Small Fish Models for Identifying and Assessing the Effects of Endocrine-disrupting Chemicals

Gerald T. Ankley and Rodney D. Johnson

Abstract

Endocrine-disrupting chemicals (EDCs), particularly those that affect the hypothalamic-pituitary-gonadal (HPG) axis of vertebrates, have become a focus of regulatory screening and testing throughout the world. Small fish species, principally the fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*), are used as model organisms for several of these testing programs. Fish are appropriate models for testing EDCs, not only from the perspective of existing ecological impacts, but also in terms of species extrapolation. Specifically, there is a significant degree of conservation of basic aspects of the HPG axis across vertebrates, which provides a technically robust basis for using results from fish tests to predict likely modes/mechanisms of action of potential EDCs in other vertebrates. Different experimental designs/endpoints for partial- and full-life cycle tests with fish that enable a consideration of a broad range of EDCs are described. Examples of results with specific chemicals in tests with the fathead minnow, medaka, and zebrafish are presented and discussed in terms of sensitivity and specificity for different classes of EDCs.

Key Words: comparative endocrinology; development; endocrine-disrupting chemicals; fathead minnow; medaka; reproduction; test methods; zebrafish

Background

Since the early 1990s, an international effort has focused on identifying possible adverse effects of endocrine-disrupting chemicals (EDCs[1]) on reproduction and development in both humans and wildlife. The hypothalamic-pituitary-gonadal (HPG[1]) axis, especially aspects of the system directly related to steroid hormones (estrogens, androgens), has been of particular concern (EPA 1998; Huet 2000). A number of chemicals with the potential to affect the HPG axis of animals enter aquatic systems through a variety of point and nonpoint source discharges. Not surprisingly, therefore, some of the better documented examples of adverse effects of EDCs in the environment are for aquatic animals, particularly fish (for reviews see Ankley and Giesy 1998; Fairbrother et al. 1999; Tyler et al. 1998; WHO 2002). For example, some of the pioneering work of Sumpter and coworkers in the United Kingdom from the early- through the mid-1990s documented the presence of feminized male fish in rivers downstream of municipal waste water treatment plants, and then associated this response with the occurrence of steroidal estrogens and some types of industrial chemicals (alkylphenols) (Desbrow et al. 1998; Purdom et al. 1994; Routledge et al. 1998). Similar research from a number of other countries suggests that the presence of estrogenic materials in municipal effluents is a relatively common phenomenon (e.g., Christiansen et al. 2000; Folmar et al. 1996; Knudsen et al. 1997; Larsson et al. 1999). Discharges from pulp and paper mill effluents also have been directly associated with alterations in endocrine function in fish from the field, suggesting the occurrence of plant byproducts with the capacity to affect processes controlled by estrogens and androgens (Larsson et al. 2000; Parks et al. 2001; Van Der Kraak et al. 1998a). Other examples of possible adverse effects of EDCs on native fish populations may be related to tetrachlorodibenzo-p-dioxin and polychlorinated biphenyls in the Great Lakes, and polycyclic aromatic hydrocarbons at marine sites (see Fairbrother et al. 1999 and references therein).

ILAR Journal recently published a series of papers describing the technical basis and benefits of using small fish models for biomedical research focused on human health issues (Law 2001; Moorman 2001; Reimschuessel 2001; Stoskopf 2001; Walter and Kazianis 2001; Winn 2001). Fish can offer significant advantages over mammalian systems for certain types of toxicological studies, for example, in terms of cost and length of assays, as well as efficiency of chemical delivery/exposure. The technical rationale for use of fish as "surrogates" for clinical toxicology research is that much of the basic molecular machinery involved in initiation of toxic responses is highly conserved across vertebrate species. For example, chemicals that are alkylating agents (e.g., diethylnitrosamine) are carcinogenic both in mammals and fish, although actual tissues affected/tumor types may differ (Law 2001). Hence, chemical assessment scenarios for which the mode(s) of action of concern is (are)

Gerald T. Ankley, Ph.D., and Rodney D. Johnson, Ph.D., are a Toxicologist and Research Biologist, respectively, at the US Environmental Protection Agency Office of Research and Development, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division, Duluth, Minnesota.

[1]Abbreviations used in this article: EDC, endocrine-disrupting chemical; EPA, Environmental Protection Agency; FSH, follicle-stimulating hormone; HPG, hypothalamic-pituitary-gonadal; LH, luteinizing hormone; OECD, Organisation for Economic Cooperation and Development.

well defined and known to be phylogenetically conserved are particularly well suited for the use of fish as test models for prediction of risk across species. As discussed in more detail below, the HPG axis is quite similar in all vertebrates and hence serves as a reasonable system for conducting studies focused on extrapolation across species (Ankley et al. 1997).

In recognition of the utility of fish as surrogate models for other vertebrates, as well as documentation of impacts of EDCs on this class of animals in the field, different testing approaches utilizing fish are being developed and validated for regulatory programs for EDCs both nationally and internationally. In the United States, a 1996 congressional mandate directed the Environmental Protection Agency (EPA[1]) to develop a formal screening and testing program for EDCs (EPA 1998). Five test systems were recommended by an advisory committee for Tier 1 screening for the EPA program (EPA 1998). Three systems use rats, one uses an amphibian (*Xenopus laevis*), and the other uses a small fish, the fathead minnow (*Pimephales promelas*), in a short-term (21-day) assay (EPA 1998). Chemicals identified as possible EDCs in Tier 1 screening might then be subjected to more intensive Tier 2 tests, which could include full-life cycle or even multigenerational assays with a number of vertebrate species including fish such as the fathead minnow, Japanese medaka (*Oryzias latipes*), or sheepshead minnow (*Cyprinidon variegatus*). From an international perspective, the Organisation for Economic Cooperation and Development (OECD[1]) has formed a task group focused on developing internationally harmonized test methods for EDCs for both mammalian and nonmammalian species (Huet 2000). A subcommittee within the task group is currently focusing specifically on fish tests. Three small fish species (fathead minnow, medaka, and zebrafish [*Danio rerio*]) are being evaluated for screening (partial-life cycle assays) as well as more extensive (full-life cycle) testing of EDCs (OECD 1999, 2000, 2004).

This article provides a general overview of the use of small fish species for EDC testing. We initially describe the HPG axis in fish in the context of a generalized vertebrate system, with an emphasis on the role of the axis in sexual development and reproduction. We then present basic experimental design and methodological considerations germane to fish testing, and discuss the advantages and limitations of three commonly used small fish species—the fathead minnow, medaka, and zebrafish—in the context of EDC testing. Finally, we briefly summarize the results of EDC testing with these three species in terms of test design and relevant endpoints.

Endocrinology of Fish: Role of the HPG Axis in Reproduction and Development

Overview

This overview of the structure and function of the HPG axis of teleost fishes is a synthesis of information described in much greater depth in several excellent books or book chapters. For more comprehensive coverage of this topic, especially in the context of EDCs, we suggest additional reading (e.g., Kime 1998; Norris 1996; Van Der Kraak et al. 1998b; WHO 2002).

