4.2 Primary diagnoses in fathead minnow, Japanese medaka and zebrafish following exposure to endocrine disruptors

4.2.1 Primary diagnoses in male fathead minnow, Japanese medaka and zebrafish following exposure to endocrine disruptors

*Increased proportion of spermatogonia in male fathead minnow*

*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.

A proportional increase in spermatogonia was observed consistently in the testes of fathead minnow and zebrafish (and less dependably in Japanese medaka) as an exposure response to, e.g., the estrogenic compound 4-tert-pentylphenol. Other experiments have also linked exposure to estrogens, or substances with estrogenic activity, to increases in spermatogonia (Condeça and Canario, 1999; Sohoni et al., 2001; Wester et al., 2003). Proportional increases in spermatogonia are often associated with increases in the thickness of the testicular germinal epithelium, but this is not always the case. Testes exhibiting Grade 4 severity for this change may be difficult, if not impossible, to distinguish from juvenile testes, and in truth, the distinction may not be important. Other potential ruleouts for gonads that contain large numbers of immature gonial cells would include germ cell neoplasms such as seminomas and dysgerminomas.

Fig. 26. Increased cells (spermatogonia) in fathead minnow testis: (a) Testis from adult male FHM negative control. (b) Spermatogonia dominate the germinal epithelium in this testis from adult male FHM exposed to 10 nM 17β-estradiol for 10 days. Other diagnoses for this section include “decreased cells, spermatocytes” and “decreased cells, spermatids” (GMA, H&E, bars = 25 µm).
Fig. 27. Testis from an adult male fathead minnow: There is a minimal (Grade 1) increase in the proportion of spermatogonia (→; GMA, H&E).

Fig. 28. Testis from an adult male fathead minnow: There is a slight/mild (Grade 2) increase in the proportion of spermatogonia throughout the germinal epithelium (→; GMA, H&E).

Fig. 29. Testis from an adult male fathead minnow: There is a moderate (Grade 3) increase in the proportion of spermatogonia (→; GMA, H&E).

Fig. 30. Testis from an adult male fathead minnow: There is a severe (Grade 4) increase in the proportion of spermatogonia (GMA, H&E).
Fig. 31. Spermatogonia, increased. (a) Testis of an adult control fathead minnow. (b) Testis of an adult male fathead minnow exposed to 1000 µg/L flutamide. In this minimally affected testis, spermatogonia dominate the germinal epithelium, but many spermatocytes are evident also. (c) Testis of an adult male fathead minnow exposed to 100 ng/L estradiol. Multiple layers of spermatogonia surround seminiferous tubules in this mildly affected testis. (d) Moderately affected testis. (e) Severely affected testis: Due to their longevity, spermatozoa may be abundant despite the lack of intermediate germ cell phases (i.e., spermatocytes and spermatids; paraffin, H&E, bars = 25 µm)
Fig. 32. Spermatogonia, increased: A proportional increase in spermatogonia has been observed in the testes of male zebrafish exposed to 17β-estradiol. (a) Testis from adult male zebrafish (negative control). (b) Testis from adult male zebrafish exposed to 17β-estradiol for 3 weeks. Again, spermatogonia are present in greater numbers compared to control fish (paraffin, H&E).
**Presence of testis-ova in male fish**

The presence of testis-ova defines a state in which fully formed male and female gonad tissues are present in the same individual. The phrase “fully formed” indicates that (1) the male and female gonadal tissues are in discrete compartments; (2) the organizational architecture of the gonads is maintained; and/or (3) there is visible evidence of supportive structures (e.g., tunica albuginea, ducts) in addition to germinal cells. The testicular and ovarian tissues may be present within confines of the same gonad (ovotestis) as defined by the tunica albuginea, or they may exist as completely separate organs (e.g., left and right, rostral and caudal).

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**Fig. 33.** Hermaphroditism in young adult fathead minnow: This very rare finding occurred spontaneously in an untreated fish. The small arrows (→) indicate perinucleolar stage oocytes. This case should not be diagnosed merely as “testicular oocytes”, because this gonad has additional ovarian features, such as the presence of an ovarian cavity (OV) that is lined by an ovarian wall-type epithelium (open arrow). As further evidence for hermaphroditism, the contralateral gonad (not shown) was a normal-appearing ovary (whole-body cross section, paraffin, H&E, bar = 500 µm).

**Fig. 34.** Hermaphroditism in young adult fathead minnow: example of spontaneous testicular oocytes. Whether spontaneous or induced, testicular oocytes are a rare finding in fathead minnow (paraffin, H&E, bar = 100 µm).
Fig. 35. Testis-ova in adult male Japanese medaka: Testicular oocytes (paraffin, H&E). Photomicrographs from four different negative control fish from several studies. The large arrows indicate perinucleolar oocytes, whereas the small arrows are chromatin nucleolar oocytes (paraffin, H&E).

