Histopathology Guidance Document for the Medaka Extended One-Generation Reproduction Test (MEOGRT)

TABLE OF CONTENTS

I. Histopathology Guidance for the Medaka Extended One Generation Reproduction Test

(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 Medaka Extended One-generation Reproduction Test. The goal of this document is to serve as guidance for the collection, histological preparation, and pathological evaluation of gonads, kidney, and liver specimens from Japanese medaka (*Oryzias latipes*) in support of the OECD Medaka Extended One-generation Reproduction Test (MEOGRT) which is a long-term test generating data on adverse effects of test chemicals on various life-stage of the fish. The test is indicated at Level 5 of the OECD Conceptual Framework on Endocrine Disrupters Testing and Assessment, comprising in vivo assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle. 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 Procedures.**

At the conclusion of the exposure, fish are anesthetized by transfer to an oxygenated solution of MS-222 (100 mg/L buffered with 200 mg NaHCO3/L) for sampling. If potency of the solution is not adequate, additional MS-222 (<10 mg) may be added to strengthen effectiveness.

If other observations and measurements are to be made at this time (e.g., length and body weight, blood collection, and evaluation of secondary sex characteristics), these tasks must be performed rapidly in order to avoid tissue autolysis which occurs rapidly in fish. To further avoid autolysis caused by delays in data collection, the number of fish euthanized at any one time should be kept to a minimum.

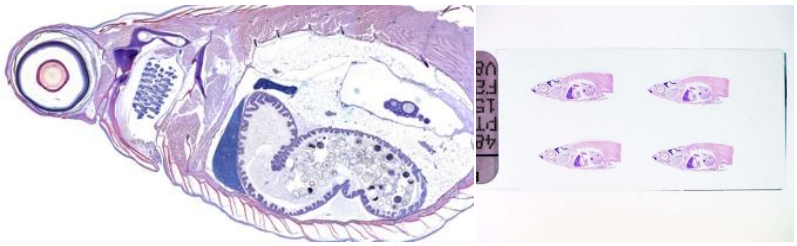
Prior to fixation, the tail is excised caudal to the abdominal cavity and discarded. Using a #11 blade or micro scissors, a slit incision approximately 3 mm in length is made carefully through the ventral abdominal wall to allow penetration of viscera by the fixative. Each fish is placed in an individual tissue cassette along with its corresponding identification label, and the cassette is placed in modified Davidson's solution for 24-48 hours. After the initial fixation period, the tissues are rinsed thoroughly in 70% ethanol, after which they may be stored in 10% neutral buffered formalin prior to histological processing.

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

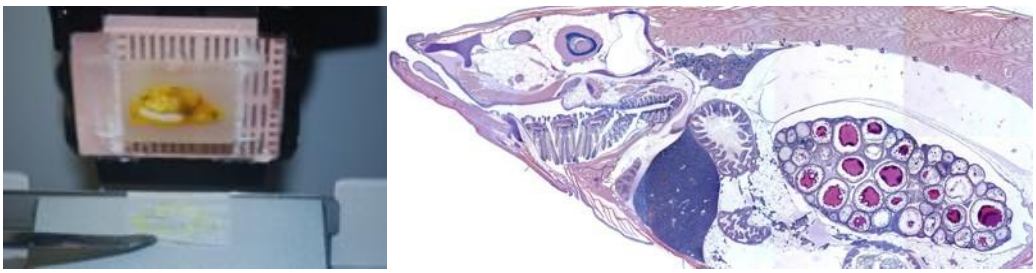
(1) **Decalcification.** Decalcification of the specimens is usually not required due to the presence of acetic acid in the modified Davidson's fixative. However, it is usually prudent to process and microtome a few control specimens ahead of the rest to ensure that decalcification is complete. If further decalcification is necessary, specimens may be immersed in a commercial formic acid/EDTA decalcifying solution for a short interval (e.g., several hours or overnight) prior to processing.

(2) **Processing and Embedding.** Each whole fish specimen (i.e., minus the tail) is processed in an automated tissue processor and infiltrated with paraffin according to routine methods. Fish are embedded in paraffin to allow sectioning in the parasagittal / sagittal plane, with the left side being cut first. The cassette should include an appropriate label.

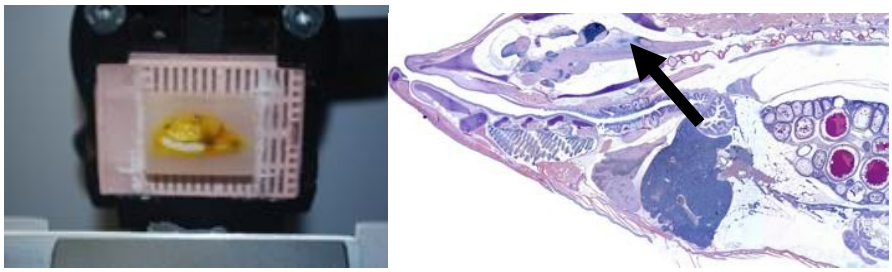
(3) **Microtomy.** Section thicknesses will be set at 4-5 microns. Each fish will be step sectioned in the parasagittal / sagittal plane at five distinct levels. Each of the five sections acquired in this fashion will be placed on a single slide. A duplicate set of sections, which will remain unstained, will be obtained at the same five levels, and these will be placed on five additional slides. Specific landmarks for each of the five levels are listed below.



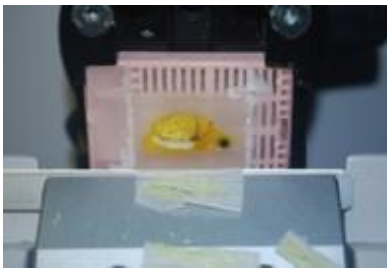
Level 1: The block is faced from the left toward the right side of the fish. Sections are trimmed away until the left eye is revealed, and then trimming continues to the mid-portion of the lens. The lens will be visible as a ring within the eye. The ring can be seen in the sections and the block. The lens will be hard, the microtome blade will produce a scratch that can be heard and felt as the blade cuts through. Sections acquired at this level should reveal the visceral cavity. A ribbon of 3-4 serial sections is obtained and mounted on a single slide.
A second ribbon of 3-4 sections should be obtained and mounted on a second slide. The first slide will be stained and the second slide will be left unstained for possible future reference. This will be done for all sectioning levels.

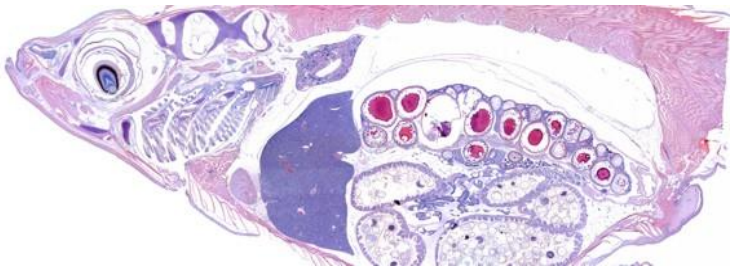


Level 2: Trimming is continued until the left eye is no longer present in the sections, and the dark brown pigment of the retinal epithelium has diminished. A ribbon of 3-4 serial sections is obtained at that point and mounted on a single slide. A second ribbon of 3-4 sections should be obtained and mounted on a second slide. The first slide will be stained and the second slide will be left unstained for possible future reference.

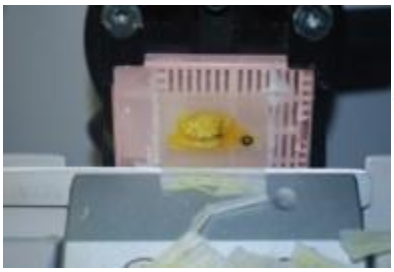


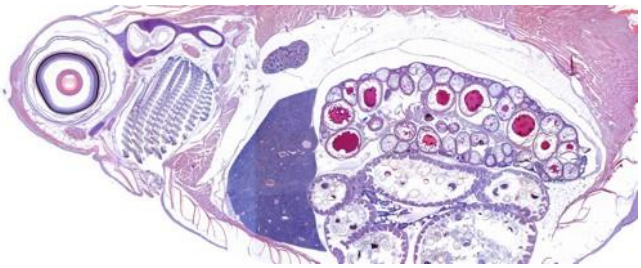
Level 3: The target organ for this slide is the pituitary gland, which is located at a level that is midway between the eyes. Trimming is continued until the brain begins to elongate, leading into the spinal cord (arrow). Four step sections are then obtained at 200 micron intervals, collecting one good quality section at each step (sectioning halts before the right eye is reached). All four sections are mounted on a single slide in the order they were obtained. A second ribbon of 3-4 sections should be obtained and mounted on a second slide. The first slide will be stained and the second slide will be left unstained for possible future reference.





