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Endocrine disrupting chemicals in fish: Developing exposure indicators and predictive models of effects based on mechanism of action

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ABSTRACT

Knowledge of possible toxic mechanisms (or modes) of action (MOA) of chemicals can provide valuable insights as to appropriate methods for assessing exposure and effects, thereby reducing uncertainties related to extrapolation across species, endpoints and chemical structure. However, MOA-based testing seldom has been used for assessing the ecological risk of chemicals. This is in part because past regulatory mandates have focused more on adverse effects of chemicals (reductions in survival, growth or reproduction) than the pathways through which these effects are elicited. A recent departure from this involves endocrine-disrupting chemicals (EDCs), where there is a need to understand both MOA and adverse outcomes. To achieve this understanding, advances in predictive approaches are required whereby mechanistic changes caused by chemicals at the molecular level can be translated into apical responses meaningful to ecological risk assessment. In this paper we provide an overview and illustrative results from a large, integrated project that assesses the effects of EDCs on two small fish models, the fat-head minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*). For this work a systems-based approach is being used to delineate toxicity pathways for 12 model EDCs with different known or hypothesized toxic MOA. The studies employ a combination of state-of-the-art genomic (transcriptomic, proteomic, metabolomic), bioinformatic and modeling approaches, in conjunction with whole animal testing, to develop response linkages across biological levels of organization. This understanding forms the basis for predictive approaches for species, endpoint and chemical extrapolation. Although our project is focused specifically on EDCs in fish, we believe that the basic conceptual approach has utility for systematically assessing exposure and effects of chemicals with other MOA across a variety of biological systems.

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1. Background

Prospective ecological risk assessments of most chemicals typically are conducted with little consideration for toxic mechanisms

(or modes) of action (MOA). Testing for ecological effects usually includes a wide array of species and endpoints, with a focus primarily on apical responses. When little is known about the properties of a test chemical, this is a pragmatic approach; however, substantial benefits can be realized by basing testing and subsequent risk management decisions on known or probable MOA. For example, a priori knowledge of MOA can lead to identification of mechanism-based (and, hence, stressor-specific) molecular indicators that can potentially be linked to environmental concentrations and used to inform exposure assessments. Furthermore, knowledge of MOA can

