OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads

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(1) Wolf, J., Johnson, R. (2008) Guidance Document for Diagnostic Histopathology for Screening Endocrine Disruptors in Small Fish. Draft of December 2008.

(2) Grim, C. (2006) Histopathology Guidelines for the Fathead Minnow (*Pimephales promelas*) 21-day reproduction assay. Draft dated 6 June 2006.

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Contents

Summary ◄

Exposure of fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) to endocrine disrupting chemicals may result in histopathological alterations of gonadal structure and microarchitecture. The diagnosis of such histopathological changes may, therefore, assist in the detection of endocrine disrupting chemicals. Given the long historical background of piscine histopathology and diverse traditions in the terminology in different fish species and in different regions, there is a need for harmonization in order to facilitate non-biased comparisons of results from different laboratories and, thus, to optimize reproducibility and reliability of histopathological diagnoses. The purpose of this document is to provide guidance for the preparation and histopathological evaluation of gonads from fathead minnow, Japanese medaka and zebrafish following exposure to chemicals potentially disrupting the endocrine system.

Throughout this document, the proposed procedures were derived from consensus opinions of various fish pathologists, recommendations from the Bilthoven (2002), Paris (2003) and Heidelberg (2004) workshops, from information distilled from previous guidelines, and the scientific literature. Major sections of the guidance document comprise (1) post-mortem and histotechnical procedures (chapter 2), (2) primary, secondary and additional gonadal histopathology glossary and diagnostic criteria (chapters 3 and 4), and (3) gonadal staging criteria (chapter 5).

Note: It is the intention of the authors to make this document available on the internet, preferably on the OECD website. Further experience in the use of histopathological diagnoses of endocrine-disruptor-related changes in testes and ovaries of fathead minnow, Japanese medaka and zebrafish should be incorporated into future updates of this guidance document.

1. Purpose of this document ◄

The purpose of this document is to provide guidelines for the preparation and histopathological evaluation of gonads from fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) following exposure to chemicals potentially disrupting the endocrine system.

The goals of these guidelines are

a) to harmonize techniques for the preparation of histological specimens for the diagnosis of endocrine-related histopathology in fish gonads,

b) to provide guidance for the diagnosis of histopathological alterations in fish exposed to potential endocrine-disrupting chemicals according to

   (1) the OECD "Fish Screening Assay" (FSA),

   (2) the US "Fathead Minnow 21-d Short-term Reproduction Assay", and

   (3) any other assay designed for the detection of endocrine-disrupting chemicals and requiring histological analyses of the gonads.

c) to supply template text for laboratory protocols, and (d) to facilitate non-biased comparisons of results from different laboratories.

Throughout this document, the proposed procedures were derived from consensus opinions of various fish pathologists, recommendations from the Bilthoven (2002), Paris (2003) and Heidelberg (2004) workshops, information distilled from previous guidelines, and the scientific literature.

This guidance document is divided into three main sections:

(1) post-mortem and histotechnical procedures (chapter 2),

(2) gonadal histopathology glossary and diagnostic criteria (chapters 3 and 4),

(3) gonadal staging criteria (chapter 5).

In order to facilitate interspecies comparisons, this document is *not partitioned according to species*; instead, for each diagnosis, all three fishes (fathead minnow, Japanese medaka, zebrafish) are considered simultaneously whenever possible.

2. Post-mortem and histotechnical procedures ◄

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

2.1 Fixation of tissue specimens 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 3 step sections of both gonads on a single glass slide.

In all three fish species, the gonads are excised from the fish. In case very young individuals were used, the gonads may alternatively be fixed inside the carcass. However, in order, to allow optimal penetration of the fixative, head and tail portions should be removed by means of sharp razor blades.

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 as follows:

(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.

Formulation of Davidson´s fixative (1 L; 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

Formulation of Modified Davidson's Fixative (1 L):

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

For a photographic comparison of specimens fixed in Davidson's versus Bouin's fixatives, see 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 decalcification solution (EDTA, acetic acid) may be used. 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.

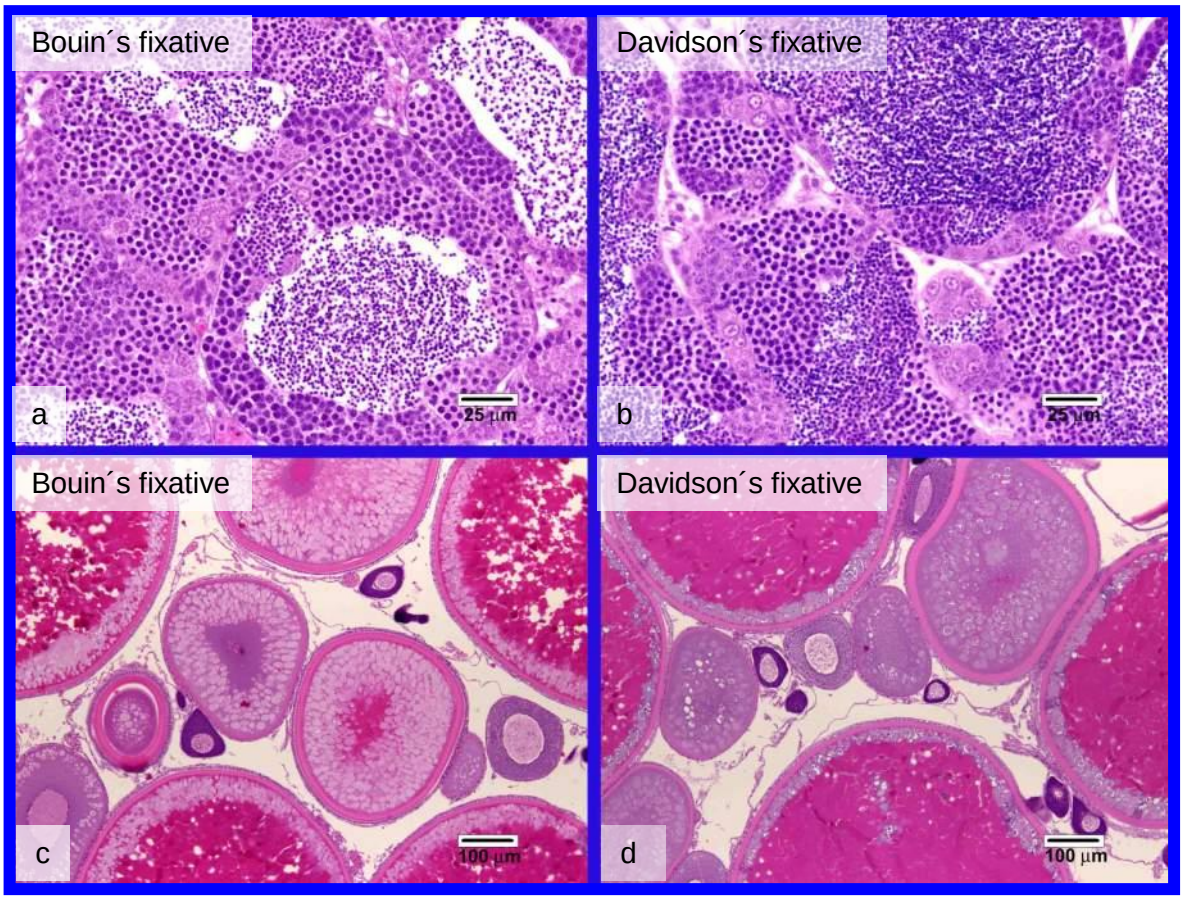


Fig. 1. Fathead minnow, testis (a, b) and ovary (c, d) fixed in Bouin's fixative (a, c) and modified Davidson's fixative (b, d). Color contrast was slightly superior in testes fixed with Davidson's fixative and was clearly superior in ovaries fixed with Bouin's fixative. Either fixative is satisfactory for diagnostic purposes; however, Davidson's fixative was eventually selected.

2.2 Euthanasia and necropsy ◄

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 (**not required for Japanese medaka and zebrafish**).

(5) Evaluate secondary sex characteristics (**not required for Japanese medaka and zebrafish**).

(6) Provide for adequate fixation of the gonads and/within 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 (see below).

(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 (see 2.1).

(15) Plastic syringe (3 ml).

(16) Standard plastic tissue cassettes (one per fish).

(17) Fixation containers (100 ml, one per fish).

Euthanasia solution (MS-222; tricaine methanesulfonate)

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

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 single 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 (fathead minnow: presence or absence of nuptial tubercles, dorsal pad; medaka: shape of dorsal and anal fins; zebrafish: coloration, body shape).

(3) The fish is placed into buffered MS-222 (tricaine methanesulfonate) 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 (Figs. 2, 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) ***Fathead minnow***: 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.

   Japanese medaka, zebrafish: Using the microdissection scissors and forceps, the liver is dissected from the viscera and retained for vitellogenin analysis. **Go to step (11).**

(9) ***Fathead minnow***: 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) ***Fathead minnow***: 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. **Go to step (12).**

(11) ***Japanese medaka, zebrafish***: The abdominal cavity is ***gently*** flushed with 0.5 ml of Davison's fixative, and then the carcass and abdominal viscera are placed in an individual container of Davidson's fixative. The volume of fixative in the container should be at least 10 times the approximated volume of the tissues. **Go to step (12).**

(12) **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).