Level 4: Trimming is continued to the medial edge of the right eye, where the dark brown retinal epithelial pigment is visible. A ribbon of 3-4 serial sections is obtained at that point and mounted on a single slide. A second ribbon of 3-4 sections should be obtained and mounted on a second slide. The first slide will be stained and the second slide will be left unstained for possible future reference.





Level 5: Trimming is continued to the midpoint of the right lens, where light can be seen through the lens. A ribbon of 3-4 serial sections is obtained at that point and mounted on a single slide. A second ribbon of 3-4 sections should be obtained and mounted on a second slide. The first slide will be stained and the second slide will be left unstained for possible future reference. Following microtomy, each paraffin block is sealed with paraffin.

(4) **Staining and Coverslipping**. Slides destined for staining are stained with hematoxylin and eosin, and are covered with glass cover slips using an appropriate permanent mounting medium.

(5) **Labeling.** Slides are labeled with at least the following information:

(i) Study number
(ii) Name of the test chemical
(iii) Generation and age of the specimen (i.e., F1, 16 wpf)
(iv) Treatment or dose level
(v) Individual animal identification number

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

(1) **General Approach to Pathologic Evaluations.** Studies are to be read by individuals experienced in reading toxicologic pathology studies, and who are familiar with normal, small fish gonad histology, with gonadal physiology, and with general responses of the gonads 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, fish 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 gonadal histopathology for the MEOGRT assay. If an individual has toxicological pathology experience and is familiar with gonadal histology in small fish species, he/she may be trained to read the fish 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., without knowledge of the treatment group status of individual fish). 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 prior to conducting their evaluations. 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., 2004).

(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 the tissue section being not remarkable. This is not to mean "Grade 0." This practice provides continuity with subsequent statistical analyses.

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 fish are considered to be exacerbations of "normal", physiologic findings (e.g., oocyte atresia [Nagahama, 1983; Tyler and Sumpter, 1996]). 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 interlaboratory results comparisons.

(3) **Data Recording.** The pathologist records the results on a spreadsheet template. For each fish, 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 fish, 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, organ and body weights, secondary sex characteristics, genotypic sex, reproductive performance data, and biochemical analyses (e.g., reproductive or thyroid hormones, vitellogenin).

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 article. 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 at 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; 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 theories 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 theories 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.

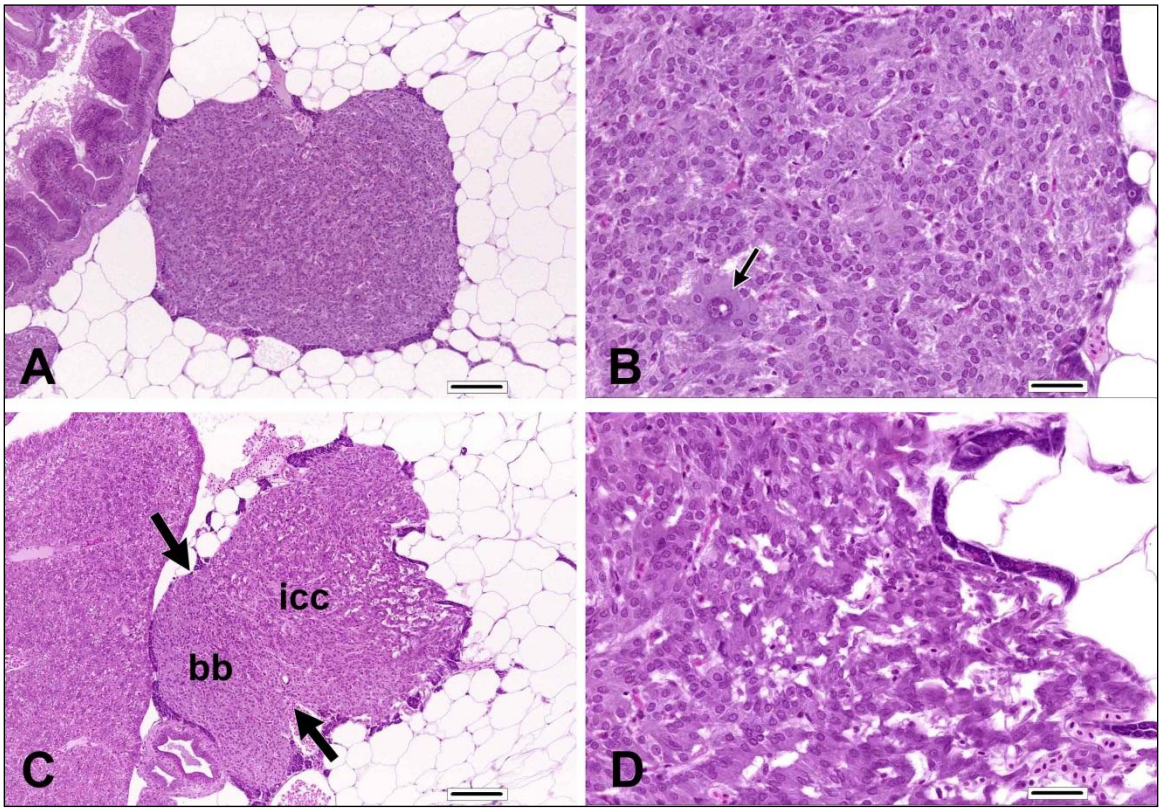
(ii) **Spreadsheet**. In addition to the recorded histopathology findings, the completed spreadsheet should indicate the animals from which figure images were photographed, and the number of images obtained per photographed fish.

(iii) **Figures.** A complete set of unembedded and unannotated photomicrographic figures should be submitted electronically on portable media as uncompressed TIFF files.

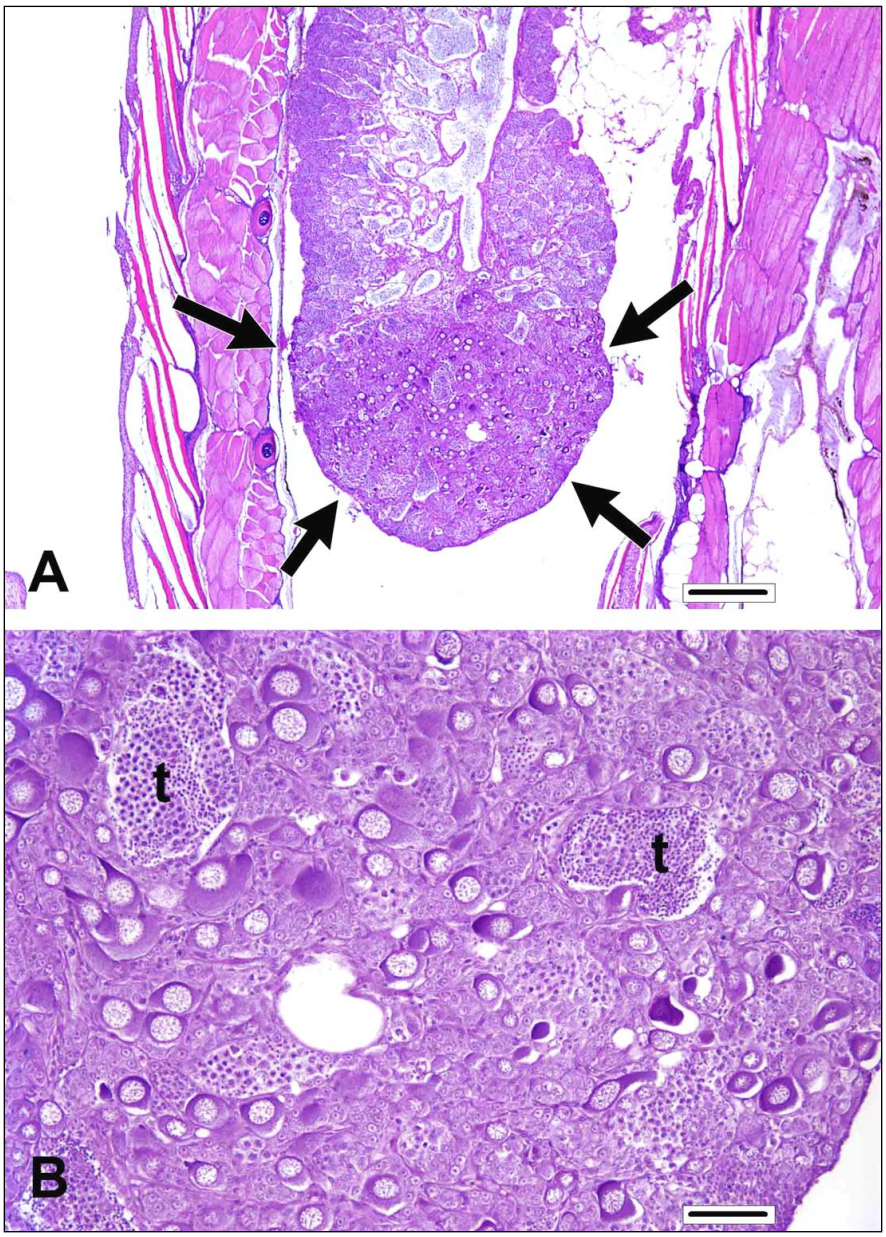
(7) **Pathology Peer Review**. Following the initial slide evaluation and creation of a draft report by the study pathologist (SP), it is encouraged

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).

