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

Cancels & replaces the same document of 22 September 2015

**Guidance Document on Medaka Histopathology Techniques and Evaluation for the Medaka Extended  
One-Generation Reproduction Test (MEOGRT) - Part 4**

**Series on Testing & Assessment  
No. 227**

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**OECD Environment, Health and Safety Publications**

**Series on Testing and Assessment**

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Reproduction Test (MEOGRT) - Part 4**

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## FOREWORD

The project to develop a Medaka One Generation Reproduction Test (MEOGRT) was initiated by Japan and the United States and included in the work plan of Test Guidelines Programme in 2000, originally under the name; Medaka Life Cycle (MLC)/Multi-generation Test (MMT).

The Integrated Summary Report and first draft TG were submitted to the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) in 2013, with subsequent commenting rounds in 2013 and 2014. The draft guidance document on Medaka histopathology was prepared to accompany the draft Test Guideline and help users of the test become more proficient in applying tissue sampling and preparation techniques, evaluation techniques and in the interpretation of the slides.

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

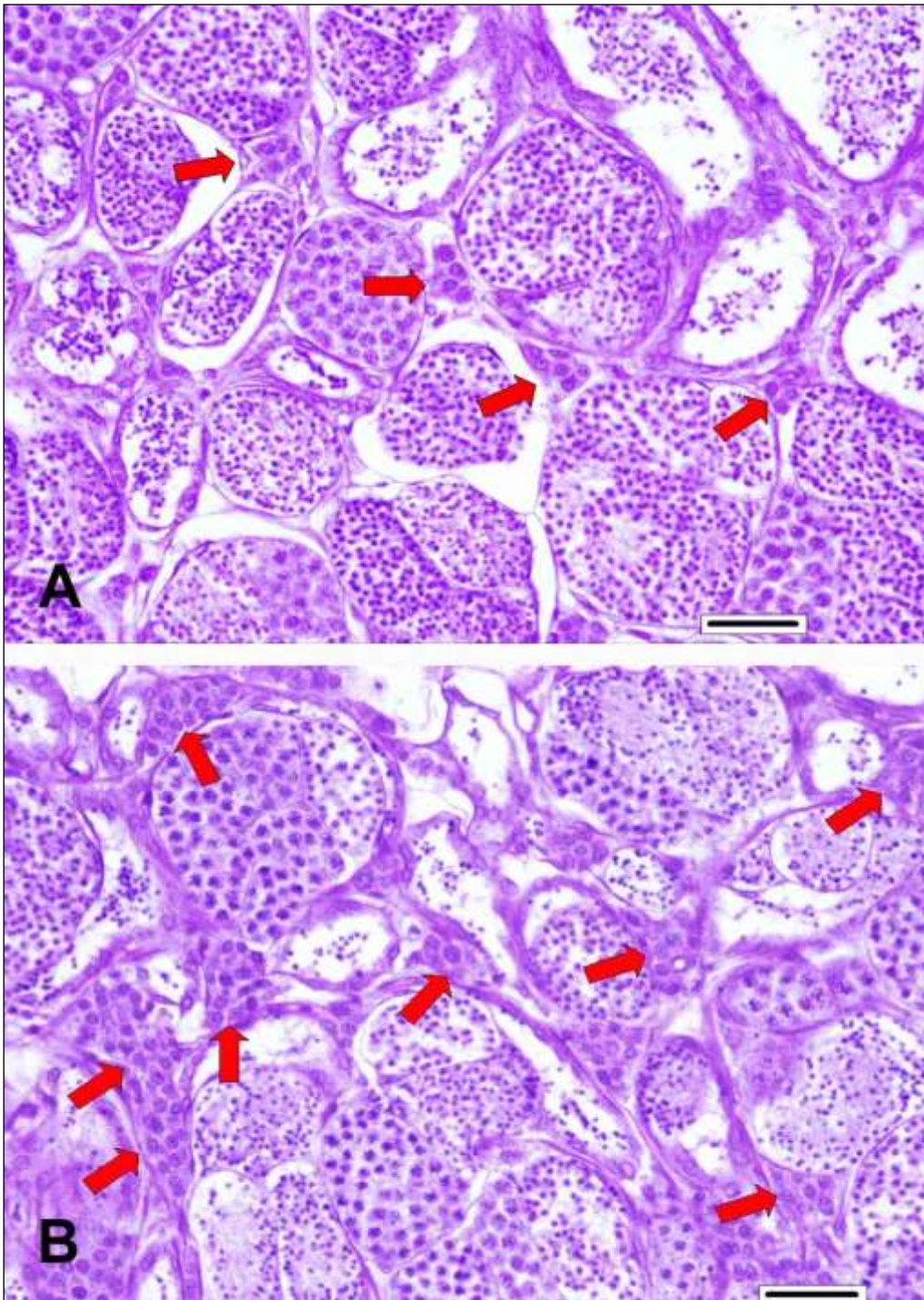
This document presents **Part 4** of the guidance document which in total consists of four parts.