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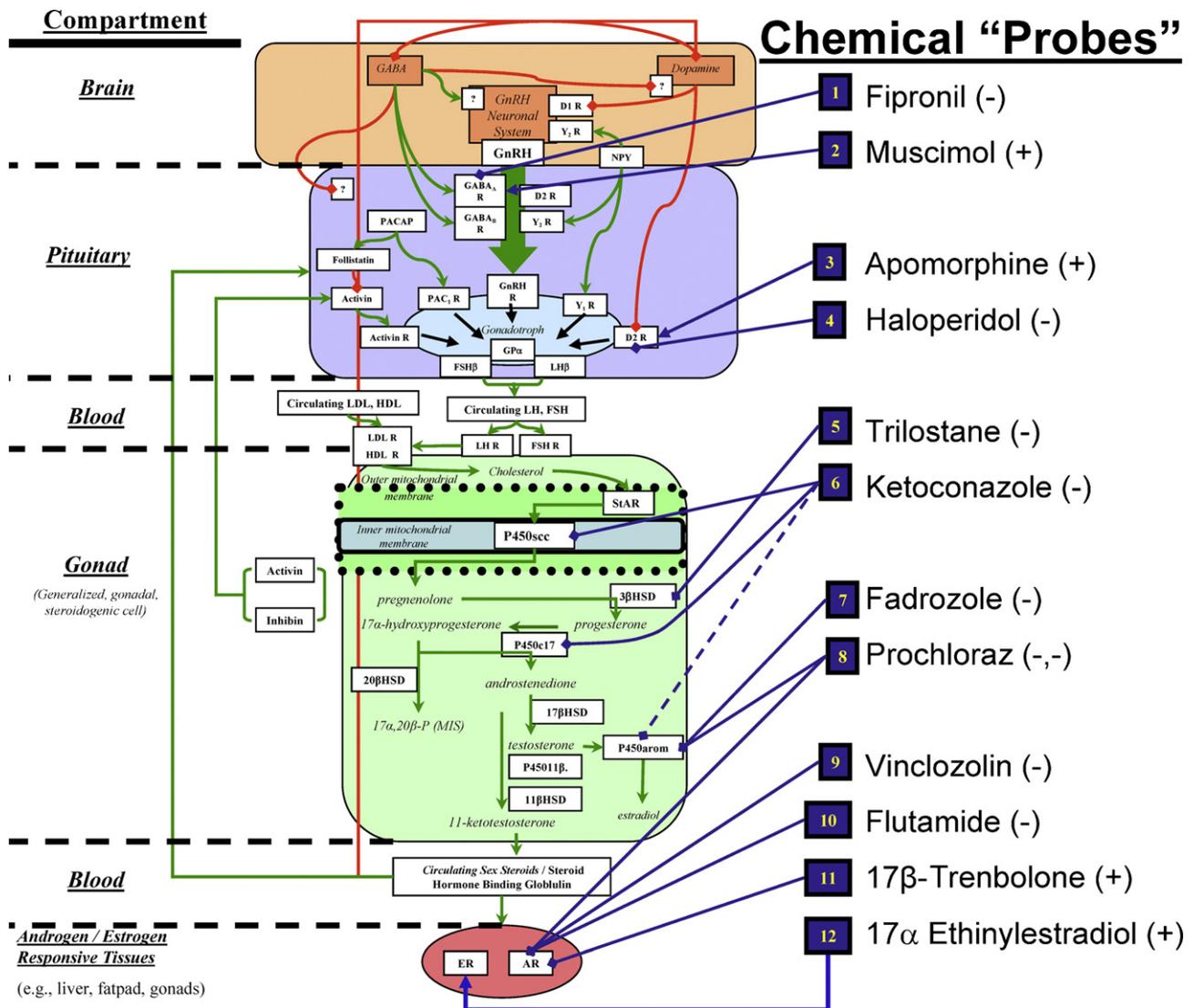


Fig. 1. Overview of the fish hypothalamic–pituitary–gonadal (HPG) axis, and experimental chemical “probes” with different mechanisms of action. The “+” or “-” shown in parentheses indicate, respectively, stimulation or inhibition of a particular target (enzyme or receptor) by the test chemical. See text for further details. Depiction of the HPG axis is adapted from Villeneuve et al. (2007b).

serve as a basis for effective extrapolation of biological effects across species, biological levels of organization, and chemical structures. This information can help identify potentially sensitive responses, and even species prior to extensive testing, thereby optimizing time and resource use (Bradbury et al., 2004).

Endocrine-disrupting chemicals (EDCs) represent a comparatively recent departure from past regulatory activities with toxic compounds in that there is a need to know both MOA and potential adverse effects. There have been several definitions of EDCs from a MOA perspective, ranging from (in the most limited sense) chemicals which are estrogenic (specifically, estrogen receptor agonists) to (in the broadest sense) “an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes” (Kavlock et al., 1996). From a regulatory perspective, the definition currently most widely used for EDCs encompasses agents that cause alterations in reproduction or development through direct effects on the vertebrate hypothalamic–pituitary–thyroidal or hypothalamic–pituitary–gonadal (HPG) axes (USEPA, 1998).