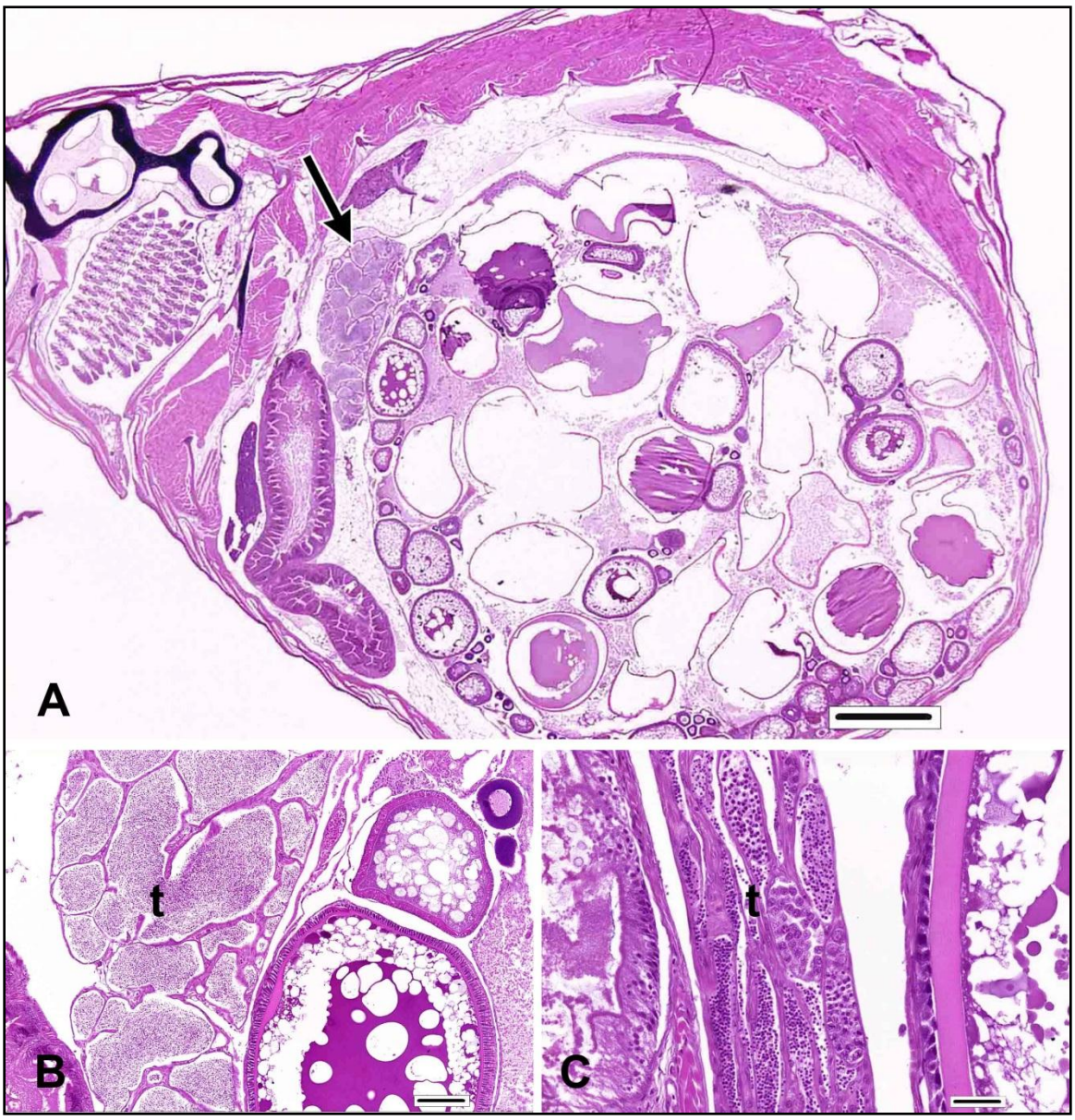
(8) **Atlas of Histopathologic Findings.** The purposes of this section are: 1) to provide a common technical “language” and 2) to create a reference atlas of both microanatomical structures and potential pathological findings. Listed alphabetically are a number of terms followed by working definitions or descriptions. 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 fish pathologists.



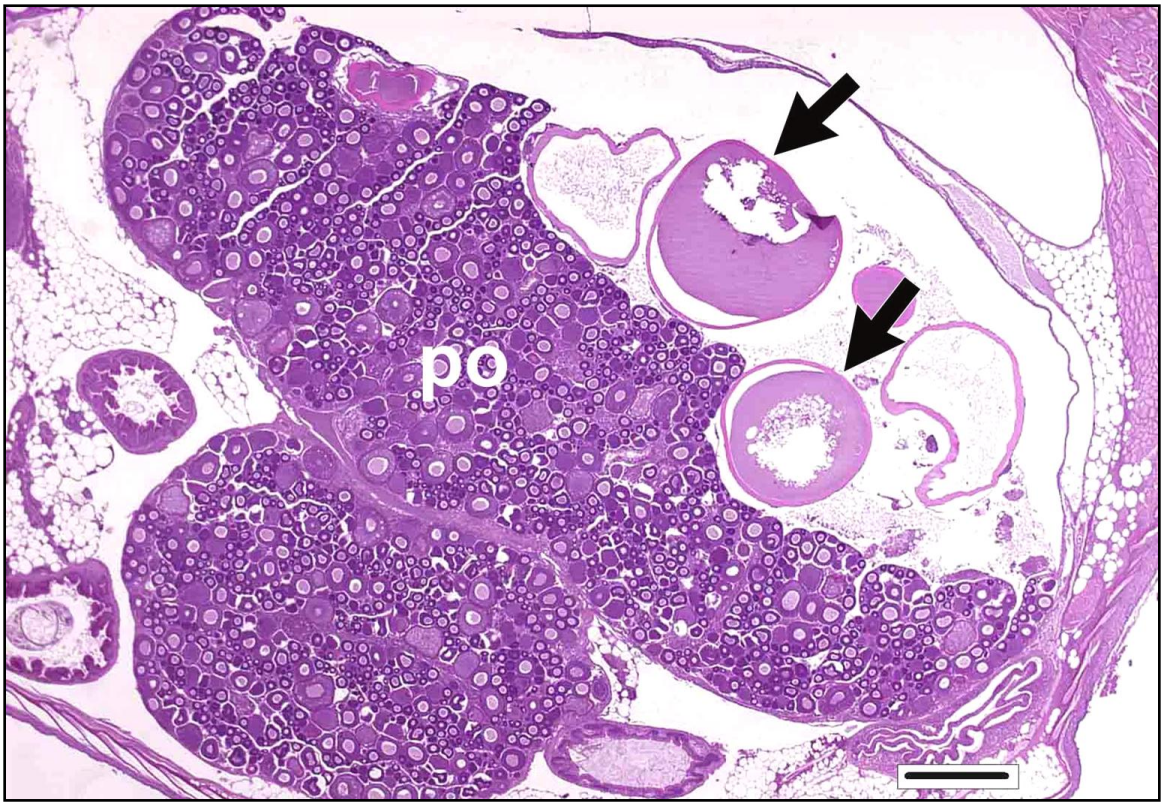
Endocrine pancreas, islet cell lesions. A and B: Large islet (Brockman body) from a control medaka. It is not uncommon to observe large or bizarre looking cells in normal islets. Bar = 25 µm. C and D: Islet cell carcinoma. Arrows indicate the line of demarcation between the unaffected area of Brockman body (bb) and the islet cell carcinoma (icc). This was an incidental finding in this study. Bar = 100 µm (A and C), 25 µm (B and D).