Fig. 36. Testicular oocytes in adult male Japanese medaka: Red arrows indicate oocytes in the testis of this fish treated with 17ß-estradiol (paraffin, H&E, bar = 30 μm).
Fig. 37. Examples of testis-ova severity grading in male Japanese medaka: It is important to note that the severity grade can vary dramatically from section to section within the same testis; therefore, the value of severity grading becomes questionable when it is based on the evaluation of only one or two sections per fish. (a) A minimally affected testis with a single perinucleolar oocyte (→) in one section. (b) Mildly affected fish with a number of scattered, individual perinucleolar oocytes (→). (c) In this moderately affected testis the oocytes occur within large lobular nests that also contain oogonia. (d) Small remnants of spermatogenic tissue and the bilobed configuration of this gonad are evidence that this is a testis rather than an ovary. Another potential ruleout for this finding would be dysgerminoma. This is an 8 weeks old genetically male fish that was exposed to 100 ng/L 17ß-estradiol for approximately eight weeks (all images are paraffin, H&E, bar = 100 µm).
Fig. 38. Hermaphroditism in zebrafish: In these sections from two different adult fish, each section contains fully-formed male and female gonad tissues. According to a convention established herein, (see Gender), the fish in image “a” would be designated as a male, whereas the fish in “b” would be designated as a female. (whole-body cross-section, H&E; bar = 500 µm).

Fig. 39. Testicular oocytes in adult male zebrafish: Red arrows indicate oocytes. Note that the fish on the right is a control animal. The testis in the lower image would be considered mild (Grade 2) severity. (paraffin, H&E).
**Increased testicular degeneration in male fathead minnow**

**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.

Fig. 40. Testicular degeneration in adult male fathead minnow: (a) Multiple clusters of apoptotic germ cells (→) and vacuolated germ cells (red →) within the germinal epithelium. (b) Another mildly-affected testis with three large germ cell syncytia (→; GMA, H&E, bars: 25 µm).
Fig. 41. Increased testicular degeneration in adult male fathead minnow: There are low numbers of germ cell syncytia (→) in this minimally affected testis. This degree of degeneration is occasionally seen in control males; therefore, this diagnosis should be made relative to the degree of degeneration evident among the majority of the concurrent controls (paraffin, H&E, bar = 100 µm).

Fig. 42. Increased testicular degeneration in adult male Japanese medaka: (a) In most studies, an occasional degenerating cell, like this multinucleated germ cell (arrow), would be below the threshold for “increased” and therefore would be considered non-remarkable. (b) There are several clusters of apoptotic cells (arrows) in a relatively small area of this mildly affected testis (paraffin, H&E, bar = 25 µm).
**Interstitial cell (Leydig cell) hyperplasia/hypertrophy in male fish**

Fig. 43. Testis from an adult male fathead minnow: Interstitial areas contain small aggregates of interstitial (Leydig) cells (→). Most interstitial cells have wispy, pale cytoplasm (GMA, H&E).

Fig. 44. Testis from an adult male fathead minnow: Interstitial cell aggregates (→) in the testis of this fish are larger than in control fish, and the cytoplasm of these cells is slightly denser. This was diagnosed as “Increased Cells, Interstitial Cells”, Grade 1 (minimal) severity (GMA, H&E).

Fig. 45. Testis from an adult male fathead minnow: Interstitial cell aggregates (→) 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 (GMA, H&E).
Fig. 46. Interstitial (Leydig) cell hyperplasia in adult male fathead minnow: (a) Unaffected testis from control fathead minnow. Arrowheads (►) indicate occasional interstitial cells as single cells or small clusters. (b) Testis with a minimal increase in the number of interstitial cells. Interstitial cells are present in small to medium-sized aggregates (►). Note that the cell nuclei are small and condensed, and the cytoplasm of these cells is clear. (c) Testis with mild interstitial cell (IC) hyperplasia. (d) Testis with moderate interstitial cell hyperplasia/hypertrophy (►) associated with ketoconazole exposure (unpublished data). There are relatively large aggregates of interstitial cells, and these cells have rounded nuclei and dense cytoplasm; paraffin, H&E, bar = 25 µm).
4.2.2 Primary diagnoses in female fathead minnow, Japanese medaka and zebrafish following exposure to endocrine disruptors

**Increased oocyte atresia**

**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. 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 appear to have been interrupted in either the late vitellogenic oocyte phase or the mature/spawning phase of development.

**Fig. 48.** Ovary from an adult female fathead minnow: Oocyte atresia, mature oocytes – Note clumping and pore formation in the vitelline envelope (chorion) of the early atretic oocyte (large →), and the vacuolar hypertrophy of its surrounding granulosa cells (small →). Compare these with granulosa cells that surround a non-atretic late vitellogenic oocyte (►). In FHM, granulosa cells of atretic oocytes often appear to contain phagocytized material, whereas the granulosa cells of non-atretic oocytes do not (paraffin, H&E).