The HPG axis of the teleost fishes is, in general, similar to that found in other oviparous (egg laying) vertebrates. The principal components of the axis include the hypothalamus and hypophysis (pituitary gland) in the brain, the gonads (testis, ovary), and the liver. These tissues are structurally connected in one or both of the following mechanisms: a relatively fast neuronal linkage and a slower vascular linkage. The functional signals that affect the linkages between these components are somewhat diverse in chemical structure and how they travel among tissues. In some cases, the signal, a hormone, is secreted by specialized cells within the tissue into the extracellular space. From there the hormone diffuses into the blood stream and is carried throughout the body to specific cells within the target tissue, thereby modulating activity of the tissue in some fashion. In other cases, the signal, a neurotransmitter or neurohormone, is secreted by a neuron directly onto or near the surface of the cells receiving the signal. Finally, in some cases, the signal (termed a paracrine) is synthesized and secreted by one cell type into the extracellular space where it diffuses to adjacent cells of a different type that respond in some fashion to the signal.

The target cells within target tissues respond to these signaling molecules in various ways, but in general the responses are agonistic (i.e., activation of cellular processes) or antagonistic (i.e., inhibition of cellular processes). Depending on the developmental status of the organism, the ultimate effects of these signals on the whole animal can be categorized as organizational or activational. In other words, during the early developmental stages of fish and other vertebrates, the differentiation of tissues and cells (such as the gonads) into organs with the proper structure and capability of responding to external and internal cues is controlled, at least in part, by the hormones of the HPG axis. These responses are considered to be organizational. In later life-stages, these same tissues are capable of responding to one or more external signals (e.g., photoperiod, temperature) with responses such as the initiation of reproduction. These responses are termed activational.

It should be noted that the HPG axis functions as a dynamic system throughout each life-stage of the organism, early in development, through gonadal development, and finally into adult life-stages. As illustrated in Figure 1, each component is linked via positive and/or negative feedback loops into a dynamic but stable control system. This system is capable of maintaining the organism so that when the proper external cues are received, a new dynamic state resulting in the development of viable gametes, reproductive behavior, and finally reproduction is achieved. It is advantageous to think of these systems as dynamic loops rather than linear systems that when perturbed, respond to bring the systems back to a stable dynamic state (equilibrium). "Natural perturbations" such as photoperiod or temperature

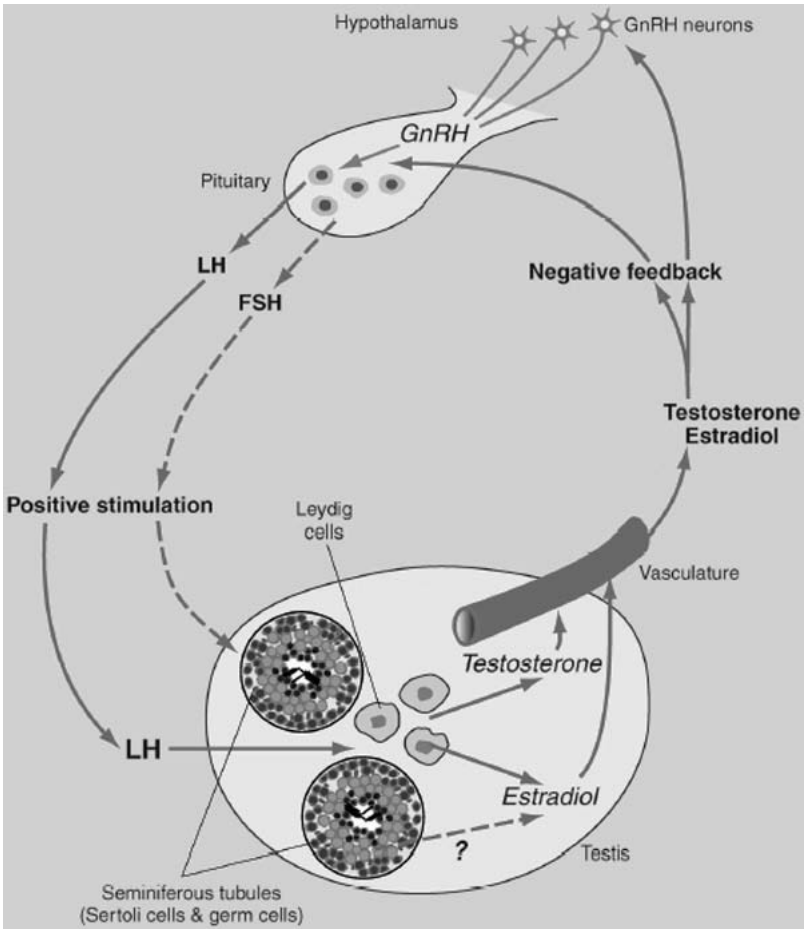

Figure 1 Overview of the teleost hypothalamic-pituitary-gonadal axis. The cells of the testis are diagramed; however, cells with similar roles are present in the ovary. The linkages between components of the axis simplistically illustrate how the system maintains a dynamic equilibrium. See the text for more detail. GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone. Adapted from WHO [World Health Organization]. 2002. Global Assessment of the State-of-the-Science of Endocrine Disruptors. Geneva: International Programme on Chemical Safety.

change activate the dynamic system into a new state resulting in reproduction. However, exogenous chemical signals (xenobiotic molecules) are capable of interfering with the dynamic equilibrium of the HPG axis, either by perturbing it into a new state when inappropriate or by rendering the system incapable of responding properly to normal environmental cues. In general, organisms are most susceptible to these perturbations during the organizational (or developmental) phases of the life-cycle, or during activational phases leading to reproduction in mature animals. Hence, the bioassays being developed to detect chemicals capable of disrupting this dynamic system often are relatively short-term, but incorporate early life-stages for assessing organizational disruptors, or adult reproductive life-stages for assessing activational effects.

HPG Axis and Reproduction

The following description of the components of the HPG axis and their role in actively reproducing adult fish is provided to afford the reader a better understanding of the endpoints used in short-term tests to assess chemicals for endocrine-disrupting activity. All three of the small fish species (fathead minnow, medaka, zebrafish) on which we focus in this review are considered asynchronous spawners. When fish of this type are provided with the appropriate cues, they will spawn successively for a relatively long period, with each spawning event producing another batch of eggs. The frequency of spawns is specific to the species and the environmental condition; for example, optimally medaka can spawn every day, fathead minnows every 3 days, and the zebrafish approximately every other day. The endocrinology of the HPG axis of asynchronous spawners is not as well characterized as for other types of reproductive strategies (e.g., synchronous, or one event, spawners such as salmonids), but there appears to be a high degree of conservation of the system across fishes, thereby enabling generalized comparisons. The teleost hypothalamus/hypophysis complex is unique among vertebrates in that the neurohormone secretions of the hypothalamus do not travel via a

vascular portal system to the adenohypophysis (pituitary). Instead, the pituitary is directly inervated by hypothalamic neurons that secrete directly into the intercellular space of the hypophysis. The principal HPG axis neurohormone secreted by the hypothalamus is gonadotropin-releasing hormone. This decapeptide hormone stimulates the production and/or release of two types of glycoprotein gonadotropic hormones: one that is similar to a follicle-stimulating hormone (FSH[1]), which primarily induces oogenesis and spermatogenesis, and one that is similar to a lutenizing hormone (LH[1]), whose primary activity causes final gamete maturation and induction of ovulation or sperm release.