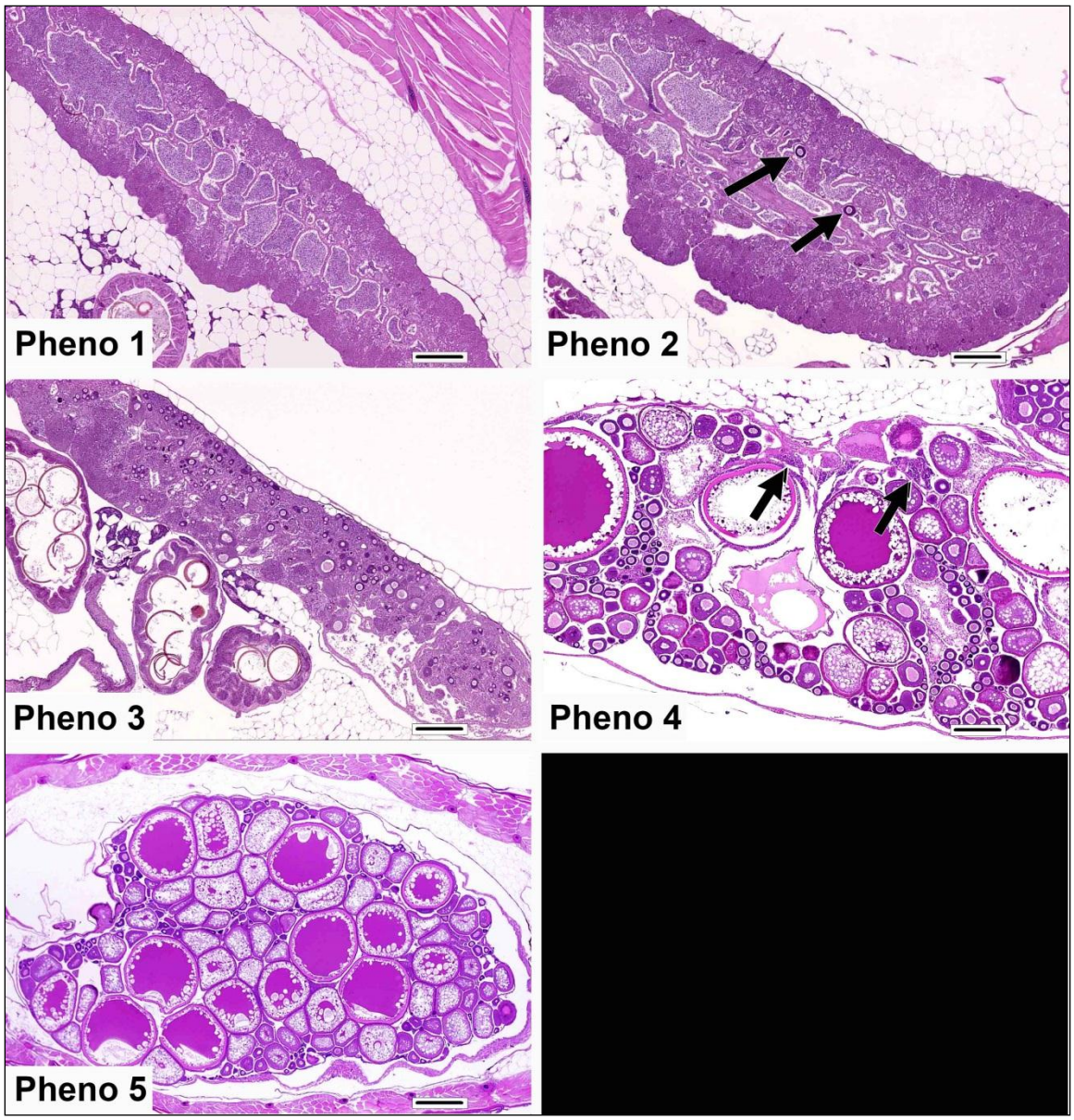
***Gonads, germ cell neoplasms*.** A: Dysgerminoma in the testis of an adult male. The caudal pole of the testis is effaced by a mass (arrows) consisting of oogenic tissue. B: A higher magnification of the tumor in A. The disorganization of the oogenic tissue is apparent. Germ cell neoplasms such as seminomas and dysgerminomas are rare spontaneous findings in medaka. There is currently little evidence to support the idea that such tumors are linked to EDC exposure, and control animals seem to be affected as often as chemically-exposed individuals. Distinguishing features of germ cell neoplasms include haphazard anatomic organization and progression of cell development, and a tendency to form mass-like lesions that distort the gonad architecture. In early life stage studies in which fish are exposed to potent hermaphroditic chemicals such as 17β-estradiol or ethinylestradiol, it may be difficult to distinguish germ cell neoplasms from malformed intersex gonads. It is also important to differentiate this neoplasm from other findings such as: 1) asynchronous development of the gonad in which different areas of the gonad are in different stages of development that blend almost imperceptibly and do not form a mass; 2) testicular oocyte formation, in which the scattered oocytes do not form a mass capable of distorting the gonad); and 3) possibly from hermaphroditism, in which the anatomic arrangement and developmental progression of the aberrant tissue is orderly and essentially resembles the normal gonad. H&E, bar = 250 µm (A), 50 µm (B).



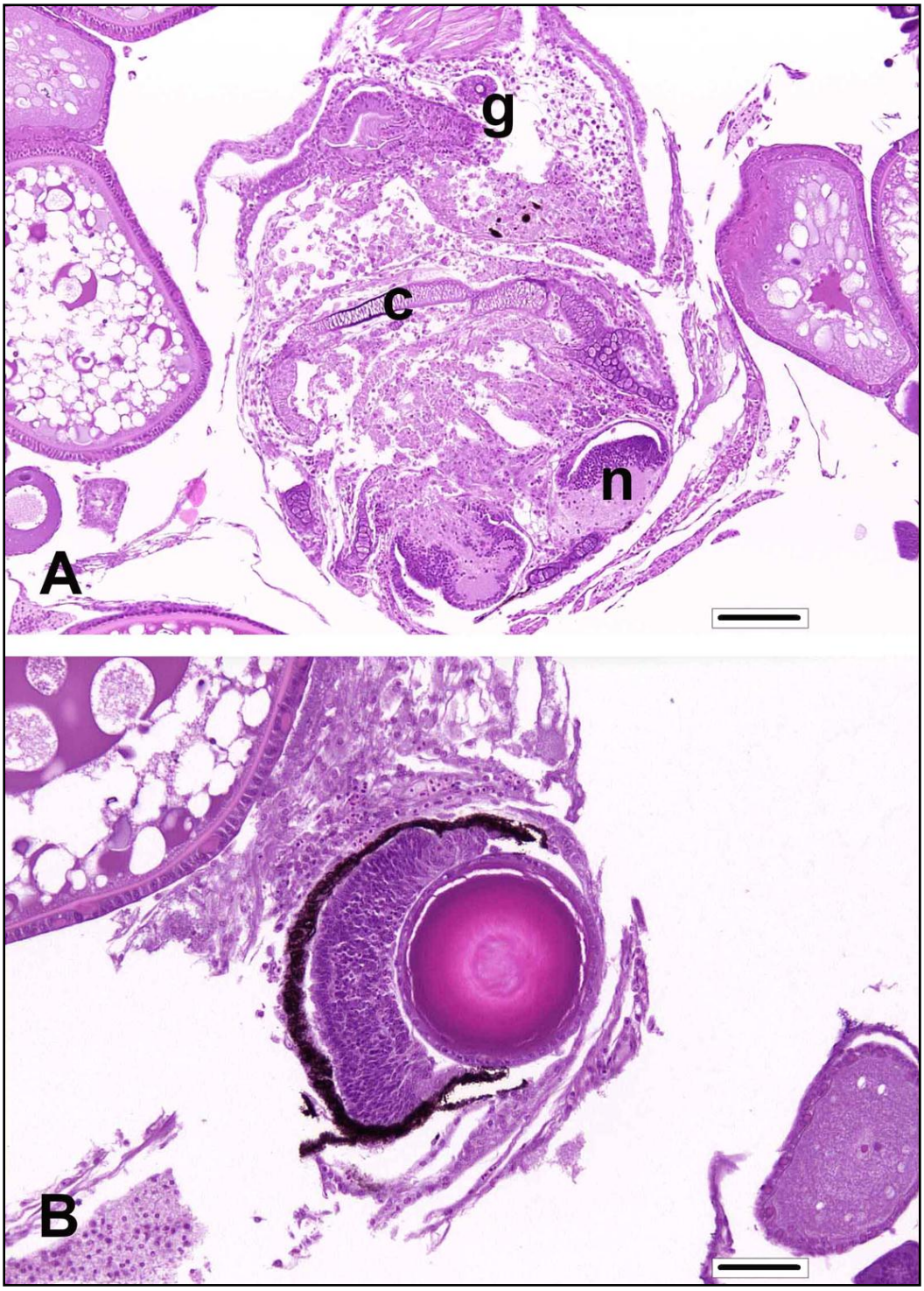
Gonads, hermaphroditism. A: Sagittal section of the abdomen of an adult medaka. Most of the abdomen is occupied by a massively enlarged ovary that contains primarily atretic oocytes. Anterior to the ovary is a separate testis (arrow). B and C: Higher magnifications of the testis (t) in A, and of the same testis (t) in another section. Hermaphroditism is a state in which *fully formed* male and female gonad tissues are present in the same individual. The phrase "fully formed" indicates that: 1) the male and female gonadal tissues are in discrete compartments; 2) the organizational architecture of the gonads is maintained; and/or 3) there is visible evidence of supportive structures (e.g., tunica albuginea, ducts) in addition to germinal cells. Bar = 800 µm (A), 100 µm (B), 25 µm (C).