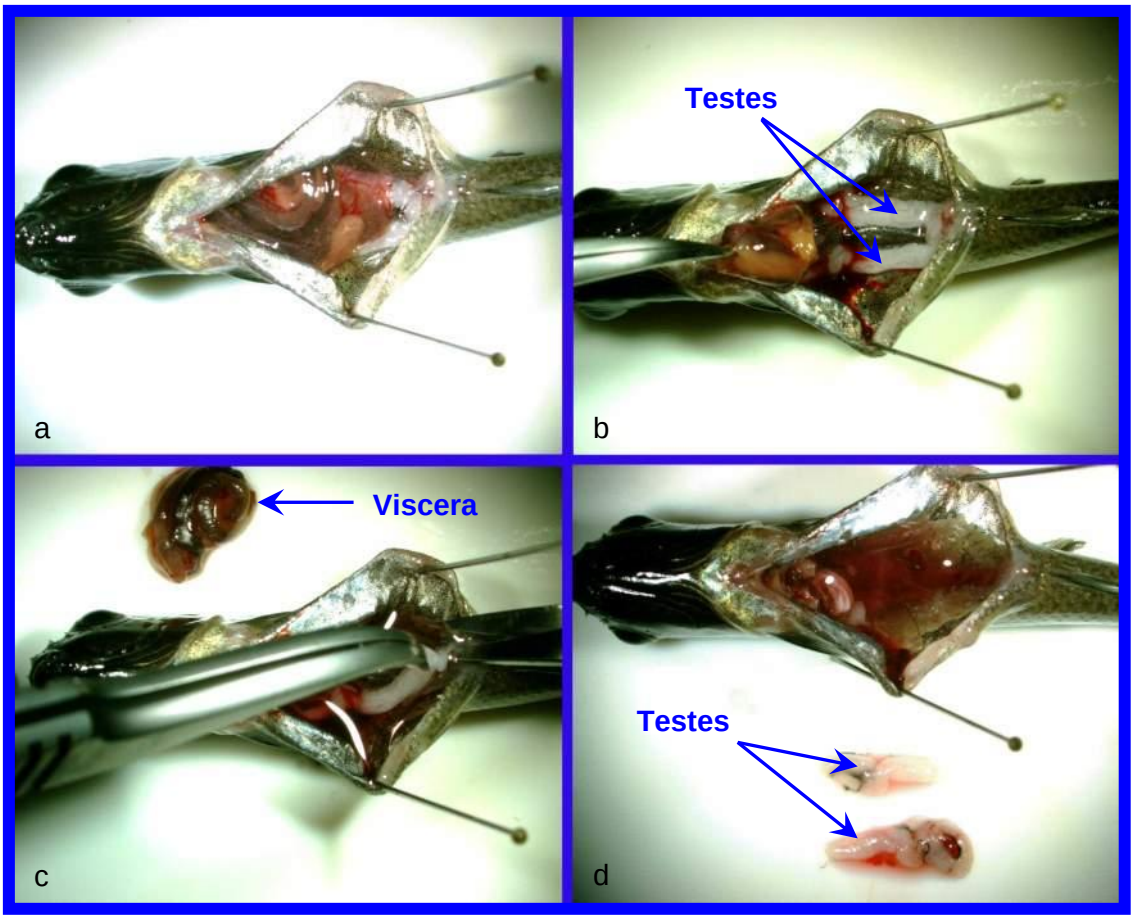


Fig. 2. Fathead minnow, male: Excision of the testes during necropsy. (a) The abdominal wall is pinned laterally. (b) The terminal intestine is severed and retracted prior to removal. (c) The testes are grasped near the spermatic ducts. (d) Removal of the testes is complete.

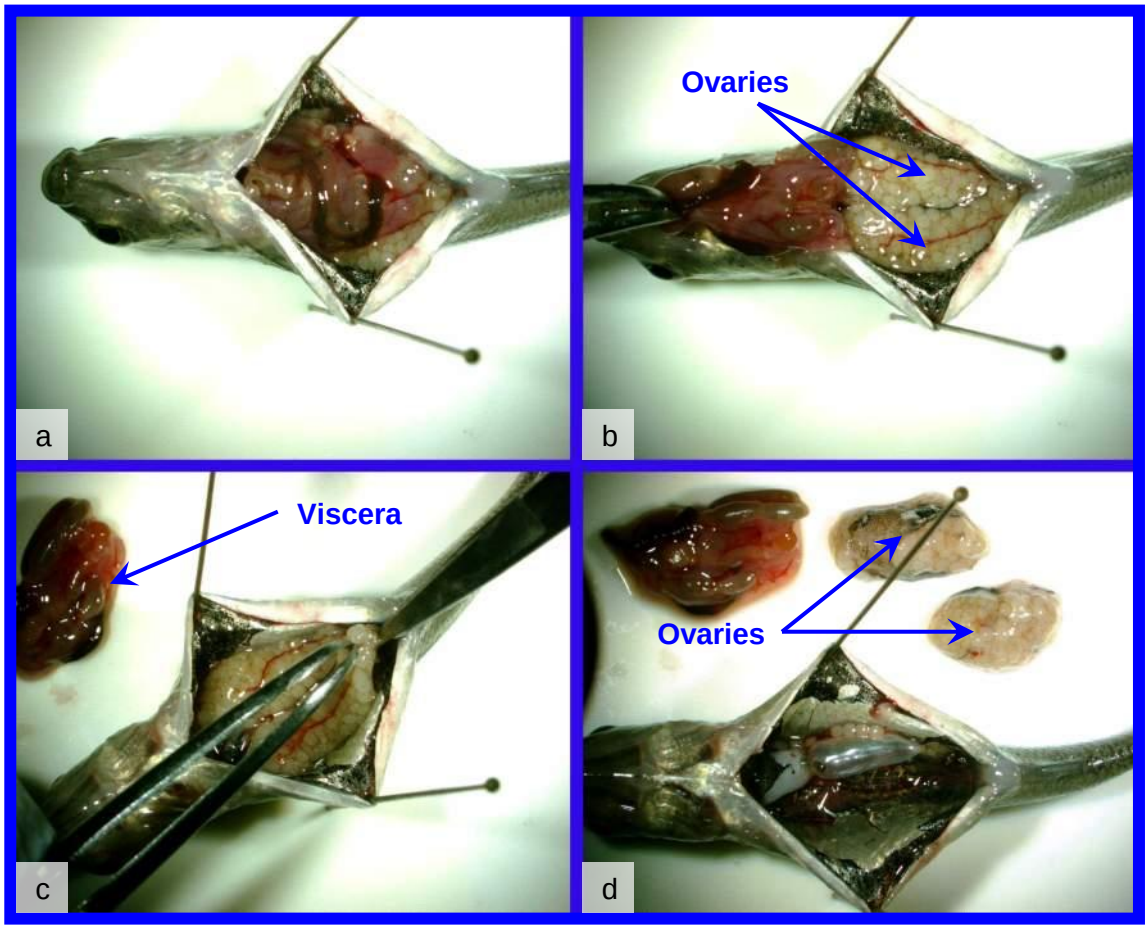


Fig. 3. Fathead minnow, female: Excision of the ovaries during necropsy. (a) The abdominal wall is pinned laterally. (b) The terminal intestine is severed and retracted prior to removal. (c) The ovaries are grasped near the oviducts. (d) Removal of the ovaries is complete.

2.3 Tissue processing

Objectives

(1) **Japanese medaka and zebrafish**: Trim carcass for embedding and microtomy.

(2) Dehydrate tissue to provide for adequate penetration of paraffin.

(3) 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^{TM}, or equivalent).

(6) 10 % neutral buffered formalin.

(7) Ethyl alcohol (absolute and dilutions as required).

(8) Proprietary clearing agent (Clear Rite-3™ or equivalent).

(9) Xylene.

Procedures

(0) **Japanese medaka, zebrafish**: Head and tail are severed and removed prior to embedding.

(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 baskets are loaded into the tissue processor.

(3) The processing schedule is selected (see Appendix B, Schedule 1). The "Gonad Program" or equivalent is selected for fathead minnow.

(4) After the tissue processor has completed the processing cycle, the basket(s) may be transferred to the embedding station.

Schedule 1: Tissue Processing

Station No.	Reagent	Pressure/Vacuum Cycle	Heat (°C)	GONAD program (min.)	WHOLE-FISH program (min.)
1	10 % NBF[a]	On	Ambient	40	60
2	70 % Ethanol	On	Ambient	40	60
3	80 % Ethanol	On	Ambient	40	60
4	95 % Ethanol	On	Ambient	40	60
5	95 % Ethanol	On	Ambient	40	60
6	100 % Ethanol	On	Ambient	40	60
7	100 % Ethanol	On	Ambient	40	60
8	100 % Ethanol	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.

2.4 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 cryoconsole 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.

2.5 Microtomy ◄

Objective

(1) Create and mount histological 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 face down 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.

h. **Japanese medaka**: The block is faced from the dorsum towards the ventrum (through the majority of the swim bladder) until the fused left and right gonads are reached.

Zebrafish: The block is faced from the ventrum and towards the dorsum until the level is reached at which the following anatomic landmarks are observed:

i. Testis: disappearance of the liver, prior to the appearance of the rostral swim bladder. There is greater than 500 microns of tissue thickness at this level. Sections at this level do not contain intestine.

ii. Ovary: the appearance of the swim bladder.

(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 µm, 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.

2.6 Staining, cover-slipping, and slide labeling ◄

Objectives

(1) Differential staining of intra- and inter-cellular components of the gonads to facilitate diagnostic examination by bright field 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 cover-slipping 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).

(6) Hematoxylin-2 (Richard Allen or equivalent, Appendix C).

(7) Xylene.

(8) Absolute ethyl alcohol (100% ETOH).

(9) 95% ETOH.

(10) 80% ETOH.

(11) Cover-slipping mountant (Permount®, DPex® or equivalent).

(12) Glass cover-slips, No. 1, 24 x 50 (or 60) mm.

(13) Slide flats.

Gill´s 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

Procedures

(1) Staining

a. Slides are routinely air-dried overnight before staining.

b. An example H&E staining schedule for automated stainers is given below. A similar schedule can be adapted for manual staining.

(2) Cover-slipping

a. Cover-slips 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 cover-slip 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).

Schedule 2. Hematoxylin and eosin Staining

Reagent	**Minutes in Reagent**	**Reagent Maintenance**	
		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

3. 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.

3.1 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 Fish Screening Assay (FSA) or 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(s). 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). This is because endocrine effects on histomorphology tend to be incremental, and subtle differences between exposed and unexposed animals may not be recognizable unless tissue sections from high dose animals can be knowingly compared to those from controls. Thus, the aim of the initial evaluation is to ensure that diagnoses are not missed (i.e., to avoid false-negative results). 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 al., 2004).