Due to the emphasis on MOA, consideration of EDCs in current testing and regulatory frameworks has been challenging. For exam-

ple, it is important that tests include responses other than apical endpoints if the assays are to be indicative of specific MOA. However, mechanism-specific endpoints are not necessarily predictive of an adverse biological outcome, and it is problematic to intensively regulate a chemical that does not cause adverse effects even if it does, for example, activate the estrogen receptor (ER). A common (and logical) approach to addressing this seeming dilemma has been the development of tiered testing frameworks that use short-term assays first to identify chemicals as possessing a MOA of concern before proceeding with longer-term tests better suited to quantifying adverse effects (e.g., USEPA, 1998). However, even relatively efficient tiered testing programs for EDCs may not be sustainable in terms of resources (or timeliness) if hundreds or thousands of chemicals need to be assessed using long-term assays.

The efficiency of EDC testing programs could be enhanced through the use of emerging technologies in the areas of genomics and computational biology to provide mechanistic insights as to exposures and possible adverse effects in animals, such as fish (e.g., Ankley et al., 2006; Hoffmann et al., 2006, 2008; Hook et al., 2006; Samuelsson et al., 2006; Filby et al., 2007; Martyniuk et al., 2007). This type of approach is consistent with recent recommendations from the National Research Council (NRC, 2007), who suggest a

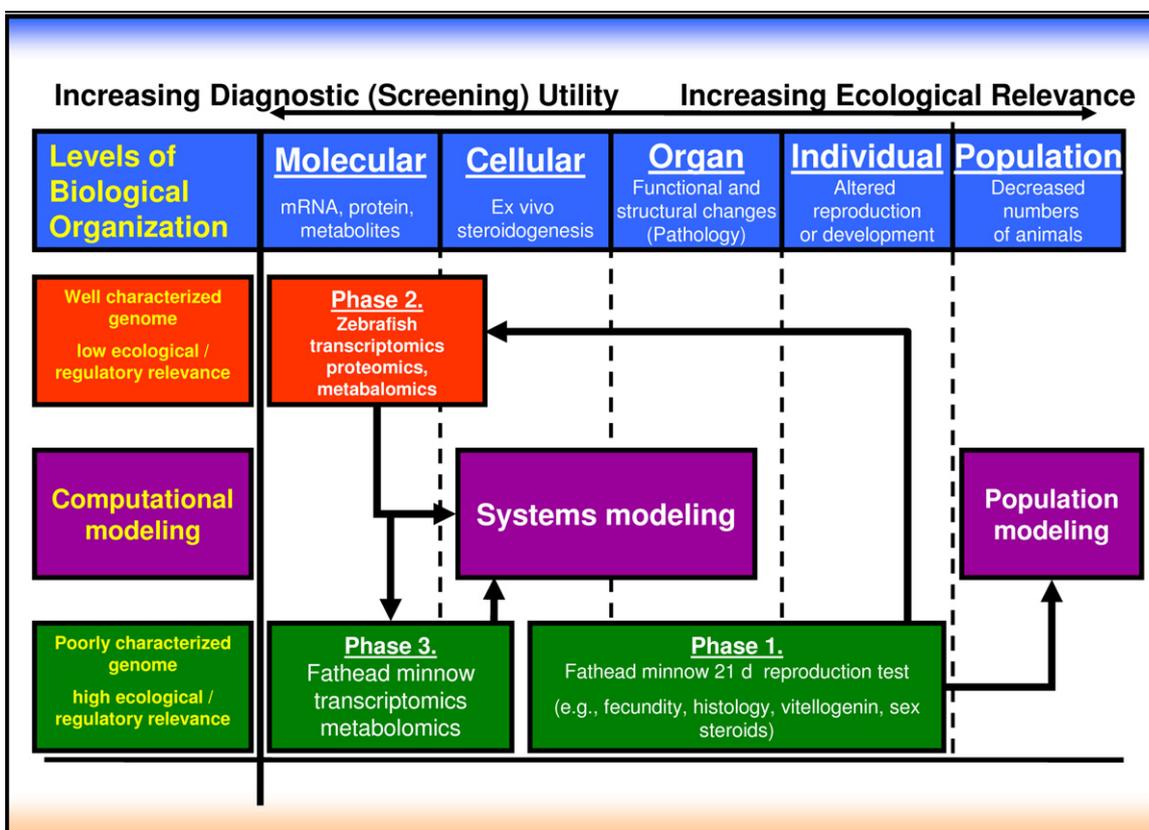


Fig. 2. Conceptual linkages across biological levels of organization for effects of endocrine-disrupting chemicals in fish. Different research phases of the project are aligned to reflect where/how they address these linkages and arrows reflect the flow of information. See text for further details.