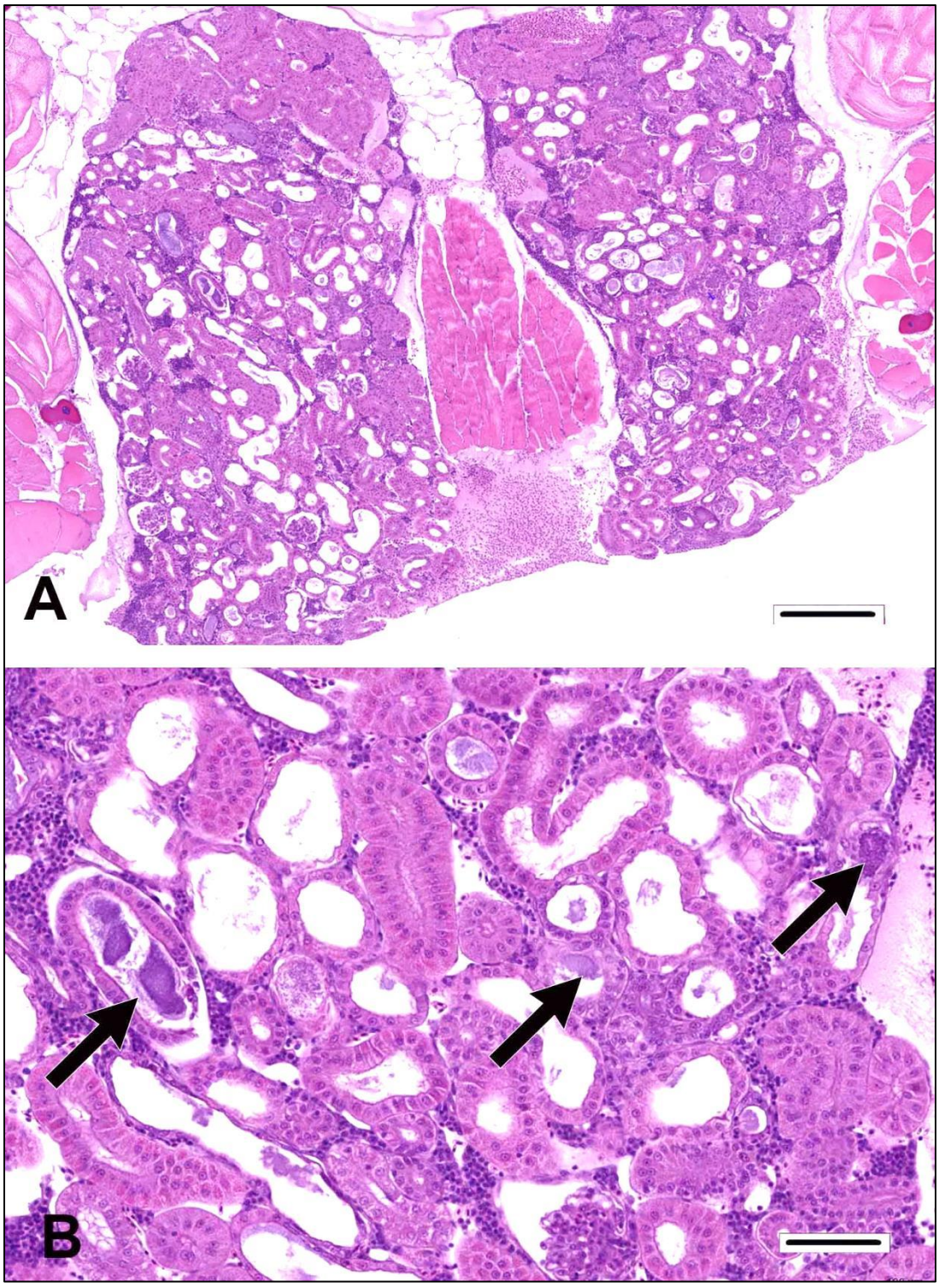
Gonad, increased / decreased cells, [insert cell type], (testis or ovary). In this case, perinucleolar oocytes (po) dominate this ovary, which also contains a few degenerating mature follicles (arrows). Another possible rule-out to consider in this particular case would be a germ cell neoplasm (dysgerminoma). It is recognized that endocrine active compounds may alter the proportional distribution of gametogenic and supportive cell types in the testis or ovary. Certain types of alterations (for example, the proliferation or absence of single cell population) may not be adequately documented by gonadal staging. This diagnostic term provides a mechanism for documenting such changes. For consistency, the pathologist should presume that these semi-quantitative changes are: 1) relative to other cell types in the gonad; 2) relative to cell numbers in control animals; and 3) estimates only, versus actual cell counts. Bar = 500 µm.



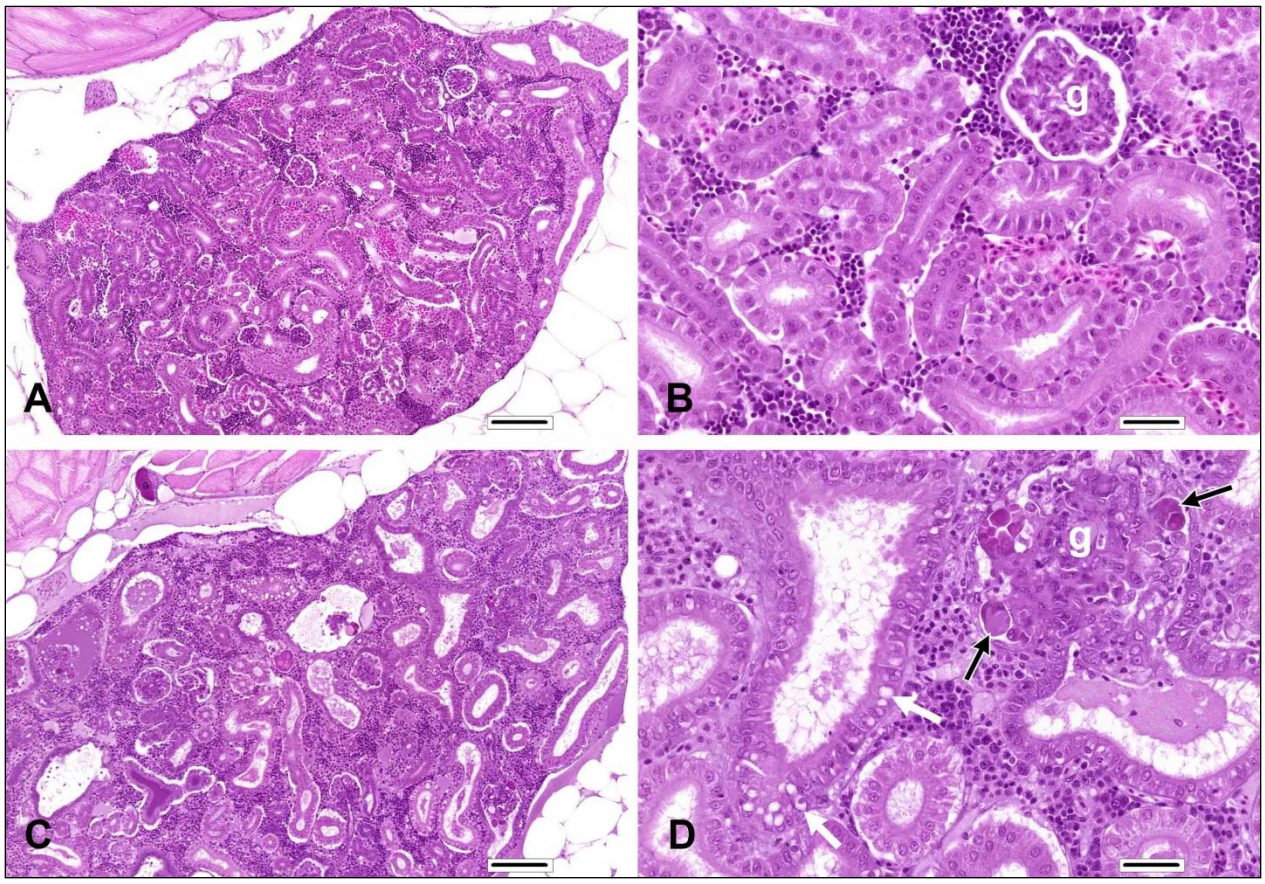
Gonads, phenotype scoring. For the purpose of the MEOGRT assay, gonads are scored for histologic phenotype according to the following criteria: Phenotype 1 = entirely testicular tissue; Phenotype 2 = predominantly testicular tissue; Phenotype 3 = approximately equal testicular and ovarian components; Phenotype 4 = predominantly ovarian tissue; Phenotype 5 = entirely ovarian tissue. Bar = 250 µm.