The ovaries and testes of adult fathead minnow, medaka, and zebrafish are paired or bilobed organs. The principal cell types of the ovary are the germinative cells, in various stages of development starting with the primary oogonia, which develop into mature oocytes. At any particular time, the asynchronous ovary contains oocytes at all stages of development. Surrounding the developing oocyte is a spherical layer of cells called the granulosa, which assists in the translocation of the egg-yolk lipoprotein vitellogenin into the developing oocyte. Finally, in the stromal layers of the ovary resides a cell type called thecal cells, which are involved in the synthesis of steroid hormones. The primary effects in the ovary of the FSH- and LH-like hormones are to stimulate the thecal and granulosa cells to produce the female steroid hormone 17β-estradiol. However, the synthetic pathway of this hormone includes the male hormone testosterone as an intermediate. Apparently, the thecal cells, under the stimulatory influence of the LH-like gonadotrope, synthesize testosterone, which diffuses to the granulosa cells. The FSH-like gonadotrope induces the enzyme aromatase in the granulosa cells, which converts testosterone to estrogen. The estrogen that is produced has several critical roles, including oocyte development and stimulation of the liver to produce and secrete vitellogenin into the bloodstream. Once in the ovary, the vitellogenin is translocated via the granulosa cells into the developing oocyte. Other estrogen effects include induction of female secondary sex characteristics and negative feedback linkages to the hypothalamus and hypophysis.

Three principal pituitary hormone-responsive cell types are also in the male testis. The first cell type are germinal spermatogonia, which differentiate into sperm. Like the female asynchronous spawners, developing sperm can be seen histologically at all stages of development, with the difference being that groups of sperm, all at the same stage, develop within a cyst. The perimeter of each testicular lobule is lined with Sertoli or "nurse" cells that respond to the gonadotropic hormones by fostering the production and maturation of sperm. Finally, there are interstitial or Leydig cells within the interlobular space that produce the male fish androgens testosterone and 11-ketotestosterone. These androgens induce masculine secondary sex characteristics, and provide negative feedback to the hypothalamus and hypophysis. Although the liver is a functioning endocrine organ in the female fish, it is not the same in the male fish under normal conditions. Yet male fish have retained the cellular receptors as well as the synthetic machinery for producing vitellogenin in their hepatocytes. Thus, when male fish are exposed to chemicals that bind to these hepatocyte estrogen receptors, the liver synthesizes vitellogenin and secretes it into the bloodstream. Unfortunately, the male fish cannot easily remove vitellogenin from the bloodstream, so under physiologically abnormal conditions it can accumulate to very high levels causing reproductive dysfunction and kidney failure.

The paragraphs above provide a brief overview of the male and female HPG axis in fish during reproduction. Although the discussion is somewhat linear, starting with the secretions from the neurons in the brain hypothalamus and ending with the gonadal negative feedback signal to the hypothalamus-hypophysis, it is clear that this system is dynamic (Figure 1). Numerous other sources of stimulatory and inhibitory input are both internal (i.e., from other parts of the brain as well as from other tissues) and external (e.g., light and temperature). It is also clear that there must be temporal synchronization of these various signals for the system to operate smoothly. For instance, little is known about the daily hormonal cycles that regulate the various oocyte developmental stages, ovulation, and spawning behavior in asynchronous spawners. Toxicity tests such as those described below provide information regarding the integrated function or dysfunction of this complex system. Systems models of the HPG axis in small fish would assist the development of our ability to predict where in the system EDCs are likely to act. This information would be useful in developing tools for prioritizing which chemicals are most likely to be EDCs and through which one or more endocrine pathways the effects are likely to be observed.

HPG Axis and Development

Further research is needed to delineate fully the role of the HPG axis during sexual development and differentiation in the fathead minnow, medaka, and zebrafish. However, as is true for reproduction, there is a general understanding of the system relative to organizational events. Slightly more is understood about the process of sexual differentiation in medaka than in the other two species, because the male sex determining gene has been identified and sequenced in medaka (Matsuda et al. 2002). However, even with this ability to identify the genotypic sex of adult and embryonic medaka, it is not known how the gene programs sex differentiation. With respect to gonadal differentiation of the three species discussed in this review, the gonad of both the fathead minnow and medaka begins to differentiate into ovary and testis in early development. By hatch, the gonad can be identified as either male or female. In contrast, all zebrafish embryos first develop a female gonad. Then, approximately 45 days after hatching, the ovarian tissues of a proportion of the young fish begin to regress and redevelop into a testis. Apparently, this process is under the control of the HPG axis; however, it is not clear where the primary

signal leading to this transformation originates. When complete, the HPG axis of the 60-day posthatch zebrafish is remodeled to be structurally and functionally similar to that observed in the other two species. Under standard culturing conditions, the sex ratio of the medaka and fathead minnow appears to be nearly 50:50, whereas that of the zebrafish is commonly different from unity, sometimes approaching, or even exceeding, 60:40 ratios in either direction.

Although it is not clear exactly how the HPG axis is involved in sexual differentiation of small fish, there is a wealth of experimental evidence that the sex of all three of these species can be skewed in either direction, (i.e., male to female and vice versa) by treating the developing larval fish, at the right time, with estrogenic or androgenic chemicals. Hence, estrogens and androgens, whether natural or synthetic, are capable of inducing organizational effects on the sexual differentiation of developing fish. In some cases, these effects are not complete; for example, the gonads of treated fish contain developing germ cells of both sexes, resulting in a testis-ova, or ova-testis. To date, little work has been done to look at the effects of other HPG axis hormones on sexual development.

There are suggestions that a number of activational parameters (i.e., for hormone action in the adult life-stage) are influenced during early development. For example, medaka exposed to octylphenol (an estrogen agonist) from larval stages through sexual maturity have a reduction in some courtship activity (Gray et al. 1999b). Apparently, activational set-points, organizational effects, or both can be influenced by EDCs in early development of the HPG axis. With the advent of genomic and proteomic technologies, it should be possible to elucidate how components of the HPG axis develop. These data will provide insights into the interdependency (temporal and otherwise) of the HPG axis in organizing its own development. In time, these tools will help provide an understanding of how EDCs interfere with this highly evolved system.

Experimental Designs for Small Fish EDC Testing

A number of different designs ranging from acute (≤96-hr) lethality to full-life cycle multiendpoint protocols have been used for toxicity testing with fish. Due to the nature of the developmental and reproductive processes affected, and specificity with respect to mode/mechanism of action (i.e., interactions with discrete enzymes or receptors), EDCs are more likely to cause long-term sublethal, rather than acute, effects (Ankley et al. 2004). Consequently, short-term lethality assays are of little utility for assessing EDCs. As described above, early gonadal development and active reproduction are two windows of enhanced sensitivity during which chemicals might cause adverse effects through disruption of the HPG axis. Hence, full-life cycle tests with a variety of developmental and reproductive endpoints are ideal for detecting all possible effects of EDCs on fish. However, full-life cycle toxicity tests, even with small fish that mature relatively rapidly, require several months to complete and are very resource intensive (>US $100,000 per test). For this reason, they are not feasible for large-scale routine testing of all chemicals.