3.2 Diagnostic criteria ◄

Histopathology is a descriptive and interpretive science, and therefore somewhat subjective. However, histopathological evaluations of the same study by any qualified pathologist should identify the same treatment-related findings (Crissman et al., 2004). Therefore, we aim to define the diagnostic criteria

that will likely be encountered during the histopathological analysis of the FSA or the 21-day reproduction assay in fathead minnow, medaka and/or zebrafish.

In the following text, a consolidated set of diagnostic criteria will be introduced. These criteria are based on pathologists' experience with certain consistent histopathological changes that occur in fathead minnow, medaka and/or zebrafish gonads in response to chemical exposure; however, any additional/novel findings that are exposure-related shall also be reported.

The criteria below have been divided into two categories (Table 1):

(1) primary criteria,

(2) secondary criteria and

(3) additional criteria.

The criteria are graded for severity on a numerical scale. Likewise, any novel findings are either graded on a numerical scale, or are qualitatively described.

Table 1. Primary and secondary diagnoses for histopathological changes in male and female fathead minnow, Japanese medaka and zebrafish after exposure to potential endocrine disruptors

Primary Diagnoses		
	For males:	**For females:**
1	Increased proportion of spermatogonia	Increased oocyte atresia
2	Presence of testis-ova	Perifollicular cell hyperplasia/hypertrophy
3	Increased testicular degeneration	Decreased vitellogenesis
4	Interstitial (Leydig) cell hyperplasia/hypertrophy	Changes in gonadal staging

Secondary Diagnoses		
	For males:	**For females:**
1	Decreased proportion of spermatogonia	Interstitial fibrosis
2	Increased vascular or interstitial proteinaceous fluid	Egg debris in the oviduct
3	Asynchronous gonad development	Granulomatous inflammation
4	Altered proportions of spermatozoa or spermatocytes	Decreased post-ovulatory follicles
5	Gonadal staging	
6	Granulomatous inflammation	

3.2.1 Primary criteria in the gonads of male fathead minnow, Japanese medaka and zebrafish ◄

The following criteria have been defined in male fish as diagnoses of primary interest:

(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 dependent 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. Whenev-

er applicable, the term testis-ova should be used in preference to less precise terms such as "intersex" or "hermaphrodite".

> Terms such as *hermaphrodism*, *hermaphroditism*, *intersex*, *mixed sex*, *sex reversal*, *ovotestis*, *ovatestis*, *ova-testis*, *testis-ova*, *testicular oocytes* or *testicular oogenesis* abound in the lower vertebrate scientific literature. Three of these terms are listed in standard medical dictionaries (Dorland's, 1981; Illustrated Stedman's, 1982), and their consensus definitions are: (1) *intersex*: the presence of both male and female characteristics in the same individual; (2) *hermaphrodism* (= *hermaphroditism*): the presence of male and female gonadal elements within the same individual; (3) *ovotestis*: the presence of male and female gonadal elements within the same gonad. The validity of many other terms is largely based on traditional usage, which has not always been consistent.

(3) ***Increased testicular degeneration***: Testicular degeneration is characterized by

a) individual or clustered apoptotic germ cells;

b) vacuolated germ cells; and/or

c) 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 (destructive nuclear fragmentation) or pyknosis (shrinkage of nuclei in conjunction with chromatin condensation of the chromatin to a central mass), 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.

3.2.2 Primary criteria in the gonads of female fathead minnow, Japanese medaka and zebrafish ◄

The following criteria have been defined in female fish as diagnoses of primary interest:

(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) ***Changes 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. Since 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).

3.2.3 Secondary criteria in the gonads of male fathead minnow, Japanese medaka and zebrafish ◄

The following criteria have been defined in male fish as diagnoses of secondary interest:

(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 dependent 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 cells 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 spermatocytes (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).

(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.

3.2.4 Secondary criteria in the gonads of female fathead minnow, Japanese medaka and zebrafish ◄

The following criteria have been defined in female fish as diagnoses of secondary interest:

(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).

3.3 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 histomorphological 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:

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

(2) ***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.

(3) ***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 occurrences per microscopic field, or 1 - 2 occurrences per section. For multifocal or diffusely-distributed alterations, this grade is used for processes where ≤ 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 20 - 50 % of the tissue in the section are 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 50 - 80 % of the tissue in the section are 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 > 80 % of the tissue in the section are involved.

At least some of the histomorphological changes that have been associated with EDCs in fish are considered to be exacerbations of "normal", physiologic findings (e.g., oocyte atresia [Nagahama, 1983; Tyler and Sumpter, 1996]). 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 section 3.5: **Histopathology report format**). By convention, severity grading should *not* be influenced by the estimated physiologic importance of the change, since this would add a further layer of subjectivity to the findings that complicates inter-laboratory results comparisons. 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.

3.4 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 section 3: **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.

3.5 Histopathology report format ◄

Each histopathology narrative report should contain the following five sections:

Introduction
Materials and Methods
Results
Discussion
Summary/Conclusions
(References)

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 histomorphological descriptions need only be included for findings that differ substantially from diagnoses presented in Section 4 (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.

4. Glossary and diagnostic criteria ◄

The purposes of this section are:

(1) to provide photomicrographs of normal gonadal structure in fathead minnow, medaka and zebrafish,

(2) to provide a common technical "language" and

(3) to create a reference atlas of both microanatomical structures and potential pathological findings.

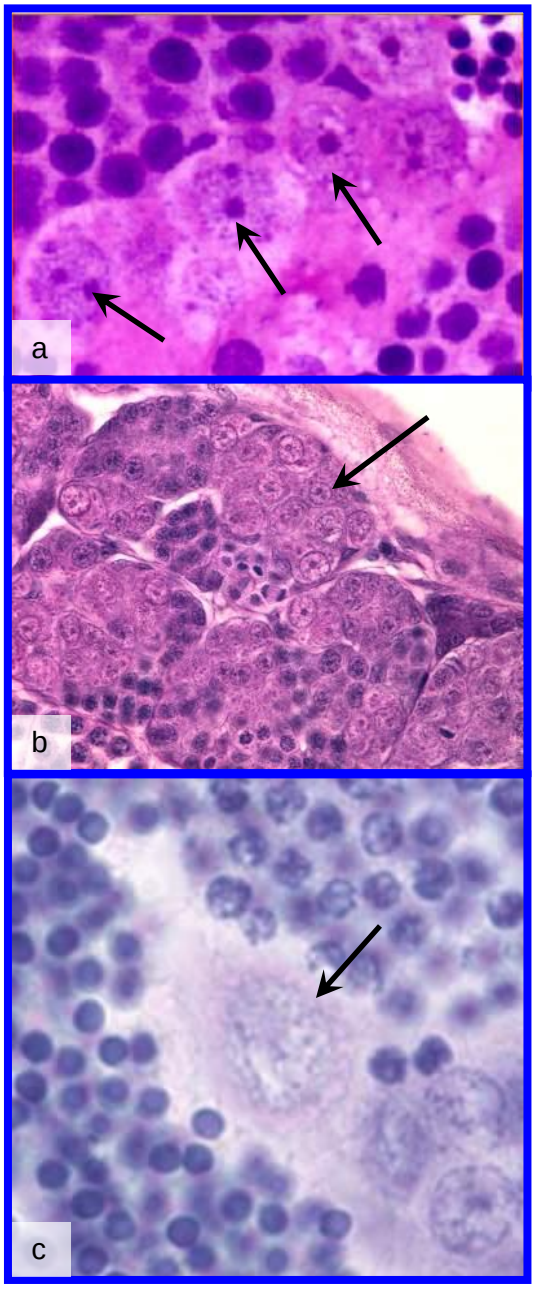
The information in this section is derived from a number of sources including scientific articles, conference proceedings, related guidelines, toxicologic pathology textbooks, medical dictionaries, and the personal experience of various fish pathologists. Regarding the last, opinions were solicited *via* 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. Additional guidance was provided by pathologists attending the 2nd OECD meeting of the Fish Pathologist Subcommittee at the University of Heidelberg, Germany, November 22 - 23, 2004. Other considerations include traditional usage and scientific precedence, and attributes such as clarity and brevity.

In addition to illustrating standard microanatomical features of Japanese medaka, fathead minnow, and zebrafish gonads, this document presents an array of histopathological diagnoses and their associated morphologic criteria. Whereas a few of these diagnoses have been consistently linked to endocrine disruptor exposure under certain specified laboratory conditions, many other findings are less well-established as EDC markers. It is also important to recognize that the utility of a given diagnosis for detecting EDC effects may depend greatly on the species and age of the test subjects, the effective dose of the test compound, and other factors such as husbandry practices. For example, juvenile and adult Japanese medaka seem to be relatively more susceptible to estrogen-induced testis-ova formation than either fathead minnow or zebrafish; however, another excellent response to estrogenic endocrine disruptors that has been primarily reported in juvenile fathead minnow and zebrafish is retained peritoneal attachments/gonadal duct feminization of the testis. On the other hand, while preliminary evidence indicates that decreased yolk formation may be a reliable marker for aromatase inhibitors and non-aromatizable androgens in reproductively active adult female Japanese medaka and zebrafish, this effect seems to be less robust in similarly-treated female fathead minnow. Conversely, adult male fathead minnow appear to be particularly well-suited for observing interstitial cell hyperplasia/hypertrophy of the testis as a reaction to the steroidogenesis inhibitor ketoconazole. One should also keep in mind that endocrine disruptors may induce different effects at high *versus* low exposure concentrations.

Chapter 4 is arranged as follows:

4.1 Normal gonadal architecture in fathead minnow, Japanese medaka and zebrafish ◄

4.1.1 Normal testicular architecture in male fathead minnow, Japanese medaka and zebrafish ◄



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".

Fig. 4. Spermatogonia A in male fathead minnow (a; GMA, H&E), Japanese medaka (b; paraffin, H&E) and zebrafish (c; paraffin, 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.