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

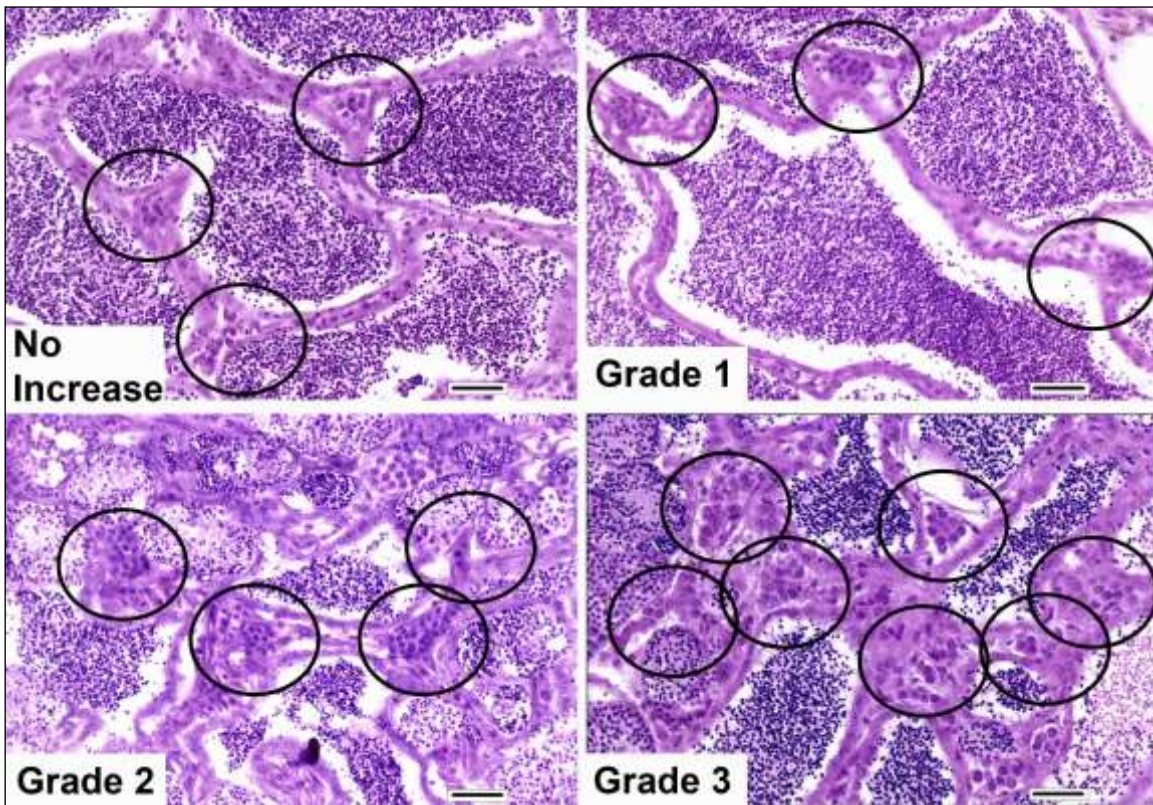
**GUIDANCE DOCUMENT ON MEDAKA HISTOPATHOLOGY TECHNIQUES AND  
EVALUATION (PART 4)**

