Histopathology guidelines for the Fathead Minnow (*Pimephales promelas*) 21-day reproduction assay

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INTRODUCTION

The purpose of this document is to provide guidelines for the preparation and histopathological evaluation of gonads from fathead minnow (*Pimephales promelas*).

The goals of these guidelines are to provide an updated source of direction for the participating laboratories, to supply template text for laboratory protocols, and most importantly, to facilitate non-biased comparisons of inter-laboratory results.

Throughout this document, the proposed procedures were derived from the consensus opinions of various fish pathologists, recommendations from the Bilthoven 2002 and Paris 2003 workshops, information distilled from previous guidelines, and the scientific literature.

This guidance document is divided into three sections: I) Post-mortem and Histotechnical Procedures; II) Gonadal Histopathology Glossary and Diagnostic Criteria; and III) Gonadal Staging Criteria.

I. POST-MORTEM AND HISTOTECHNICAL PROCEDURES

The purpose of this section is to outline all of the post-mortem steps and procedures that occur prior to the evaluation of histologic sections on glass slides, to include euthanasia, necropsy, tissue fixation, decalcification, tissue trimming, processing, embedding, microtomy, staining, coverslipping, and slide labeling.

1. Substrate obtained for vitellogenin analysis.
   Fathead Minnow (FHM): blood sample from the caudal vein/artery or heart
2. Tissue specimen for gonad histopathology. Techniques were selected that would most optimally: 1) preserve the cellular structure of the gonads; 2) maximize the amount of gonad tissue available for analysis; 3) sample the gonads in a representative and consistent fashion; and 4) allow the pathologist to examine at least three step sections of both gonads on a single glass slide. In FHM, the gonads are excised from the fish.

Davidson's fixative is the recommended fixative. Compared to other common fixatives, such as 10% neutral buffered formalin or Bouin's fixative, the advantages of Davidson's fixative are: 1) the morphologic appearance of gonad sections is generally considered to be comparable to sections fixed in Bouin's fixative and superior to sections fixed in formalin; 2) compared to Bouin's fixative, which contains picric acid, Davidson's fixative is generally considered to be less noxious, less hazardous, and more easily disposed of; 3) there is anecdotal information which suggests that Bouin's fixative may be difficult to obtain in the near future; 4) specimens fixed in Bouin's fixative require multiple rinses prior to transfer to alcohol or formalin. Please see photographic comparison of specimens fixed in Davidson's versus Bouin's fixatives (Appendix A, Fig. 1). Please be aware that different recipes and products that are designated as "Davidson's fixative" may actually be modifications of the original formula (Appendix C); if a modified Davidson's fixative is used, this should be noted by the laboratory. If necessary, a recommended decalcification solution is listed (Appendix D). Factors that may affect the need for decalcification include the size of the individual fish, the length of time that the carcass was immersed in fixative, and the extent to which the abdominal cavity came into contact with the fixative.

I. Fathead Minnow

1. Euthanasia, Necropsy, and Tissue Fixation

Objectives:

1. Provide for the humane sacrifice of fish.
2. Obtain necessary body weights and measurements.
3. Obtain specimens for vitellogenin analysis.
4. Excise gonad specimens.
5. Evaluate secondary sex characteristics.
6. Provide for adequate fixation of the gonads and the carcass.

Materials:

1. Fish transport container (~500 ml, contains water from the experimental tank or system reservoir).
2. Small dip net.
3. Euthanasia chamber (~500 ml vessel).
4. Euthanasia solution (Appendix C or FA-100 [Japan]).
5. Electronic slide caliper (minimum display: ≤ 0.1mm)
6. Electronic analytical balance (minimum display: ≤ 0.1mg) and tared vessels.
7. Stereoscopic microscope.
8. Pins and corkboard.
9. Small scissors (e.g., iris scissors).
10. Small forceps.
11. Microdissection forceps.
12. Microdissection scissors.
13. Gauze sponges.
14. Davidson's fixative (Appendix C & Appendix D).
15. Plastic syringe (3ml).
16. Standard plastic tissue cassettes (one per fish).
17. Fixation containers (100 ml, one per fish).

Procedures:

1. Fish should be sacrificed within one to two minutes prior to necropsy. Therefore, unless multiple prosectors are available, multiple fish should not be sacrificed simultaneously.
2. Using the small dip net, a fish is removed from the experimental chamber and transported to the necropsy area in the transport container. For each test chamber, all male fish are sacrificed prior to the sacrifice of female fish; the sex of each fish is determined by external body characteristics (e.g., presence or absence of nuptial tubercles, dorsal pad, etc.).
3. The fish is placed into buffered MS-222 solution. The fish is removed from the solution when there is cessation of respiration and the fish is unresponsive to external stimuli.

4. The fish is wet weighed, measured according to protocol, and a blood sample is obtained from the caudal artery/vein or heart.
5. The fish is placed on a corkboard on the stage of a dissecting microscope. Using iris scissors and small forceps, the abdomen is opened via a carefully made incision that extends along the ventral midline from the pectoral girdle to a point just cranial to the anus.
6. The fish is placed in dorsal recumbency and the opposing flaps of body wall are pinned laterally to expose the abdominal viscera (Appendix A, Fig. 2 & Fig. 3).
7. Using the small forceps and small scissors, the abdominal viscera (liver, gastrointestinal tract, spleen, pancreatic tissue, and abdominal mesentery) are carefully removed *en masse* in the following manner:
    a. The intestine is severed proximal to the anus.
    b. A forceps is applied to the terminal portion of the intestine. Using gentle traction and taking care not to disturb the gonads, the viscera are dissected out of the abdominal cavity in a caudal to cranial direction.
    c. The distal esophagus is severed just proximal to the liver.
8. Using a syringe, approximately 0.5 ml of Davidson's fixative is then gently applied to the gonads *in situ*. Approximately 90 seconds following the application of fixative, the liquid fixative within the abdomen is removed with a gauze sponge, and the gonads are excised in a manner similar to the abdominal viscera:
    a. Using the microdissection scissors, the spermatic ducts or oviducts are severed proximal to the genital pore.
    b. Microdissection forceps are then applied to the spermatic ducts/oviducts. Using gentle traction, the gonads are dissected out of the abdominal cavity in a caudal to cranial direction, severing the mesorchial/mesovarial attachments as needed using the microdissection scissors. The left and right gonads may be excised individually or they may be excised simultaneously and subsequently divided at their caudal attachment.
9. The gonads (right and left) are placed into a pre-labeled plastic tissue cassette which is then placed into an individual container of Davidson's fixative accompanied by the abdominal viscera. The volume of fixative in the container should be at least 10 times the approximated volume of the tissues. The fixative container is gently agitated for five seconds to dislodge air bubbles from the cassette.
10. Using the carcass, the secondary sex characteristics are assessed (e.g., dorsal nape pad, nuptial tubercles). The carcass is then added to the fixative container.
11. All tissues remain in Davidson's fixative overnight, followed by transfer to individual containers of 10% neutral buffered formalin the next day. Containers with cassettes are gently agitated for 5 seconds to ensure

adequate penetration of formalin into cassettes (it is not necessary to rinse with water or perform multiple changes in formalin).

2. Tissue Trimming

Tissue trimming is not required for FHM.

3. Tissue Processing

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.

Materials:

1. Tissue processor.
2. Paraffin heating pots.
3. Processing unit oven.
4. Activated charcoal.
5. Paraffin (Paraplast®, or equivalent, Appendix D).
6. 10% neutral buffered formalin.
7. Ethyl alcohol (absolute and dilutions as required).
8. Proprietary clearing agent (Clear Rite-3™ or equivalent, Appendix D).
9. Xylene.

Procedures:

1. Labeled tissue cassettes are removed from formalin storage and are washed in tap water.
2. The cassettes are placed in the processing basket(s) in a single layer. The processing basket is loaded into the tissue processor.
3. The processing schedule is selected (see Appendix B, Schedule 1). The "Gonad Program" or equivalent is selected for FHM.
4. After the tissue processor has completed the processing cycle, the basket(s) may be transferred to the embedding station.

4. Embedding

Objective:

1. Properly orient the tissue in solidified paraffin for microtomy.

Materials:

1. Embedding station (thermal, dispensing and cryo consoles).
2. Paraffin heating pots.
3. Paraffin transfer pots.
4. Laboratory oven.

5. Thermometer.
6. Embedding molds.
7. Block drawers.
8. Forceps.
9. Scraper.
10. Standard paraffin.

Procedures:

1. The cryo console of the embedding station is turned on. (Power to the dispensing console and thermal console should remain on at all times.)
2. 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.
3. 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.
4. An appropriately sized embedding mold is selected.
5. The mold is held under the spout of the dispensing console and filled with molten paraffin.
6. The gonads are removed from the base of the cassette and are placed in the molten paraffin in the mold. The two gonads (left and right) are oriented horizontal to their long axis in the mold to allow for longitudinal sectioning.
7. The base of the cassette is placed on top of the mold. Additional paraffin is added to cover the bottom of the base.
8. The mold with the cassette base is placed on the cooling plate of the cryo console.
9. After the paraffin has solidified, the block (i.e., the hardened paraffin containing the tissues and the cassette base) is removed from the mold.
10. Steps 3 through l0 are repeated for each cassette to be embedded.

5. Microtomy

Objective:

1. Create and mount histologic sections for staining.

Materials:

1. Microtome.
2. Disposable microtome knives.
3. Lipshaw Pike® oil (or equivalent lightweight, machine oil).
4. Temperature-controlled water bath.
5. Ice.

6. Microscope slides.
7. Staining racks.
8. Permanent slide marking pen.
9. Forceps.
10. Fine-tipped paint brush.
11. Temporary labels.
12. 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.
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.
    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, 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:
    a. 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.
    b. The block is removed from the ice and placed in the chuck of the microtome.

c. With the section thickness on the microtome set to 4-5 microns, the chuck is advanced by rotating the microtome wheel. Sections are cut from the block until a "ribbon" containing at least one acceptable section 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.

d. Each ribbon is floated flat on the surface of the water in the water bath. An attempt is made to obtain at least one section in the ribbon that contains no wrinkles and has no air bubbles trapped beneath it.

e. A microscope slide is immersed beneath the best section in the floating ribbon. The section is lifted out of the water using the slide. This process is referred to as "mounting" the section on the slide.

f. A single slide is prepared for each fish. A total of three step sections (each section consisting of both the right and left gonad) are mounted on each slide. The first section is obtained at the point where approximately half of the gonad has been removed and the size of the section is maximized. For both the testis and the ovary, the second and third sections are taken at 50 micron intervals following the first section.

g. With a slide-marking pen, the block number from which the slide was produced is recorded on the slide.

h. The slide is placed in a staining rack.

i. The block is removed from the chuck and placed facedown for storage.

j. Steps a. through h. are repeated for all blocks to be microtomed.

6. Staining, Coverslipping, and Slide Labeling

Objectives:

1. Differential staining of intra- and inter-cellular components of the gonads 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. Clarifier solution (Richard Allen or equivalent).
4. Bluing reagent (Richard Allen or equivalent).
5. Eosin-Y (Richard Allen or equivalent, Appendix C & Appendix D).
6. Hematoxylin-2 (Richard Allen or equivalent, Appendix C & Appendix D)
7. Xylene.
8. Absolute ethyl alcohol (100% ETOH).
9. 95% ETOH.

10. 80% ETOH.
11. Coverslipping mountant (Permount or equivalent, Appendix D).
12. Glass coverslips, No. 1, 24 x 50 (or 60) mm (Appendix D).
13. Slide flats.

Procedures:

1. Staining
    a. Slides are routinely air-dried overnight before staining.
    b. An example H&E staining schedule for automated stainers is in Appendix B, Schedule 2. A similar schedule can be adapted for manual staining.
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
    a. Each slide label should contain the following information:
        i. Laboratory name
        ii. Species
        iii. Specimen No. / Slide No.
        iv. Chemical / Treatment group
        v. Date (optional)

II. GONADAL HISTOPATHOLOGY GLOSSARY AND DIAGNOSTIC CRITERIA

The purposes of this section are:

1) To provide general guidance for the light microscopic evaluation of tissue sections;

2) To promote a common awareness of various pathological findings that may be observed; and

3) To foster consistency in the use of diagnostic terminology.

General approach to reading studies

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 21-day reproduction 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 the studies non-blinded (i.e. with knowledge of the treatment group status of individual fish). 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 relative increases or decreases in cell populations, cannot be read in a blinded manner due to the diagnostic dependence on control gonads. 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 gross morphologic abnormalities, mortality rates, and general test population performance and health are useful for pathologists to provide comprehensive reports and to aid in the interpretation of findings. For a more comprehensive discussion of standard reading approaches for toxicologic pathology studies, please refer to the Society of Toxicologic Pathology Best Practices for reading toxicologic histopathology studies (Crissman JW et al. 126-31).

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 (Crissman JW et al. 126-31). Therefore, we aim to define the diagnostic criteria that will likely be encountered during the histopathologic analysis of the 21-day reproduction assay in fathead minnow.

A consolidated set of diagnostic criteria follow. These criteria are based on pathologists' experience with certain consistent histopathologic changes that occur in fathead minnow gonads in response to chemical exposure, however novel findings that are exposure-related shall also be reported.

The criteria below have been divided into two sections: 1. Primary criteria, and 2. Additional criteria. The criteria are graded for severity on a numerical scale. Any novel findings are either graded on a numerical scale, or are qualitatively described.

Primary Criteria - Males:

1. *Increased proportion of spermatogonia*: Increases in the proportion of spermatogonia are consequent of changes of the relative ratios of spermatogenic cells. This could be due to an increase in the number of spermatogonia, or a decrease in the number of other cell types, such as spermatocytes, spermatids, and spermatozoa. Because the diagnosis of increased proportion of spermatogonia is dependant on a comparison to controls, it is necessary to establish the normal range of the ratios of spermatogenic cells in control male fish testes prior to making determinations on relative proportions in dose groups.

2. *Presence of testis-ova*: The presence of one or more individualized or clustered oogenic cells within the testis. Oocytes within the testis may be determined to be perinucleolar, cortical alveolar, vitellogenic, or atretic. There is little or no evidence of ovarian architecture. Whenever applicable, the term testis-ova should be used in preference to less precise terms such as "intersex" or "hermaphrodite".

3. *Increased testicular degeneration*: Testicular degeneration is characterized by 1) individual or clustered apoptotic germ cells; 2) vacuolated germ cells; and/or 3) multinucleated (syncytial) cells in the germinal epithelium or testicular lumen. Apoptotic germ cells are characterized by cell shrinkage, nuclear condensation, and fragmentation into spherical, membrane-bound bodies, which are often phagocytized by neighboring cells. There is no inflammation associated with these cells. If possible, testicular degeneration should be differentiated from necrosis, which is characterized morphologically by cytoplasmic coagulation or swelling, nuclear karyorrhexis or pyknosis, associated inflammation, a locally extensive pattern of tissue involvement, and/or the involvement of different local

tissue elements (e.g., both germinal and stromal tissues). Extensive testicular degeneration may lead to localized or generalized loss of the germinal epithelium.

4. *Interstitial cell (Leydig cell) hyperplasia/hypertrophy*: An increase in number and/or size of the interstitial cells responsible for producing androgens. Interstitial cells may have larger, more rounded nuclei, and interstitial cell aggregates may occupy and expand some interstitial spaces.

Primary Criteria – Females:

1. *Increased oocyte atresia*: An increase in degradation and resorption of oocytes at any point in development. Atresia is characterized by clumping and perforation of the chorion, fragmentation of the nucleus, disorganization of the ooplasm, and/or the uptake of yolk materials by perifollicular cells.

2. *Perifollicular cell hyperplasia/hypertrophy*: Increase in the size or number of granulosa, theca, and/or surface epithelium cells involved in a developing follicle. Abnormal perifollicular cell hypertrophy must be distinguished from the normally enlarged granulosa and theca cells of a post-ovulatory follicle.

3. *Decreased yolk formation*: A decrease in the amount of vitellogenic/yolk material that is deposited in the developing oocyte. Decreased vitellogenesis is characterized by the presence of oocytes in which yolk material is not present despite their relatively large size. Note that oocytes may be affected to varying degrees. Some affected oocytes have extremely fine vitellogenic granules, and this is interpreted as ineffective vitellogenesis.