Information concerning potentially sensitive life stages has been used as a basis for developing more abbreviated test designs (and associated endpoints) suitable for detecting EDCs. Specifically, two types of partial-life cycle tests with fish have been utilized to detect effects associated with exposure to EDCs. One is initiated with embryonic or larval life-stages exposed to the test chemical(s) of concern, and usually extends through periods of gonadal differentiation/development. Endpoints used in this type of design focus on aspects of sexual differentiation, particularly the potential for deviations in phenotypic sex (based on external morphology and gonadal characteristics) relative to expected (or known) genotypic sex. A second partial-life cycle protocol that has been used to detect EDC effects in fish involves exposure of reproductively mature adults to chemicals of concern, after which endpoints related to status of the reproductive endocrine system (e.g., fecundity, secondary sex characteristics, vitellogenin, steroids) are assessed. Aspects of full-life cycle and different partial-life cycle designs (and endpoints) relative to testing known/suspected EDCs in fathead minnows, medaka, and zebrafish are presented in greater detail below.

Protocols for fish partial- and full-life cycle tests necessarily vary in a species-specific manner, as well as with the physicochemical properties of the test material under consideration. A full exploration of test design characteristics and their possible variations across species/chemicals is well beyond the scope of this review; however, several sources of information on the topic are available (e.g., ASTM 2000a,b; EPA 1994, 2002; OECD 1992a,b). In this article, we highlight only a few key considerations that should be considered when conducting partial- and full-life cycle assays with fish.

Short-term tests with fish that focus on lethality and growth are not particularly difficult to conduct, even for laboratories with a minimal level of experience. Many laboratories purchase test animals from suppliers and use them relatively quickly (within days to weeks) for toxicity testing. In general, this particular approach does not work well for longer-term partial- and full-life cycle tests. Even subtle variations in factors such as water or diet quality, temperature or photoperiod control, and/or handling of animals can adversely affect outcomes of chronic tests that would not (seemingly) affect the results of shorter-term assays. To conduct partial- and full-life cycle fish tests successfully, it is generally necessary to have established conditions and expertise consistent with actual maintenance of a reproducing culture of the fish species used in the toxicity tests. This is not to say that laboratories with little culture experience cannot conduct the types of partial- and full-life tests described in this review. Rather, we believe that the likelihood

of successful tests is significantly increased in situations of established competence with a particular species.

Fish can be exposed to test chemicals using any of a number of routes of exposure, including the water, orally (generally via addition to the diet), or through intraperitoneal injections. All three approaches have been used successfully with various EDCs (Ankley et al. 1998). Because of relative simplicity, exposure via the water is the most common route utilized; however, there are circumstances (e.g., when chemicals are available only in limited quantities, or are poorly soluble in water) that necessitate a dietary or injection route of exposure (Kahl et al. 2001). When administering organic chemicals to fish via the water, it has been historically common to enhance solubility of the chemicals through the use of carrier solvents such as acetone or methanol. However, the use of carrier solvents in aquatic toxicity testing can be problematic in several ways, including the following:

1. It is possible to enhance solubility of the test chemical far beyond what could actually be present in water in an environmental setting;
2. The solvent could contribute to observed responses of the animal through either direct toxicity or interactions with the test material;
3. Use of the solvent requires additional animals/test chambers to incorporate the necessary solvent and non-solvent controls; and
4. Solvents can contribute to enhanced microbial/algal growth in the test system, hence increasing labor in terms of maintenance and/or potentially compromising water quality, particularly in longer term (e.g., >7-day) tests.

In many cases, however, it is not necessary to use solvents for testing organic chemicals. Numerous approaches based on "saturator" systems have been developed and used successfully in long-term tests with fish exposed to EDCs via the water (EPA 2002; Kahl et al. 1999).

A particularly critical test variable with respect to both biology and chemical exposure is water renewal rate. In the past, toxicity tests with fish have been conducted under regimes ranging from completely static conditions, where chemical/water is added once to the system, to static-renewal situations, where solutions are removed/replaced periodically (often every 24 hr), to variations on a continual flow/renewal of water to the test system. In any test longer than 1 or 2 days, completely static conditions are impractical from the standpoint of maintaining water quality that is suitable for the fish—principally adequate concentrations of dissolved oxygen and minimal concentrations of ammonia, a potentially toxic metabolic by-product excreted by the fish. Even in static-renewal situations, slight decreases in water quality can significantly affect test results in situations where loading rates are suboptimal and/or where very sensitive endpoints during early development or active reproduction are under consideration (Tietge et al. 2000). The water renewal regime also can significantly affect exposure of fish to test chemicals administered through the water. Specifically, if test chemicals are volatile or prone to degradation, static or static-renewal designs will result in a fluctuating exposure scenario, which complicates interpretation of test results in terms of predicting environmental risk Hence, given both biological and chemical exposure considerations, the optimal course of action is to use continual-flow designs during partial- or full-life cycle testing of EDCs with fish.

In summary, full- or partial-life cycle tests focused specifically on development or reproduction are suitable for detecting chemicals that might affect the HPG axis in fish. These types of tests can be challenging, however, in that laboratories conducting the tests need to have resources and expertise consistent with successfully culturing the animals. From both biological performance and chemical exposure perspectives, EDC testing with fish ideally should be conducted under flow-through conditions in which carrier solvent use is minimized.

Small Fish Models for EDC Testing: Strengths and Limitations

The three species addressed in this review—fathead minnow, medaka, and zebrafish—have many of the same positive attributes, but there are also specific advantages and limitations for each of the three. All three have a comparatively rapid life-cycle (≤4-mo generation time from embryos to reproducing adults) relative to large species that have been used for reproductive endocrinology and/or toxicity testing, such as rainbow trout (*Oncorhynchus mykiss*), goldfish (*Carassius auratus,*) and carp (*Cyprinus carpio*). Methods for maintaining all three species in continuous cultures in a laboratory setting are well established, and the fish are amenable to the level of handling necessary for routine test procedures. Spawning activity in fathead minnow, medaka, and zebrafish can be controlled precisely through temperature and photoperiod manipulations, so they can be used successfully for partial- and full-life cycle testing focused on reproductive endpoints. Finally, although there are clear regional preferences for a particular species, the results of toxicity tests with each species have been used for regulatory programs in North America (fathead minnow), Europe (zebrafish, fathead minnow), and Japan (medaka). The specific biological attributes of each of the three species are discussed below.

Fathead Minnow

The fathead minnow is in the Cyprinidae family of fish, and has a ubiquitous distribution across North America. Adults are approximately 50 to 75 mm long, weigh 2 to 5 g, and are large enough for collection of blood for measurement of endpoints such as vitellogenin and sex steroids (Jensen et al.