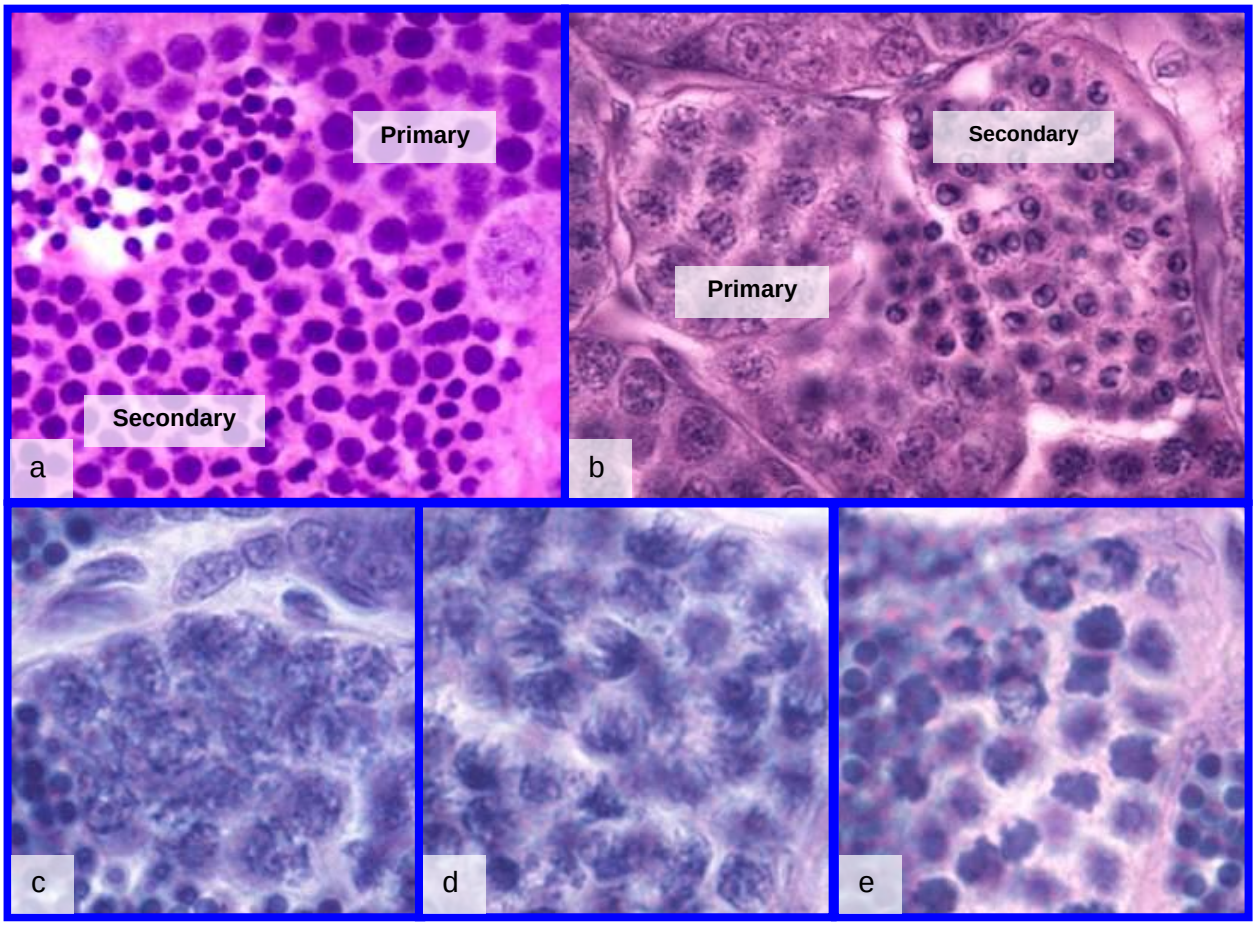
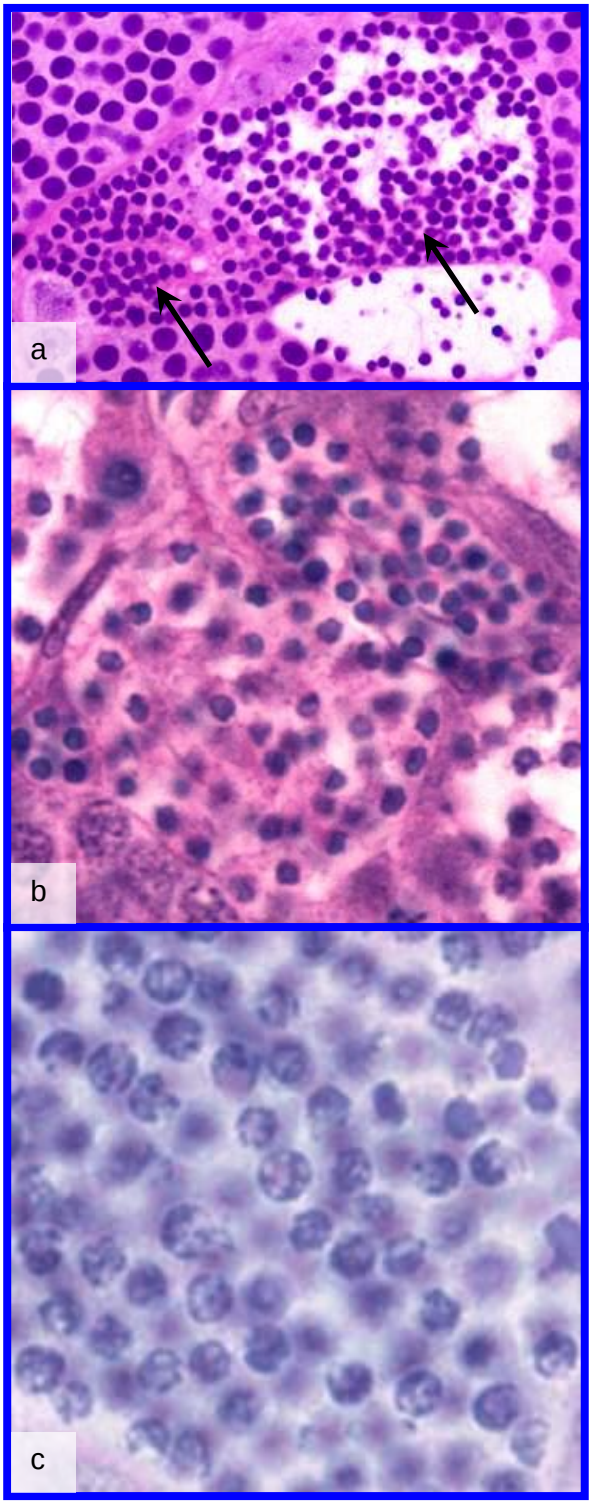
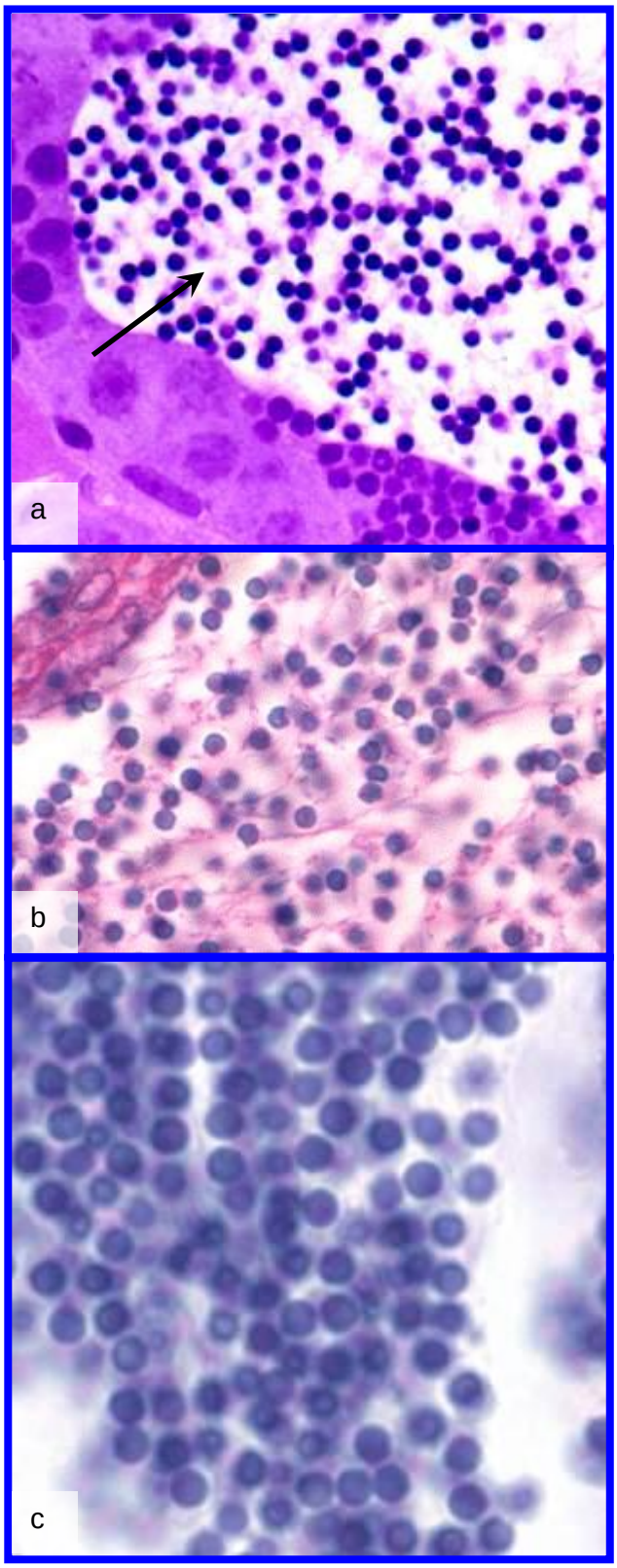


Fig. 5. Spermatocytes A: (a) male fathead minnow (GMA, H&E); (b) male Japanese medaka (paraffin, H&E), and (c) in the leptotene, zygotene, and pachytene phases, respectively, during the first meiosis in the zebrafish testis (paraffin, H&E).