**FOR THE**

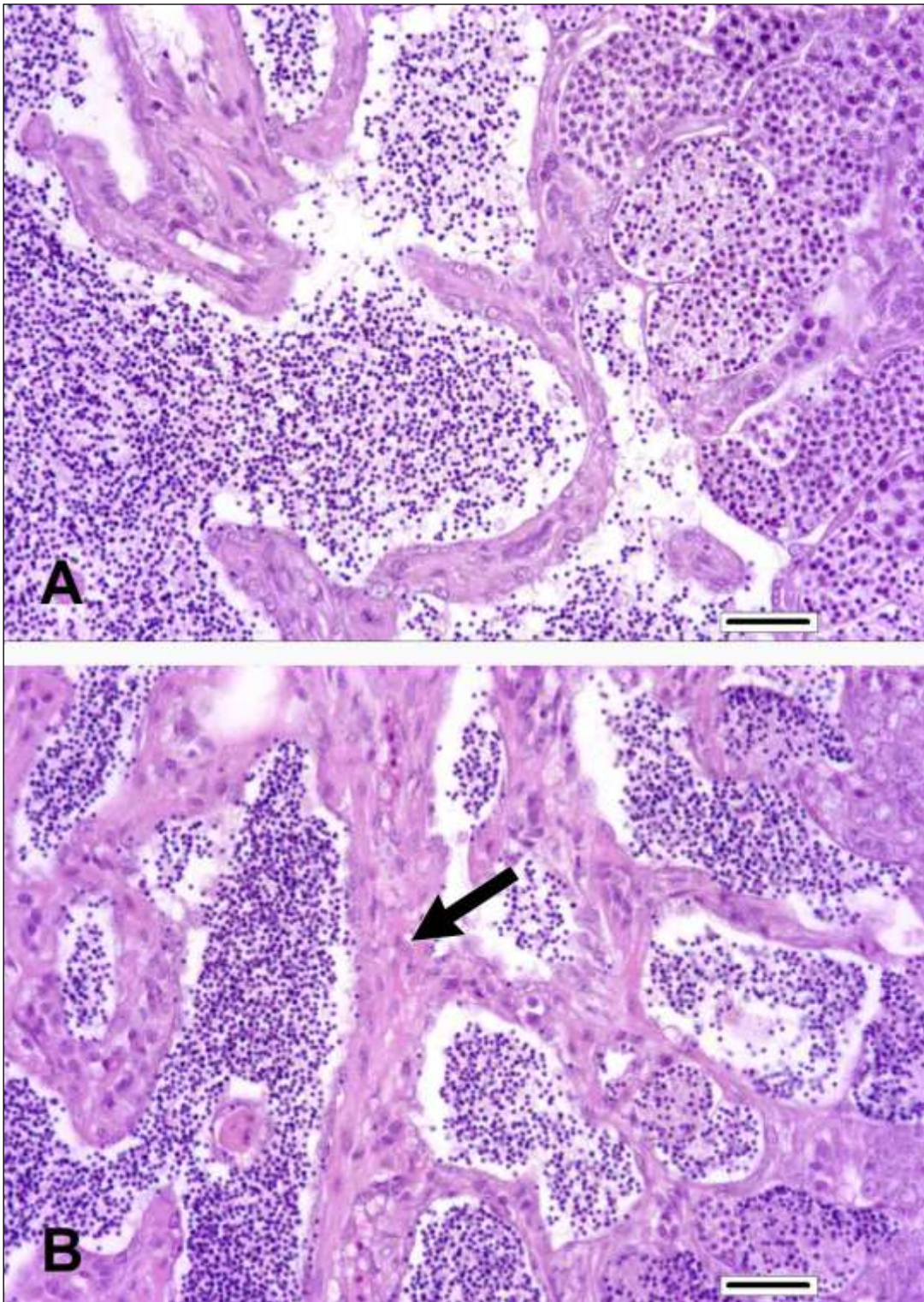
**MEDAKA EXTENDED ONE-GENERATION REPRODUCTION TEST (MEOGRT)**



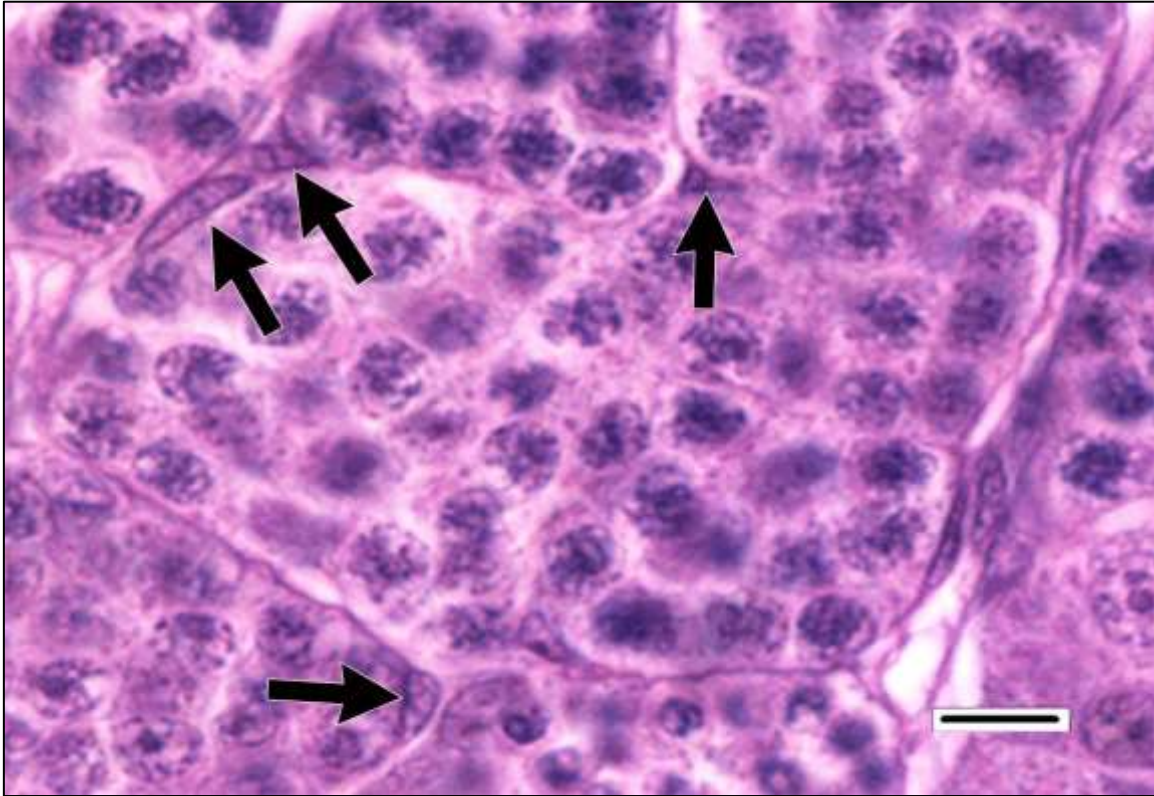
**Testis, interstitial cell hyperplasia / hypertrophy.** A: Testis from an adult male control. Scattered small clusters of interstitial cells (arrows) are located between tubules. B: Interstitial cell aggregates (arrows) are larger and more numerous in a testis from an adult male exposed to fadrozole at 100 ppm. This finding is characterized by a relative increase in the number and/or size of interstitial cells in the testis, as compared to the testes in the majority of control males. In moderate to severe hyperplasia, the testicular interstitium may be expanded due to the proliferation of these cells. Hypertrophic interstitial cells feature enlarged rounded nuclei with increased nuclear detail, and relatively abundant dense cytoplasm as compared to non-hypertrophic interstitial cells. Bar = 25  $\mu$ m (A and B).



**Testis, interstitial cell hyperplasia / hypertrophy, grading.** Testes of compound-treated males are scored relative to the typical appearance of testes among concurrent controls. Bar = 25 μm (all).



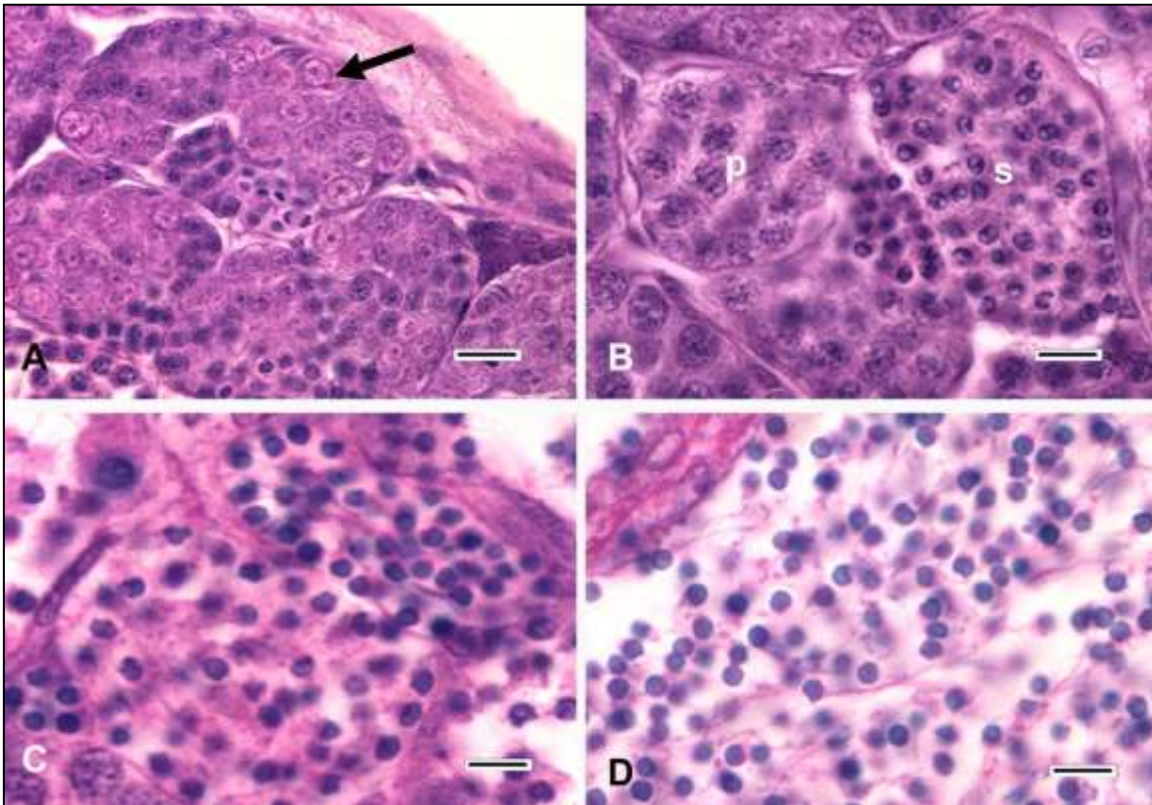
**Testis, interstitial fibrosis.** Whether in the testis or ovary, fibrosis is characterized by the presence of increased fibrous connective tissue (collagenous fibers and fibrocytes or fibroblasts) within the testicular or ovarian interstitium (stroma). Due to a high degree of inter-animal variability among controls, it may be difficult to reliably distinguish subtle fibrosis in treated fish. Bar = 25  $\mu$ m (A and B).