2001; Korte et al. 2000). Under optimal conditions, the generation time for the fathead minnow is roughly 4 mo (Denny 1987), which is longer than for the medaka or zebrafish. The species is sexually dimorphic, an important attribute from two perspectives: (1) It is possible to initiate reproduction assays with known phenotypic males and females; and (2) the occurrence of secondary sex characteristics in reproductively active adults is under control of sex steroids and can be an excellent endpoint for identifying EDCs (EPA 2002).

Although male fathead minnows exhibit territorial behavior, the species is relatively flexible in that reproduction tests can be conducted using either paired- or group-spawning designs, provided there is an adequate number of spawning substrates when multiple fish of the same sex are present (Ankley et al. 2001; Harries et al. 2000). Group spawning (e.g., two males and four females per tank [Ankley et al. 2001]) is logistically easier than paired spawning because fewer discrete experimental units (tanks) are needed to maintain a given number of fish compared with paired animals. However, the quality of reproductive endocrinology data from paired fish can be superior to that generated from group designs in that it is possible to relate biochemical and histological information for individual animals directly to their reproductive output. A reproductively active female fathead minnow typically deposits clutches of 50 to 200 eggs on the bottom of spawning substrate (e.g., a tile) about every 3 days (Jensen et al. 2001). Determination of fertility of the eggs can be achieved easily using a microscope, and the larva hatch in approximately 4 to 5 days at 25°C.

Recent studies have shown that it is possible to visually differentiate male versus female fathead minnows based on gonad appearance within about 80 to 90 days after hatch (Panter et al. 2002b); however, the exact timing and genetic basis of sexual differentiation in the fathead minnow is currently poorly understood. Of the three species considered in this review, the greatest amount of traditional aquatic toxicology research has been conducted with the fathead minnow (EPA 2002).

Japanese Medaka

Although not used as extensively in toxicological research as the fathead minnow, the Japanese medaka has served as an experimental model since the early 1900s (Yamamoto 1975), and literally thousands of papers have been written concerning different aspects of its biology (http://biol1.bio.nagoya-u.ac.jp/~mdkfish/; http://w-edcdb.nies.go.jp/SHf/index.html). The medaka is a member of the Adrianichthyidae (rice fish) family, and has a wide distribution throughout southeastern Asia. Adult medaka are considerably smaller than fathead minnows, with an average length of 25 to 50 mm and approximate weight of 0.7 to 0.8 g. It is difficult to collect blood from individual animals for determination of endpoints such as sex steroids and vitellogenin. Hence, steroids usually have not been determined in EDC experiments with medaka, and vitellogenin is typically measured in liver homogenates from this species (Kang et al. 2002a,b; Seki et al. 2002; 2003a).

The generation time for medaka is comparatively rapid (i.e., 2-3 mo). Like the fathead minnow, mature medaka are sexually dimorphic, which facilitates their use in reproduction studies and allows for consideration of external secondary sex characteristics as an endpoint in EDC studies. The medaka also can be used in either group- or paired-spawning experimental designs. Female medaka produce about 10 to 30 eggs per day, for periods of time up to 4 mo (Yamamoto 1975). In the laboratory, the eggs are deposited on artificial substrates from which they can be removed, evaluated microscopically for fertilization success, and held until hatched, typically in 8 to 10 days at 25°C.

As described above, the process of development and differentiation of the gonad in larval medaka is better understood and documented than in either the fathead minnow or zebrafish. An additional strength of the medaka model is that there are a number of potentially useful strain variants available that may be well suited for EDC screening and testing (Papoulias et al. 2000; Scholz and Gutzeit 2000; Wakamatsu et al. 2001; Winn et al. 2000).

Zebrafish

Zebrafish have been used for many years in developmental biology research, in large part because developing embryos can be observed readily through the relatively transparent egg chorion (Laale 1977). Recently, to support developmental biology research, an effort to sequence the entire genome of the zebrafish has emerged (http://www/ncbi.nlm.nih.gov/genome/guide/zebrafish/index.html). This information has the potential of providing a basis for identifying novel diagnostic endpoints related to gene expression in EDC studies with this species.

The zebrafish is a member of the Cyprinidae family, and is native to India and Burma. Adult zebrafish are slightly larger than medaka—approximately 40 to 50 mm long—and weigh about 1.5 g. However, it is still difficult to collect more than a couple microliters of blood from individual zebrafish for analysis of sex steroids or vitellogenin. In EDC studies, the latter measurement has been made using whole body homogenates (Petersen et al. 2001; Porcher et al. 2003). Compared with the fathead minnow and medaka, the zebrafish exhibits little sexual dimorphism, so this endpoint has not proven particularly useful in EDC studies with adults (OECD 2004).

The zebrafish generation time of 2 to 3 mo is similar to that of the medaka. Reproductively active female zebrafish can exhibit some level of spawning activity (egg production) almost daily, but relatively large spawns (e.g., >150 eggs) occur every 5 to 10 days. In contrast to the fathead minnow and medaka, zebrafish are "broadcast" spawners, releasing eggs that settle to the bottom of the tank. Hence,

enumerating and assessing the condition of prehatch zebrafish can be more time intensive than for the fathead minnow or medka, both of which deposit eggs as discrete egg masses. Zebrafish embryos hatch in about 3 days at 28°C. The timing and genetic basis of sexual development and differentiation in zebrafish are not well understood, especially compared with the medaka.

In summary, each of the three small fish models has specific strengths and weaknesses relative to EDC testing and screening. In following sections, we present basic designs used and endpoint responses for partial- and full-life cycle tests with the three species conducted with EDCs with different modes/mechanisms of action within the HPG axis. These examples are not intended to be exhaustive; rather, our intent is to provide the reader with a general background of the data that can be expected from the assays and associated endpoints and the appearance of typical results.

Partial Life-Cycle Reproduction Tests

As described above, active reproduction is a critical window of sensitivity to chemicals that affect the HPG axis. Partial life-cycle tests designed to exploit this window of sensitivity in the context of EDC testing start with reproductively mature animals and evaluate a suite of apical (whole organism) and diagnostic endpoints during a relatively short-term chemical exposure. This type of approach has been described and used for EDC testing with all three species, but there has been comparatively more work with the fathead minnow and medaka than the zebrafish (Ankley et al. 2001; Harries et al. 2000; Kang et al. 2002a,b; Kramer et al. 1998; Seki et al. 2002; van den Belt et al. 2001; van der Ven et al. 2003).

Tests are typically initiated with animals that have an established history of successful reproduction, as indicated by active egg production. After a short period of acclimation to the test system, chemical exposure is initiated, generally via water. During the subsequent exposure period (often 21 days), a number of apical endpoints can be assessed, including survival and size (of the adults), fecundity (number of eggs spawned), fertility (number of fertile eggs produced), hatch (number of fertile eggs that produce larvae), and, occasionally, larval viability (e.g., occurrence of malformations in hatched animals). At the conclusion of the test, endpoints more diagnostic for specific EDC-related modes or mechanisms of action can be assessed, including gonadal condition (relative gonad weight, histopathology), status of secondary sex characteristics, concentrations of vitellogenin in plasma or liver, and, occasionally, plasma sex steroid (estrogen, androgen) concentrations.