4. *Change in gonadal staging*: 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. Consequently, following the gonadal staging of individual fish, each treatment group is assessed as a whole and compared to the appropriate control group to determine if a compound-related effect has occurred. Hence, gonadal staging cannot be performed in a blinded manner. Because 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. Similarly, both gonads should be staged as a single organ according to the predominant pattern. 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).

Criteria for Staging Ovaries

- **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 of follicles 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.

Secondary criteria – males:

1. *Decreased proportion of spermatogonia*: Decreased relative proportion of spermatogonia to other spermatogenic cell types. This can be due to a decrease in the number of spermatogonia, or an increase in the number of other cell types, such as spermatocytes, spermatids, and spermatozoa. Because the diagnosis of decreased proportion of spermatogonia is dependant on a comparison to controls, it is necessary to establish the normal range of the ratios of spermatogenic cells in control male fish testes prior to making determinations on relative proportions in dose groups.

2. *Increased vascular or interstitial proteinaceous fluid*: Homogenous dark pink translucent material, presumably vitellogenin, within the testicular interstitium or blood vessels. The presence of this fluid may cause a thickening of interstitial areas that might be misinterpreted as "stromal proliferation".

3. *Asynchronous gonad development*: The presence of more than one developmental phase of spermatogenic cell within a single spermatocyst enclosed by a Sertoli cell. For example, this term may be applied to a spermatocyst that contains a mixture of spermatocytes and spermatids, or a spermatocyst that contains more than one meiotic phase of primary spermatocyte (i.e., leptotene, pachytene, and/or zygotene). It also refers to the presence of distinctly different populations (i.e. developmental phases) of gametogenic cells in the right and left gonads.

4. *Altered proportions of spermatocytes or spermatids*: A change in the relative proportions of spermatocytes or spermatids to other spermatogenic cell types. Changes in relative ratios could be due to an increase in the number of

spermatocytes or spermatids, or to a decrease in the number of other cell types. Relative changes may also occur between spermatocytes and spermatids.

5. *Gonadal staging*: 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. Consequently, following the gonadal staging of individual fish, each treatment group is assessed as a whole and compared to the appropriate control group to determine if a compound-related effect has occurred. Hence, gonadal staging cannot be performed in a blinded manner. Because 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. Similarly, both gonads should be staged as a single organ according to the predominant pattern. 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).

   Criteria for Staging Testes

   - **Juvenile**: gonad consists of spermatogonia exclusively; it may be difficult or impossible to confirm the sex of these individuals.
   - **Stage 0 – Undeveloped**: entirely immature phases (spermatogonia to spermatids) with no spermatozoa.
   - **Stage 1 – Early spermatogenic**: immature phases predominate, but spermatozoa may also be observed; the germinal epithelium is thinner than it is during Stage 2.
   - **Stage 2 – Mid-spermatogenic**: spermatocytes, spermatids, and spermatozoa are present in roughly equal proportions; the germinal epithelium is thinner than Stage 1 but thicker than Stage 3.
   - **Stage 3 – Late spermatogenic**: all stages may be observed, however, mature sperm predominate; the germinal epithelium is thinner than it is during Stage 2.
   - **Stage 4 – Spent**: loose connective tissue with some remnant sperm.

6. *Granulomatous inflammation*: This process is characterized by the presence of epithelioid macrophages that typically form sheets or nodules (granulomas) due to desmosome-like cytoplasmic attachments (Noga et al., 1989). When compared to histiocytic-type macrophages, epithelioid macrophages have larger, more open-faced, centralized nuclei and less abundant cytoplasm. During resolution of inflammation, the epithelioid macrophages may become flattened into fibrocyte-like cells. Lymphocytes, granulocytes, and multinucleated giant cells may also be components of granulomatous inflammation. Granulomatous inflammation is intrinsically a pathologic process that is often associated with reactions to infectious agents, foreign materials or the aftermath of necrosis; therefore, it is important to distinguish this, if possible, from the presence of histiocytic cells in the lumen of the testis.

Secondary criteria –females:

1. *Interstitial fibrosis*: The presence of increased fibrous connective tissue (collagenous fibers and fibrocytes or fibroblasts) within the ovarian interstitium (stroma). Collagen may be difficult to appreciate in early phases of fibrosis.

2. *Egg debris in the oviduct*: The presence of inspissated-appearing, homogenous, irregular, dense pink material, presumed to be yolk, within the oviduct.

3. *Granulomatous inflammation*: This process is characterized by the presence of epithelioid macrophages that typically form sheets or nodules (granulomas) due to desmosome-like cytoplasmic attachments (Noga et al., 1989). When compared to histiocytic-type macrophages, epithelioid macrophages have larger, more open-faced, centralized nuclei and less abundant cytoplasm. During resolution of inflammation, the epithelioid macrophages may become flattened into fibrocyte-like cells. Lymphocytes, granulocytes, and multinucleated giant cells may also be components of granulomatous inflammation. Granulomatous inflammation is intrinsically a pathologic process that is often associated with reactions to infectious agents, foreign materials or the aftermath of necrosis; therefore, it is important to distinguish this, if possible, from the presence of macrophage aggregates in the ovary.

4. *Decreased post-ovulatory follicles*: A decrease in the number of collapsed perifollicular sheaths, or membranous structures lined by granulosa cells, theca cells and surface epithelium, following release of oocytes, in comparison to control fish. The granulosa cells are often hypertrophic, although this appears to be species dependent (Saidapur, 1982).

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 will employ the following system:

Not remarkable
Grade 1 (minimal)
Grade 2 (mild)

Grade 3 (moderate)
Grade 4 (severe)

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:

Discrete: these are changes that can be readily counted. Examples include atretic follicles, oocytes in the testis, and clusters of apoptotic cells.

Spatial: these are changes that can be quantified by area measurements. This includes lesions that are typically classified as focal, multifocal, coalescing, or diffuse. Specific examples include granulomatous inflammation and tissue necrosis.

Global: these are generalized changes that would usually require more sophisticated measurement techniques for quantification. Examples include increased hepatocyte basophilia, Sertoli cell/interstitial cell hypertrophy, or quantitative alterations in cell populations.

General severity grading scale:

- **Not Remarkable:** This grade is used if there are no findings associated with a particular diagnostic criterion.

- **Grade 1: Minimal.** Ranging from inconspicuous to barely noticeable but so minor, small, or infrequent as to warrant no more than the least assignable grade. For discrete changes, grade 1 is used when there are fewer than 2 occurances per microscopic field, or 1-2 occurances per section. 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 2: Mild.** A noticeable feature of the tissue. For discrete changes, grade 2 is used when there are 3-5 occurrences per microscopic field or per tissue section. For multifocal or diffusely-distributed alterations, this grade is used for processes where 30-50% of the tissue in the section is involved.

- **Grade 3: Moderate.** A dominant feature of the tissue. For discrete changes, grade 3 is used when there are 6-8 occurrences per microscopic field or per tissue section. For multifocal or diffusely-distributed alterations, this grade is used for processes where 60-80% of the tissue in the section is involved.

- **Grade 4: Severe.** An overwhelming feature of the tissue. For discrete changes, grade 4 is used when there are more than 9 occurrences per microscopic field or per tissue section. For multifocal or diffusely-distributed alterations, this grade is used for processes where greater than 80% of the tissue in the section is involved.

At the discretion of the pathologist, **the severity of a given change should be scored**

according to one of the following two methods: 1) score compound-exposed animals relative to the severity of the same change in control animals, or 2) score all animals relative to "normal" as determined by the pathologist's experience. For each important (i.e., treatment-associated) finding, the method that was used should be stated in the Materials and Methods section of the pathology narrative report (see **Histopathology Report Format**). By convention, 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.

Data recording

An Excel worksheet form has been created that includes worksheets for primary, secondary, and additional diagnoses to facilitate histopathology data collection. In this worksheet, each data entry cell represents an individual fish. Additional sheets are available for comments and additional findings. For each fish, the pathologist records a severity score associated with the diagnosis (see **Severity Grading**). Diagnostic criteria with non-remarkable findings shall be denoted using (-). If there is no reasonably appropriate diagnostic term for a particular finding, the pathologist can create a term that can be recorded in the "Additional diagnoses" worksheet. If insufficient tissue is available for diagnosis, this should be recorded as **IT** (insufficient tissue). If a target tissue is missing, this should be recorded as **MT** (missing tissue).

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

Histopathology Report Format

Each histopathology narrative report should contain the following five sections: Introduction, Materials and Methods, Results, Discussion, Summary/Conclusions. A References section can also be included if applicable. The **Introduction** section briefly outlines the experimental design. The **Materials and Methods** section describes any items or procedures that are essentially different from Section 1: Post-mortem and Histotechnical Procedures. As applicable, specific severity grading criteria (see **Severity Grading**) should also be listed in this section. The **Results** section should report findings that are: 1) compound-related; 2) potentially compound-related; 3) novel or unusual. Detailed histomorphologic descriptions need only be included for findings that differ substantially from diagnoses presented in Section IIB, Glossary and Diagnostic Criteria. 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.

Glossary / Diagnostic Criteria

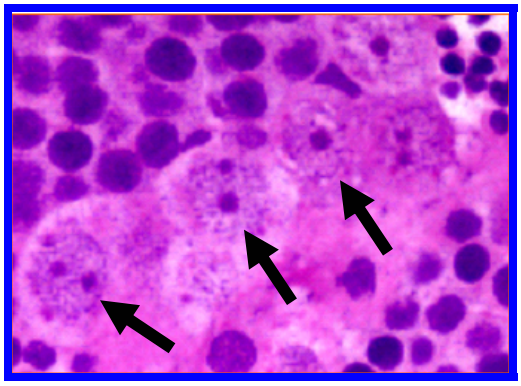
The purposes of this section are: 1) to provide photomicrographs of normal gonadal structure in fathead minnow, 2) to provide a common technical "language" and 3) to create a reference atlas of both microanatomical structures and potential pathological findings. The information in this section is derived 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. Regarding the last, opinions were solicited via a questionnaire that was circulated among conference participants following the October 2003 meeting of the histopathology subcommittee of the Fish Discussion Group in Paris. Consensus replies to this questionnaire form the basis for naming many of these terms. Other considerations include traditional usage and scientific precedence, and attributes such as clarity and brevity.

The section is arranged as follows:

1. Normal testicular architecture in fathead minnow
2. Normal ovarian architecture in fathead minnow
3. Primary diagnoses – males
4. Primary diagnoses – females
5. Additional diagnostic criteria and an illustrated glossary of microanatomical and diagnostic terms

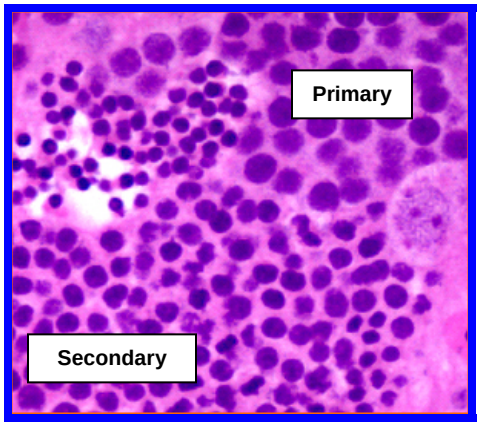
Normal Testicular Architecture in Fathead Minnow

Spermatogenic Cell Types:



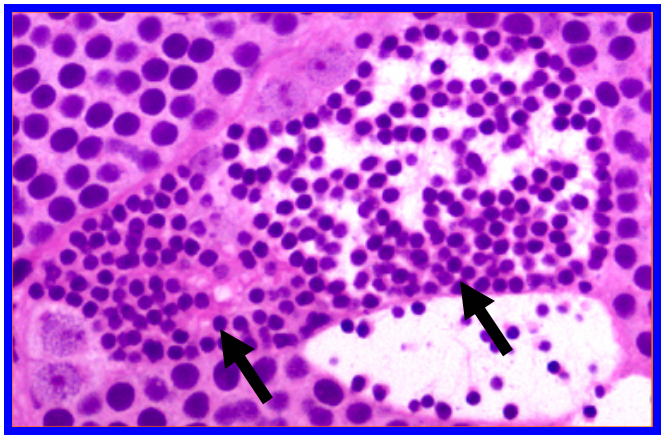
Spermatogonia. Spermatogonia A in a male FHM (GMA, H&E).

***Spermatogonia*:** The largest of the spermatogenic cells (~ 5-10 µm), spermatogonia generally have pale vesicular nuclei, prominent nucleoli, variably distinct nuclear membranes, perinuclear cytoplasmic granules, and moderate amounts of granular cytoplasm. Spermatogonia B are smaller than spermatogonia A, and spermatogonia B are usually present in larger clusters (e.g., > 4 cells). If at all possible, an attempt should be made to classify these cells as spermatogonia rather than to label them with a non-specific term such as "pale cells" or "light cells".



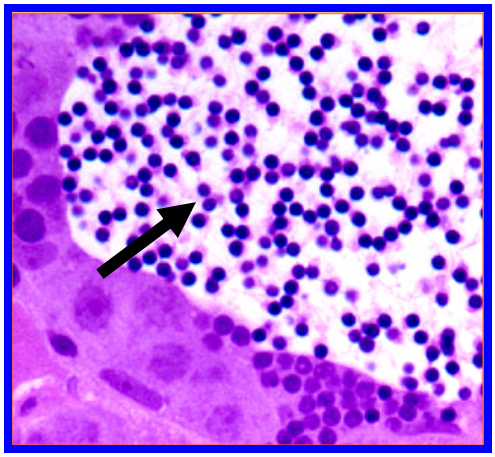
Spermatocytes. FHM testis (GMA, H&E).

Spermatocytes: Derived from spermatogonia, spermatocytes are of intermediate cell size (~ 4-6 µm), and have comparatively dense nuclei and minimal to moderate amounts of indistinct cytoplasm. Spermatocyte nuclei are usually evident in one of three meiosis phases: pachytene, leptotene, or zygotene. Primary spermatocytes are larger than secondary spermatocytes, 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.



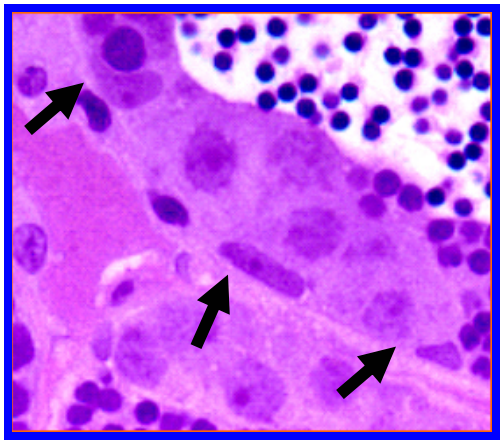
Spermatids. Spermatids in a male FHM. Intercellular attachments are lost just prior to rupture of the spermatocyst and release of these cells as spermatozoa (GMA, H&E).

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 µm), and the cells lose their cytoplasmic attachments to one another during spermiogenesis.



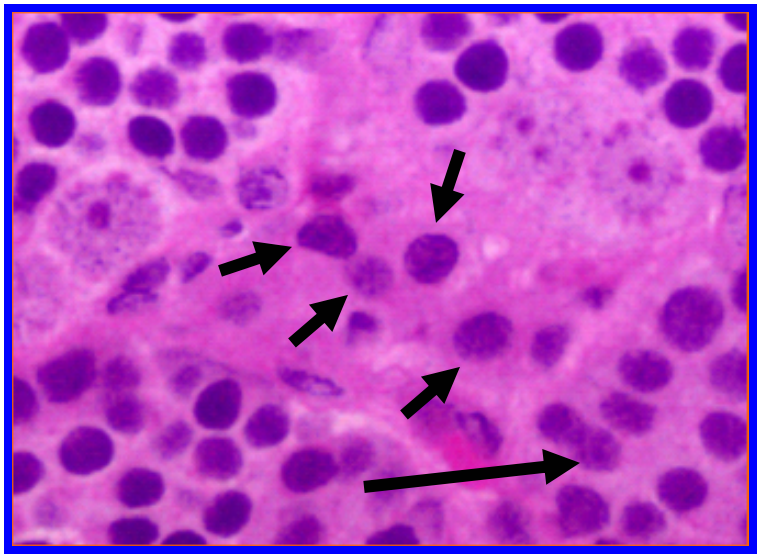
Spermatozoa. Spermatozoa in a male FHM (GMA, H&E).

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 µm), and they exist as scattered individual cells within tubular lumen.