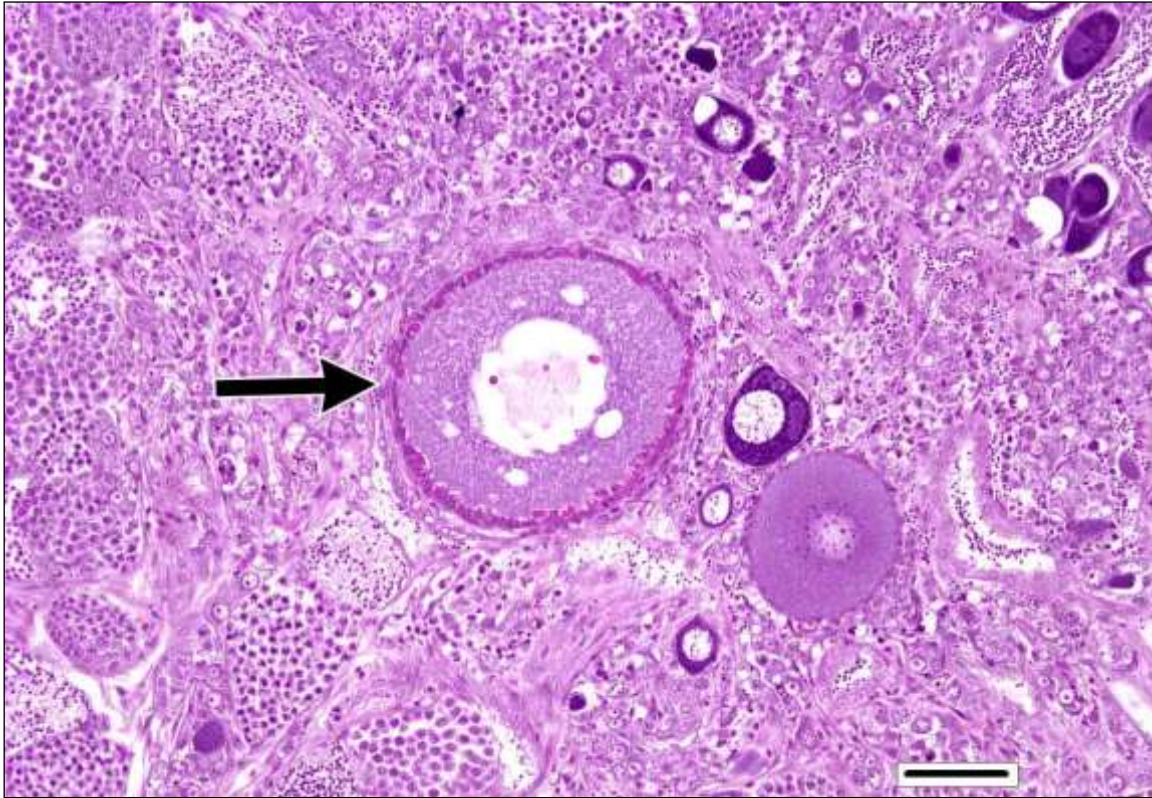
**Testis, Sertoli cells.** Sertoli cells (arrows) tend to have sharply-defined elongated or triangular nuclei, variably evident nucleoli, and cytoplasm that is often indistinct. The cytoplasmic arms of a Sertoli cell encircle a clonal group of spermatogenic cells, forming a spermatocyst. Compared to germinal cells, Sertoli cells are usually present in low numbers, usually as single cells located adjacent to lobular septa. Bar = 8  $\mu$ m.



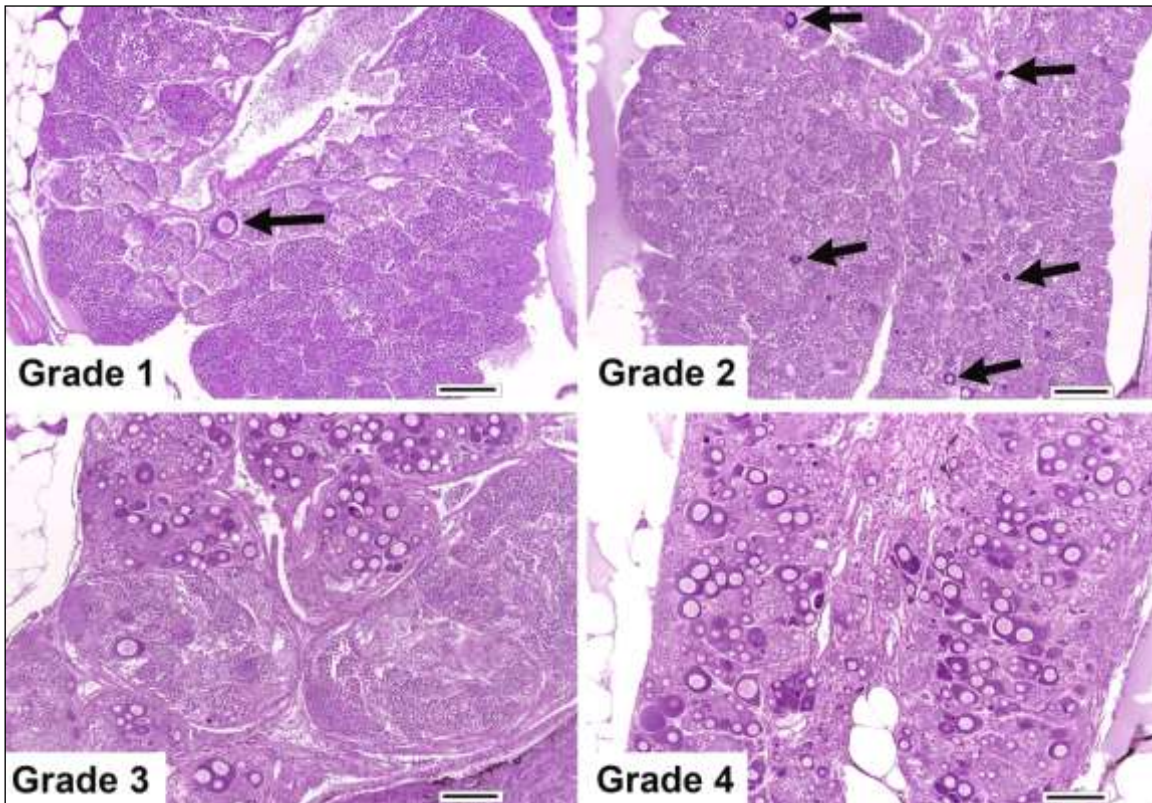
**Testis, spermatocysts.** The functional unit of the testis, this structure consists of a clonal group of spermatogenic cells (spermatogonia, spermatocytes, or spermatids) that are surrounded by the cytoplasmic arms of (usually) one Sertoli cell. Cells within spermatocysts exist as syncytia, maintained by intercellular attachments (cytoplasmic bridges), until final maturation and release of spermatozoa occurs (spermiogenesis) (Grier, 1976). Each spermatocyst (packet of cells) represents a cohort of germ cells in approximately the same developmental phase. Circled is a spermatocyst containing spermatogonia, and the arrow indicates the Sertoli cell that appears to be associated with that particular spermatocyst. Bar = 15  $\mu$ m.