This type of protocol has been utilized to assess a number of chemicals known or suspected to act as estrogen agonists, including the potent natural and synthetic steroidal estrogens estradiol and ethinylestradiol, as well as weaker nonsteroidal chemicals (e.g., alkylphenols, methoxychlor). Strong estrogen agonists can cause changes in secondary sex characteristics of adult fathead minnows and medaka; specifically, decreases in the degree of expression of sex characteristics normally present in spawning males have been reported (e.g., Harries et al. 2000; Miles-Richardson et al. 1999a; OECD 2004). In reproduction studies, strong estrogen agonists invariably reduce fecundity of the exposed animals, often stopping reproduction altogether, usually concurrent with alterations in condition of the gonads in both sexes (Harries et al. 2000; Kang et al. 2002a; Kramer et al. 1998; Miles-Richardson et al. 1999a; Seki et al. 2002; Van den Belt 2001; van der Ven et al. 2003).

Of particular note from a histological perspective is a condition in which the gonad contains developing gametes of both sexes, termed testis-ova. This condition is commonly observed in medaka exposed to estrogenic materials in both early-life stage and adult studies, although there is evidence that animals are more susceptible to the condition during early developmental exposures than during later life stages (Egami 1955; Gray et al. 1999a; Koger et al. 2000). Testis-ova has been noted only infrequently in zebrafish and fathead minnows exposed to strong estrogens (e.g., Hill and Janz 2003; Länge et al. 2001).

Not unexpectedly, weaker estrogen agonists cause changes in fish similar to, but more subtle than, the strong estrogens. These changes include decreases in fecundity, changes in gonadal histopathology, and alterations in circulating sex steroids (e.g., Ankley et al. 2001; Kang et al. 2002b; Miles-Richardson et al. 1999b; Van den Belt et al. 2001).

Probably the single most important diagnostic endpoint for identifying weak or strong estrogens in partial life-cycle reproduction tests with the fathead minnow, medaka, and zebrafish has been induction of vitellogenin in the adult male (Ankley et al. 2001; Harries et al. 2000; Kang et al. 2002a,b; Kramer et al. 1998; Seki et al. 2002; Sumpter and Jobling 1995; Van den Belt et al. 2001). As described above, vitellogenin production in actively reproducing oviparous animals is a normal process in females stimulated principally by estradiol. Although vitellogenin is not present in males normally, they nonetheless produce the lipoprotein in response to exposure to exogenous estrogens. Thus, vitellogenin induction is highly specific for estrogen agonists, and because the vitellogenin "baseline" in control males is low (often nondetectable), the response is comparatively sensitive and can be quite rapid. Furthermore, because males do not have the capacity to eliminate vitellogenin readily, concentrations of the protein can remain elevated for a relatively lengthy time after even a brief exposure to an estrogen.

In Figure 2, a time course of vitellogenin induction seen in male fish exposed to a strong estrogen agonist is illustrated. In this study, male fathead minnows were treated with a single intraperitoneal injection of 0.5 or 5 mg of estradiol/kg, and determinations of vitellogenin mRNA (in liver) and protein (in blood) were made over the course of about 3 wk (Korte et al. 2000). Plasma concentrations of

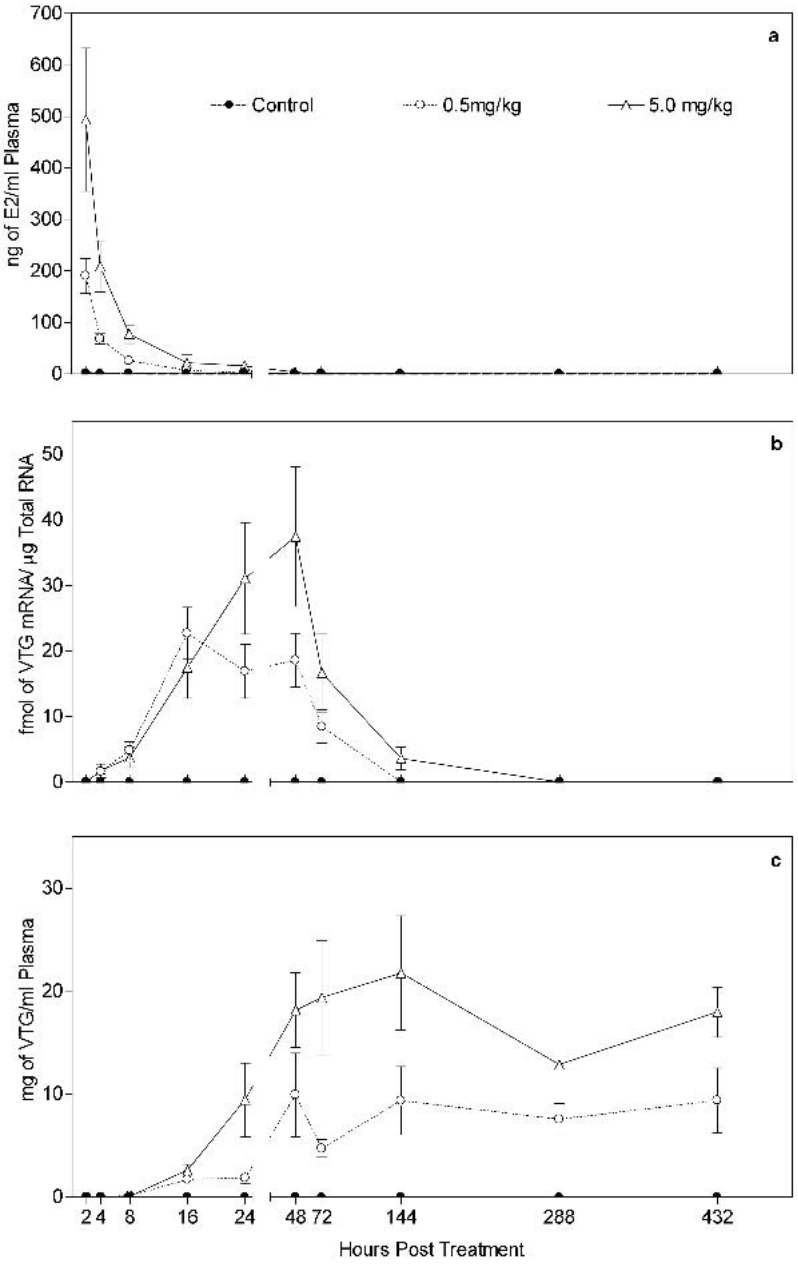

Figure 2 Induction of vitellogenin (VTG) in fathead minnows exposed to 17β-estradiol (E2). Male fish were treated with single intraperitoneal injections of 0.5 or 5 mg of E2/kg. Indicated data are mean (± SE, n = 5). (a) Plasma E2; (b) liver VTG mRNA; (c) plasma VTG protein. The X-axis was split into segments of 0 to 25 hr and 25 to 500 hr to enable presentation of results on the same plot. Reprinted from Korte JJ, Kahl MD, Jensen KM, Pasha MS, Parks LG, LeBlanc GA, Ankley GT. 2000. Fathead minnow vitellogenin: Complementary DNA sequence and messenger RNA and protein expression after a β-estradiol treatment. Environ Toxicol Chem 19:972-981, with permission granted by the Society of Environmental Toxicology and Chemistry (SETAC®), Pensacola, Florida.

estradiol peaked rapidly in the fish (after the injection) but returned to control levels within about 24 hr (Figure 2a). There was evidence of induction of vitellogenin mRNA production within 8 hr of exposure (Figure 2b). The mRNA returned to control levels within 6 days. Plasma concentrations of the vitellogenin protein increased within about 24 hr, achieved maximal concentrations within about 48 hr, and remained at these levels throughout the remainder of the test (Figure 2c).