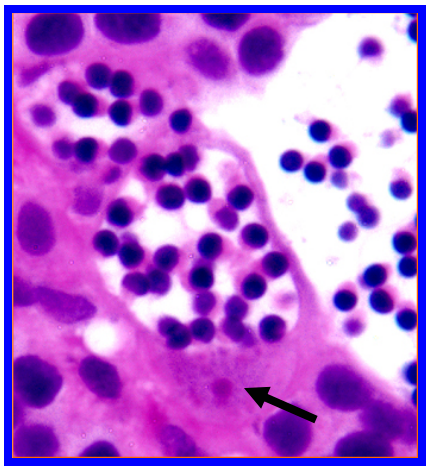
Sertoli cells (FHM, GMA, H&E).

Sertoli cells: These cells 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. In some instances, hypertrophic (enlarged, swollen) Sertoli cells may resemble spermatogonia.



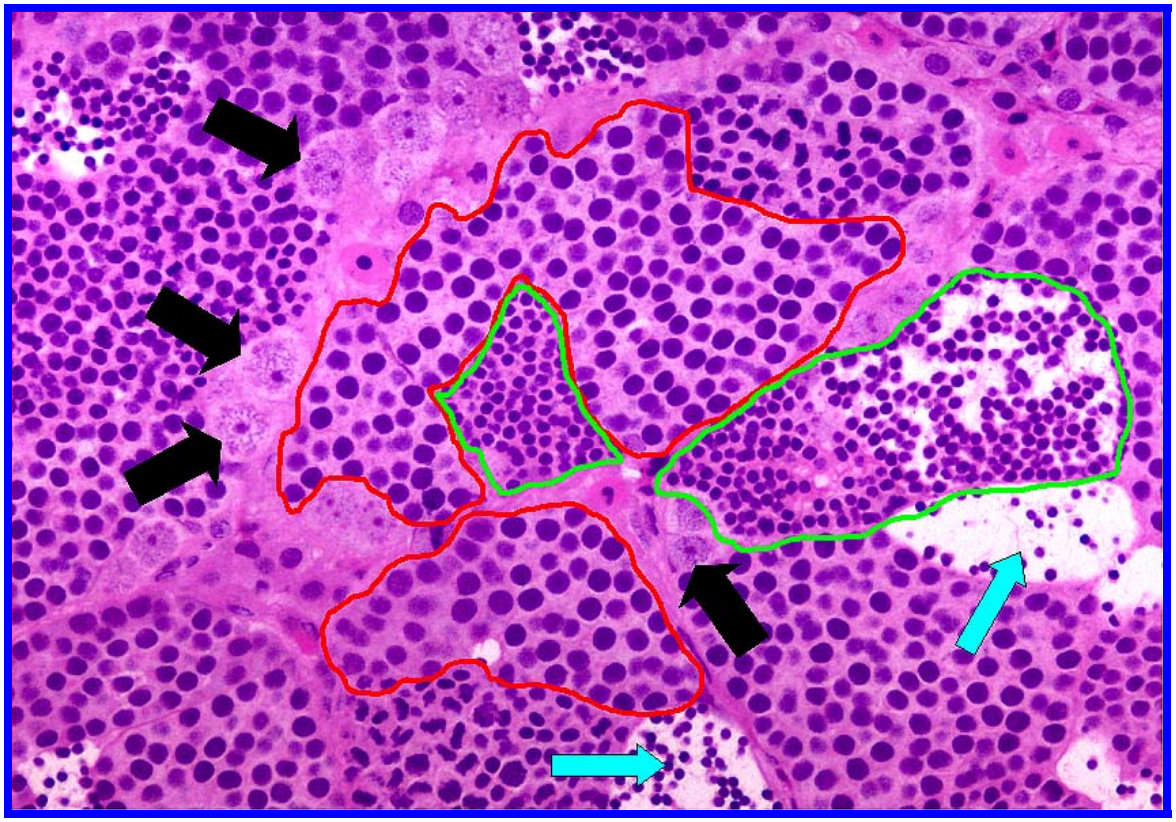
***Interstitial (Leydig) cells*:** These cells have dense, dark round or oval nuclei with little detail and moderate amounts of variably-evident, faintly vacuolated cytoplasm. Compared to germinal cells, interstitial cells are usually present in low numbers, usually as single cells or small aggregates, within the interlobular interstitium. Although they may resemble spermatocytes, interstitial cells are only present in interlobular areas.

Interstitial cells (FHM, H&E). Interstitial cells (small arrows) are only found in interlobular areas. Note the resemblance between these cells and spermatocytes (large arrow).

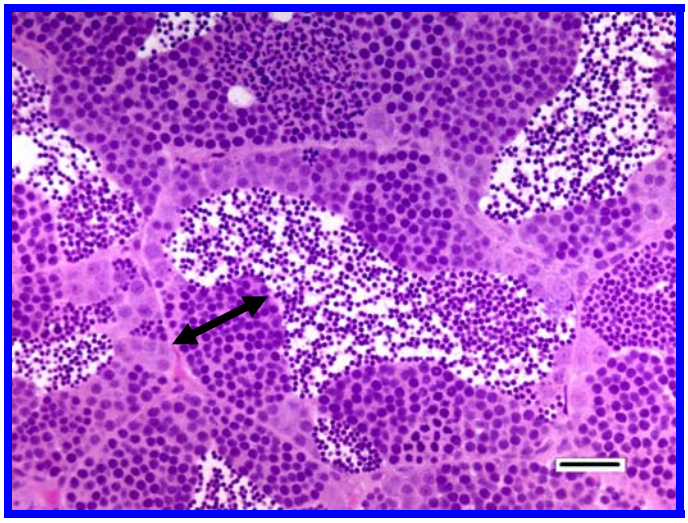


***Spermatocyst*:** The functional unit of the testis, this structure consists of a clonal group of spermatogenic cells (spermatogonia, spermatocytes, or spermatids) that are surrounded by the cytoplasmic arms of (usually) one Sertoli cell. Cells within spermatocysts exist as syncytia, maintained by intercellular attachments (cytoplasmic bridges), until final maturation and release of spermatozoa occurs (spermiogenesis) (Grier, 1976).

Spermatocyst (FHM, adult male, plastic, H&E). A group of dissociated spermatids are surrounded by the cytoplasmic "arms" of a single Sertoli cell (arrow). This arrangement is usually not as obvious as it is in this photograph. The nucleus of this particular Sertoli cell appears enlarged (hypertrophic).



Spermatocysts (FHM, adult male, GMA, H&E). Spermatocysts outlined in red and green contain spermatocytes and spermatids, respectively. Spermatogonia (black arrows) and spermatozoa within tubular lumina (blue arrows) are also indicated.

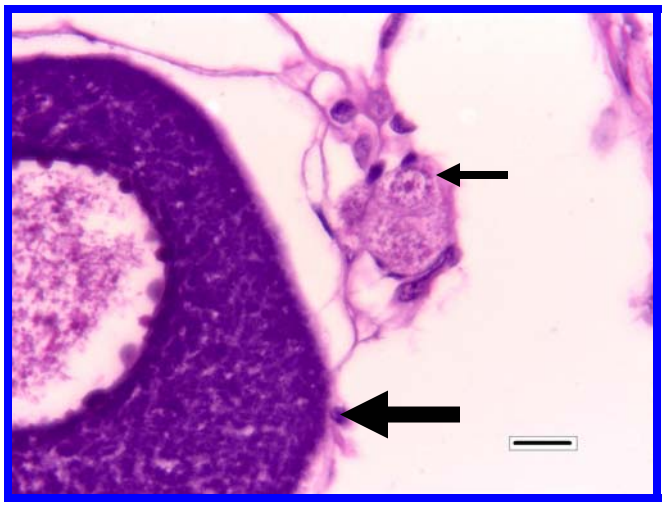


Germinal epithelium, male. Normal testis from an adult FHM. Double arrow indicates width of germinal epithelium, which extends from the interlobular interstitium to the lobular lumen (GMA = glycol methacrylate, H&E, bar = 25 µm).

Germinal epithelium (male): The germinative intratubular (intralobular) parenchyma of the testis, this membrane-bound structure consists of multiple spermatocysts in various phases of development. For FHM, boundaries of the germinal epithelium at various locations throughout the testis include the interlobular interstitium, the lobular lumina, collecting ducts, and the tunica albuginea.

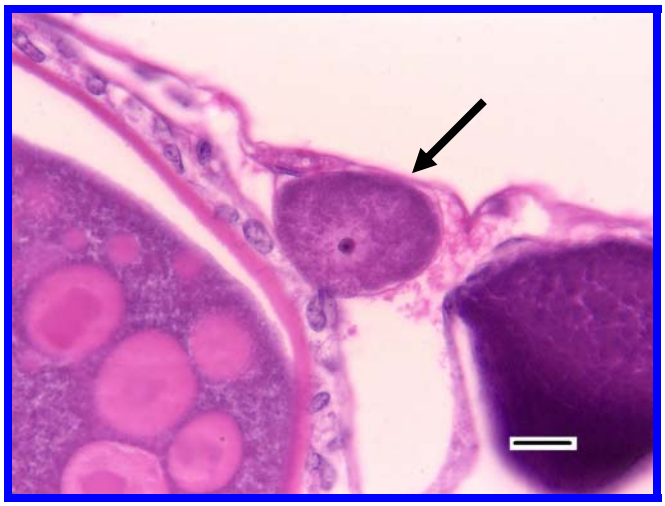
Normal Ovarian Architecture in Fathead Minnow

Oogenic Cell Types:



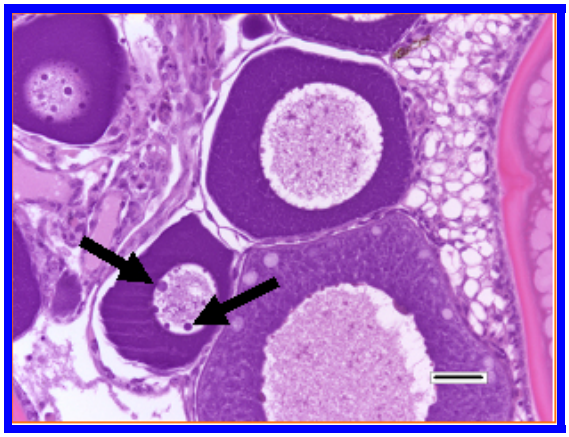
Oogonia: These cells represent the replicative pool of the ovary. Unlike mammalian oogonia (although this dogma may soon change based on recent data from rodent research), piscine oogonia continue to divide in juveniles and adults. The smallest of the oocytic cells, oogonia reside within the ovarian germinal epithelium, usually in comparatively low numbers. Oogonia are characterized by a relatively large nucleus with small or inapparent nucleolus, and minimal amounts of cytoplasm.

Oogonia (FHM, paraffin, H&E, bar = 10 µm). A small cluster of oogonia reside within a portion of germinal epithelium; the nucleus of only one oogonium is visible (small arrow). The oogonia are dwarfed by a perinucleolar oocyte (large arrow).



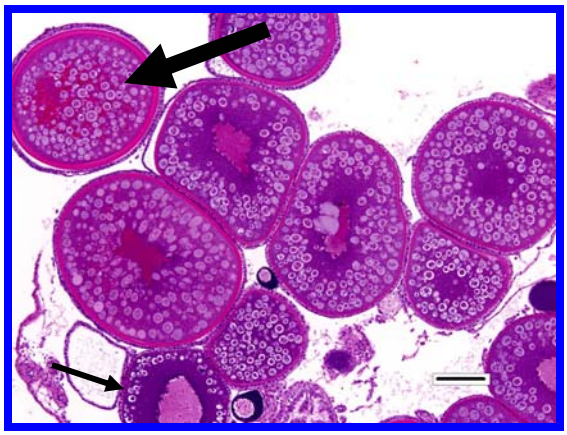
Chromatin nucleolar oocytes: Slightly larger than an oogonium, this oocyte is formed when an oogonium becomes surrounded by prefollicle cells (presumptive granulosa cells) and the resulting complex buds from the germinal nest as a primordial follicle. The chromatin nucleolar oocyte has a relatively large nucleus that contains a single, large nucleolus. Compared to an oogonium, there is more cytoplasm which is slightly more dense and finely granular.

Chromatin nucleolar oocyte (FHM, paraffin, H&E, bar = 10 µm). A single chromatin nucleolar oocyte protrudes from the germinal epithelium (arrow).



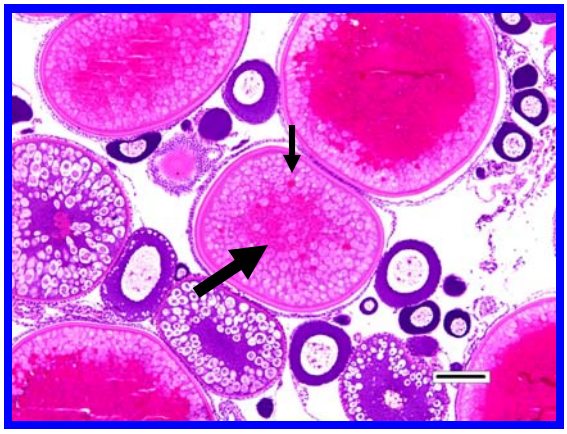
Perinucleolar oocytes. Several perinucleolar oocytes in the ovary of a FHM. Arrows indicate nucleoli at the periphery of the germinal vesicle (paraffin, H&E, bar = 25 µm).

Perinucleolar oocytes: Concomitant with oocyte growth, the nucleus (germinal vesicle) increases in size and multiple nucleoli appear, generally at the periphery of the nucleus. The cytoplasm stains uniformly dark, although late perinucleolar oocytes may have small clear or amphophilic vacuoles in the cytoplasm. These cells tend to be abundant in normal adult ovaries.



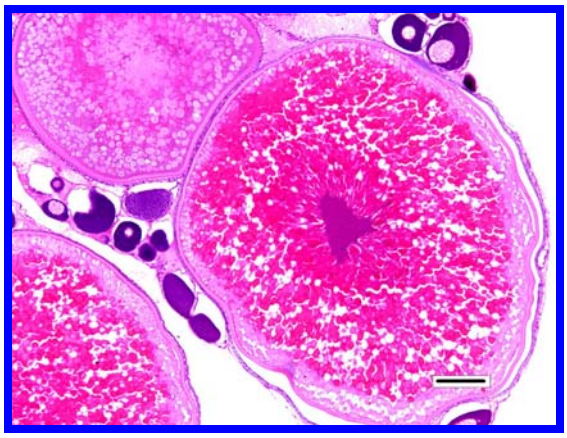
Cortical alveolar oocytes. FHM ovary demonstrating multiple cortical alveolar oocytes. The cytoplasm is predominately filled by numerous cortical alveoli, which are amphophilic with this preparation. Evident are oocytes in transition from the perinucleolar to cortical alveolar phase (small arrow), and from the cortical alveolar to early vitellogenic phase (large arrow) (paraffin, H&E, bar = 100 µm).

Cortical alveolar oocytes: Generally larger than perinucleolar oocytes, this phase is characterized by the appearance of cortical alveoli (yolk vesicles) within the ooplasm. The cortical alveoli are technically not yolk, as they do not provide nourishment for the embryo (Selman and Wallace, 1989). The chorion becomes distinctly evident in this phase, and the perifollicular cells are more easily visualized.



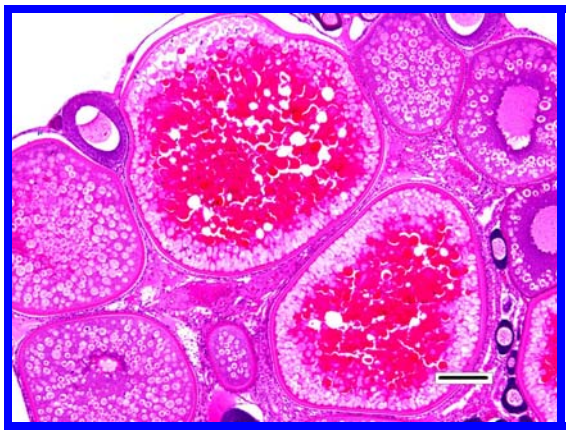
Early vitellogenic oocytes. In this FHM ovary, numerous fine pale pink granules (large arrow), and a few larger dark red granules (small arrow), are evident in the central region of an early vitellogenic oocyte. Although nuclei are present they are not apparent in every oocyte due to the comparatively vast amount of cytoplasm (paraffin, H&E, bar = 100 µm).