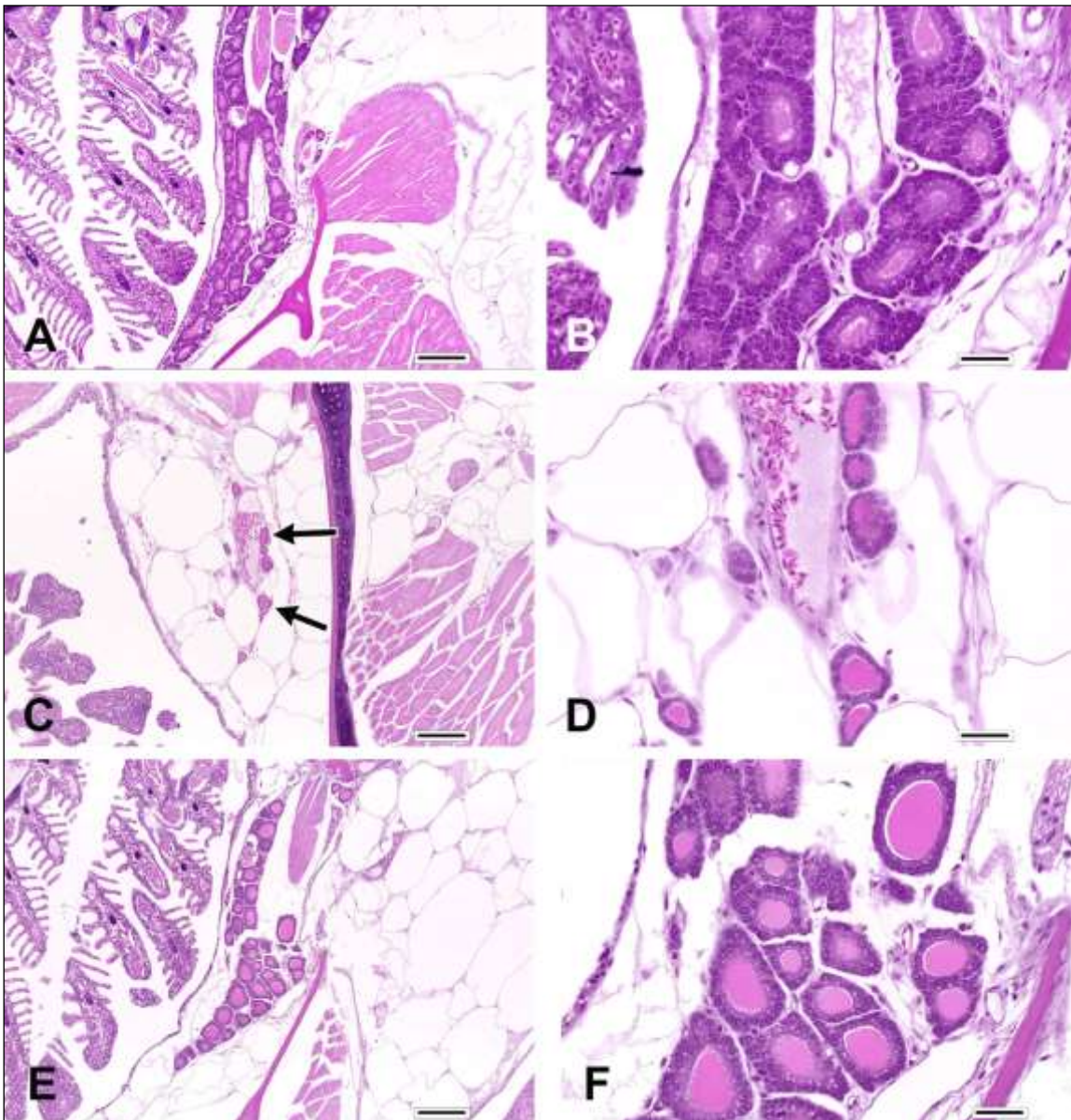
**Testis, spermatogenic cell types.** **A: Spermatogonia.** The largest of the spermatogenic cells (~ 5-10  $\mu\text{m}$ ), spermatogonia generally have pale vesicular nuclei, prominent nucleoli, variably distinct nuclear membranes, perinuclear cytoplasmic granules, and moderate amounts of granular cytoplasm (arrow). Spermatogonia B are smaller than spermatogonia A, and spermatogonia B are usually present in larger clusters (e.g., >4 cells). **B: Spermatocytes.** Derived from spermatogonia, spermatocytes are of intermediate cell size (~ 4-6  $\mu\text{m}$ ), and have comparatively dense nuclei and minimal to moderate amounts of indistinct cytoplasm. Spermatocyte nuclei are usually evident in one of three meiotic phases: pachytene, leptotene, or zygotene. Primary spermatocytes (p) are larger than secondary spermatocytes (s), and the latter are derived from primary spermatocytes following the first meiotic division. Spermatocytes are usually one of the most abundant spermatogenic cells, and they tend to contribute to the largest spermatocysts. **C: Spermatids.** Derived from spermatocytes following the second meiotic division, these cells have dense nuclei and narrow rims of eosinophilic cytoplasm. They are the smallest cells within the germinal epithelium (~2-3  $\mu\text{m}$ ), and the cells lose their cytoplasmic attachments to one another during spermiogenesis. **D: Spermatozoa.** These cells have dark, round nuclei and minimal or no apparent cytoplasm. Tails are generally not apparent in histologic sections. Spermatozoa are the smallest spermatogenic cells (~ 2  $\mu\text{m}$ ), and exist as scattered individual cells within tubular lumen. Bar = 15  $\mu\text{m}$  (A), 8  $\mu\text{m}$  (B), 4  $\mu\text{m}$  (C and D).