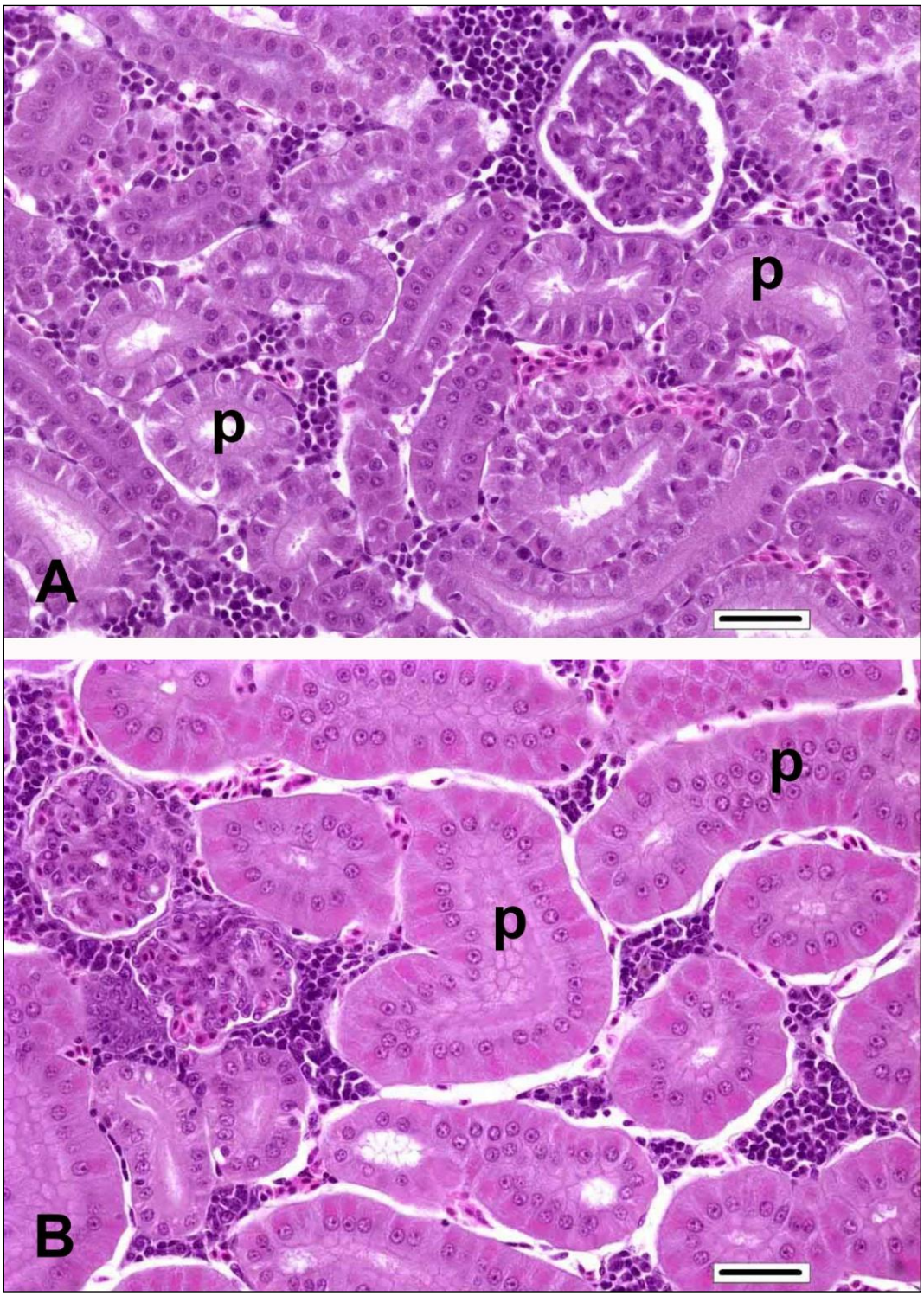
Gonads, stromal tumors. These seem to be even rarer in medaka than germ cell neoplasms. Examples include Sertoli cell tumors, granulosa cell tumors, and teratomas. A: Teratoma in the ovary of an adult female medaka. Various embryonic tissue types are represented including cartilage (c), neural tissue (n), and gonad tissue (g). B: Another area of the tumor from A. The dominant feature in this section is a developing ocular mass in which the lens and retinal tissue are recognizable. Bar = 100 µm (A), 50 µm (B).



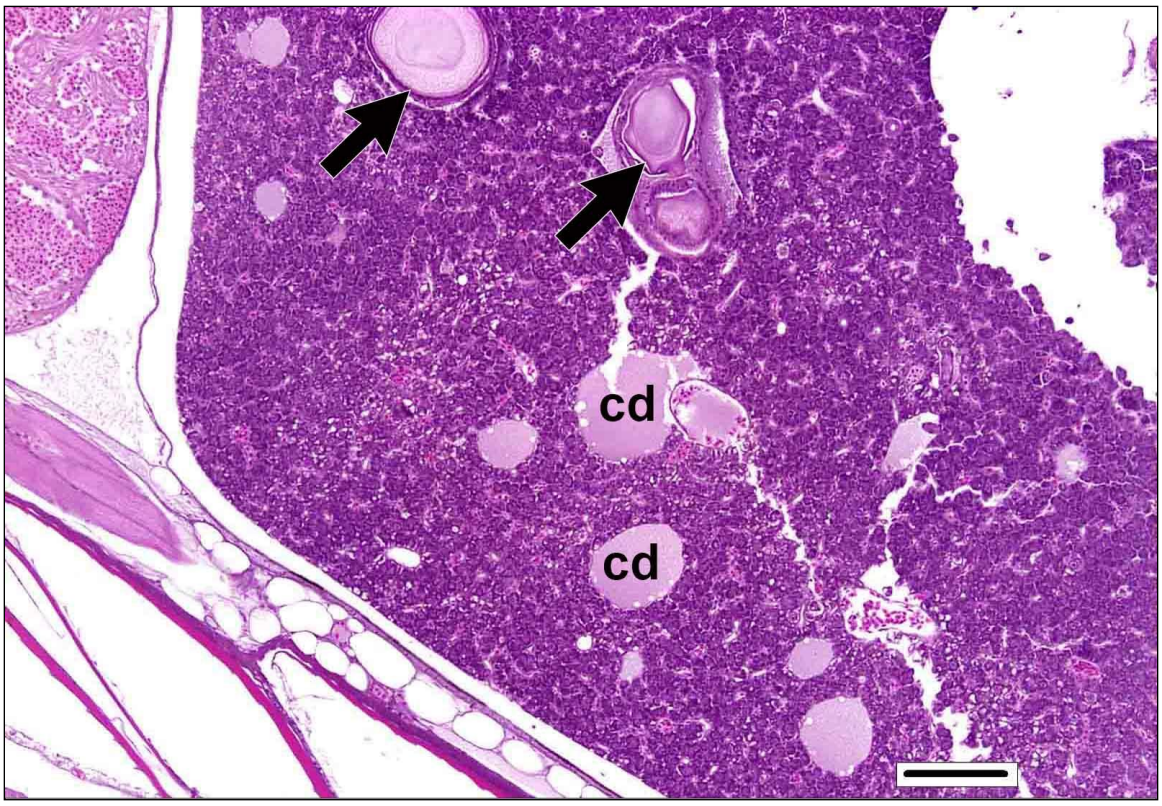
Kidney, mineralization. A: Renal tissue from an adult male. At low magnification, extensive dilation of tubular lumina and Bowman's spaces is evident. B: Intratubular mineralization is obvious at higher magnification. The tubular dilation is likely due to obstruction. It may be important in a study to differentiate this lesion from the nephropathy that can be induced by exposure to estrogenic substances. Bar = 200 µm (A), 50 µm (B).



