CHAPTER 1

Introduction

Histopathology is a tool employed routinely in disease diagnosis for the detection and assessment of xenobiotically mediated adverse effects in laboratory animals [1–19]. Recently, histopathological analysis has become a method applied more frequently for the assessment of potential effects of endocrine-active compounds (capable of endocrine modulation, often referred to as EDCs (*endocrine-disrupting chemicals* [20]) in fish. EDCs have been defined by the WHO-IPCS [21] as follows: "An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations" and "A potential endocrine disruptor is an exogenous substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations."

EDCs can have an effect at both the cellular and whole-organ levels and have been demonstrated to induce histopathological changes within the gonads of exposed fish within the confines of routine aquaculture procedures or in an environmental risk assessment (ecotoxicology) setting (see Figure 1.1) [20,22–26]. Chemicals with endocrine-active properties and proven potential for adverse effects are considered chemicals of concern and are subject to authorization under recent European legislation.

Concurrent with the definition of EDCs, effects of EDCs on the level of the cell or the organ could then be derived. Exposure of small laboratory fish to estrogens [e.g., 17β-estradiol (E_2) and 17α-ethinylestradiol (EE_2)] has been reported to induce gonadal changes, such as atresia of vitellogenic oocytes [27–33] and inhibition of spermatogenesis (via various modes of action) [28,33–45]. Additional effects have been reported, such as pronounced interstitial fibrosis [34,46] and induced interstitial accumulation of protein, in some cases identified as vitellogenin [47,48], or Sertoli cell hypertrophy [49]. However, systematic evaluation of the effects of hormonally active compounds, or any other xenobiotic for that matter, on the gonads of small laboratory fish based on peer-reviewed publications has been hampered to some extent by procedural restrictions, interpretational problems (i.e., no clear nomenclature of observed structures and effects), and graphical issues (figures inadequate for proper assessment of the effect), thus limiting the use and extrapolation potential of the data

Histological Analysis of Endocrine-Disruptive Effects in Small Laboratory Fish
By Daniel R. Dietrich and Heiko O. Krieger

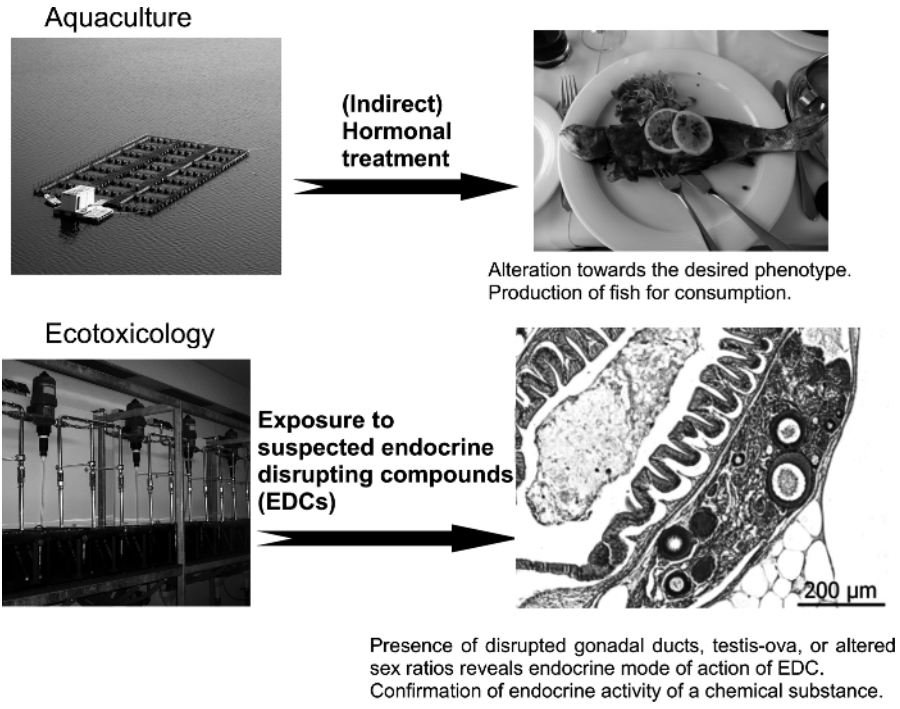

FIGURE 1.1 Control of sexual differentiation in fish: various applications. (Picture upper left courtesy of Kevin Frost, upper right courtesy of Susanne Beeck, pixelio).

provided. High stringency and standardization in the evaluation process of effects are prerequisites if the findings from different studies with endocrine-active xenobiotics in small fish are to be compared reliably [22].