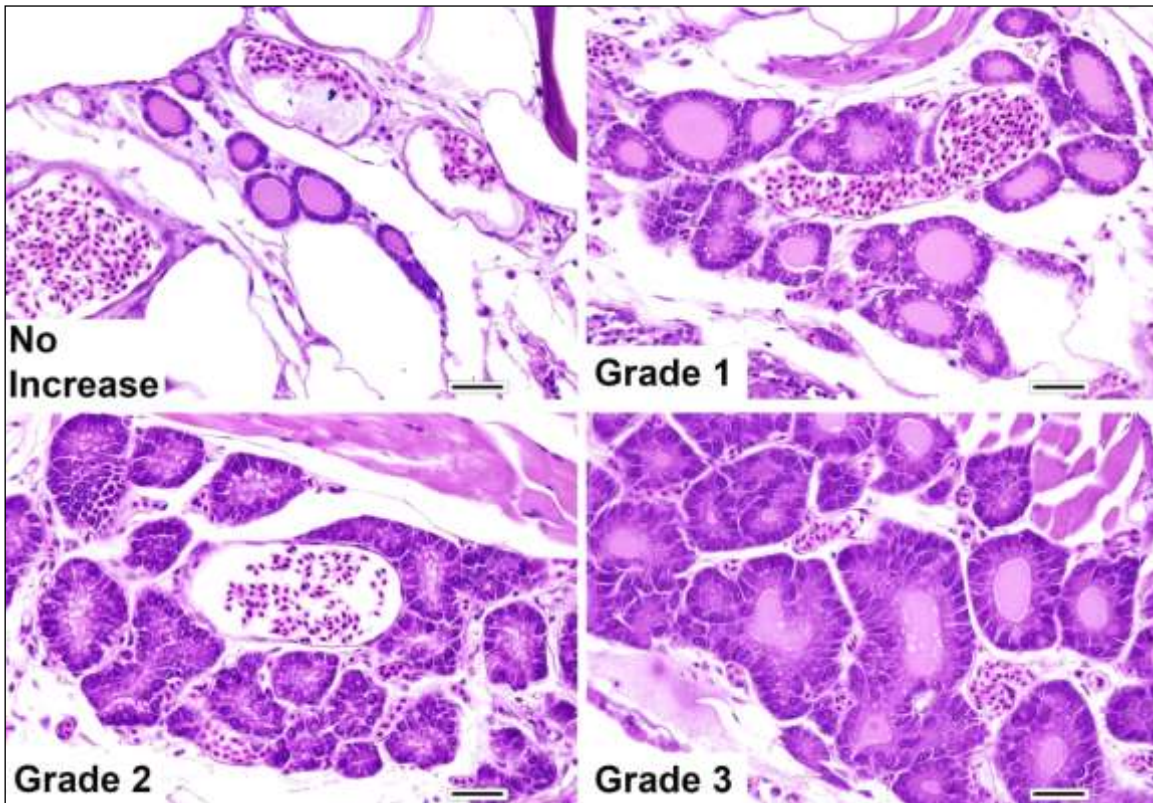
***Testis, testicular oocytes (testis-ova).*** This finding is characterized by the presence of one or more individualized or clustered oogenic cells, usually immature, within the testis. There is little or no evidence of ovarian architecture. Testicular oocytes may be chemically-induced or spontaneous; the incidence of spontaneous testicular oocytes in control fish may vary according to test facility. This particular example is fairly unusual in that one of the oocytes (arrow) has progressed to the early cortical alveolar phase. Bar = 50  $\mu$ m.



**Testis, testicular oocytes, grading.** Grade 1 is characterized by a single oocyte (arrow) per histologic testis section. Arrows indicate multiple oocytes in the Grade 2 image. Note the progressive loss of testicular ductal architecture with increasing grade score. Small remnants of spermatogenic tissue and the bi-lobed configuration of the Grade 4 testis provide evidence that this is a testis rather than an ovary. This fish had been exposed to an estrogenic substance; otherwise, another potential rule-out for this gonad might be dysgerminoma. Bar = 100  $\mu$ m (all).



**Thyroid glands.** In medaka, the bilaterally symmetrical thyroid tissue is located caudal and somewhat lateral to the branchial chamber, and similar to other fishes, the thyroid tissue is not a discrete encapsulated structure. Medaka differ from other fishes in that the thyroid tissue of reproductively active adult males is often proliferative-looking. A and B: Thyroid tissue from a control male. Not all males have thyroid tissue that appears this hyperplastic. C and D: Thyroid tissue from a control female. Here the follicles relatively small and lined by flattened epithelium. E and F: Thyroid glands from a female exposed to 102 µg/L of 4-tert-octylphenol. The severity of follicular cell hypertrophy / hyperplasia in this fish was recorded as grade 1, which is reduced compared to most control males. Bar = 100 µm (A, C, and E), Bar = 25 µm (B, D, and F).



**Thyroid glands, hypertrophy / hyperplasia, grading.** Most control females will demonstrate little or no increase in thyroid follicular cell size or number, whereas the thyroids of most reproductively active adult males will score as Grades 1 or 2. Bar = 50  $\mu\text{m}$  (all).

## II. GONADAL STAGING CRITERIA

The goal of gonadal staging is to determine if the administration of a particular endocrine-active substance affects the reproductive cycle status of adult male and female fish. The purpose of this section is to describe a rapid, semi-quantitative method for assessing the proportions of various gametogenic cell types (gonadal staging) based on the light microscopic examination of hematoxylin and eosin-stained histologic sections.