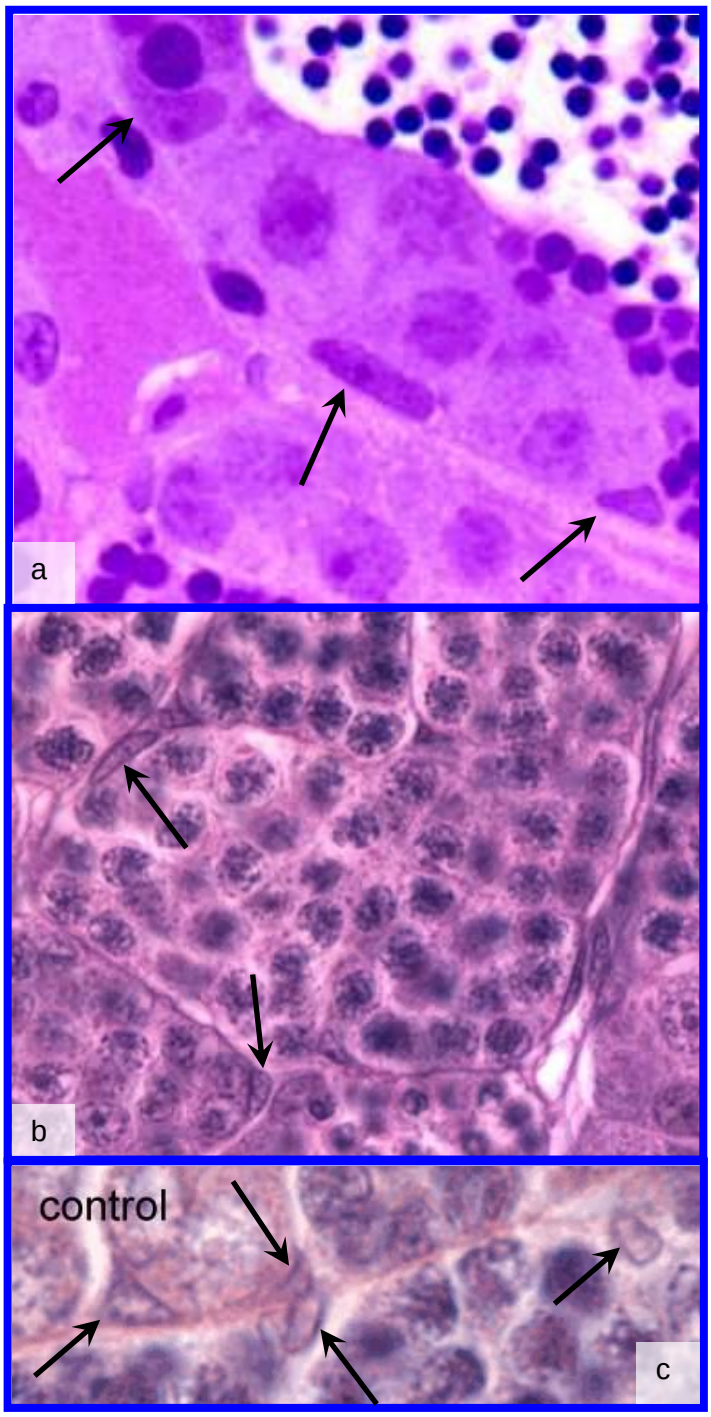
Spermatids: Derived from spermatocytes following the second meiotic division, these cells have dense nuclei and narrow rims of eosinophilic cytoplasm. They are the smallest cells within the germinal epithelium (~ 2 - 3 µm), and the cells lose their cytoplasmic attachments to one another during spermiogenesis.

Fig. 6. (a) Spermatids in male fathead minnow: Intercellular attachments are lost just prior to rupture of the spermatocyst and release of these cells as spermatozoa (GMA, H&E). (b) Spermatids in a Japanese medaka testis (paraffin, H&E). (c) Spermatids in a zebrafish (paraffin, H&E).



Spermatozoa: These cells have dark, round nuclei and minimal or no apparent cytoplasm. Tails are generally not apparent in histological sections. Spermatozoa are the smallest spermatogenic cells (~ 2 µm), and they exist as scattered individual cells within tubular lumen.

Fig. 7. (a) Spermatozoa in male fathead minnow (GMA, H&E). (b) Spermatozoa in a Japanese medaka testis (paraffin, H&E). (c) Spermatozoa in a zebrafish (paraffin, 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.

Fig. 8. In all three species, Sertoli cells are flattened. (a) Fathead minnow testis (GMA, H&E), (b) Japanese medaka testis (paraffin, H&E), and (c) zebrafish testis (paraffin, H&E).

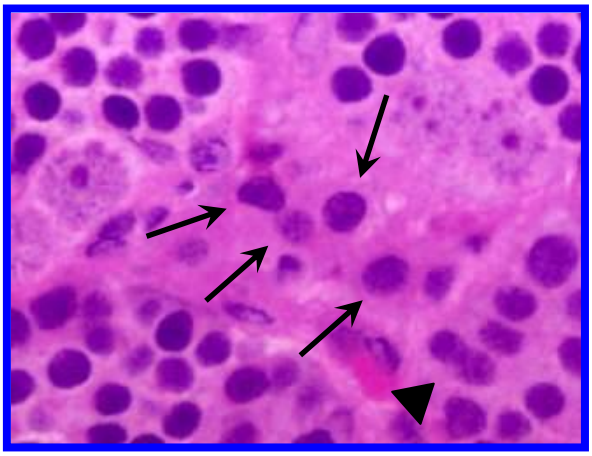


Fig. 9. Interstitial cells (→) in male fathead minnow are only found in interlobular areas. Note the resemblance between these cells and spermatocytes (►) (GMA, H&E).

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.

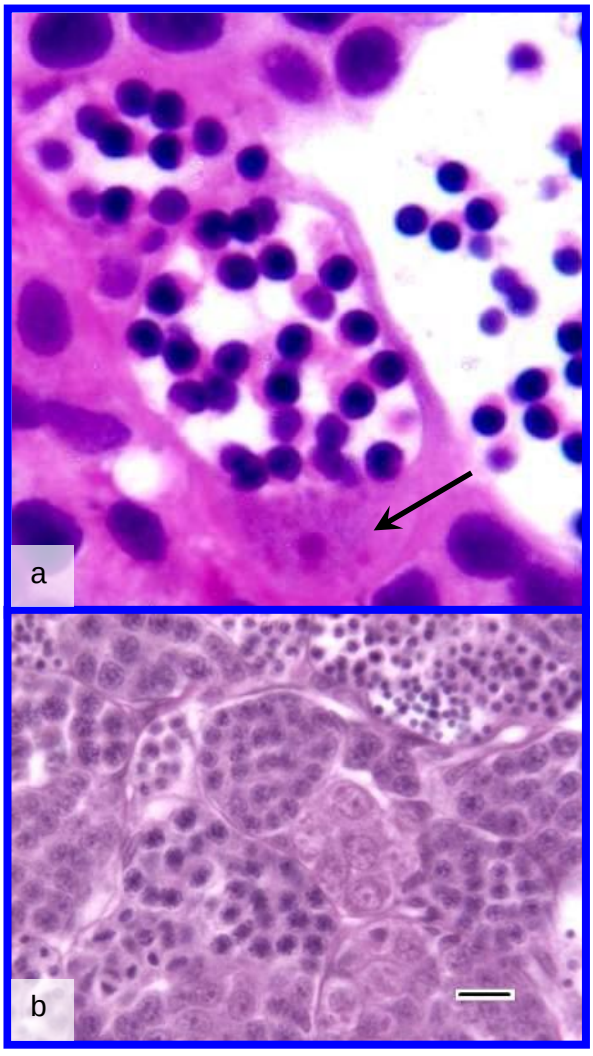


Fig. 10. (a) Spermatocyst in male fathead minnow: A group of dissociated spermatids are surrounded by the cytoplasmic "arms" of a single Sertoli cell (→). This arrangement is usually not as obvious as it is in this photograph. The nucleus of this particular Sertoli cell appears enlarged (hypertrophic; GMA, H&E). (b) Testis from adult male Japanese medaka: Each packet of cells (spermatocyst) represents a cohort of germ cells in approximately the same developmental phase (paraffin, H&E, bar = 10 µm).

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 occur (spermiogenesis; Grier, 1976).