shift toward greater use of short-term (e.g., in vitro) assays and predictive toxicology tools for assessment of human health risks of chemicals. In this paper we describe a research effort to support the development of approaches for assessing chemicals with the potential to impact the HPG axis of fish. These approaches, which could encompass techniques ranging from computational models to in vitro assays and short-term in vivo tests, would help provide regulatory agencies throughout the world with cost-effective, predictive tools for monitoring and testing EDCs.

This is a large, highly-integrated project that includes government, academic and industry scientists from several laboratories across North America. In this paper, we describe the conceptual basis of the approach we have employed and present illustrative results. The information provided herein is necessarily brief; for further detail on methods and results, interested readers should consult the indicated citations or contact us directly, as many aspects of the data collection/analyses are ongoing.

2. Experimental overview

The basic approach used for our work involves perturbation of the HPG axis with chemical probes known or hypothesized to impact different key control points, ranging from neurotransmitter receptors in the brain to steroid hormone receptors in gonads (Fig. 1). Following perturbation of the axis by chemicals with different MOA, information is collected at multiple biological levels of organization, ranging from molecular changes to apical responses (i.e., reproductive success), and even (via modeling) to likely population-level effects (Fig. 2). This type of integrated analysis facilitates a mechanistic understanding of the effects of HPG-active chemicals from the molecular to whole-organism levels from a toxicity pathway perspective (Bradbury et al., 2004).

2.1. Organisms

The experimental organisms for this research are small fish. There are several different EDC testing programs being implemented throughout the world, and most include fish assays (Ankley and Johnson, 2004). A pragmatic reason for this is that there are clearly documented adverse impacts of EDCs on fish populations in the field; this differs from the situation in humans where exposure to, and subsequent effects of environmental EDCs tend to be more uncertain (WHO, 2002). In addition, in terms of animal availability (e.g., generation of large numbers of high-quality organisms at suitable life-stages), chemical exposure dynamics and biological flexibility, small fish species are well suited for mechanistic studies with chemicals such as EDCs (Stoskopf, 2001, and references therein; Ankley and Johnson, 2004). Significantly, although there are some unique aspects of fish reproductive endocrinology, the basic structure and function of the HPG axis across all vertebrates tends to be well conserved. Hence, the results of fish studies with EDCs potentially can serve as the basis for effective cross-species extrapolation of potential effects.

Our research utilizes the zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*), two small cyprinids that have complementary attributes that make them useful for this work. The genome of the zebrafish is fully sequenced, thus reducing bioinformatic challenges when evaluating alterations in gene and protein expression (Hill et al., 2005). As such, the zebrafish is a useful model for exploratory or hypothesis-generating work focused on the effects of EDCs with different MOA on response profiles of genes and proteins (Hoffmann et al., 2006, 2008). In contrast to zebrafish, the fathead minnow has a rich history of use in regulatory programs in the US, including testing for EDCs (Ankley and Villeneuve, 2006). In addition to its relevance to regulatory activities, a fair

amount is known about basic reproductive biology in the fathead minnow, thus providing a basis for “anchoring” observed alterations in gene, protein or metabolite expression caused by test chemicals to phenotypic changes in gonad histology and reproductive success.