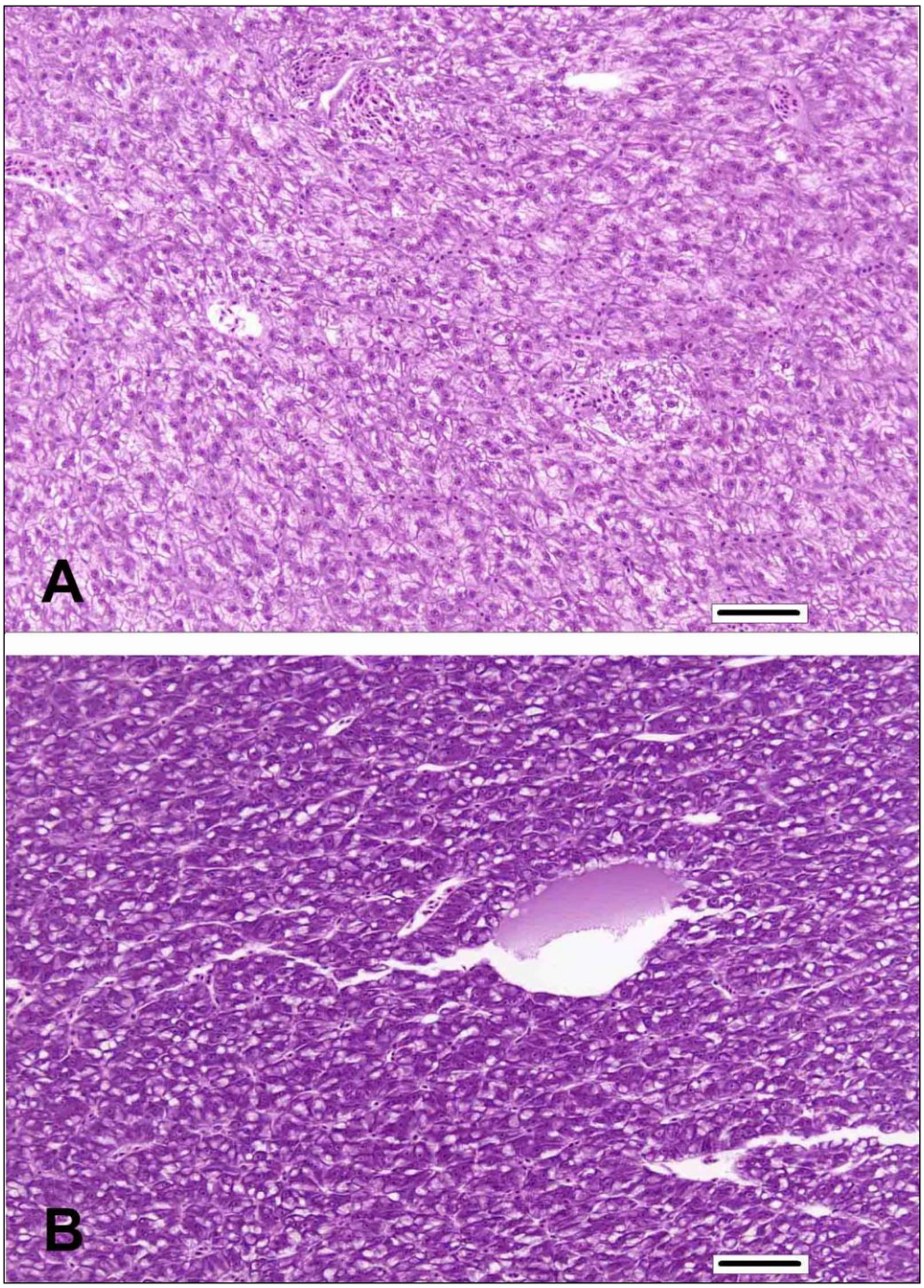
Kidney, nephropathy. A and B: Kidney from an adult female control. g = glomerulus. C and D: Kidney from an adult female exposed to a compound with estrogenic activity. Dilation of tubules and Bowman's space is evident at low magnification (C). At higher magnification (D), changes include marked enlargement of glomeruli (g), eosinophilic deposits of proteinaceous material in glomerular capillaries (black arrows), and vacuolation of the tubular epithelium (white arrows). Degenerative renal disease has been observed in a variety of fishes that have been exposed to compounds with estrogenic activity (Herman & Kincaid, 1988; Zillioux et al., 2001; Palace et al., 2002). Renal impairment presumably occurs due to increased production of vitellogenin that damages the kidney via protein overload. Such kidney changes are more likely to be observed in males, presumably because there is no physiological outlet for the excess vitellogenin, but nephropathy can also be seen in females exposed to high concentrations of estrogen-active substances. Microscopic lesions may include swelling of tubular epithelial cells, tubular necrosis, dilation of Bowman's capsule, interstitial fibrosis, casts, and hyaline droplets in tubules or glomeruli. For EDC studies, the pathologist may elect to group these lesions under an umbrella diagnosis of "nephropathy", if the data suggests that such changes are associated with estrogenic activity. Alternatively, the pathologist may choose to record these types of changes as individual findings (e.g., kidney, tubular necrosis). Bar = 100 µm (A and C), 25 µm (B and D).



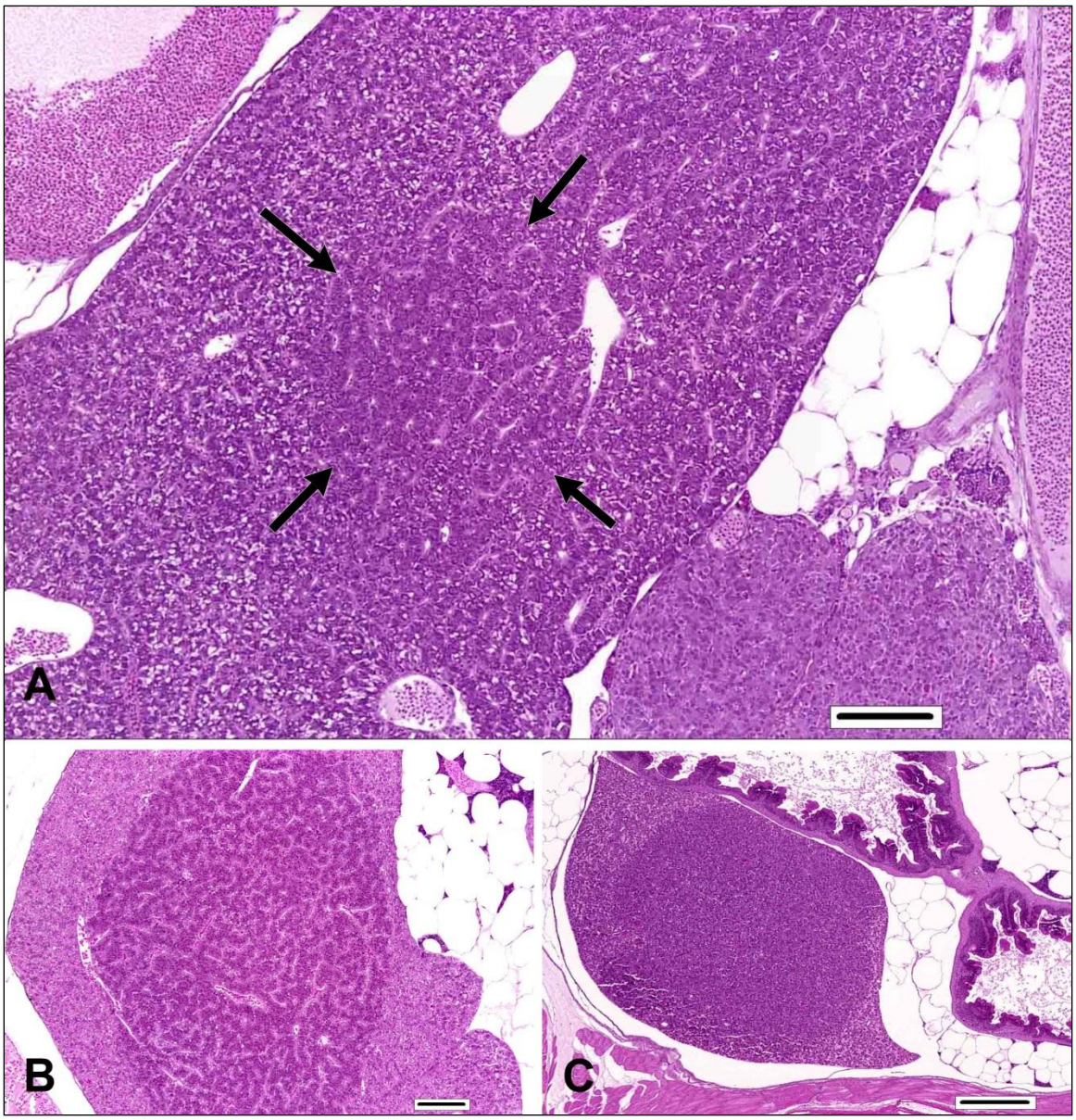
Kidney, tubular eosinophilia. A: Renal tissue from an adult female. Relative to the male (B), epithelial cells of the proximal tubules (p) are smaller and have more basophilic cytoplasm. B: Renal tissue from an adult male. The plump epithelial cells of the proximal tubules (p) have very fine granular eosinophilic material in their basal cytoplasm. Severity grading of tubular eosinophilia is as follows: Grade 1 = essentially no eosinophilia; Grade 2 = small amount of eosinophilia; Grade 3 = abundant eosinophilia. The kidney in B was scored Grade 3. Bar = 250 µm.