Semi-quantitative gonadal staging has been proposed for, or employed in, studies involving fathead minnows (Ankley et al., 2002; Jensen et al., 2001; Miles-Richardson et al., 1999a; Nichols et al., 2001; US EPA, 2002) and zebrafish (Van den Belt et al., 2002), among other fishes. Although such studies generally included excellent descriptions of the different gametogenic maturation stages (e.g., spermatogonium through spermatozoa for the testis), they did not incorporate pre-defined categorical guidelines for evaluating and reporting the reproductive cycle status of an individual fish. To maintain scientific integrity across the board in a program that involves multiple studies, multiple laboratories, and large numbers of animals, it is essential that observations are recorded on a fish-by-fish basis. The use of a categorization system can improve the consistency and objectivity of reported observations within and among experiments; consequently, comparisons of the results are more meaningful. Categorization systems also have some drawbacks and limitations, the most significant of which are: 1) the potential loss of discriminatory data when similar, but not identical, types of observations are combined (binned) into a single class; 2) the questionable biological relevance of the classification criteria in some cases; and 3) the inability of any single classification system to address every type of observation (either predicted or unforeseen). To address this last limitation, gonadal staging is accompanied by a complete histopathological evaluation of the gonads; in this manner, the loss or overabundance of a specific gametogenic cell type, for example, can be documented. It should be emphasized that gonadal staging results are virtually meaningless in terms of individual fish (versus treatment groups). This is because considerable animal-to-animal variation in gonad cell proportions is to be expected, even among fish of the control groups, as a consequence of spawning cycle asynchrony. For example, the cellular composition of fathead minnow ovaries was observed to vary substantially according to the day that each fish was sacrificed relative to spawning (Jensen et al., 2001). Consequently, following the gonadal staging of individual fish, each treatment group should be assessed as a whole and compared to the appropriate control group to determine if a compound-related effect has occurred.

The semi-quantitative gonadal staging scheme proposed here is a modification of a system adopted by the United States Department of the Interior, U.S. Geological Survey, Biological Resources Division as part of the "U.S. Biomonitoring of Environmental Status and Trends (BEST) Program" (McDonald et al., 2000). The authors of the BEST system credit previous work by Treasurer and Holiday (1981), Nagahama (1983), Rodriguez et al. (1995), and Goodbred et al. (1997). The foremost benefits of this system are speed and ease of use, especially when compared to fully-quantitative staging. The basis of the BEST system is a visual assessment of the density of gametogenic precursors as compared to mature gametocytes in one or more gonad sections. Accordingly, the stage numbers (testis: Stages 0 to 4; ovary: Stages 0 to 5) increase in direct relationship to the relative proportion of mature cells. Although the BEST system was initially developed to assess reproductive function in seasonal spawners such as carp (Cyprinidae) and black basses (Centrarchidae), the same stage categories can be applied to fractional spawners such as medaka.

A few modifications have been made to the BEST system to adapt it for use in small aquarium-sized fishes. For example, there is currently no provision in the system for gonads that are

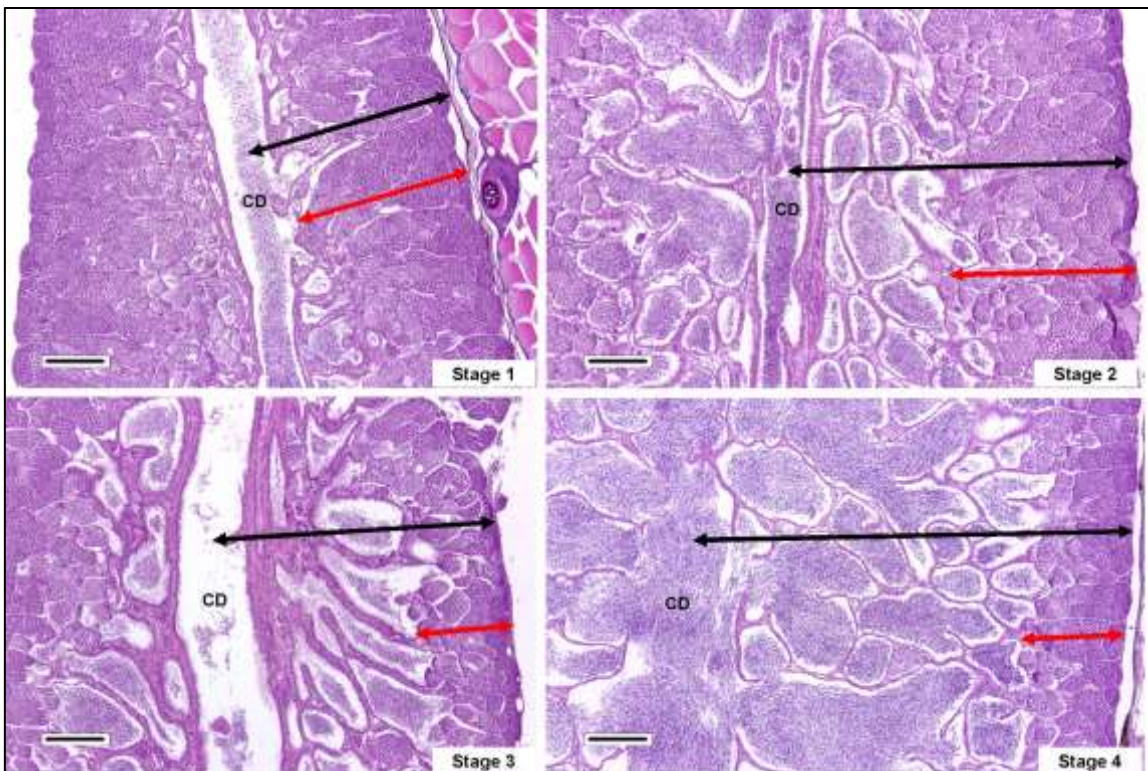
comprised entirely of spermatogonia or oogonia. Although many experiments will use reproductively mature fish, it is possible that an occasional animal may not attain sexual maturity by the time that the experiment is terminated, or that certain test compounds might cause reversion of the gonads to a juvenile phenotype. Therefore, as one modification of the BEST system, a pre-staging category called "juvenile" has been added for both male and female fish. Another modification to the system involves an apparent discrepancy between the BEST system and Goodbred et al. concerning the thickness of the testicular germinal epithelium as a staging criterion. As indicated by Goodbred et al., the germinal epithelium becomes thinner as the testis stage increases, whereas, the reverse occurs according to the BEST system (as presented in McDonald et al.). Although it is difficult to find corroborating statements in the scientific literature, empirical evidence indicates that Goodbred et al. is correct on this point. A third modification to the system is the option to subdivide a stage into two subordinate stages (e.g., Stages 3A and 3B) if the pathologist believes that this tactic would reveal a subtle, compound-related effect that might otherwise be missed. Other modifications to the system are relatively minor and primarily involve rewording for clarification.