2.2. Test chemicals

Test chemicals used for the work include those that (could) impact HPG function relatively “high” in the axis, such as muscimol (a pharmaceutical) and fipronil (an insecticide) which act, respectively, as an agonist and antagonist of specific GABA (gamma-amino butyric acid) receptors (Fig. 1). The drugs apomorphine and haloperidol act as an agonist and antagonist, respectively, of dopamine receptors (D2) involved in the release of gonadotrophic hormones from the pituitary. The fungicides ketoconazole and prochloraz, and the pharmaceuticals trilostane and fadrozole inhibit one or more enzymes involved in steroid biosynthesis in the gonad, including reactions catalyzed by 3β -hydroxysteroid dehydrogenase (3β HSD) and different cytochromes P450 (CYPs). Finally, several chemicals that directly impact hormone receptors located in the gonad and other steroid-responsive tissues are being tested, including 17α -ethinylestradiol and 17β -trenbolone, potent synthetic steroidal agonists of the ER and androgen receptor (AR), respectively, and vinclozolin (a fungicide) and flutamide (a pharmaceutical), which antagonize the AR (Fig. 1). Although several of these chemicals do occur in the environment as contaminants (e.g., the pesticides and synthetic steroids), others are less likely to do so (some of the drugs). Overall, our strategy in selection of test chemicals was not necessarily to focus on known environmental contaminants, but to perturb HPG pathways of known (or potential) biological relevance.

2.3. Phased testing

Sexual development, including gonad differentiation, during larval and juvenile life-stages, and reproduction in mature adults offer two “windows” of enhanced sensitivity of fish to EDCs (Ankley and Johnson, 2004). For this research, the adult life-stage was chosen because we felt that the substantial alterations in gene and protein expression and metabolite profiles that occur during early development might complicate understanding of the effects produced by the test chemicals. However, due to the known sensitivity of fish to EDCs during sexual development, studies of the type described herein encompassing this life-stage also would be desirable.

For our research, three different types of studies—termed Phases 1, 2 and 3—are conducted on adult fish exposed to the various HPG-active chemicals (Fig. 2). In Phase 1 studies, each chemical is tested in a standardized 21-d reproduction assay with the fathead minnow using flow-through (water) exposures and measured chemical concentrations to produce a high-quality exposure/effects dataset. Endpoints measured in the Phase 1 studies span a wide range of biological levels of organization, including determination of plasma concentrations of sex steroids (testosterone, 17β -estradiol, 11-ketotestosterone) and vitellogenin (Vtg; egg yolk protein precursor), gonad size and histopathology, secondary sex characteristics, reproductive behavior, fecundity, fertility and hatchability (Ankley et al., 2001). In addition, the Phase 1 studies incorporate analyses of a small complement of genes (measured via quantitative real-time polymerase chain reaction; PCR) known to be involved in HPG function/control, and hypothesized to be impacted by the chemical exposure (Villeneuve et al., 2007a,b). Information from the Phase 1 studies is subsequently used for three primary purposes, to: (a) aid in design (e.g., selection of test chemical concentrations) for subsequent, shorter-term Phase 2 and 3 assays, (b) generate information for systems and population modeling, and (c) provide

a robust phenotypic dataset for anchoring the various genomic responses collected in subsequent testing (Fig. 2).

Phase 2 tests are short-term assays conducted with zebrafish in which samples from multiple tissues (gonad, liver and brain) are collected after 1, 2 and 4 d of exposure to the test chemicals. The samples are used for genomic measurements, with an emphasis on gene expression determined using commercially-available 22,000 or $4 \times 44,000$ gene microarrays (Agilent, Palo Alto, CA, USA; Wang et al., 2008a,b) as well as hypothesis-driven and microarray-confirmatory PCR analyses. A subset of the zebrafish samples also are analyzed for alterations in protein expression using two-dimensional (2-D) Fluorescence Difference Gel Electrophoresis (Ettan™ DIGE) technology (G.E. Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Overall, information from the Phase 2 studies provides insights on relationships between gene and protein expression, and helps identify candidate indicators/markers of EDC exposure and effects for subsequent evaluation in Phase 3 fathead minnow studies focused on temporal changes in the HPG axis (Fig. 2).