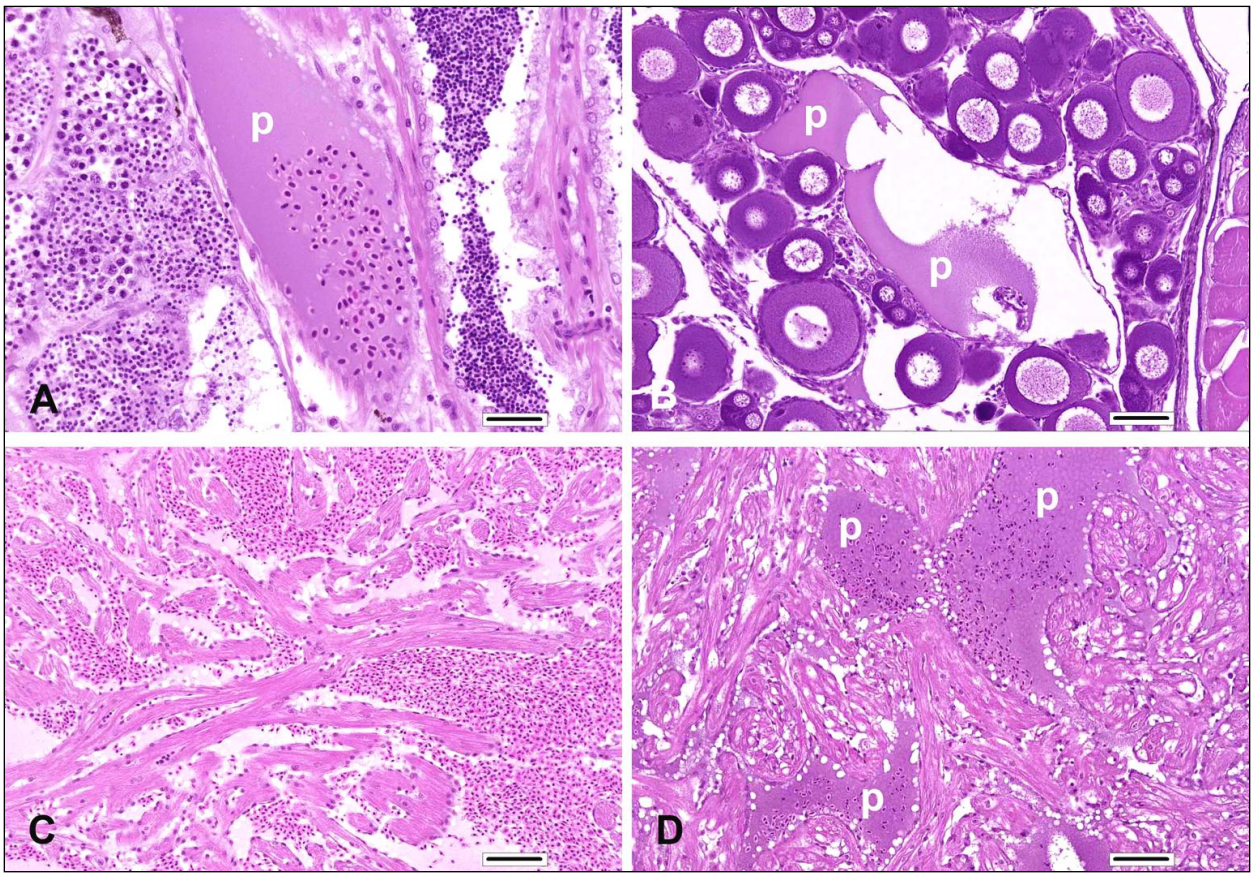
Liver, cystic degeneration. Liver from an adult female medaka. Cystic degeneration (cd) is characterized by various numbers of single or multilocular, roughly spherical, fluid-filled spaces that are scattered throughout the hepatic parenchyma. Individual lesions may or may not be associated with blood vessels, but the cysts themselves are not lined by endothelial cells. Based on morphologic criteria, such lesions have also been termed hepatic cysts or spongiosis hepatis, although empirical evidence suggests that these merely represent different stages in the progression of cystic degeneration. Cystic degeneration tends to be relatively common in medaka, and especially older females. This particular liver also features bile duct concretions (arrows). Bar = 100 µm.



Liver, hepatocyte basophilia, increased. A: Liver from an adult control male. B: Liver from an adult male exposed to 100 µg/L 4-*tert*-octylphenol, an estrogenic substance. There is a diffuse increase in hepatocyte basophilia, a loss of cytoplasmic vacuolization, and hepatic blood vessels contain proteinaceous fluid. A generally diffuse increase in hepatocyte cytoplasmic basophilia has been observed in male fish that have been exposed to compounds that are able to interact with hepatic estrogen receptors, including E2 and 17β-methyldihydrotestosterone (Wester et al., 2003). This increase in basophilia, which is correlated with increased vitellogenin production, presumably mimics the heightened metabolic state (e.g., increased endoplasmic reticulum) that is required for the production of vitellogenin in the reproductively-active female fish. Bar = 50 µm.



Liver, primary proliferative lesions. A: **Focus of hepatocellular alteration** (altered focus). This is a non-neoplastic, but likely pre-neoplastic, lesion that can be observed as a spontaneous or induced finding. Morphologic characteristics include changes in hepatocyte size and color relative to the surrounding liver parenchyma, and blending with unaffected hepatic tubules at the periphery of the lesion. B and C: **Hepatocellular adenomas**. Morphologic characteristics include distinct margins, peripheral compression of unaffected hepatic tissue, little cytologic atypia (relative to carcinomas), and generally larger size than foci. Hepatocellular carcinomas are less common but can occur also. Bar = 100 µm (A and B), 250 µm (C).



Multiple tissues, proteinaceous fluid. This finding is characterized by the presence of homogeneous dark pink translucent material, presumably vitellogenin, within the vascular and/or interstitial compartments of the testis, ovary, and other tissues in fish that have been exposed to estrogenic substances. A: Intravascular proteinaceous fluid (p) in the testis of an adult male exposed to 17β-estradiol at 100 ng/L for 4 weeks. B: Intravascular proteinaceous fluid (p) in the ovary of an adult female exposed to 4-*tert*-octylphenol at 90 µg/L for eight weeks. C: Heart from an untreated control fish. D: Heart with intravascular proteinaceous fluid (p). Bar = 25 µm (A), 50 µm (B, C, and D).