The HPG axis can, of course, be affected via modes/mechanisms of action other than by chemicals that mimic estrogens. Responses to several chemicals reflective of different modes/mechanisms of action have been assessed in partial life-cycle reproduction assays with the fathead minnow and, to a somewhat lesser extent, the medaka. For example, exposure of both species to strong androgen agonists, such as the synthetic steroids methyltestosterone and trenbolone, inhibits reproductive output (fecundity), affects

gonadal histology in both males and females, and can influence circulating concentrations of vitellogenin and steroid hormones (Ankley et al. 2001, 2003; Peterson et al. 2001). To date, the most diagnostic endpoint identified for androgen receptor agonists in adults of sexually dimorphic fish species is external masculinization of females. For example, in female fathead minnows exposed to methyltestosterone, there is a marked induction of dorsal nuptial tubercles normally present only in males (Figure 3; Ankley et al. 2001). In medaka, exposure to both natural (11-ketotestosterone) and synthetic (trenbolone) androgens causes females to develop male-like papillary processes on the anal fin (Hishida and Kawamoto 1970; OECD 2004).

Figure 3 Occurrence of male secondary sex characteristics (nuptial tubercles) in female fathead minnows exposed to the synthetic androgen methyltestosterone. (A) Control male; (B) control female; (C) exposed female. Reproduced from Ankley GT, Jensen KM, Kahl MD, Korte JJ, Makynen EA. 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). Environ Toxicol Chem 20:1276-1290, with permission granted by the Society of Environmental Toxicology and Chemistry (SETAC®), Pensacola, Florida.

There have also been studies characterizing effects of androgen antagonists on responses in fish partial life-cycle reproduction tests. For example, the antiandrogens vinclozolin and flutamide have been shown to affect vitellogenin and steroid concentration in the fathead minnow during 21-day tests (Jensen et al. 2004; Makynen et al. 2000). Flutamide also reduced fecundity (egg production) of the fish (Jensen et al. 2004). Diagnostic endpoints for androgen antagonists are not as well defined as for estrogen or androgen agonists. The most consistent response appears to be in the ovaries of exposed females, where decreases in mature oocytes and increases in atretic follicles have been observed (Jensen et al. 2004; Leino et al. 2004).

Alterations in pathways controlled by estrogens and androgens can occur not only through direct chemical interactions with the respective receptors but also via stimulation or inhibition of enzymes responsible for the synthesis or degradation of the hormones. One class of environmental contaminants of considerable interest in this regard comprises those that inhibit CYP19 aromatase, a key enzyme in the vertebrate steroid synthesis pathway that converts testosterone to estradiol (Callard et al. 1978). In a 21-day partial life-cycle reproduction assay with the fathead minnow, the prototype aromatase inhibitor fadrozole (a pharmaceutical) decreased fecundity, affected gonadal histology and, consistent with the expected mechanism of action, decreased circulating estradiol concentrations in females (Ankley et al. 2002). The decrease in circulating estrogen, not surprisingly, was accompanied by a significant decrease in vitellogenin in the females. So in this case, decreases in vitellogenin in females (in contrast to increases in males caused by estrogen agonists) appear to be, in conjunction with decreases in plasma estradiol, a suitable diagnostic endpoint for EDCs that inhibit aromatase.

Partial Life-Cycle Early Developmental Tests

Larval development, especially during critical stages of sexual differentiation and gonadal development, can be uniquely sensitive to the effects of chemicals that affect the HPG axis. A number of slightly different study designs have involved use of the fathead minnow, medaka, and zebrafish to exploit this life-stage for EDC screening and testing. Tests are initiated with embryos or, occasionally, juveniles that have not yet undergone sexual differentiation, and are continued through periods of time of established sensitivity. Chemical exposures may be continuous for several weeks or, when critical windows of differentiation are well-known (e.g., the medaka), may last only a few days (Koger et al. 2000). An important test endpoint is assessment of morphological and/or gonadal differentiation (phenotypic sex),

typically in subadult animals, relative to expected (or known) genotypic sex. In the case of the fathead minnow and zebrafish, genetic sex determination is not well understood, so deviation from the expected sex ratios serves as the basis for identifying potential EDCs. In the medaka, it is possible to determine genetic sex directly, for example, using color-sex linked strain for testing (Papoulias et al. 2000; Scholz and Gutzeit 2000), so direct comparisons of phenotypic to genotypic sex are possible.

Other endpoints that have been evaluated commonly in conjunction with early developmental partial-life cycle tests with EDCs include assessment of gonadal development (in addition to an analysis of presence of ovarian versus testicular tissue) or vitellogenin concentrations in different tissues. As is true in male fish, larval/juvenile animals typically have very low concentrations of vitellogenin but possess a capacity to produce the protein in response to stimulation by exogenous estrogen agonists.

Strong estrogens can cause sex reversal in the species of concern (i.e., development of a phenotypic sex not reflective of genotypic sex). For example, exposure of medaka to estradiol or ethinylestradiol for 1 to 2 mo after hatch produced exclusively female populations of the fish (Nimrod and Benson 1998; Scholz and Gutzeit 2000). Similar results have been described in juvenile zebrafish exposed to ethinylestradiol (Örn et al. 2003), although longer term studies suggest that the effects of early-developmental exposures to estrogen agonists on phenotypic sex in the zebrafish may be reversible to some extent (Hill and Janz 2003; Weber et al. 2003). Weak estrogens such as some alkylphenols also have been shown to feminize male medaka exposed during the first 2 mo of development (Gray et al. 1999b; Seki et al. 2003a; Yokota et al. 2000). Seki and colleagues (2003a) noted that external secondary sex characteristics of males exposed to 4-nonylphenol and 4-*tert*-octylphenol during early development reverted from female back to male after a holding period in clean water, but intersex gonads (testis-ova) persisted in the fish. Comparative studies are very limited, but it appears that fathead minnows are somewhat less sensitive than medaka or zebrafish to the sex-reversing effects of strong estrogens, although a study by van Aerle and coworkers (2002) indicated feminization of male fathead minnow gonads associated with short-term exposures to ethinylestradiol during early development.

Strong androgen agonists also can cause sex reversal during early developmental exposures. For example, studies with medaka and zebrafish have shown the production of 100% male populations of fish associated with early developmental exposures to the synthetic androgen methyltestosterone (Örn et al. 2003; Papoulias et al. 2000). From the perspective of antiandrogenic EDCs, Kiparissis and colleagues (2003) demonstrated that both ovarian and testicular development in medaka could be affected significantly by developmental exposures to the model androgen receptor antagonists cyproterone acetate and vinclozolin.

