 1		

____ Dose ____ Animal ID - replicate 2		

____ Dose ____ Animal ID - replicate 1		
____ Dose ____ Animal ID - replicate 2		