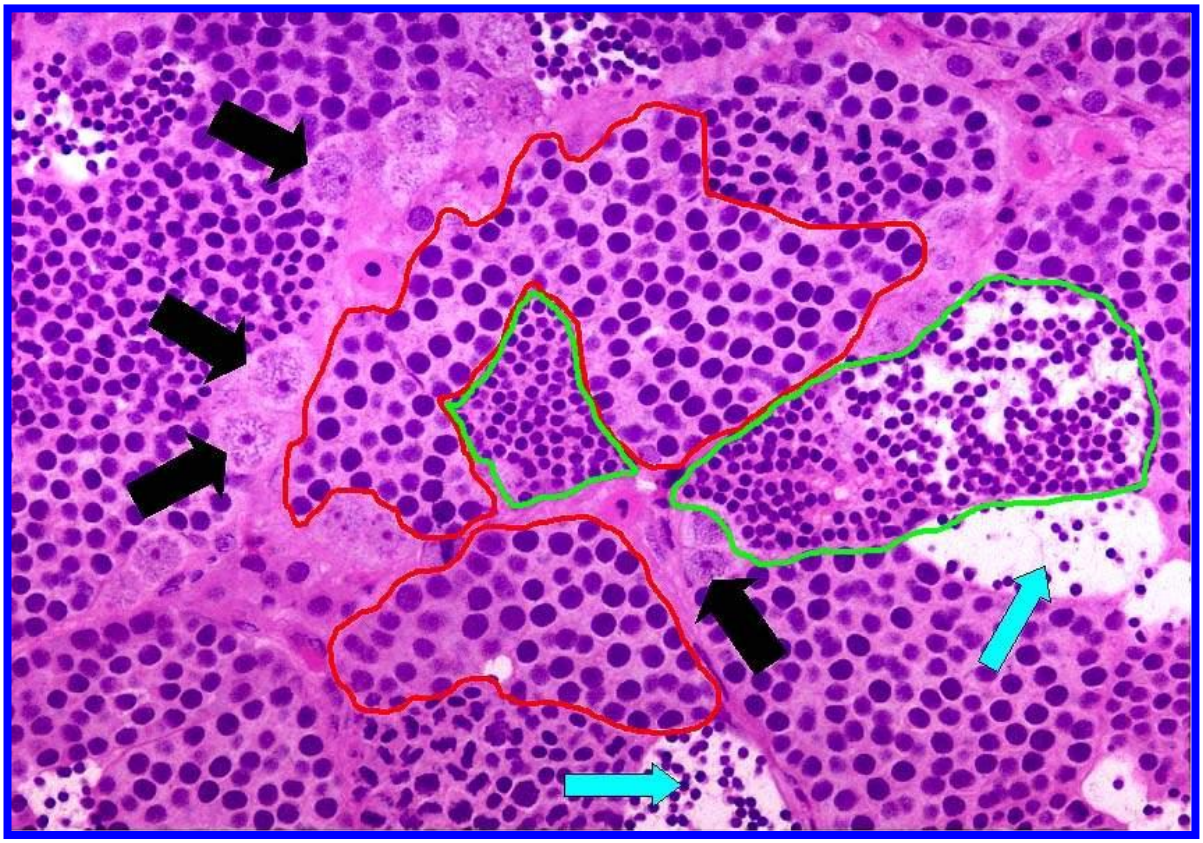


Fig. 11. Spermatocysts in adult male fathead minnow are outlined in red and green contain spermatocytes and spermatids, respectively. Spermatogonia (black arrows) and spermatozoa within tubular lumina (blue arrows) are also indicated (GMA, H&E)

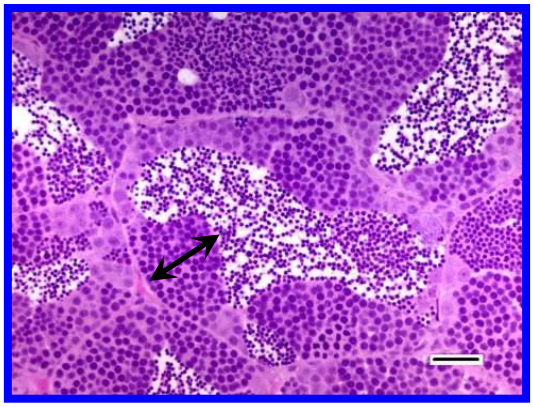


Fig. 12. Male germinal epithelium in the testis of normal fathead minnow: Double arrow indicates width of germinal epithelium, which extends from the interlobular interstitium to the lobular lumen (GMA, H&E, bar = 25 µm).

Male germinal epithelium: 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.

4.1.2 Normal ovarian architecture in female fathead minnow, Japanese medaka and zebrafish ◄

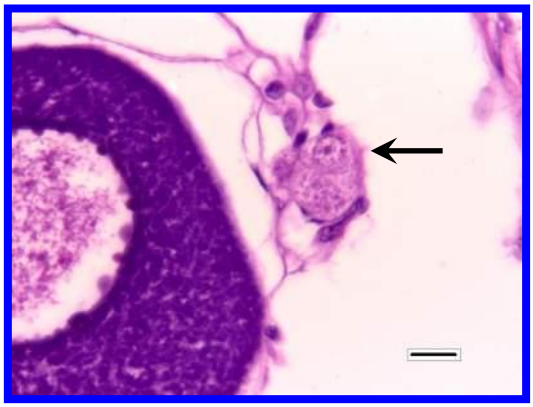


Fig. 13. Oogonia in fathead minnow: A small cluster of oogonia reside within a portion of germinal epithelium; the nucleus of only one oogonium is visible (small arrow). The oogonia are dwarfed by a perinucleolar oocyte (*; paraffin, H&E, bar = 10 µm).

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.

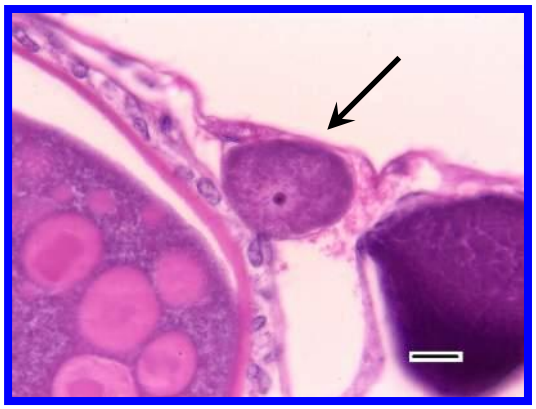
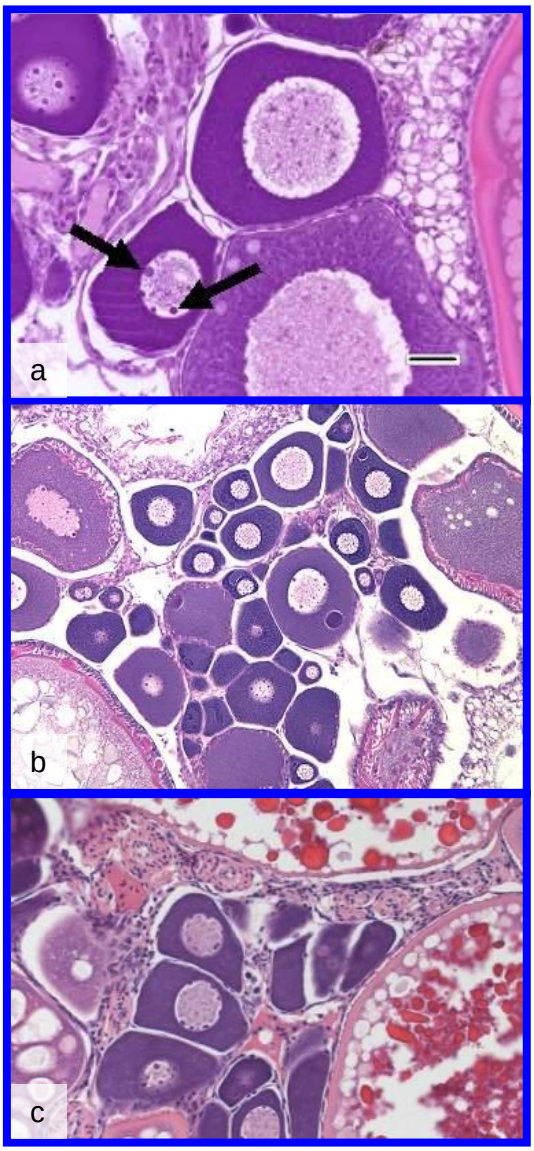


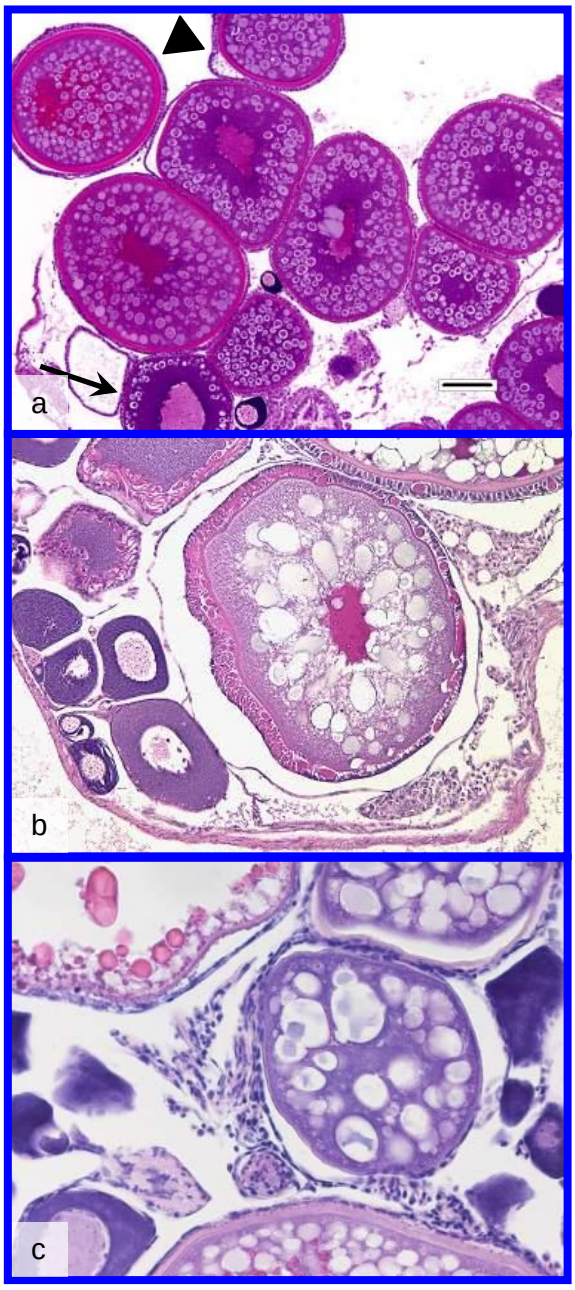
Fig. 14. Chromatin nucleolar oocyte in fathead minnow: A single chromatin nucleolar oocyte protrudes from the germinal epithelium (paraffin, H&E, bar = 10 µm).

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 denser and finely granular.