Granting that the cell distribution pattern is likely to vary throughout a given tissue section, the gonad should be staged according to the predominant pattern in that section. This is especially important for the medaka testis in which spermatogenesis progresses along axial centrifugal and rostro-caudal gradients ("restricted spermatogonial" type testis). Gonads that cannot be reasonably staged for various reasons (e.g., insufficient tissue, or extensive necrosis, inflammation, or artifact) should be recorded as **UTS** (unable to stage).

## IIA. Criteria for Staging Testes

To derive each stage score, the estimated width of the germinal epithelium (EWG) can be compared to the estimated width of the testis (EWT) in the following manner:

- Stage 1:  $EWG > \frac{2}{3} EWT$
- Stage 2:  $EWG \frac{2}{3}$  to  $> \frac{1}{2} EWT$
- Stage 3:  $EWG \frac{1}{2}$  to  $> \frac{1}{4} EWT$
- Stage 4:  $EWG < \frac{1}{4} EWT$

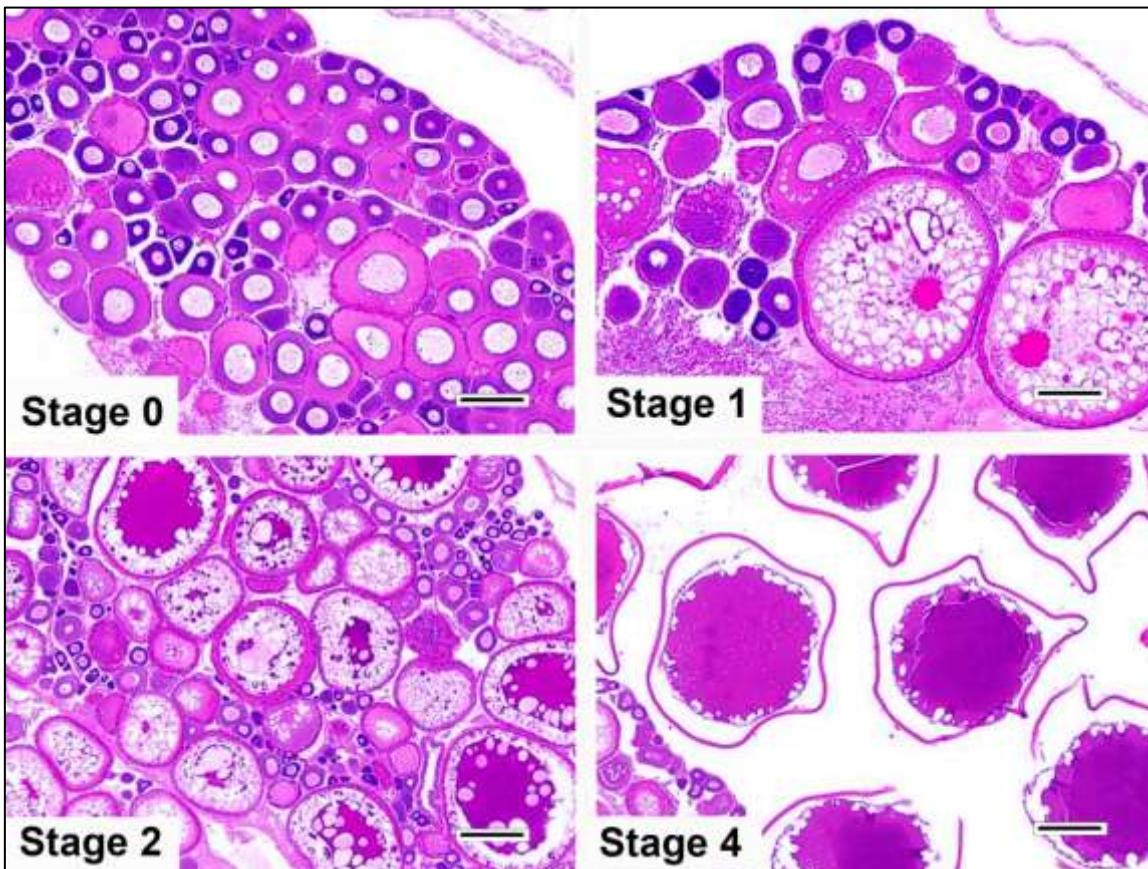


**Staging the testis.** Testes from four adult male medaka (transverse oblique sections). Black arrows represent the EWT, and red arrows represent the EWG (measurements are illustrated unilaterally for simplicity). In order to obtain comparable sections, it is imperative that each section contains a portion of the central duct (CD), preferably at its widest and longest extent (paraffin, H&E). Bar = 100  $\mu$ m (all).

## **IIB. Criteria for Staging Ovaries**

The following are morphologic criteria for staging female fish:

- **Juvenile**: gonad consists of oogonia exclusively; it may be difficult or impossible to confirm the sex of these individuals.
- **Stage 0 – Undeveloped**: entirely immature phases (oogonia to perinucleolar oocytes); no cortical alveoli.
- **Stage 1 – Early development**: vast majority (e.g., >90%) are pre-vitellogenic follicles, predominantly perinucleolar through cortical alveolar.
- **Stage 2 – Mid-development**: at least half of observed follicles are early and mid-vitellogenic.
- **Stage 3 – Late development**: majority of developing follicles are late vitellogenic.
- **Stage 4 – Late development/hydrated**: majority are late vitellogenic and mature / spawning follicles; follicles are larger as compared to Stage 3.
- **Stage 5 – Post-ovulatory**: predominately spent follicles, remnants of theca externa and granulosa.



**Staging the ovary.** Ovaries from four adult female medaka. Bar = 100  $\mu\text{m}$  (Stages 0 and 1), 250  $\mu\text{m}$  (Stages 2 and 4).

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