Early vitellogenic oocytes: Larger than cortical alveolar oocytes, these cells are characterized by the centralized appearance of spherical, eosinophilic, vitellogenic yolk granules / globules. In H&E sections, accumulations of fine yolk granules in the central region of the oocyte may somewhat resemble (and thus be confused with) the reddish nucleus.



Late vitellogenic oocytes. Late vitellogenic oocyte in a FHM ovary. The yolk granules almost fill the ooplasm. The nucleus has not yet begun to migrate peripherally (paraffin, H&E, bar = 100 µm).

Late vitellogenic oocytes: These cells are characterized by an increased accumulation of vitellogenic granules that displace the cortical alveolar material to the periphery of the cytoplasm. It is during this stage that the nucleus begins to migrate toward the periphery of the cell.



Mature/spawning oocytes. Two mature/spawning oocytes in a FHM ovary. The oocytes and the yolk granules have attained their maximum size just prior to spawning, and the nucleus is not evident (paraffin, H&E, bar = 100 μm).

***Mature/spawning oocytes*:** In this phase of development, vitellogenesis has reached its peak, the cell has become larger and more hydrated, and the nucleus has migrated toward the periphery of the cell and is in the process of dissolution. The loss of nucleus is not a very helpful diagnostic feature, however, as the nucleus is often not visible in larger oocytes due to the plane sectioning. Because of the transient nature of these cells in fractional spawning fish, mature/spawning oocytes are uncommonly observed.

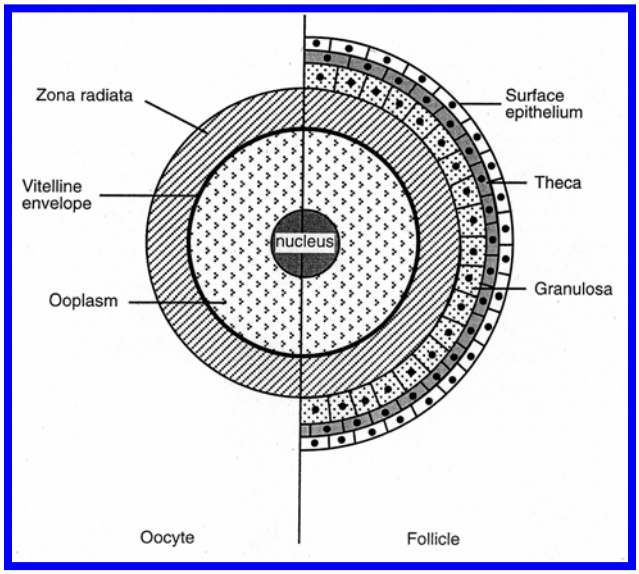
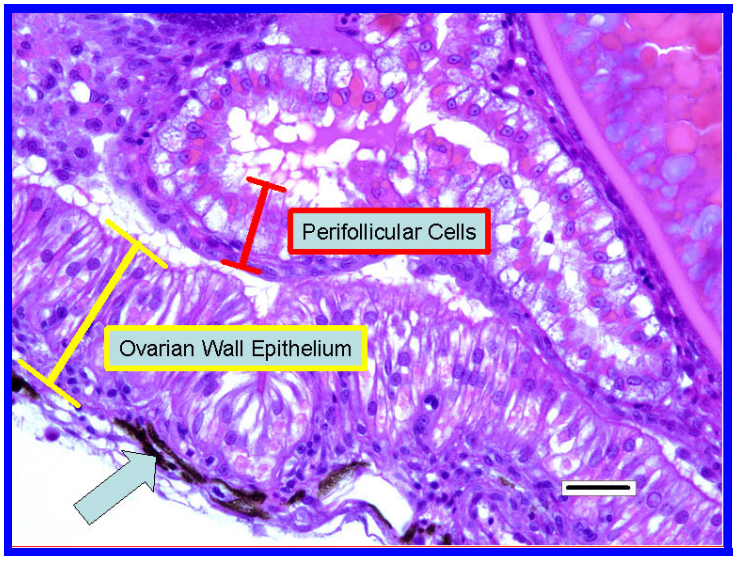


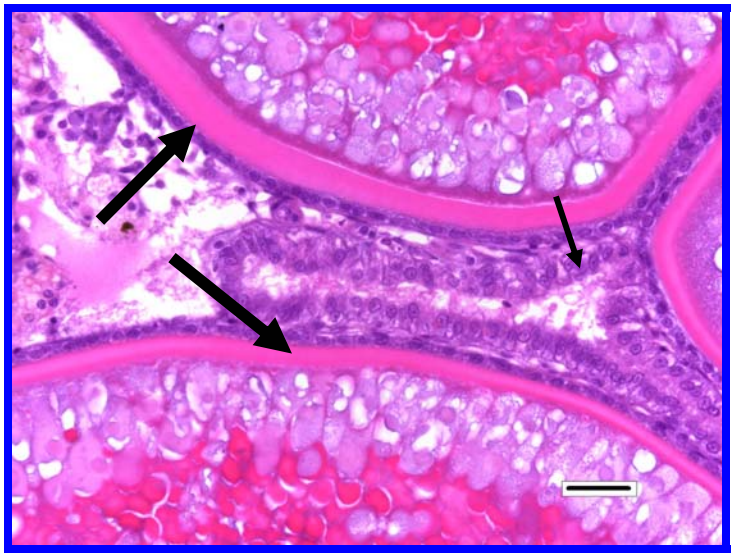
Diagram of an **ovarian follicle**. From Tyler and Sumpter, 1996.

***Ovarian follicle*:** The functional unit of the ovary, this term generally refers to an oocyte plus its surrounding sheath of perifollicular cells (granulosa cells, theca cells, and surface epithelium cells) (Tyler and Sumpter, 1996). However, there are subtypes of follicles in which the oocyte is not present or may be difficult to appreciate; these include post-ovulatory (spent), empty, and atretic follicles. A post-ovulatory follicle (the follicle has ruptured to release an oocyte during spawning) is collapsed and often has enlarged (hypertrophic) granulosa and theca cells. Conversely, an empty follicle (in which the oocyte has been dislodged from the histologic section as a post-mortem artifact) generally retains the shape of the oocyte and may or may not have enlarged granulosa and theca cells. An atretic follicle must be distinguished from both spent follicles and empty follicles; the presence of at least some ooplasmic material (often heterochromatic) within a follicle indicates that it contains an atretic oocyte.



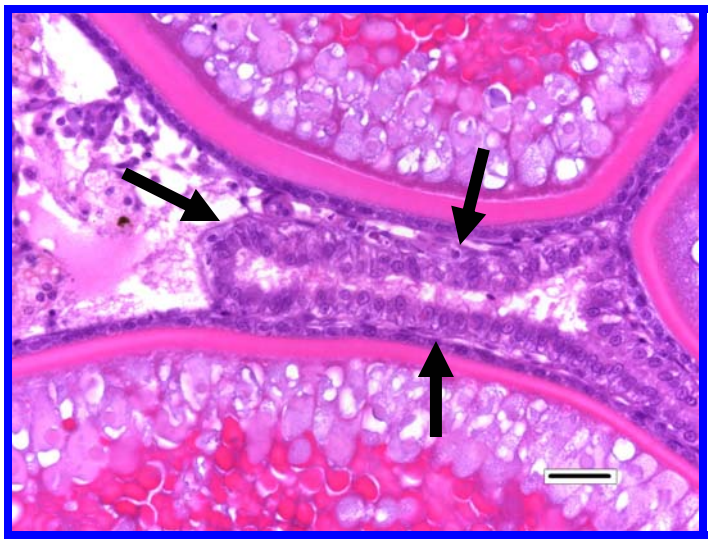
Perifollicular cells. In this photomicrograph, the perifollicular cells are compared to the cells of the ovarian wall epithelium, which contains dark brown (melanin) pigment (arrow) and is comprised of ciliated columnar cells in FHM. FHM, adult female, paraffin, H&E, bar = 25 µm)

***Perifollicular cells*:** These cells form a three-layered sheath around each oocyte, and combined with the oocyte itself comprise the ovarian follicle. These layers are more easily visualized as the oocyte matures. The innermost layer consists of the granulosa cells, the middle layer consists of the theca cells, and the outermost layer consists of the surface epithelial cells. The granulosa cells especially may become enlarged and vacuolated following ovulation or during oocyte atresia. The perifollicular sheath should not be confused with folds of the ovarian wall epithelium.



Chorion. The chorions of two oocytes are indicated by large arrows. A smaller arrow denotes a post-ovulatory follicle. (FHM, paraffin, H&E, bar = 25 µm).

***Chorion*:** Usually pale to dark eosinophilic and refractile, the chorion is the thick external layer of an oocyte that surrounds the ooplasm. The terms zona radiata and vitelline envelope have been used synonymously. In mature, unspent follicles, the chorion is noticeably surrounded by perifollicular cells (granulosa cells, theca cells, and surface epithelial cells). As viewed by light microscope, the chorion is often minimally apparent or inapparent prior to the cortical alveolar phase of oocyte development.

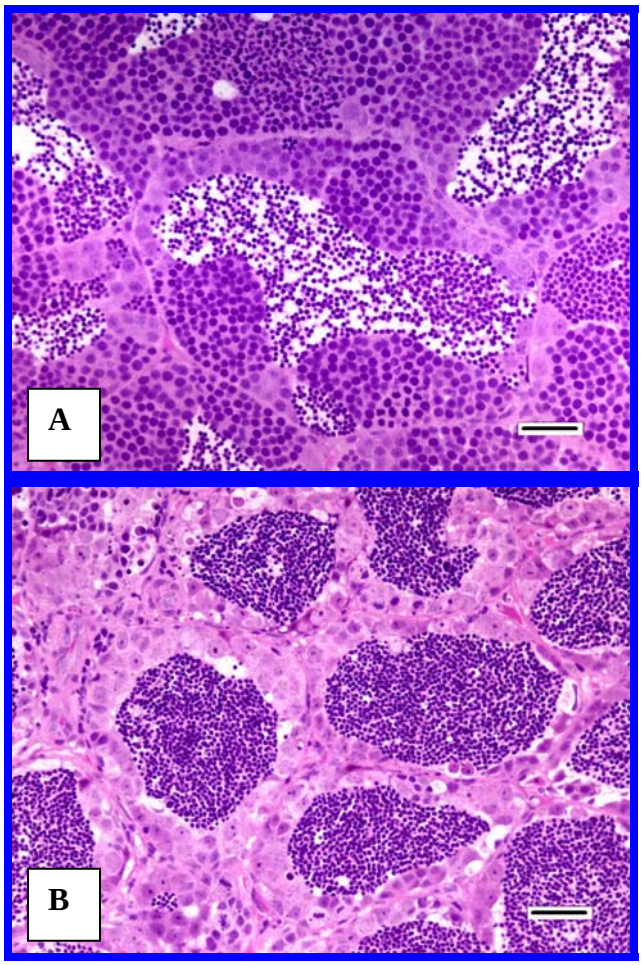


Post-ovulatory follicle. Situated between three oocyte-containing follicles is a collapsed follicle that does not contain oocyte remnants (arrows) (FHM, adult female, paraffin, H&E, bar = 25 µm).

***Post-ovulatory follicle*:** A collapsed perifollicular sheath following release of the oocyte; this is a membranous structure lined by granulosa cells, theca cells, and surface epithelium. The granulosa cells are often hypertrophic, although this appears to be species dependent (Saidapur, 1982). Mammalian terms such as "corpus lutea" and "Graafian follicles", are probably less desirable, due to structural and functional differences between these entities and piscine post-ovulatory follicles. Whenever possible, post-ovulatory follicles should be differentiated from atretic follicles, the latter of which contains oocyte debris.

Primary Diagnoses – males:

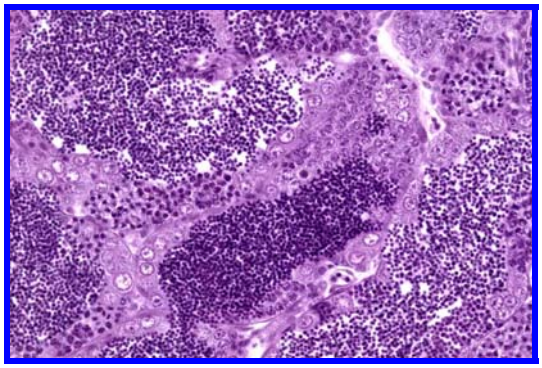
Increased proportion of spermatogonia:



Increased cells, spermatogonia. **A:** Testis from adult male FHM negative control (GMA, H&E, bar = 25 µm).
B: Spermatogonia dominate the germinal epithelium in this testis from adult male FHM exposed to10 nM E2 for 10 days. Other diagnoses for this section include "Decreased cells, spermatocytes", "Decreased cells, spermatids" (GMA, H&E, bar = 25 µm).

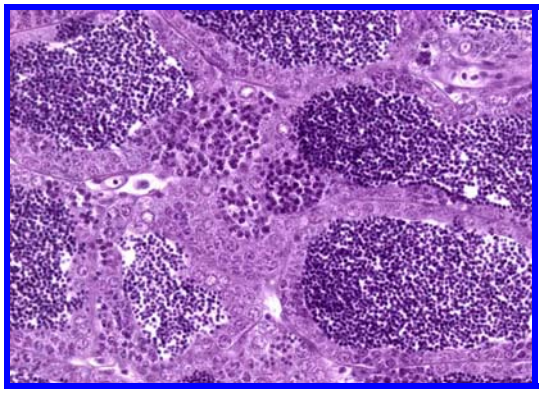
***Increased proportion of spermatogonia*:** It is recognized that endocrine active compounds may alter the proportional distribution of gametogenic cell types in the testis or ovary. Certain types of alterations (for example, the proliferation or absence of single cell population) may not be adequately documented by gonadal staging. This diagnostic term provides a mechanism for documenting such changes. Quantitative alterations 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.

Grade 1



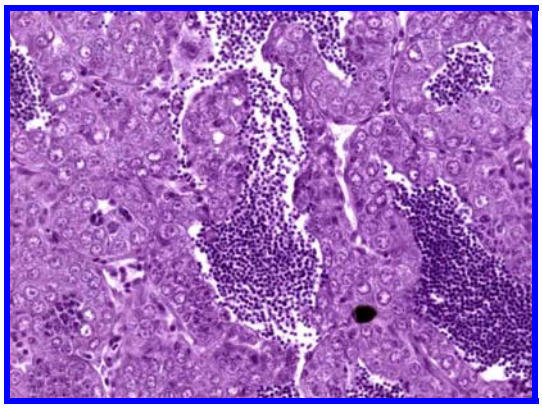
Testis from an adult male FHM. There is a minimal (Grade 1) increase in the proportion of spermatogonia (arrows). H&E.

Grade 2



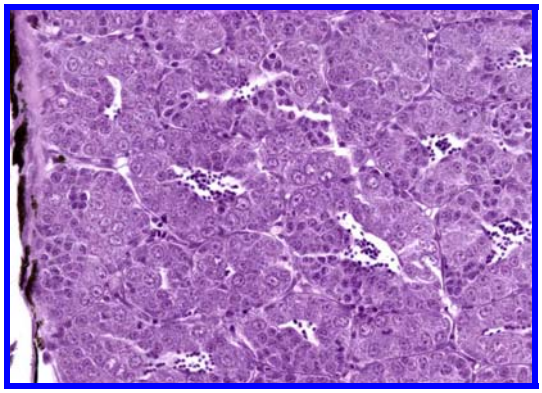
Testis from an adult male FHM. There is a slight/mild (Grade 2) increase in the proportion of spermatogonia throughout the germinal epithelium. H&E.

Grade 3



Testis from an adult male FHM. There is a moderate (Grade 3) increase in the proportion of spermatogonia. H&E.

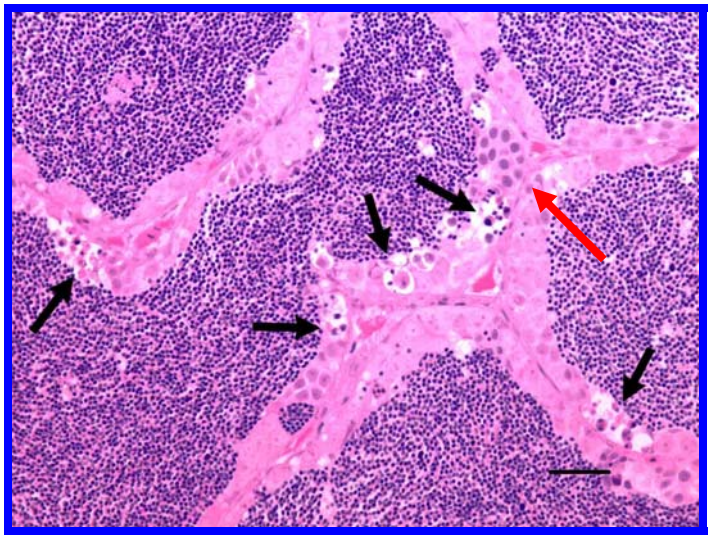
Grade 4



Testis from an adult male FHM. There is a severe (Grade 4) increase in the proportion of spermatogonia. H&E.