Comprehensive, reproducible, and objective histopathological analysis will be greatly facilitated if the following basic standards of pathological reporting are included, while by the same token, lack of the following factors will restrict the quality and thus the value of the histopathological analysis:

1. A standardized and optimized histological gonad preparation method (fixation, embedding, sectioning, and staining techniques), which would allow clear distinction of individual gonadal cell types and interpretation of changes.
2. A standardized gonadal staging and evaluation system and a clear nomenclature system for the various cell types, which would allow a qualitative and/or quantitative assessment of the effects of endocrine-modulating compounds.
3. A standardized methodology to allow quantification of the occurrence and frequency of individual gonadal cell types for effect assessment.
4. Agreement on reporting pathological findings using representative photographs with adequate resolution and magnification and, wherever possible, the inclusion of a size bar.

In the Appendix we provide standardized protocols and procedures as well as guidance for possible quantification and reporting approaches for points 1 to 4 above, and in the main body of the book we focus on an understanding of the processes visualized via histopathological changes and the interpretation of these findings. Indeed, a thorough assessment of histopathological tissue changes can provide key diagnostic information and will form the basis for an understanding of the underlying mechanism(s) of endocrine active substances [50,51]. A more detailed morphological and mechanistic understanding of effects observed will allow enhanced reproducibility and comparability of the studies conducted. However, the description of chemically induced changes in gonadal histology can be of profound value only if these changes can be put into context with and interpreted in light of the normal reproductive biology of the particular fish. Consequently, a prerequisite for the histopathological analysis of gonadal changes in fish is an understanding of the reproductive biology and normal gonadal histology of the fish such that an induced (e.g., chemical-mediated) effect can be distinguished from naturally occurring gonadal changes in fish during development, spawning, gonadal regression or recrudescence, or during aging. The distinction of such effects will then also allow better prediction of the ramifications (type and magnitude of consequences) of the chemically mediated gonadal effects for fish reproductive capacity and thus probably also for the fish population. It must be noted, however, that beyond the obvious—the complete lack of gonadal development or the destruction of the reproductive tissue and thus reproductive capacity *in toto*—no agreement has been found to date as to what small or moderate gonadal changes, whether the occurrence of intersex (testis–ova/ovo–testis) or outright pathological changes, in an individual's gonad will translate into at the level of the population.

Our goal in this book is to provide a detailed understanding of the normal gonad physiology, development, anatomy, and histopathology as well as of changes induced by xenobiotics (e.g., endocrine-active substances) primarily in three small fish species: fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*). These three species are employed predominantly in routine experiments used for environmental risk assessment, including hazard and consequently, risk assessment of chemicals (e.g., endocrine-active compounds) [52]. However, wherever possible and considered of additional value for understanding differences or exceptions to the general concept developed with the three species chosen, references to other species, notably carp, trout, bream, stickleback, guppy, platyfish, eelpout, and sheepshead minnow are included.

REFERENCES

1. Wester, P.W., J.H. Canton, and A. Bisschop, Histopathological study of *Poecilia reticulata* (guppy) after long-term β-hexachlorocyclohexane exposure. *Aquatic Toxicology*, 1985, **6**(4):271–296.
2. Wester, P.W. and J.H. Canton, Histopathological study of *Oryzias latipes* (medaka) after long-term β-hexachlorocyclohexane exposure. *Aquatic Toxicology*, 1986, **9** (1):21–45.