The HPG axis is a highly dynamic system capable of responding to environmental stressors, including contaminants, through various feedback mechanisms to maintain conditions conducive to reproduction. These types of compensatory responses can occur both during exposure to the stressor, and after the stressor has been removed. This, coupled with the fact that changes in some endpoints (e.g., gene expression) can be rapid and/or transitory, dictates a need for temporal studies to develop robust exposure indicators and predictive models. The Phase 3 studies in our research address this through systematic time-course experiments with the fathead minnow. In these studies, animals are sampled after 1, 2, 4 and 8 d of exposure to the various test chemicals, as well as 1, 2, 4 and 8 d after cessation of exposure. A variety of endpoints are examined, including a subset of those considered in the Phase 1 studies such as plasma steroid and Vtg concentrations, gonad histopathology and secondary sexual characteristics. Gene expression is evaluated using both targeted (PCR) assays (e.g., Villeneuve et al., 2007a), and through microarray analysis, using custom microarrays constructed on an Agilent platform (N. Denslow, unpublished data). Among other uses, the time intensive microarray analyses in Phase 3 studies provide data for reverse engineering of transcriptional networks within the HPG axis (di Bernardo et al., 2005). Changes in biological networks can be particularly useful in discerning MOA and mechanisms of response and compensation. Finally, Phase 3 samples are used for examination of changes in protein expression (based on targets from the Phase 2 studies), and metabolomic analyses via nuclear-magnetic resonance (NMR) and mass spectroscopy (MS) techniques (Ekman et al., 2007, 2008, in press). Information from Phase 3 serves a number of overall purposes, including (a) directly identifying linkages between changes in gene, protein and endogenous metabolite profiles; (b) relating these genomic changes to apical endpoints such as histopathology; (c) evaluating the consistency in responses to EDCs across species (zebrafish, fathead minnows) exposed under the same conditions; (d) providing information as to temporal alterations in a stressed (and unstressed or recovering) system as a basis for modeling HPG axis function; (e) evaluating the rapidity and persistence of potential indicator responses identified in earlier phases of testing; and (f) developing dynamic models to understand feedback control and compensation for stress (Fig. 2).

3. Insights from experimental work

3.1. Phase 1

Table 1 summarizes the fathead minnow 21-d reproduction tests that have been conducted to date and, where available, provides ref-

Table 1
Overview of reproductive toxicity to the fathead minnow of chemicals with differing MOA in the hypothalamic–pituitary–gonadal (HPG) axis.

Test chemical	Presumptive HPG target(s) ^a	Reproduction LOEC ^b	Reference
Fipronil	GABA receptor antagonist	>5	Kahl et al. (2007)
Muscimol	GABA receptor agonist	NC ^c	–
Apomorphine	D2 receptor agonist	NC	–
Haloperidol	D2 receptor antagonist	>20	Villeneuve et al. (in preparation)
Trilostane	3βHSD inhibitor	1500	Villeneuve et al. (2008)
Ketoconazole	CYP11A/CYP17 inhibitor	25	Ankley et al. (2007)
Fadrozole	CYP19 inhibitor	2	Ankley et al. (2002)
Prochloraz	CYP17/19 inhibitor	100	Ankley et al. (2005)
Vinclozolin	AR antagonist	60	Martinović et al. (2008)
Flutamide	AR antagonist	500	Jensen et al. (2004)
Trenbolone	AR agonist	0.05	Ankley et al. (2003)
Ethinylestradiol	ER agonist	NC	–

^a Abbreviations used: GABA, gamma-amino butyric acid; D2, dopamine; 3βHSD, 3β-hydroxysteroid dehydrogenase; CYP11A, cytochrome P450c11 (side-chain-cleavage); CYP17, cytochrome P450c17,20-lyase; CYP19, cytochrome P450 aromatase; AR, androgen receptor; ER, estrogen receptor.