Presence of testis-ova: An example of testicular oocytes is not available for FHM.

Increased testicular degeneration:

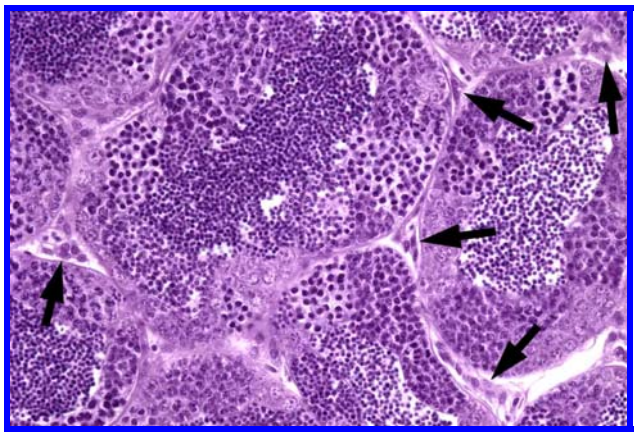


Testicular degeneration. Multiple clusters of apoptotic germ cells (black arrows) and vacuolated germ cells (red arrow) within the germinal epithelium. (FHM, adult male, GMA,H&E, bar = 25 µm).

***Testicular degeneration*:** Examples of degenerative findings in the testis include: 1) individual or clustered apoptotic germ cells; 2) vacuolated germ cells; 3) multinucleated (syncytial) cells in the germinal epithelium or testicular lumen. These diagnoses may be "lumped" together under the term testicular degeneration. Apoptotic germ cells are characterized by cell shrinkage, nuclear condensation, and fragmentation into spherical, membrane-bound bodies, which are often phagocytized by neighboring cells. There is no inflammation associated with these cells. If possible, testicular degeneration should be differentiated from **necrosis**, which is characterized morphologically by cytoplasmic coagulation or swelling, nuclear karyorrhexis or pyknosis, associated inflammation, a locally extensive pattern of tissue involvement, and/or the involvement of different local tissue elements (e.g., both germinal and stromal tissues). Extensive testicular degeneration may lead to localized or generalized loss of the germinal epithelium.

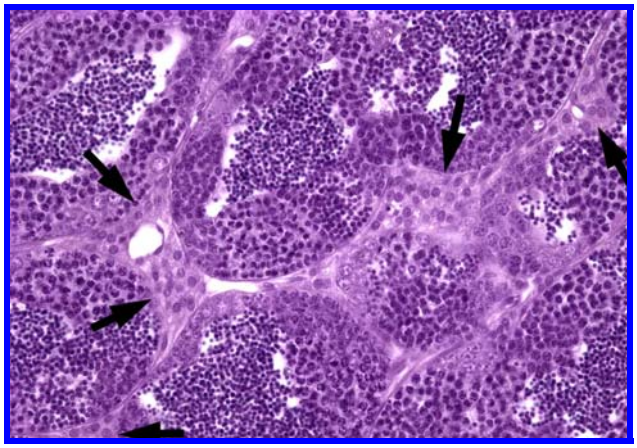
Interstitial cell (Leydig cell) hyperplasia/hypertrophy:

Non-Remarkable



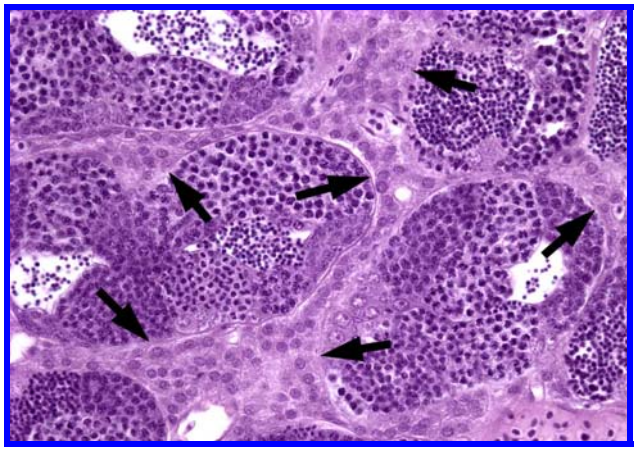
Non – remarkable testis from males FHM. Interstitial areas contain small aggregates of interstitial (Leydig) cells (arrows). Most interstitial cells have wispy, pale cytoplasm. H&E.

Grade 1



Testis from an adult male FHM. Interstitial cell aggregates (arrows) in the testis of this fish are larger than in control fish, and the cytoplasm of these cells is slightly more dense. This was diagnosed as Increased Cells, Interstitial Cells, Grade 1 (minimal) severity. H&E.

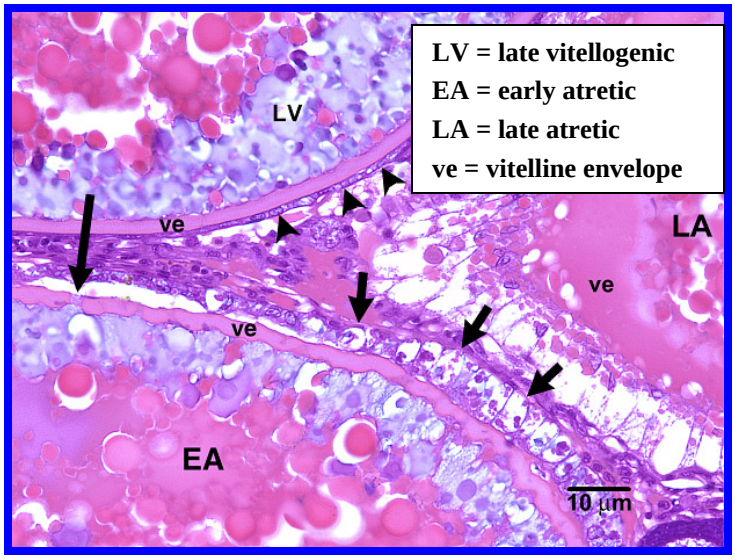
Grade 2



Testis from an adult male FHM. Interstitial cell aggregates (arrows) in the testis of this fish are larger than in control fish, and the cells tend to fill and expand the interstitial spaces. This was diagnosed as Increased Cells, Interstitial Cells, Grade 2 (mild) severity. H&E.

Primary diagnoses – females:

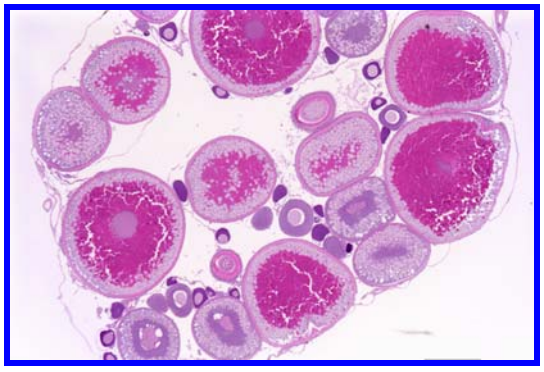
Increased oocyte atresia:



Oocyte atresia, mature oocytes. Note clumping and pore-formation in the vitelline envelope (chorion) of the early atretic oocyte (large arrow), and the vacuolar hypertrophy of its surrounding granulosa cells (small arrows). Compare these with granulosa cells that surround a non-atretic late vitellogenic oocyte (arrowheads). In FHM, granulosa cells of atretic oocytes often appear to contain phagocytized material, whereas the granulosa cells of non-atretic oocytes do not. (FHM, adult female, paraffin, H&E).

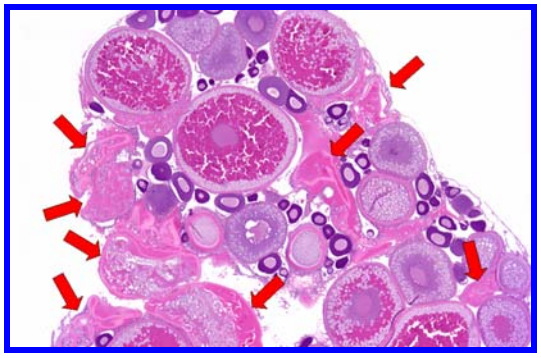
***Oocyte atresia, increased, immature / mature*:**
Degradation and resorption of an oocyte at any point in development. Histopathologically, atresia is often characterized by clumping and perforation of the chorion, fragmentation of the nucleus, disorganization of the ooplasm, and/or the uptake of yolk materials by perifollicular cells (FHM, e.g.). Separate diagnoses and severity grades can be given to atretic oocytes that are mature ("Oocyte atresia, increased, mature") versus immature ("Oocyte atresia, increased, immature"). In this context, oocytes will be considered "mature" if they are appear to have been interrupted in either the late vitellogenic oocyte phase or mature / spawning phase of development.

Non Remarkable



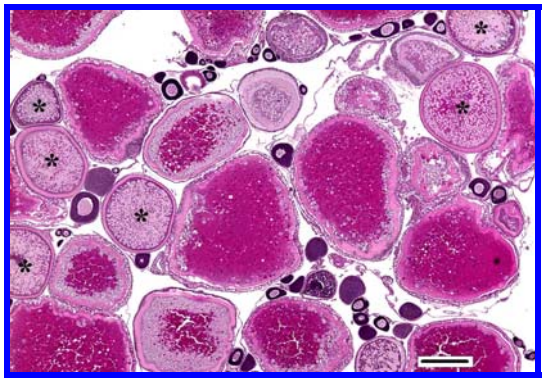
Ovary from a control group female. H&E.

Grade 3



Ovary from an adult female FHM. Numerous atretic oocytes are evident (arrows). H&E.

Grade 4

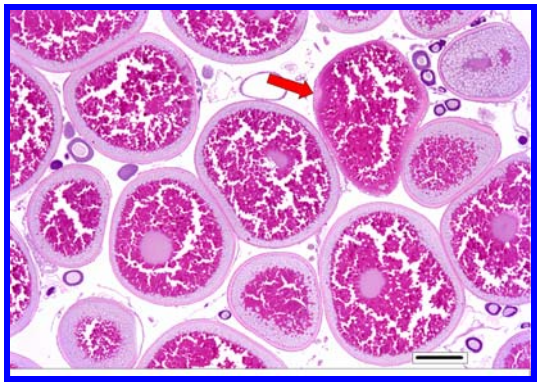


Stage 4 ovary from an adult female FHM. This ovary is characterized by severe oocyte atresia. Asterisks indicate the relatively few non-atretic oocytes. H&E.

Perifollicular cell hyperplasia/hypertrophy: An example of perifollicular cell hyperplasia/hypertrophy is not currently available for FHM.

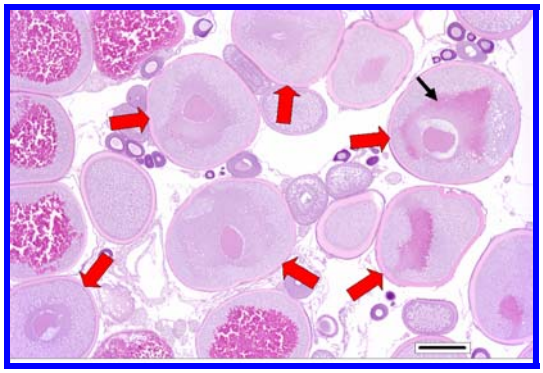
Decreased yolk formation:

Not Remarkable



Ovary (Stage 3) from a control group female. A single atretic ovary is evident (arrow). H&E, bar = 250 microns.

Grade 3



Ovary from an adult female FHM. Decreased yolk formation is characterized by the presence of oocytes in which yolk material is not present despite their relatively large size (large arrows). Note that oocytes are affected to varying degrees. Some affected oocytes have extremely fine vitellogenic granules (small arrow), and this is interpreted as ineffective yolk formation and deposition. H&E, bar = 250 microns.

Change in ovarian staging: Photographic examples are not currently available that demonstrate changes in ovarian staging.

Additional diagnostic criteria and an illustrated glossary of microanatomical and diagnostic terms

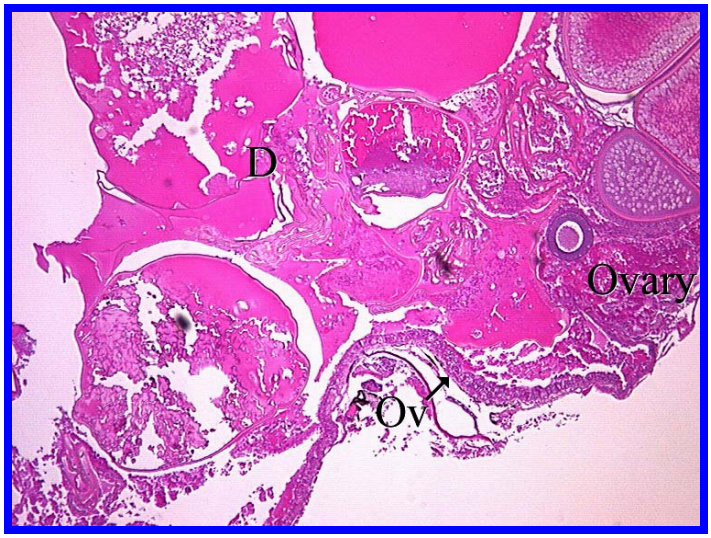
Asterisks denote "secondary" diagnoses for male and female FHM

****Asynchronous development, gonad (male or female):** The presence of distinctly different populations (i.e., range of developmental phases) of gametogenic cells in different regions of a gonad.

Asynchronous development, spermatocyst (male): The presence of more than one developmental phase of spermatogenic cell within a single spermatocyst. For example, this term may be applied to a spermatocyst that contains a mixture of spermatocytes and spermatids, or a spermatocyst that contains more than one meiotic phase of primary spermatocyte (i.e., leptotene, pachytene, and/or zygotene).

Asynchronous development, right and left gonads (male or female): The presence of distinctly different populations (i.e., developmental phases) of gametogenic cells in the right and left gonads.

****Egg debris, oviduct:** The presence of inspissated-appearing, homogenous, irregular, dense pink material, presumed to be yolk, within the oviduct.



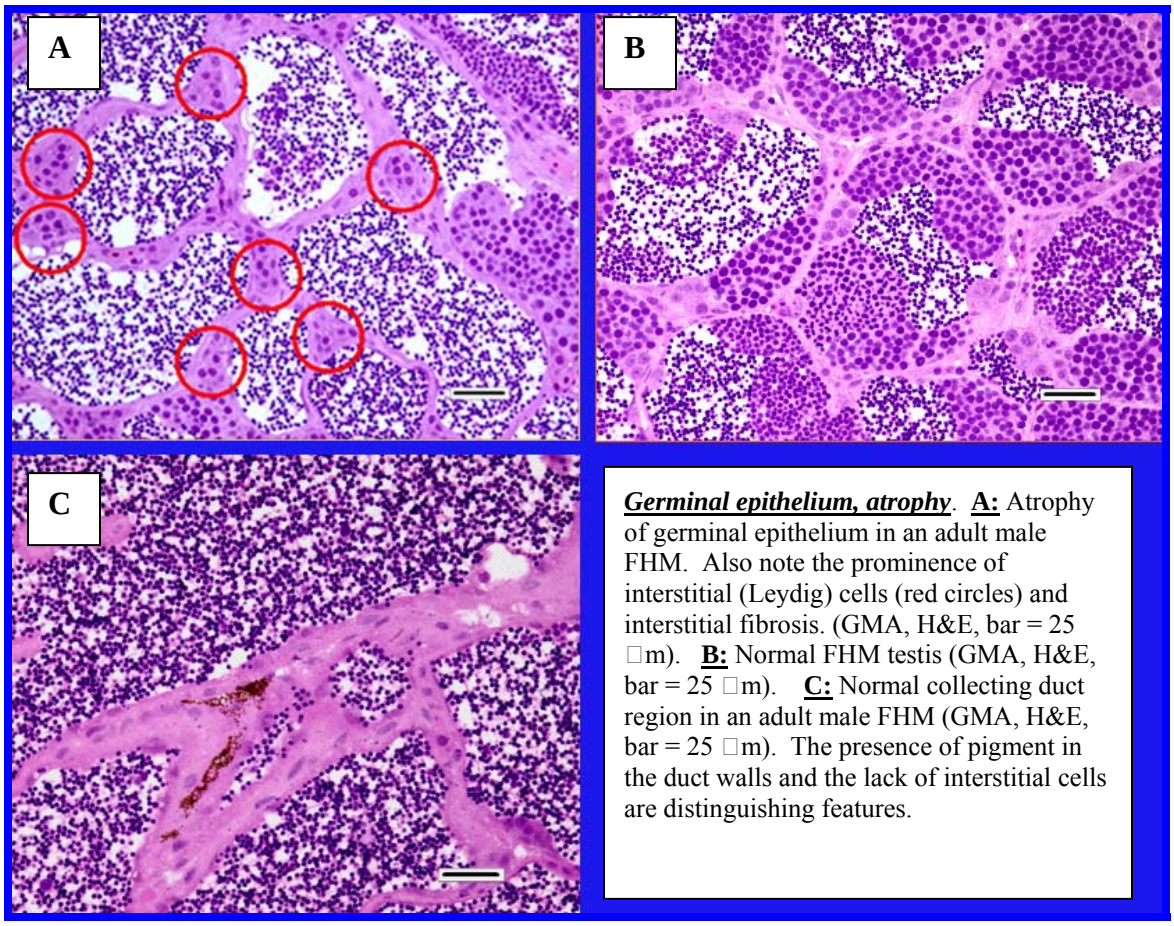
D = debris
Ov = oviduct

Egg debris, oviduct. (FHM, adult female, paraffin, H&E).