3. Wester, P.W. and J.H. Canton, Histopathological study of *Poecilia reticulata* (guppy) after long-term exposure to bis(tri-*n*-butyltin)oxide (TBTO) and di-*n*-butyltindichloride (DBTC). *Aquatic Toxicology*, 1987, **10**(2–3):143–165.
4. Wester, P.W., Histopathological study of *Poecilia reticulata* (guppy) and *Oryzias latipes* (medaka) following methyl bromide and sodium bromide exposure, in *Pathology*. University of Utrecht, Utrecht, The Netherlands, 1988, p. 208.
5. Wester, P.W., et al., The toxicity of bis(tri-*n*-butyltin)oxide (TBTO) and di-*n*-butyltin-dichloride (DBTC) in the small fish species *Oryzias latipes* (medaka) and *Poecilia reticulata* (guppy). *Aquatic Toxicology*, 1990, **16**(1):53–72.
6. Wester, P.W., J.H. Canton, and J.A.M.A. Dormans, Pathological effects in freshwater fish *Poecilia reticulata* (guppy) and *Oryzias latipes* (medaka) following methyl bromide and sodium bromide exposure. *Aquatic Toxicology*, 1988, **12**(4):323–344.
7. Wester, P.W. and H.H. Canton, Histopathological effects in *Poecilia reticulata* (guppy) exposed to methyl mercury chloride. *Toxicologic Pathology*, 1992, **20**(1):81–92.
8. Wester, P.W. and J.G. Vos, Toxicological pathology in laboratory fish: An evaluation with two species and various environmental contaminants. *Ecotoxicology*, 1994, **2**(1):21–44.
9. Wester, P.W., Histopathological effects of environmental pollutants β-HCH and methyl mercury on reproductive organs in freshwater fish. *Comparative Biochemistry and Physiology, Part C: Toxicology and Pharmacology*, 1991, **100C**(1–2):237–239.
10. Wester, P.W. and J.H. Canton, The usefulness of histopathology in aquatic toxicity studies. *Comparative Biochemistry and Physiology, Part C: Toxicology and Pharmacology*, 1991, **100**(1–2):115–117.
11. Fenske, M. and H. Segner, Aromatase modulation alters gonadal differentiation in developing zebrafish (*Danio rerio*). *Aquatic Toxicology*, 2004, **67**(2):105–126.
12. Hoeger, B., et al., Sex and low-level sampling stress modify the impacts of sewage effluent on the rainbow trout (*Oncorhynchus mykiss*) immune system. *Aquatic Toxicology*, 2005, **73**(1):79–90.
13. Hoeger, B., et al., Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (*Salmo trutta* f. *fario*). *Aquatic Toxicology*, 2005, **75**(1):53–64.
14. Hoeger, B., et al., Effects of treated sewage effluent on immune function in rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology*, 2004, **70**(4):345.
15. Schwaiger, J., et al., Toxic effects of the non-steroidal anti-inflammatory drug diclofenac: Part I. Histopathological alterations and bioaccumulation in rainbow trout. *Aquatic Toxicology*, 2004, **68**(2):141.
16. Schwaiger, J., et al., How estrogenic is nonylphenol? A transgenerational study using rainbow trout (*Oncorhynchus mykiss*) as a test organism. *Aquatic Toxicology*, 2002, **59**(3–4):177–189.
17. Schwaiger, J., et al., Chronic toxicity of nonylphenol and ethinylestradiol: Haematological and histopathological effects in juvenile common carp (*Cyprinus carpio*). *Aquatic Toxicology*, 2000, **51**(1):69.
18. Triebskorn, R., et al., Toxic effects of the non-steroidal anti-inflammatory drug diclofenac: Part II. Cytological effects in liver, kidney, gills and intestine of rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology*, 2004, **68**(2):151.
19. van der Ven, K., et al., Development and application of a brain-specific cDNA microarray for effect evaluation of neuro-active pharmaceuticals in zebrafish (*Danio rerio*).

Comparative Biochemistry and Physiology, Part B: Biochemistry and Molecular Biology, 2005, **141**(4):408.

20. Vos, J.G., et al., Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. *Critical Reviews in Toxicology*, 2000, **30**(1): 71–133.
21. Damstra, T., et al., *Global Assessment of the State-of-the-Science of Endocrine Disruptors.* International Programme on Chemical Safety. WHO-IPCS, Geneva, Switzerland, 2002, p. 180.
22. Brown, R.P., et al., A critical review of the scientific literature on potential endocrine-mediated effects in fish and wildlife. *Ecotoxicology and Environmental Safety*, 2001, **49**(1):17–25.
23. McLachlan, J.A., Environmental signaling: What embryos and evolution teach us about endocrine disrupting chemicals. *Endocrine Reviews*, 2001, **22**:319–341.
24. Jobling, S., et al., Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. *Biology of Reproduction*, 2002, **66**:272–281.
25. Jobling, S., et al., Widespread sexual disruption in wild fish. *Environmental Science and Technology*, 1998, **32**:2498–2506.
26. van Aerle, R., et al., Sexual disruption in a second species of wild cyprinid fish (the gudgeon. *Gobio gobio)* in United Kingdom freshwaters. *Environmental Toxicology and Chemistry*, 2001, **20**(12):2841–2847.
27. Nagahama, Y., The functional morphology of teleost gonads, in *Fish Physiology,* W.S. Hoar, D.J. Randall, and E.M. Donaldson, Editors. Academic Press, San Diego, CA, 1983, pp. 223–275.
28. Papoulias, D.M., D.B. Noltie, and D.E. Tillitt, An in vivo model fish system to test chemical effects on sexual differentiation and development: Exposure to ethinyl estradiol. *Aquatic Toxicology*, 1999, **48**(1):37–50.
29. Gray, M.A., K.L. Teather, and C.D. Metcalfe, Reproductive success and behaviour of Japanese medaka (*Oryzias latipes*) exposed to 4-*tert*-octylphenol. *Environmental Toxicology and Chemistry*, 1999, **18**(11):2587–2594.
30. Olsson, P.-E., et al., Effects of maternal exposure to estrogen and PCB on different life stages of zebrafish (*Danio rerio*). *Ambio*, 1999, **28**(1):100–106.
31. Ankley, G.T., et al., Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry*, 2001, **20**(6):1276–1290.
32. Miles-Richardson, S.R., et al., Effects of waterborne exposure to 4-nonylphenol and nonylphenol ethoxylate on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). *Environmental Research, Section A*, 1999, **80**(2):S122–S137.
33. van den Belt, K., R.Verheyen, and H. Witters, Reproductive effects of ethynylestradiol and 4*t*-octylphenol on the zebrafish (*Danio rerio*). *Archives of Environmental Contamination and Toxicology*, 2001, **41**(4):458–467.
34. Kang, I.J., et al., Effect of 17β-estradiol on the reproduction of Japanese medaka (*Oryzias latipes*). *Chemosphere*, 2002, **47**(1):71–80.
35. Seki, M., et al., Effect of ethinylestradiol on the reproduction and induction of vitellogenin and testis–ova in medaka (*Oryzias latipes*). *Environmental Toxicology and Chemistry*, 2002, **21**(8):1692–1698.