**Fig. 47.** Interstitial (Leydig) cell hyperplasia in control adult male Japanese medaka (a) showing scattered small clusters of interstitial cells (→) located between tubules, as well as in Japanese medaka exposed to 100 µg/L fadrozole (b), where interstitial cell aggregates are slightly larger and more numerous (Grade 1; paraffin, H&E, bar = 25 µm).
Fig. 49. Ovary from a normal (control) adult female fathead minnow (paraffin, H&E).

Fig. 50. Ovary from an adult female fathead minnow: Numerous atretic oocytes are evident – Grade 3 (→; paraffin, H&E).

Fig. 51. Stage 4 ovary from an adult female fathead minnow as characterized by severe oocyte atresia. Asterisks (*) indicate the relatively few non-atretic oocytes (paraffin, H&E).
Fig. 52. Oocyte atresia in ovaries from adult female fathead minnow: (a) Although atresia is most often observed in vitellogenic oocytes, it can occur at any phase of development, such as in this cortical alveolar oocyte (→). (b) Although not usually diagnosed as atresia, increased apoptotic-type death of early germ cells (→) could be a finding in some studies (paraffin, H&E).

Fig. 53. Moderately atretic zebrafish ovary (paraffin, H&E, original mag. 10 ×).
**Perifollicular cell hyperplasia/hypertrophy**

Exposure to aromatase inhibitors (e.g., fadrozole, prochloraz) has been associated with these perifollicular cell changes in Japanese medaka. A similar effect has also been linked with exposure to the non-aromatizable androgen, trenbolone (unpublished data). This finding is characterized by an increase in the height and number of granulosa cells, which gives this cell layer a “pseudostratified” appearance in extreme cases. A common coexisting change in affected Japanese medaka was decreased yolk formation. Since perifollicular cells (i.e., granulosa cells) are thought to be involved with aromatase production in fish (Devlin and Nagahama, 2002; Nagahama, 1987), it is possible that the increased number and size of these cells is a compensatory mechanism aimed at restoring aromatase to levels required for vitellogenesis. It is important to note that (1) normal perifollicular cells may appear hypertrophic in tangentially-sectioned oocytes, and (2) perifollicular cell changes are best identified by comparisons made with concurrent control fish.

Fig. 54. Perifollicular cell hyperplasia/hypertrophy: (a) Ovary from an untreated adult female Japanese medaka: Perifollicular cells (→) are cuboidal and have small, condensed nuclei. (b) Ovary from female Japanese medaka exposed to 3 µg/L fadrozole illustrating minimal perifollicular cell hyperplasia: Perifollicular cells surrounding some oocytes are columnar. It should be noted that in certain studies the control animals can look like this. (c) Ovary from female Japanese medaka exposed to 100 µg/L fadrozole: Ovaries graded as mildly affected, such as this, should be distinctly different from concurrent controls. (d) Ovary from female Japanese medaka exposed to 300 µg/L prochloraz: Moderately hyperplastic perifollicular cells (→) have a pseudostratified columnar appearance, and relatively enlarged oval nuclei (paraffin, H&E, bars = 25 µm).
Decreased yolk formation

Fig. 55. Stage 3 ovary from a normal (control) adult female fathead minnow: A single atretic ovary is evident (→; paraffin, H&E).

Fig. 56. Ovary from an adult female fathead minnow: Decreased yolk formation is characterized by the presence of oocytes in which yolk material is not present despite their relatively large size (large →). Note that oocytes are affected to varying degrees. Some affected oocytes have extremely fine vitellogenic granules (small →), and this is interpreted as ineffective yolk formation and deposition (paraffin, H&E, bar = 250 µm).

Fig. 57. Ovary from an adult female zebrafish: A striking example (i.e., severity Grade 4) of decreased yolk formation in an adult female zebrafish exposed to fadrozole (paraffin, H&E, bar = 500 µm).
Fig. 58. Decreased yolk formation in the ovaries of Japanese medaka: (a) Ovary from a control medaka. (b) Ovary from a female medaka exposed to 150 ng/L trenbolone (non-aromatizable androgen) shows minimal decreased yolk formation; many vitellogenic oocytes have pale, watery yolk (→). (c) Ovary from a female medaka exposed to 500 ng/L trenbolone: mildly decreased yolk formation as indicated by inadequate amounts of yolk that is excessively vacuolated. (d) Ovary from female medaka exposed to 500 ng/L trenbolone: moderately decreased yolk formation characterized by the presence of scanty yolk in relatively few oocytes. (e) Ovary from female medaka exposed to 5 µg/L trenbolone: yolk granules are absent, cortical alveoli (A) are disrupted, and there is dramatic perifollicular cell hypertrophy/hyperplasia. (f) Severely-affected ovary from a female medaka exposed to 300 µg/L prochloraz (aromatase inhibitor). All images: paraffin, H&E, bar = 500 µm except for (f): bar = 250 µm.