Gender: Because the genetic sex of a fish cannot be determined within the context of a screening assay, and because the external phenotypic sex may be an unreliable indicator and/or is not easily determined in some species, by convention the gender of a fish will be assigned according to the most abundant mature cell type that is present in the gonad.

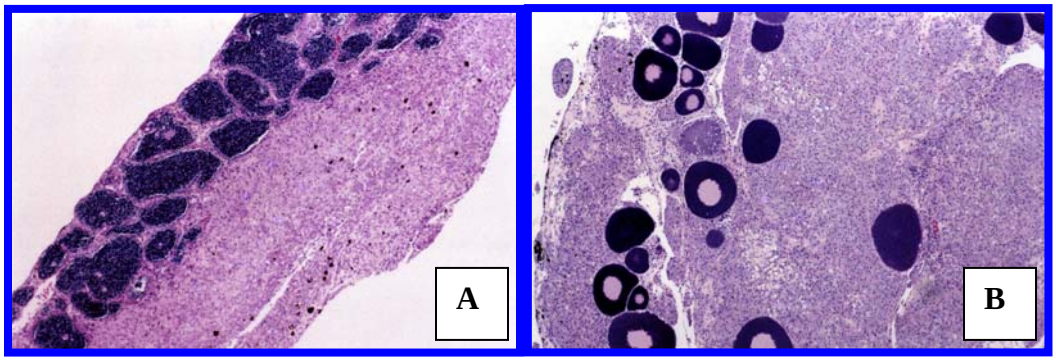
Germinal epithelium (female): The germinative parenchyma of the ovary is a membrane bound structure constitutively contains oogonia, prefollicular and prethecal cells, epithelial cells, and occasionally small chromatin nucleolar (primary growth) oocytes (Norberg et al., 2000; Parenti and Grier, 2003). The germinal epithelium separates the ovarian lumen from the stroma, the latter of which often contains perinucleolar, cortical alveolar, and vitellogenic follicles within a variably-apparent extravascular space.

Germinal epithelium, atrophy / hypoplasia (male): Indicating loss or underdevelopment of germinal epithelium, respectively, this condition may be associated with interstitial fibrosis and increased prominence of interstitial cells in affected areas of the testis. It may be difficult to distinguish atrophy from hypoplasia. Care should be taken to avoid mistaking areas of collecting ducts for atrophy. Severity of this finding can vary from Grade 1 (minimum, focal) to Grade 4 (severe, diffuse). If thinning of the epithelium appears to be caused by degenerative changes that are obvious in the section, the diagnostic term testicular degeneration should be used instead.



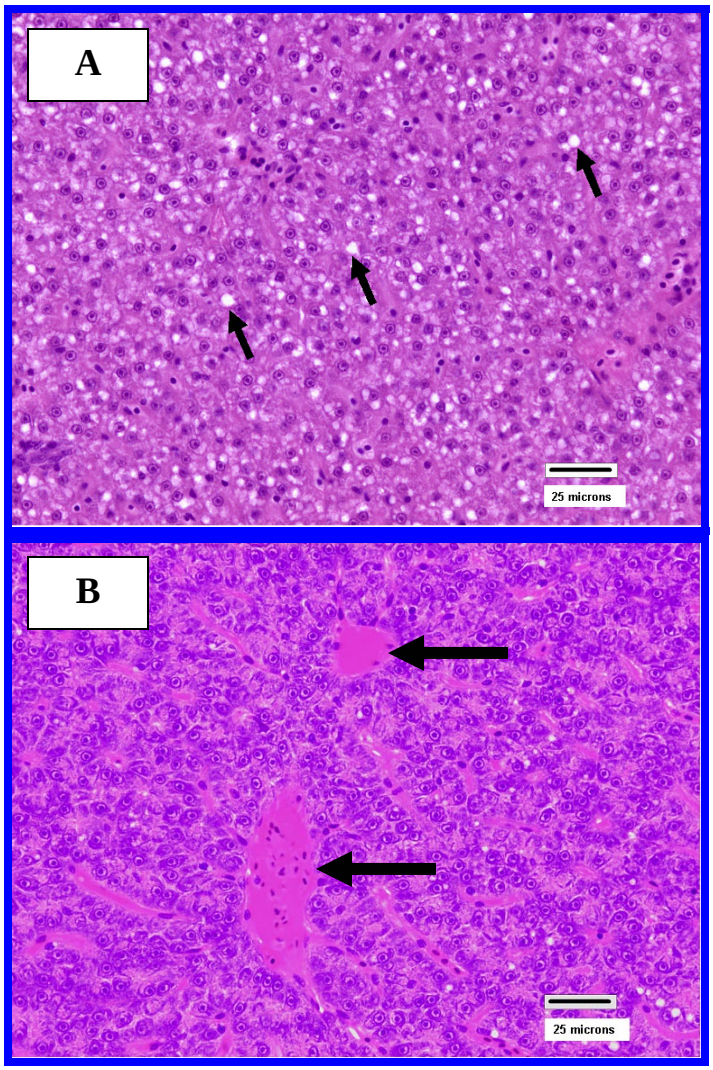
Germinal epithelium, atrophy. **A:** Atrophy of germinal epithelium in an adult male FHM. Also note the prominence of interstitial (Leydig) cells (red circles) and interstitial fibrosis. (GMA, H&E, bar = 25 □m). **B:** Normal FHM testis (GMA, H&E, bar = 25 □m). **C:** Normal collecting duct region in an adult male FHM (GMA, H&E, bar = 25 □m). The presence of pigment in the duct walls and the lack of interstitial cells are distinguishing features.

**__Granulomatous inflammation:__ In the early stages of inflammation, this process is characterized by the presence of epithelioid macrophages that typically form sheets or nodules (granulomas) due to desmosome-like cytoplasmic attachments (Noga et al., 1989). When compared to histiocytic-type macrophages, epithelioid macrophages have larger, more open-faced, centralized nuclei and less abundant cytoplasm. During resolution of inflammation, the epithelioid macrophages may become flattened into fibrocyte-like cells. Lymphocytes, granulocytes, and multinucleated giant cells may also be components of granulomatous inflammation. Granulomatous inflammation is intrinsically a pathologic process that is often associated with reactions to infectious agents, foreign materials, or the aftermath of necrosis; therefore, it is important to distinguish this, if possible, from the presence of macrophage aggregates in the ovary or histiocytic cells in the lumen of the testis.



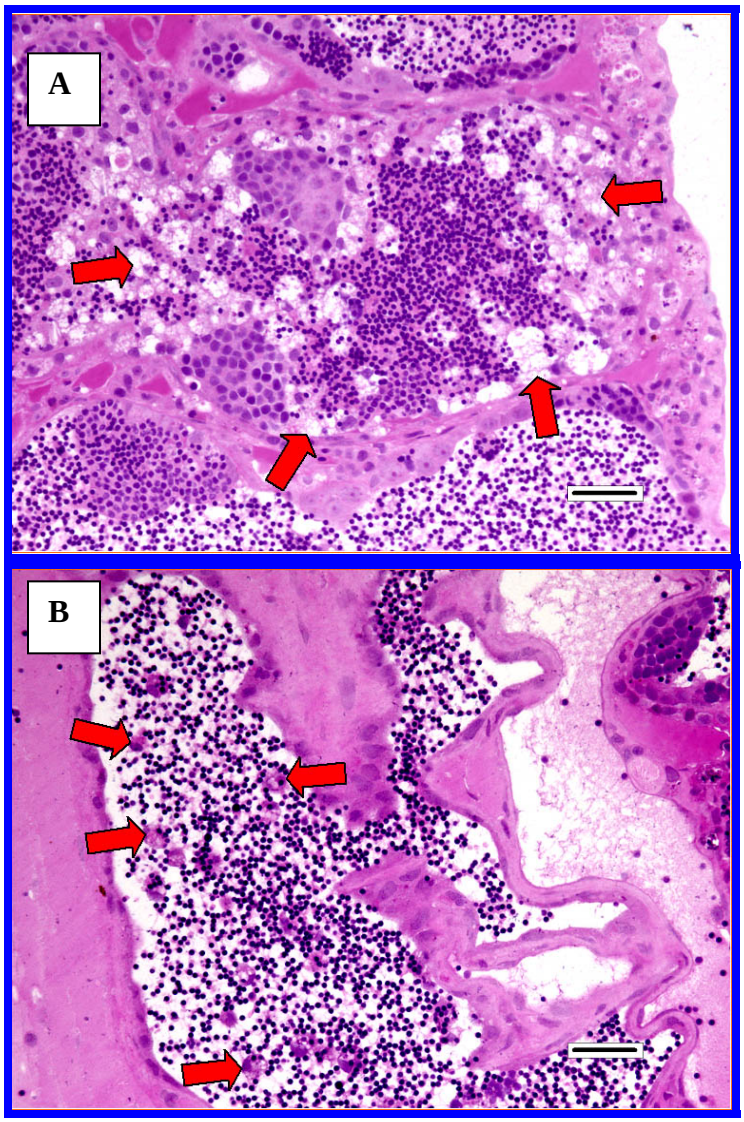
Granulomatous inflammation. **A:** Sheets of macrophages and other inflammatory cells eclipse much of the germinative tissue in this testis (FHM, adult male, paraffin, H&E). **B**: Relatively few viable-appearing oocytes remain in this ovary. As in the testis photo, the inciting cause of the inflammation is not evident at this magnification (FHM, adult female, paraffin, H&E).

__Hepatocyte basophilia, increased / decreased:__ 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 17α-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.



Hepatocyte basophilia <u>A:</u> The liver from an adult male FHM control. In addition to the overall coloration, note the hepatocyte cytoplasmic vacuolization as indicated by the arrows. **<u>B:</u>** Liver from an adult male FHM that was exposed to a compound with estrogenic activity. There is a diffuse increase in hepatocyte basophilia, a loss of cytoplasmic vacuolization, and hepatic blood vessels contain proteinaceous fluid (arrows). (FHM, paraffin, H&E).

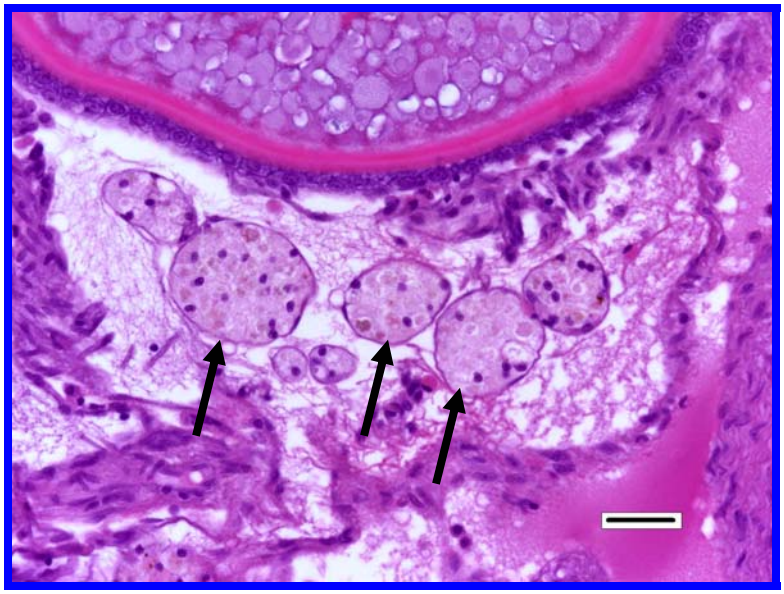
Histiocytic cells (male): The presence of individual or clustered cells with small eccentric nuclei and moderate to abundant, pale or vacuolated cytoplasm within the testicular lumen, germinal epithelium, efferent ducts and/or ductus deferens. Such cells may contain intracytoplasmic cellular debris (presumably phagocytized). The origin of the histiocytic cells in each particular case may not be clear; for example, they may be hematogenous macrophages or Sertoli cells. Histiocytic cells should be differentiated from macrophage aggregates (these variably pigmented cells are primarily interstitial) and granulomatous inflammation (which is predominately comprised of "epithelioid" macrophages and/or flattened, fibrocytic cells).



Histiocytic cells. (FHM, adult male, GMA, H&E). **A:** Cells with small peripheral nuclei and abundant vacuolated cytoplasm are present within the germinal epithelium and are scattered throughout the tubule lumen (arrows). Some of these cells contain phagocytized cellular debris. **B:** Similar cells are evident within the lumen of the collecting duct.

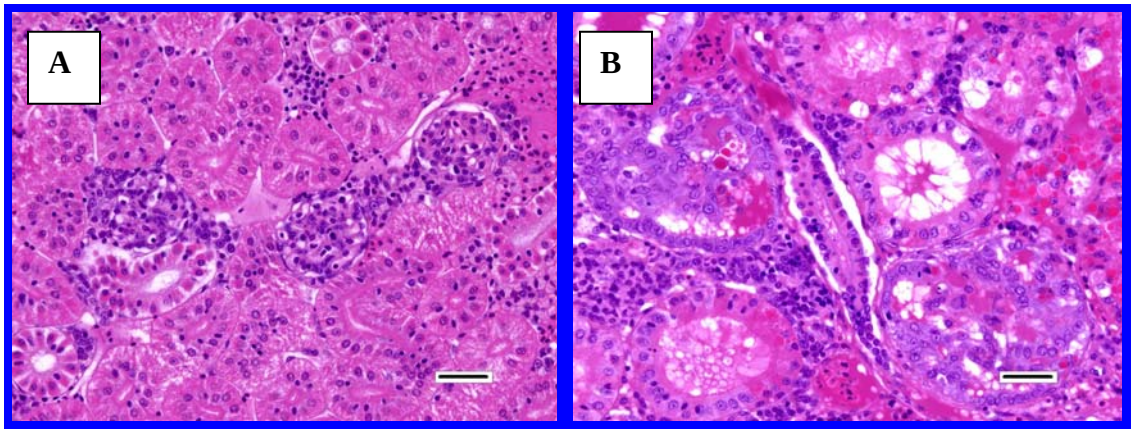
Interstitial fibrosis (male or female): The presence of increased fibrous connective tissue (collagenous fibers and fibrocytes or fibroblasts) within the testicular or ovarian interstitium (stroma). Collagen may be difficult to appreciate in early phases of fibrosis. In most cases, this term should be used in preference to terms such as "stromal hyperplasia."

Macrophage aggregates: These cell clusters are constitutively present in the interstitium of the ovary primarily, although they may also be found in the fish testis (unusual for tank-raised FHM). These phagocytes usually have small condensed eccentric or peripheralized nuclei and various brown, yellow, red, or gold pigmented granules (lipofuscin, ceroid, hemosiderin, and/or melanin) that often impart a slightly crystalline appearance to their comparatively abundant pale cytoplasm. In the normal ovary, macrophage aggregates are thought to be involved in the processing of breakdown products associated with atresia of unspawned oocytes. It has been demonstrated that macrophage aggregates may become larger and/or more numerous following exposure to certain toxicants or infectious agents (Blazer et al., 1987). Whenever possible, macrophage aggregates should be distinguished from granulomatous inflammation, which is characterized by the presence of epithelioid macrophages. This is not always easy, as macrophage aggregates often proliferate with, and become incorporated into, granulomatous inflammation.