36. van den Belt, K., et al., Effects of ethynylestradiol on the reproductive physiology in zebrafish (*Danio rerio*): Time dependency and reversibility. *Environmental Toxicology and Chemistry*, 2002, **21**(4):767–775.

37. Zillioux, E.J., et al., The sheepshead minnow as an in vivo model for endocrine disruption in marine teleosts: A partial life-cycle test with 17α-ethynylestradiol. *Environmental Toxicology and Chemistry*, 2001, **20**(9):1968–1978.

38. Laenge, R., et al., Effects of the synthetic estrogen 17α-ethynylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry*, 2001, **20**(6):1216–1227.

39. Miles-Richardson, S.R., *Effects of Estrogenic Compounds on the Fathead Minnow* (*Pimephales promelas*). PhD thesis, Michigan State University, East Lansing, MI, 1997.

40. Nimrod, A.C. and W.H. Benson, Reproduction and development of Japanese medaka following an early life stage exposure to estrogens. *Aquatic Toxicology*, 1998, **44**(1–2): 141–156.

41. Palace, V.P., et al., Induction of vitellogenin and histologic effects in wild fathead minnows from a lake experimentally treated with the synthetic estrogen, ethynylestradiol. *Water Quality Research Journal of Canada*, 2002, **37**(3):637–650.

42. Panter, G.H., R.S. Thompson, and J.P. Sumpter, Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmental relevant con centrations of the natural oestrogens, oestradiol and oestrone. *Aquatic Toxicology*, 1998, **42**:243–253.

43. van Aerle, R., T.J. Runnalls, and C.R. Tyler, Gonadal sex differentiation in fathead minnow. *Pimephales promelas. Annual Conference of SETAC Europe*, Vienna, Austria, 2002.

44. Kinnberg, K., B. Korsgaard, and P. Bjerregaard, Concentration-dependent effects of nonylphenol on testis structure in adult platyfish *Xiphophorus maculatus*. *Marine Enviromental Research*, 2000, **50**(1–5):169–173.

45. Christiansen, T., B. Korsgaard, and A. Jespersen, Effects of nonylphenol and 17β–estradiol on vitellogenin synthesis, testicular structure and cytology in male eelpout (*Zoarces viviparus*). *Journal of Experimental Biology*, 1998, **201**(2):179–192.

46. Gray, M.A., A.J. Niimi, and C.D. Metcalfe, Factors affecting the development of testis–ova in medaka, *Oryzias latipes*, exposed to octylphenol. *Environmental Toxicology and Chemistry*, 1999, **18**(8):1835–1842.

47. Wolf, J.C., et al., Qualitative and quantitative histomorphologic assessment of fathead minnow *Pimephales promelas* gonads as an endpoint for evaluating endocrine-active compounds: a pilot methodology study. *Toxicologic, Pathology*, 2004, **32**(5):600–612.

48. van der Ven, L.T.M. and P.W. Wester. http://www.rivm.nl/fishtoxpat, 2003.

49. Kinnberg, K., et al., Effects of nonylphenol and 17β-estradiol on vitellogenin synthesis and testis morphology in male platyfish *Xiphophorus maculatus*. *Journal of Experimental Biology*, 2000, **203**(2):171–181.

50. van der Ven, L.T.M., P.W. Wester, and J.G. Vos, Histopathology as a tool for the evaluation of endocrine disruption in zebrafish (*Danio rerio*). *Environmental Toxicology and Chemistry*, 2003, **22**(4):908–913.

51. Wester, P.W., et al., Aquatic toxicology: Opportunities for enhancement through histopathology. *Environmental Toxicology and Pharmacology*, 2002, **11**(3–4):289–295.

52. Ankley, G.T. and R.D. Johnson, Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. *ILAR Journal*, 2004, **45**(4):469–483.