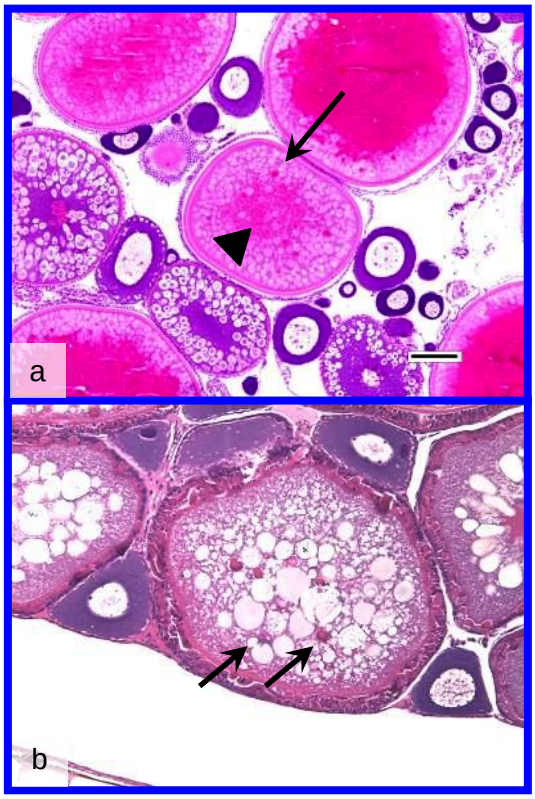
Perinucleolar oocytes: Concomitant with oocyte growth, the nucleus (germinal vesicle) increases in size and multiple nucleoli appear, generally at the periphery of the nucleus. The cytoplasm stains uniformly dark, although late perinucleolar oocytes may have small clear or amphophilic vacuoles in the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

Fig. 15. (a) Perinucleolar oocytes in fathead minnow: Arrows (→) indicate nucleoli at the periphery of the germinal vesicle (paraffin, H&E, bar = 10 µm). (b) Numerous perinucleolar oocytes in a Japanese medaka ovary appear virtually identical to perinucleolar oocytes of fathead minnow (cf. Fig. a; paraffin, H&E). (c) Perinucleolar oocytes in a zebrafish ovary (paraffin, H&E, original mag.: 20 ×).



Cortical alveolar oocytes: Generally larger than perinucleolar oocytes, cortical alveolar oocytes are 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.

Fig. 16. (a) Cortical alveolar oocytes in fathead minnow ovary: The cytoplasm is predominately filled by numerous cortical alveoli, which are amphophilic within this preparation. Evident are oocytes in transition from the perinucleolar to cortical alveolar phase (→), and from the cortical alveolar to early vitellogenic phase (►; paraffin, H&E, bar = 100 µm). (b) Japanese medaka ovary with a single large cortical alveolar oocyte adjacent to several smaller perinucleolar oocytes. Compared to fathead minnow, the cortical alveoli are fewer, larger, and are relatively clear in this preparation (paraffin, H&E). (c) Cortical alveolar oocyte in a zebrafish ovary (paraffin, H&E, original mag. 40 ×).



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.

Fig. 17. Early vitellogenic oocytes in fathead minnow ovary: Numerous fine pale pink granules (►) and a few larger dark red granules (→) 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). (b) A few eosinophilic vitellogenic granules (arrow) are present in the ooplasm of this oocyte in a Japanese medaka ovary (paraffin, H&E).

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.

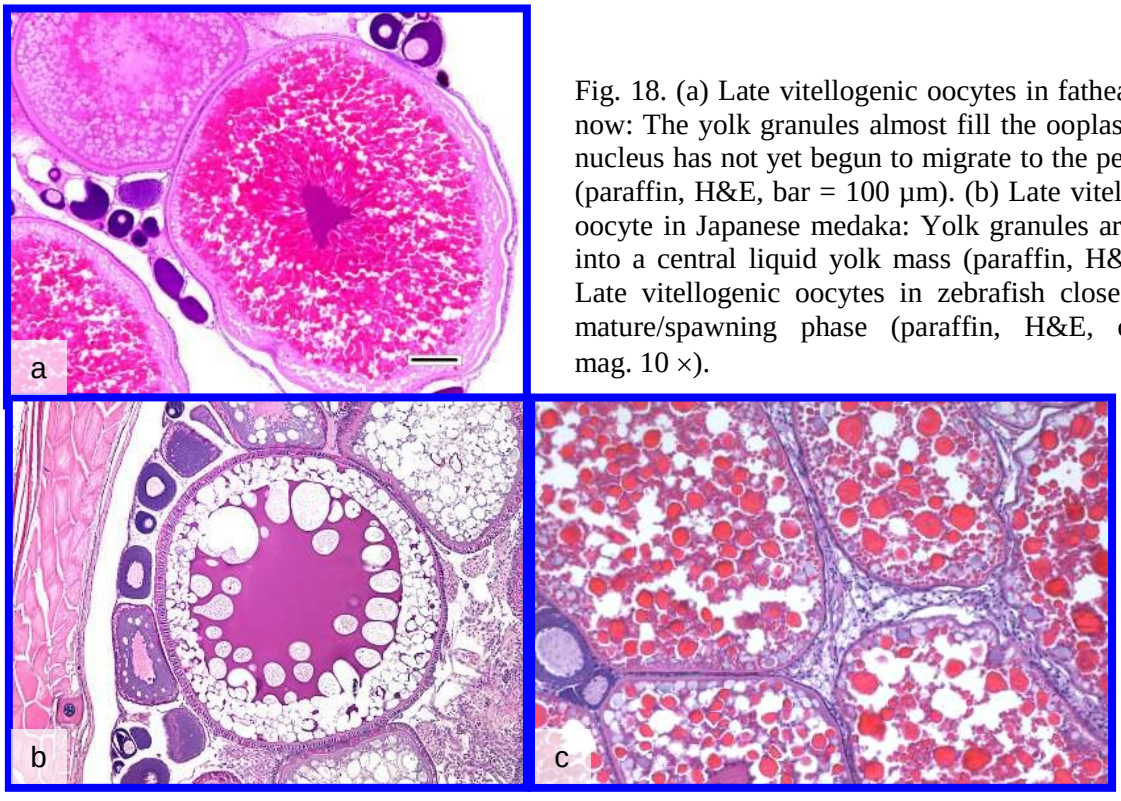
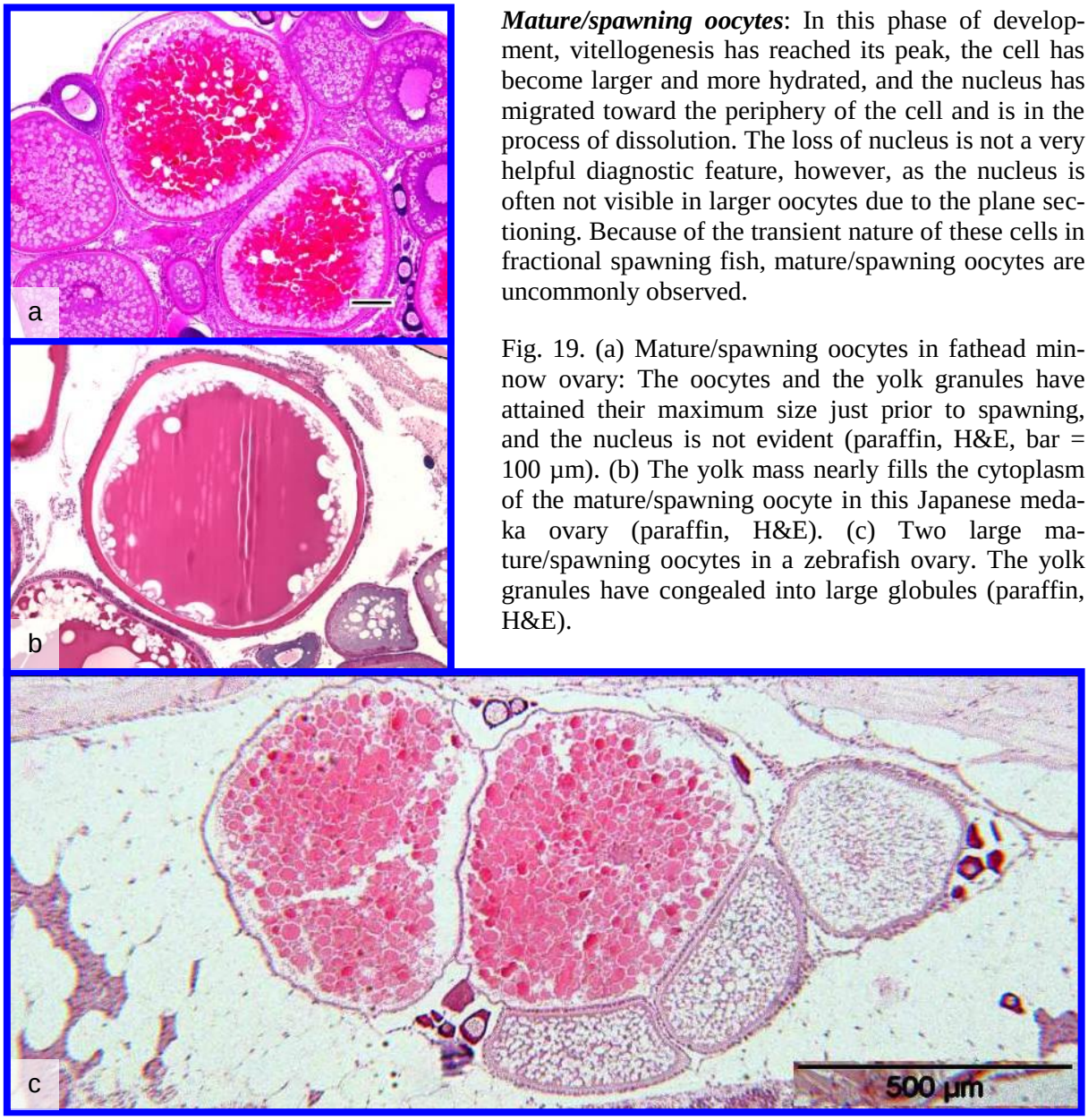


Fig. 18. (a) Late vitellogenic oocytes in fathead minnow: The yolk granules almost fill the ooplasm. The nucleus has not yet begun to migrate to the periphery (paraffin, H&E, bar = 100 µm). (b) Late vitellogenic oocyte in Japanese medaka: Yolk granules are fused into a central liquid yolk mass (paraffin, H&E). (c) Late vitellogenic oocytes in zebrafish close to the mature/spawning phase (paraffin, H&E, original mag. 10 ×).



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.

Fig. 19. (a) Mature/spawning oocytes in fathead minnow 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). (b) The yolk mass nearly fills the cytoplasm of the mature/spawning oocyte in this Japanese medaka ovary (paraffin, H&E). (c) Two large mature/spawning oocytes in a zebrafish ovary. The yolk granules have congealed into large globules (paraffin, H&E).