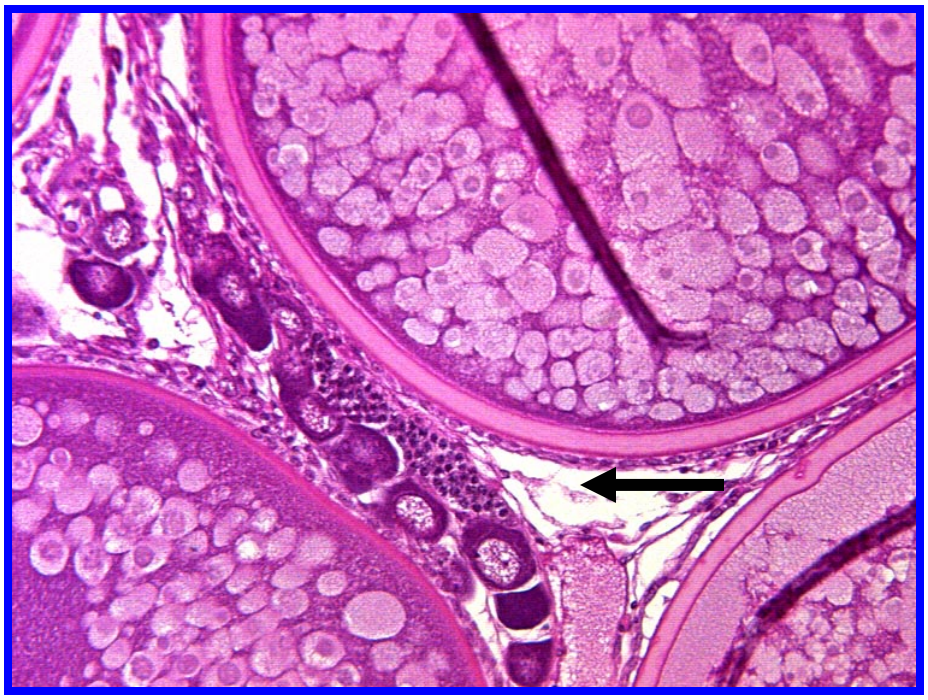
Macrophage aggregates. The arrows indicate multiple aggregates within the ovarian interstitium. (FHM, adult female, paraffin, H&E).

Nephropathy: 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 (especially in males) that stresses the kidney via protein overload. 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.



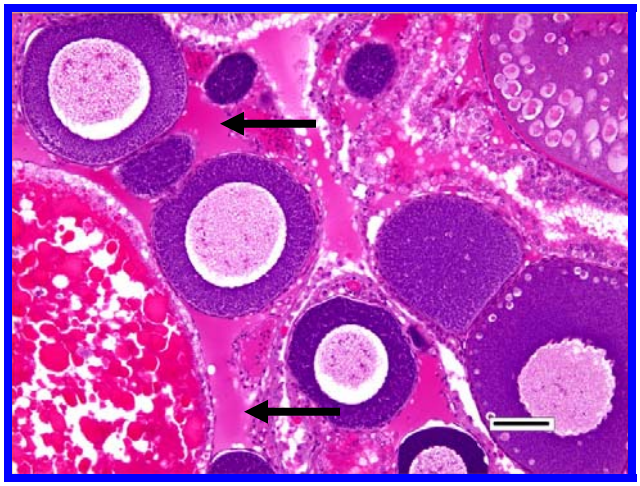
Nephropathy. **A:** Kidney from an untreated adult male FHM. **B:** Kidney from an adult male FHM exposed to a compound with estrogenic activity. Changes include glomerular epithelial cell hypertrophy, vacuolar swelling and necrosis of the tubular epithelium, and hyaline droplets within glomerular and tubular epithelia (paraffin, H&E, bar = 25 µm).

Ovarian spermatogenesis: The presence of non-neoplastic spermatogenic cells, usually immature, within the ovary. There is little or no evidence of lobular or tubular testicular architecture. Care should be taken to distinguish ovarian spermatogenesis from mitotically dividing oogonia; a key feature of ovarian spermatogenesis is the presence of multiple spermatogenic phases.



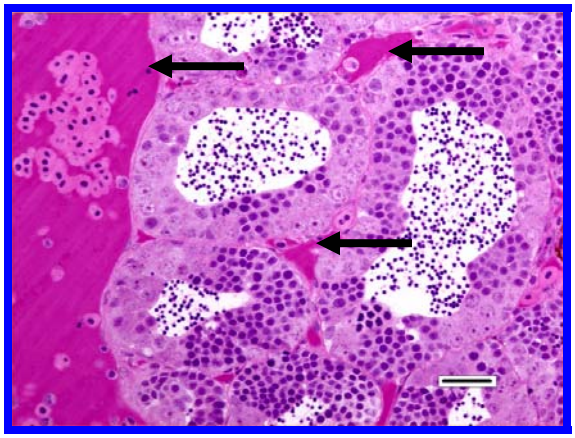
Mitotically dividing oogonia. Packets of cells that resemble spermatocytes (arrow) are situated between perinucleolar and cortical alveolar oocytes. This should not be mistaken for spermatogenesis. (FHM, adult female, paraffin, H&E).

****Proteinaceous fluid, interstitial (male or female):** Homogenous dark pink translucent material, presumably vitellogenin, within the testicular or ovarian interstitium. In male fish especially, this finding has been associated with exposure to estrogenic substances. The presence of this fluid may cause a thickening of interstitial areas that might be misinterpreted as "stromal proliferation".



Proteinaceous fluid, interstitial. There is homogenous dark pink material in interstitial spaces (arrows). (FHM, adult female, paraffin, H&E, bar = 50 µm).

****Proteinaceous fluid, intravascular (male or female):** Homogenous dark pink translucent material, presumably vitellogenin, within testicular or ovarian blood vessels. In male fish especially, this finding has been associated with exposure to estrogenic substances.



Proteinaceous fluid, intravascular. There is homogenous dark pink material within large and small blood vessels (arrows). Also note the increased proportion of spermatogonia in the testis. (FHM, adult male, GMA, H&E, bar = 25 µm).

Sertoli cell hypertrophy: Exposure of male fish to estrogen-active compounds has been reported to cause enlargement of Sertoli cells, with or without Sertoli cell proliferation (Miles-Richardson et al., 1999a; Miles-Richardson et al., 1999b; Kinnberg et al., 2000; van der Ven et al., 2003). In the scientific literature, the light microscopic appearance of hypertrophic Sertoli cells tends to be ambiguous, as Sertoli cells resemble spermatogonia in some descriptions and images.

Vitellogenic oocyte: An oocyte that contains microscopically visible yolk material. Generally, such material is strongly eosinophilic and slightly refractile in hematoxylin- and eosin-stained sections. This material may be present in the form of spherical, globular, yolk granules. In some scholarly sources (e.g., Iwamatsu, et al., 1998), the term "vitellogenic" has been applied to cortical alveolar oocytes, which lack eosinophilic yolk granules/globules (although their amphophilic or clear cortical alveoli are also known as yolk *vesicles*).

GONDAL 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 fathead minnows. 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), 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.

The semi-quantitative gonadal staging scheme selected for analysis of FHM gonads 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), Rodriquez 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 fathead minnow. This was demonstrated at the October 2003 meeting of the histopathology subcommittee of the FDG in Paris, at which the participants were asked to briefly evaluate the applicability of a modified BEST system using actual histologic specimens of these three species. In general, the participants agreed that they could readily recognize the various gonadal stages as defined by modified BEST criteria. The participants acknowledged that the terminal stages of the system (testis: Stage 4, "Spent"; ovary: Stage 5, "Post-ovulatory") would be minimally present (or possibly not present at all) among reproductively-active adult FHM because they are not seasonal spawners. Similarly, it was thought that Stage 4, "Late development/hydrated", would be rarely observed in the test fishes, due to the very transient nature of this stage in fractional spawners.

A few modifications have been made to the BEST system to adapt it for use. For

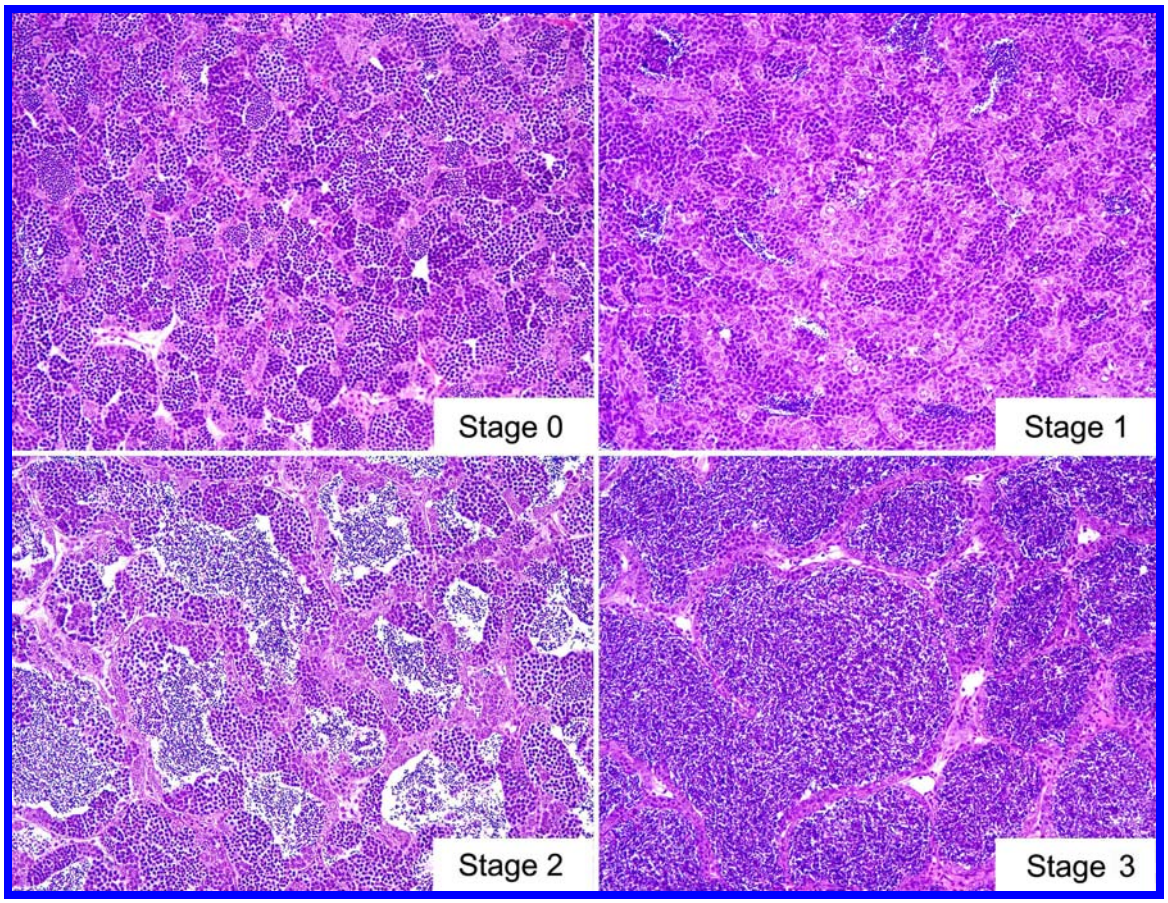
example, there is currently no provision in the system for gonads that are comprised entirely of spermatogonia or oogonia. Although it is intended that reproductively mature fish are used, it is possible that an occasional animal may not attain sexual maturity by the time the experiment is terminated, 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.

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. Both gonads should be staged as a single organ according to the predominant pattern. 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).

Criteria for Staging Testes

The following are morphologic criteria for staging male fish:

- **Juvenile**: gonad consists of spermatogonia exclusively; it may be difficult or impossible to confirm the sex of these individuals.
- **Stage 0 – Undeveloped**: entirely immature phases (spermatogonia to spermatids) with no spermatozoa.
- **Stage 1 – Early spermatogenic**: immature phases predominate, but spermatozoa may also be observed; the germinal epithelium is thinner than it is during Stage 2.
- **Stage 2 – Mid-spermatogenic**: spermatocytes, spermatids, and spermatozoa are present in roughly equal proportions; the germinal epithelium is thinner than Stage 1 but thicker than Stage 3.
- **Stage 3 – Late spermatogenic**: all stages may be observed, however, mature sperm predominate; the germinal epithelium is thinner than it is during Stage 2.
- **Stage 4 – Spent**: loose connective tissue with some remnant sperm.

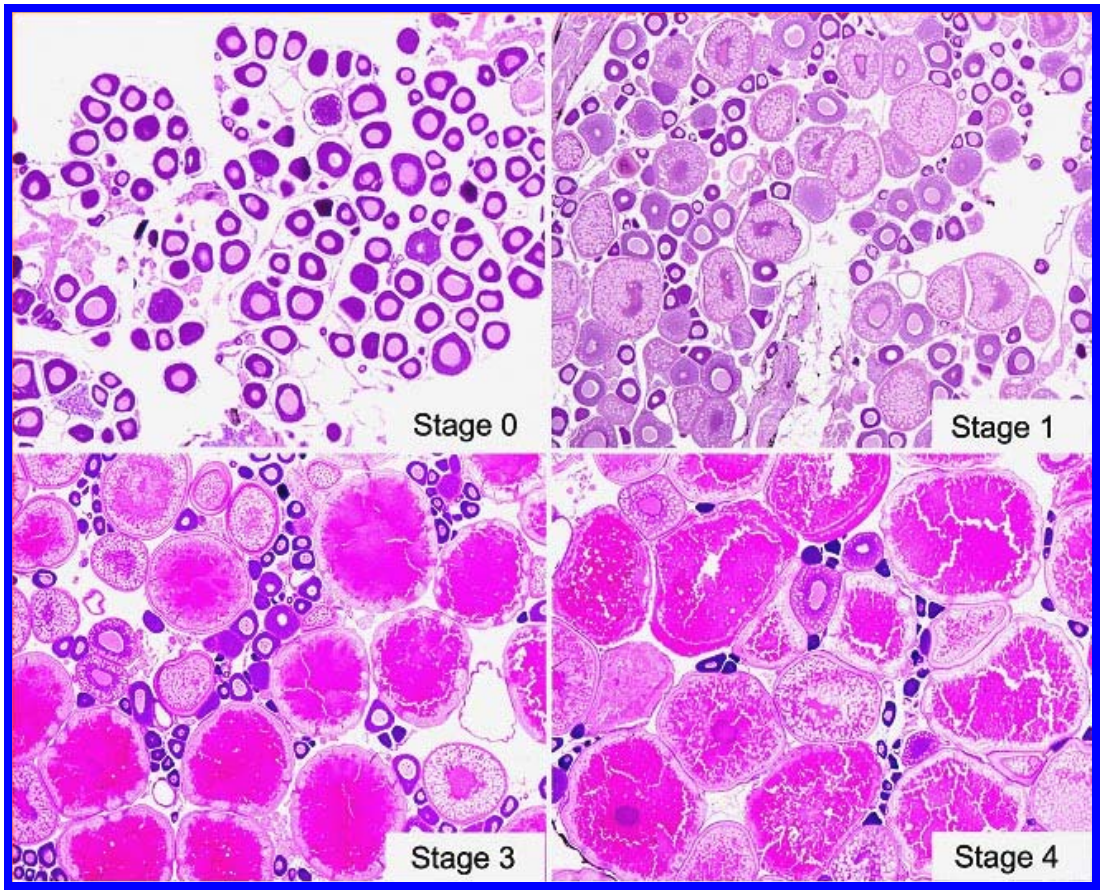


Examples of staging system applied to the FHM testis. Testes from four different adult male FHM. There is progressive thinning of the germinal epithelium and expansion of the lobular lumen with each increase in stage. Note that no spermatozoa are present at in the Stage 0 image (GMA, H&E)**.**

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.



Examples of staging system applied to the FHM ovary. Ovaries from four adult female FHM. Due its transient nature in FHM, Stage 4 is not often observed (paraffin, H&E).