Early-life stage tests with the fathead minnow, medaka, and zebrafish all have successfully used vitellogenin induction as an indicator of, primarily, estrogenic properties of chemicals (Örn et al. 2003; Panter et al. 2002a; Seki et al. 2003a; Tyler et al. 1999). For example, Tyler and colleagues (1999) found significant induction of the protein in fish exposed to estradiol from 24 hr after fertilization through 30 days after hatch. Panter and coworkers (2002a) recommended a 14-day exposure with juvenile fathead minnows for EDC screening based on vitellogenin induction caused by ethinylestradiol, several weaker estrogen receptor agonists (e.g., methoxychlor, 4-*tert*-pentylphenol), and an estrogen receptor antagonist (ZM 189,154), which decreased vitellogenin concentrations in the fish. The induction of vitellogenin during developmental exposures also has been reported in medaka exposed to alkylphenols (Seki et al. 2003a) and zebrafish exposed to ethinylestradiol (Örn et al. 2003).

Full Life-Cycle Tests

The most comprehensive approach to detecting possible effects of EDCs on fish is via full-life cycle testing. Full-life cycle test designs vary somewhat with both the species and the applicable research hypothesis/risk assessment question, but the designs utilized in EDC studies by Länge and colleagues (2001; fathead minnow), Yokota and coworkers (2001; medaka), and Segner and colleagues (2003; zebrafish) are typical. In these studies exposures were initiated with newly fertilized embryos, and the animals (termed the F_0 generation) were maintained through a period of active reproduction, ranging from weeks to months. A subset of the F_1 offspring produced by the parental generation was maintained under the same chemical exposure regime from which they were derived through hatch, and usually until sexual differentiation (based on external morphological characteristics and/or gonadal development) could be assessed. Hence, the full-life cycle design enabled consideration of EDC effects both on reproduction and development, essentially capturing all of the endpoints encompassed by the more focused partial-life cycle tests described above.

A drawback of full life-cycle tests is that they are very resource intensive. Assays with any of the three small fish models can take 6 to 9 mo to complete. The tests require sometimes relatively intensive daily attention, and even a minor problem with the test system (e.g., in terms of water/chemical delivery) or the animals (e.g., disease outbreak) can result in a failed assay. Such an event can be especially costly if it occurs several months into an otherwise successful experiment. Given this caveat, full-life cycle tests with fish have been, and likely will continue to be, relatively rarely used for routine regulatory testing for EDCs (or other classes of chemicals).

Nevertheless, full-life cycle tests have an important role in the overall testing process. First, there are/will be high-priority EDCs (e.g., in terms of production/discharge volume and/or potential toxicity) for which full-life cycle dose-response data are necessary for the purpose of ecological

risk assessment. A current example of such chemicals are high-potency steroidal estrogens (estradiol, ethinylestradiol), as well as alkylphenols, associated with feminization of fish exposed to municipal effluents (Desbrow et al. 1998; Purdom et al. 1994; Routledge et al. 1998). Second, full-life cycle tests are valuable for identifying potentially sensitive life stages/endpoints on which to base shorter-term tests, and can be used to validate the biological significance of these endpoints in terms of adverse effects on development and reproduction.

Several full-life cycle tests with fish have focused on estrogenic EDCs. For example, Länge and coworkers (2001) conducted a 305-day test with the fathead minnow and reported a no-observable effect concentration for ethinylestradiol effects on reproduction of 1 ng/L (parts-per-trillion) in the water. They reported occurrence of testis-ova and vitellogenin induction, respectively, in fish exposed to 4 and 16 ng/L of the estrogen. Yokota and colleagues (2001) described a medaka full-life cycle test with 4-nonylphenol, another chemical associated with feminization of fish collected from near outfalls of some types of effluents. Based on the occurrence of testis-ova, they reported lowest-observable and no-observable effects concentrations of 17.7 and 8.2 μg/L (parts-per-billion) in the F_0 fish. The F_1 fish appeared to be more sensitive to induction of testis-ova than the parental fish in that a significant occurrence of the pathology was noted at 8.2 μg/L.

The finding of a seemingly greater sensitivity of F_1 compared with F_0 animals exposed to another weak estrogen agonist, in this case 4-*tert*-pentylphenol, was mirrored in a medaka full-life cycle study conducted by Seki and coworkers (2003b), who also reported that vitellogenin induction was the most sensitive endpoint evaluated in both generations. Gray and colleagues (1999b) performed a medaka full-life cycle test with 4-*tert*-octylphenol, and described reductions in courtship behavior of exposed males as well as various malformations during early development of the F_1 generation. Segner and coworkers (2003) conducted a zebrafish full-life cycle test with three different estrogen receptor agonists that focused on fertilization success of eggs from the F_0 as a marker of estrogenic effects (Segner et al. 2002), and reported a rank order potency for the three chemicals of ethinylestradiol>>4-*tert*-octylphenol>bisphenol A.

Seki and colleagues (2004) also assessed one androgenic EDC in a fish full-life cycle test. They reported that methyltestosterone masculinized both parental and F_1 female medaka exposed to concentrations of the synthetic androgen on the order of about 10 to 30 ng/L.

In addition to full-life cycle designs, the literature includes descriptions of multigenerational protocols for EDC studies with fish (e.g., Nakari and Erkomaa 2003; Patyna et al. 1999). Multigenerational test methods have been proposed, in part, because of observations made in studies such as those by Yokota and coworkers (2001) and Seki and colleagues (2003b), which suggest a progressively increasing sensitivity to (estrogenic) EDCs in successive generations. Multigenerational designs typically incorporate an F_2 generation, which undoubtedly would better account for possible EDC transgenerational effects, especially those that might be related to maternal transfer of contaminants (Metcalfe et al. 2000; Nirmala et al. 1999; Olsson et al. 1999). However, the degree to which it is reasonable to justify the extra resource investments entailed by multigenerational testing for routine assessments of EDCs is uncertain. Further research on this topic is ongoing at our laboratory and others' around the world.

Summary and Conclusions

Both from ecological effects and species extrapolation perspectives, fish tests are an important component of EDC screening and testing programs. Partial- and full-life cycle tests with fish that are focused on key aspects of reproduction and development not only provide a basis for quantitative predictions of ecological risk of EDCs to fish populations but, through consideration of endpoints that are sensitive and diagnostic for different classes of EDCs, serve as effective generalized models for identifying chemicals that affect specific components of the vertebrate HPG axis. In this review, we specifically emphasize three small fish species—fathead minnow, medaka, and zebrafish—that have received a significant amount of international attention relative to EDC testing. However, other small fish species such as the guppy (*Poecilia reticulata;* Bayley et al. 2002), as well as more traditional large fish models such as the rainbow trout and carp, also could prove useful for focused studies with EDCs. Based on progress in this area, it is clear that fish models will continue to play an important role both in research and regulation of EDCs.

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