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 histological 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 (see also: *oocyte atresia*).

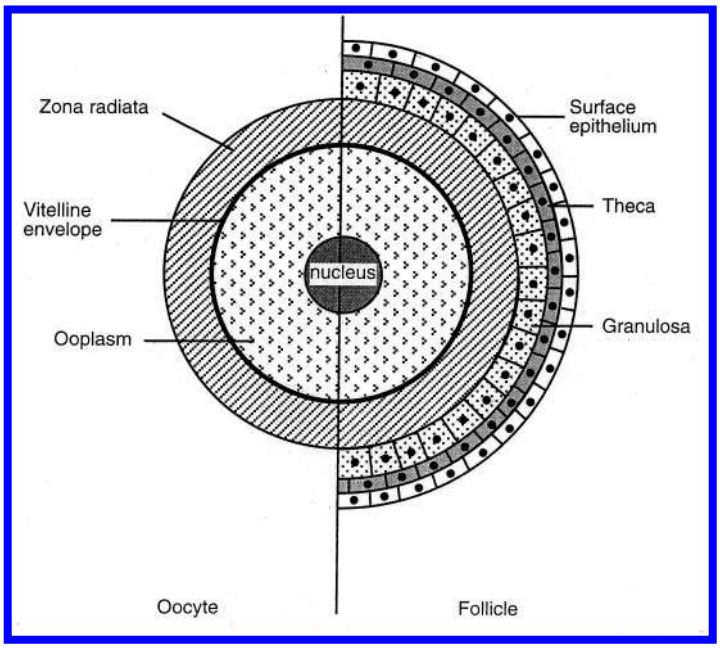


Fig. 20. Diagram of an ovarian follicle (from Tyler and Sumpter, 1996).

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.

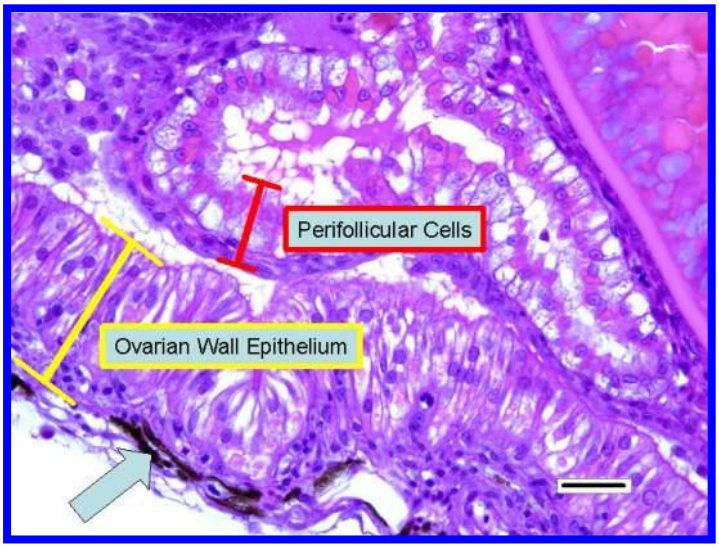


Fig. 21. Perifollicular cells in adult fathead minnow ovary, as compared to the cells of the ovarian wall epithelium, which contain dark brown (melanin) pigment (→) and are comprised of ciliated columnar cells (paraffin, H&E, bar = 25 µm).

Fig. 22. The chorions (►) of two oocytes in an adult fathead minnow ovary: A smaller arrow (→) denotes a postovulatory follicle (paraffin, H&E, bar = 25 µm).

Chorion: The chorion usually stains 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.

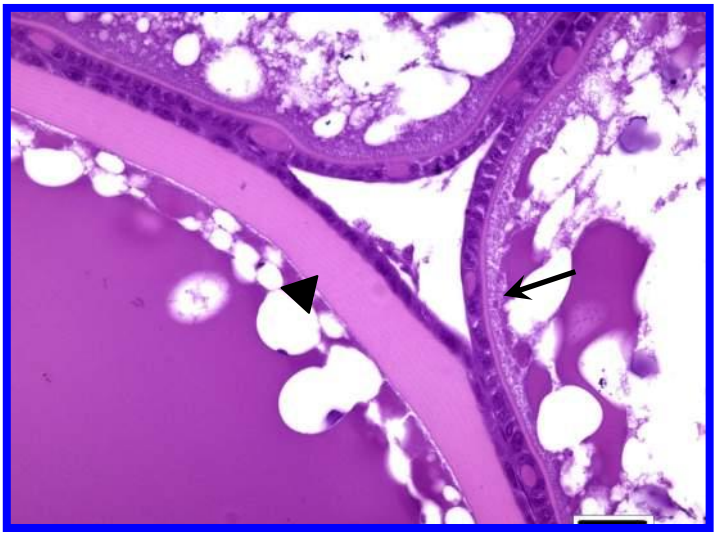


Fig. 23. Chorion in the Japanese medaka ovary: Note the vast difference in thickness between the chorion of a cortical alveolar oocyte (→) and the chorion of a mature vitellogenic oocyte (►; paraffin, H&E, bar = 25 µm.

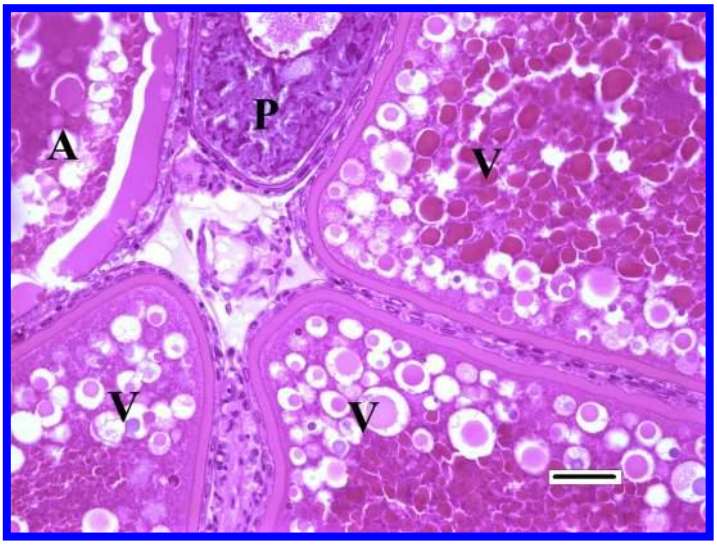
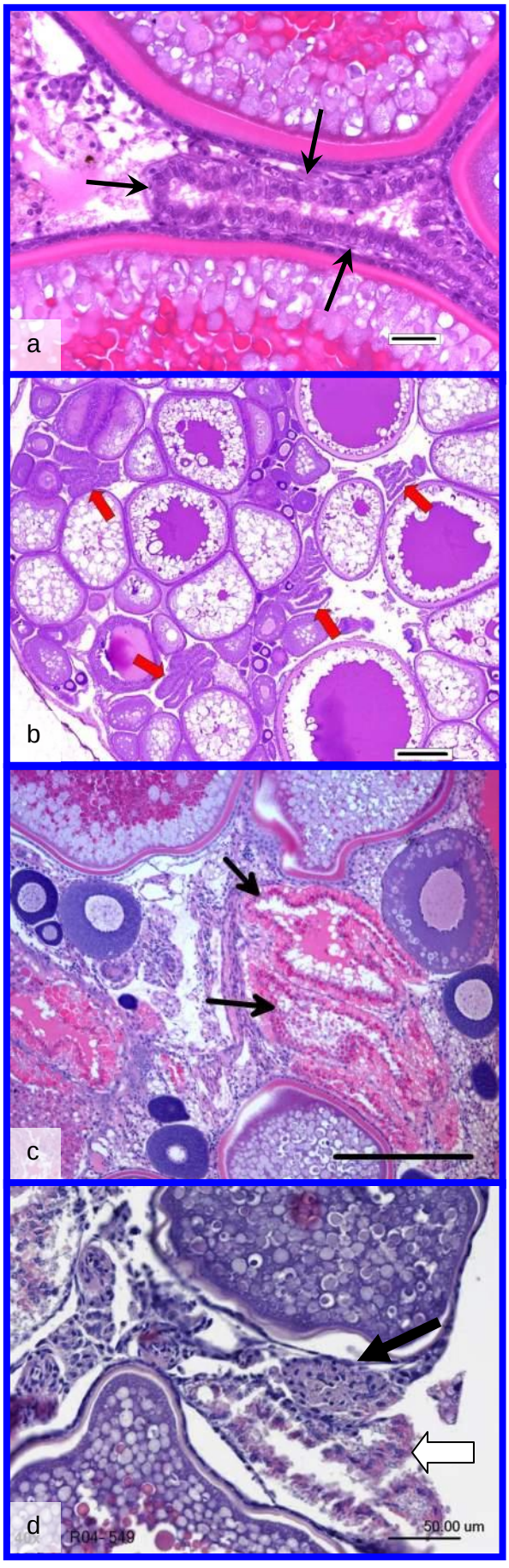


Fig. 24. Chorion in the zebrafish ovary: Three vitellogenic oocytes (V) have smooth, intact chorions. In contrast, this "membrane" is just beginning to form around a perinucleolar oocyte (P), and the chorion surrounding an atretic oocyte (A) has become thickened and fragmented (paraffin, H&E, bar = 25 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.

Fig. 25. Post-ovulatory follicle in an adult fathead minnow ovary: Situated between three oocyte-containing follicles is a collapsed follicle, which does not contain oocyte remnants (→; paraffin, H&E, bar = 25 µm). (b) Post-ovulatory follicle in an adult Japanese medaka ovary: A number of post-ovulatory follicles (→), indicating recent spawning, are evident in this ovary (paraffin, H&E, bar = 250 µm). (c) Ovary from an adult female fathead minnow. These oocytes (→) are actually *not post-ovulatory follicles*. The presence of yolk material indicates that they are late phase atretic follicles. (d) Ovary from an adult female zebrafish: The solid arrow indicates a post-ovulatory follicle, whereas the open arrow points to a late phase atretic follicle (paraffin, H&E).