APPENDIX A: HISTOLOGY FIGURES

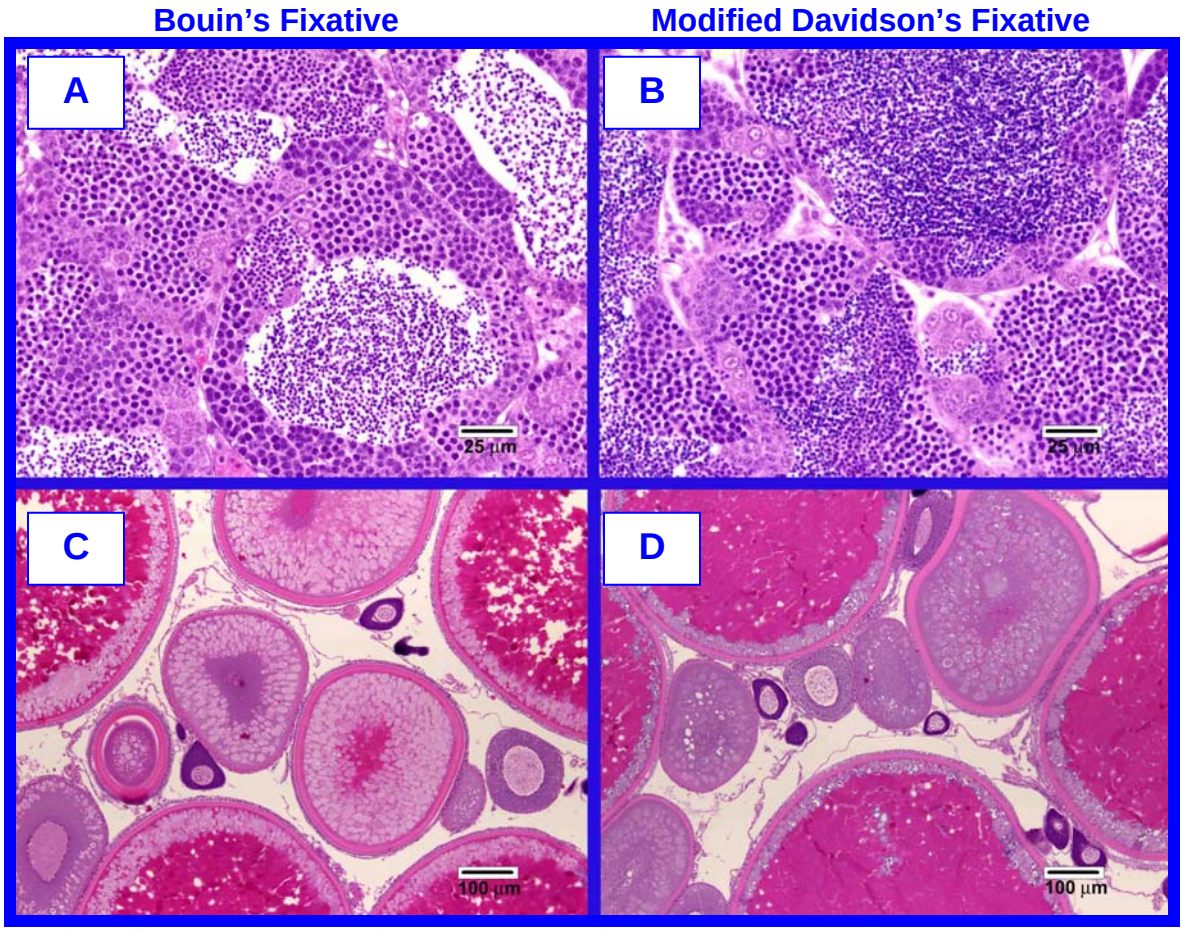


Fig. 1. Fathead Minnows, Testis (A&B) and Ovary (C&D): Gonads fixed in Bouin's fixative (A&C) and modified Davidson's fixative (B&D). Color contrast was slightly superior in testes fixed with Davidson's fixative and was clearly superior in ovaries fixed with Bouin's fixative. Either fixative is satisfactory for diagnostic purposes; however, Davidson's fixative was selected for the Phase 1B assay.

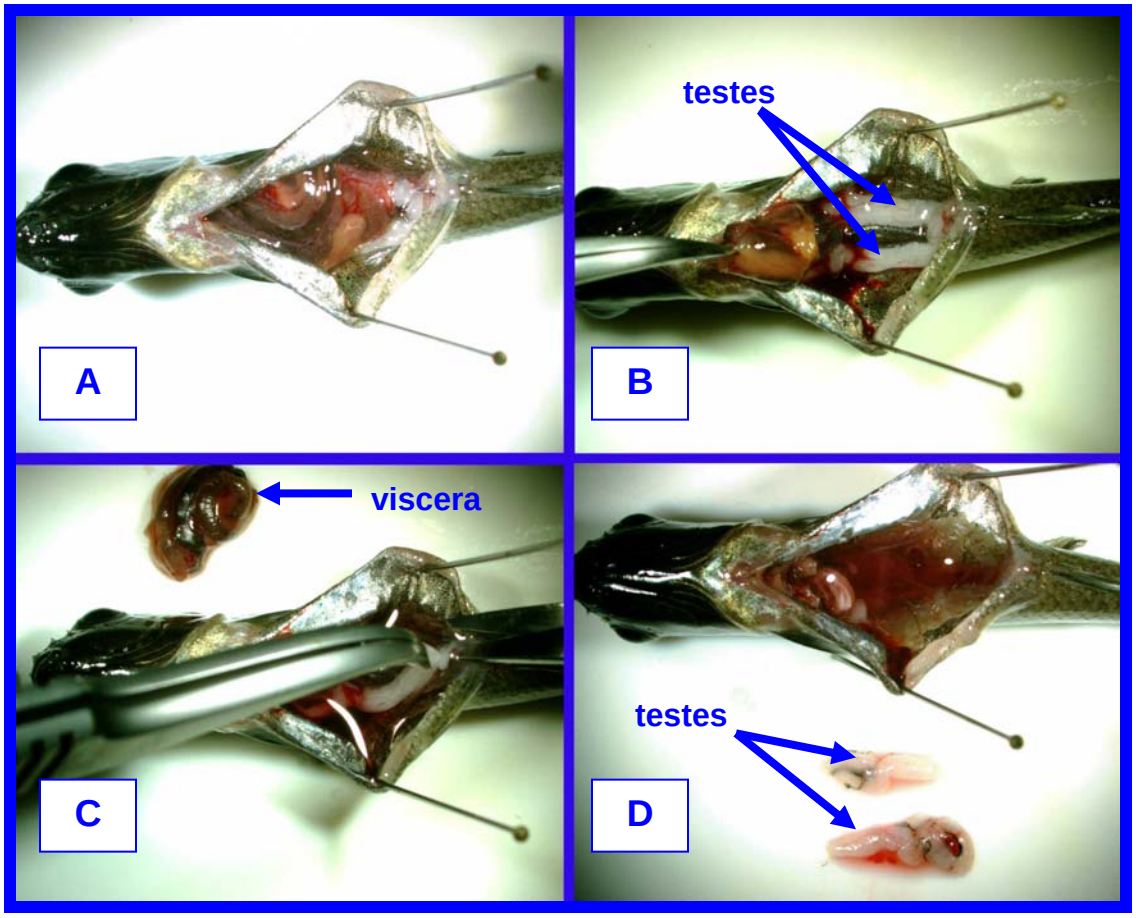


Fig. 2. Fathead Minnow, Male: Excision of the testes during necropsy. **A**. The abdominal wall is pinned laterally. **B**. The terminal intestine is severed and retracted prior to removal. **C**. The testes are grasped near the spermatic ducts. **D**. Removal of the testes is complete.

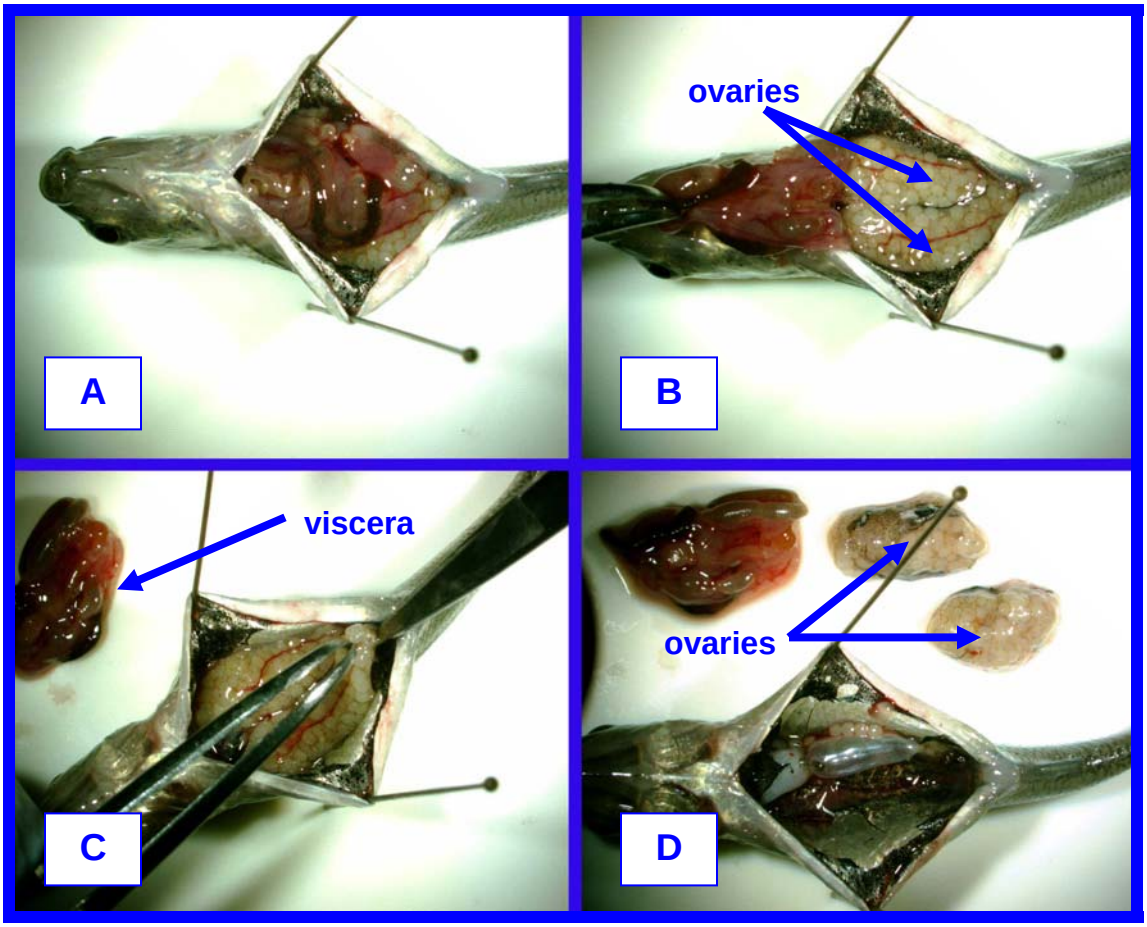


Fig. 3. Fathead Minnow, Female: Excision of the ovaries during necropsy. **A**. The abdominal wall is pinned laterally. **B**. The terminal intestine is severed and retracted prior to removal. **C**. The ovaries are grasped near the oviducts. **D**. Removal of the ovaries is complete.

APPENDIX B: SCHEDULES

Schedule 1. Tissue Processing

Station No.	Reagent	Pressure/ Vacuum Cycle	Heat (°C)	GONAD PROGRAM (minutes)	WHOLE-FISH PROGRAM (minutes)
1	10% NBF[a]	On	Ambient	40	60
2	70% ethyl alcohol	On	Ambient	40	60
3	80% ethyl alcohol	On	Ambient	40	60
4	95% ethyl alcohol	On	Ambient	40	60
5	95% ethyl alcohol	On	Ambient	40	60
6	100% ethyl alcohol	On	Ambient	40	60
7	100% ethyl alcohol	On	Ambient	40	60
8	100% ethyl alcohol	On	Ambient	40	60
9	Clear Rite 3	On	Ambient	60	80
10	Clear Rite 3	On	Ambient	60	80
11	Paraffin	On	60	45(60[b])	75(100[b])
12	Paraffin	On	60	45(60[b])	75(100[b])
13	Paraffin	On	60	45(60[b])	75(100[b])
14	Paraffin	On	60	45	75
Drain and Clean Cycle[c]					

[a] Neutral buffered formalin.
[b] Times are increased for processors that have three (versus four) final stations
[c] Automatic cleaning cycle to be run after removal of tissues from the processor. Time, temperature, and vacuum are preset by the manufacturer.

Schedule 2. Hematoxylin and Eosin Staining

		Reagent Maintenance	
Reagent	**Minutes in Reagent**	**After 1st Run**	**After 2nd Run**
Xylene	4	Remove	Remove
Absolute Alcohol	2	Remove	Remove
80% Alcohol	1	Renew	Renew
Water	1	---	---
Hematoxylin	3	---	Remove
Water	2	---	---
Clarifier	1	Renew	Renew
Water	1	---	---
Bluing	1	Renew	Renew
Water	2	---	---
95% Alcohol	1	Renew	Renew
Eosin	1	---	Renew
Absolute Alcohol	4	Remove	Remove
Xylene	3	Remove	Remove

APPENDIX C: FORMULARY

Euthanasia Solution

Tricaine methanesulfonate	100 mg
Sodium bicarbonate	200 mg
Tank or reservoir water	1 L

Davidson's Fixative (Fournie *et al*., 2000)

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

Modified Davidson's Fixative

Formaldehyde (37-40%)	220 ml
Glacial acetic acid	115 ml
95% Ethyl alcohol	330 ml
Distilled water	335 ml

It is recommended that hematoxylin and eosin be purchased as premixed solutions. Examples are the Hematoxylin-2 (Gill hematoxylin) and Eosin Y solutions that are manufactured by Richard-Allan Medical Industries (Appendix D).

Gill Hematoxylin Solution (Gill *et al*. 1974)

Distilled water	730 ml
Ethylene glycol	250 ml
Hematoxylin, anhydrous	2 g
Sodium iodate	0.2 g
Aluminum sulfate	17.6 g
Glacial acetic acid	20 ml

Eosin Solution

Eosin Y (1% aqueous solution)	100 ml
Ethyl alcohol, 95%	600 ml
Glacial acetic acid	4 ml

APPENDIX D: EXAMPLE PRODUCT GUIDE

Example Product	Catalogue #	Supplier/Manufacturer
Clear Rite-3™	6901	Richard Allen Medical Industries 8850 M89 Box 351 Richland, MI 49083 800-522-7270 http://www.rallansci.com.
Coverglass, 24x50 premier nonstick Thinness: 0.13mm-0.17mm	00145-ACS	Surgipath Medical Industries, Inc. P. O. Box 528 Richmond, IL 60071 800-225-3035
Davidson's Fixative	S2250	Poly Scientific R&D Corp. 70 Cleveland Avenue Bay Shore, NY 11706 631-586-0400
Decalcifier: Formical-2000®	1354	Decal Chemical Corp. PO Box 916 Tallman, NY 10982-0916 800-428-5856
Eosin Y (for H&E Stain) Eosin-Y Reagent Alcohol Deionized Water Glacial Acetic Acid	7111	Richard Allen Medical Industries 8850 M89 Box 351 Richland, MI 49083 800-522-7270 http://www.rallansci.com.
Hematoxylin 2 (for H&E Stain) Hematoxylin Aluminum Sulfate Sodium Iodate Ethylene Glycol Deionized Water Glacial Acetic Acid	7231	Richard Allen Medical Industries 8850 M89 Box 351 Richland, MI 49083 800-522-7270 http://www.rallansci.com.
MS-222 Fenquel™ (Tricaine Methanesulfonate)	C-FINQ-UE	Argent Chemical Laboratories, Redmond, WA 98052, USA.
Paraplast® (CSMP) Kendall Paraplast Tissue Embedding Medium 8889 501006	SHM8889-501006	Supplier: Laboratory Supply Co. 800-888-9004 Manufacturer: Sherwood Services AG Tyco Healthcare Group L 15 Hampshire Street Mansfield, MA 02048
Permount® Mounting Media Toluene 55% BHT < 1% Polymer Alpha pinene & Betapinene 45%	SP15-500	Fisher HealthCare 800-640-0640
Slide, Single Frosted, ground edge Crystal Line Premier Brand	8105	C&A Scientific Co., Inc. 7241 Gabe Court Manassas, VA 20109 703-330-1413

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