^b Lowest-observable effect concentration (LOEC) for egg production in 21-d tests. Values are nominal water concentrations provided in μg/L.

^c NC, not conducted/completed.

erence information for the completed studies. In terms of exposure concentrations that cause impacts on reproductive health, the test chemicals span a wide range of potency and efficacy, ranging from trenbolone which significantly decreased egg production at a water concentration of 0.05 μg/L (Ankley et al., 2003), to trilostane which affected egg production at a concentration of 1500 μg/L (Villeneuve et al., 2008). Some of the test chemicals (e.g., fipronil, haloperidol) did not cause marked effects on reproductive endocrine function, even when tested at concentrations at maximum water solubility, or within a factor of five of those that produced toxicity in short-term range-finding assays. When effects were observed, biochemical and apical responses in the 21-d test generally reflected the anticipated MOA of the test chemicals. For example, consistent with activation of the AR, trenbolone caused morphological masculinization of female fathead minnows, while the AR antagonist, vinclozolin, demasculinized males (Ankley et al., 2003; Martinović et al., 2008). Although different enzymes were affected, inhibitors of steroidogenesis (fadrozole, prochloraz, trilostane) all decreased Vtg concentrations in female fish due to a depression in synthesis of estradiol (Ankley et al., 2002, 2005; Villeneuve et al., 2008).

A critical role of the Phase 1 studies in the overall project is delineation of hypothesized toxicity pathways across biological levels of organization, such that the Phase 2 and 3 transcriptomic, proteomic and metabolomic data can be mechanistically linked to higher-level apical responses. The fadrozole data provide a particularly good example of how this is achieved (Fig. 3). Fadrozole was developed to treat breast cancer as a relatively specific inhibitor of CYP19 aromatase, the enzyme that catalyzes conversion of testosterone to estradiol. The pharmaceutical decreases brain and ovarian aromatase activity in vitro and in vivo in the fathead minnow, and produces a corresponding decrease in circulating plasma estradiol concentrations in female fish (Ankley et al., 2002; Villeneuve et

al., 2006). This, in turn, translates into a decreased circulating concentration of Vtg (which is produced in the liver via activation of the ER) in the females and, ultimately, decreased deposition of the lipoprotein in the developing oocytes. This corresponds with significant reductions in fecundity of fadrozole-exposed fish, resulting in complete cessation of egg production at higher fadrozole exposure concentrations (Fig. 3). As described in greater detail below, these laboratory fecundity data can then be taken, via modeling, one step further to predict likely population-level responses of fish exposed to HPG-active chemicals (Fig. 2).

In addition to providing baseline effects and toxicity pathway data for the various chemicals, several other significant observations have been made in the Phase 1 studies. One of these involves indirect changes in the HPG axis in response to certain EDCs. For example, evidence for at least some degree of compensation within the axis comes from 21-d tests with three of the test chemicals, trenbolone, vinclozolin and ketoconazole, with the latter providing the most complete demonstration of the phenomenon (Ankley et al., 2007). Ketoconazole is a pharmaceutical that decreases fungal growth through inhibition of an ergosterol (cell wall component) biosynthesis step catalyzed by CYP51. However, ketoconazole is not particularly specific to CYP51, and can inhibit a variety of vertebrate CYPs involved in xenobiotic metabolism and steroid biosynthesis. In fact, the fungicide is considered a model inhibitor of testosterone production in mammals (Feldman, 1986). In the fathead minnow, ketoconazole decreased fecundity in 21-d tests and, consistent with its anticipated MOA, inhibited testosterone production by gonad tissue from both males and females. However, after a continuous 21-d exposure, this inhibition was not translated into decreased circulating testosterone (or estradiol) concentrations in vivo in the fish, suggesting that the animals were somehow able to compensate for effects of the fungicide. This response was manifested in several

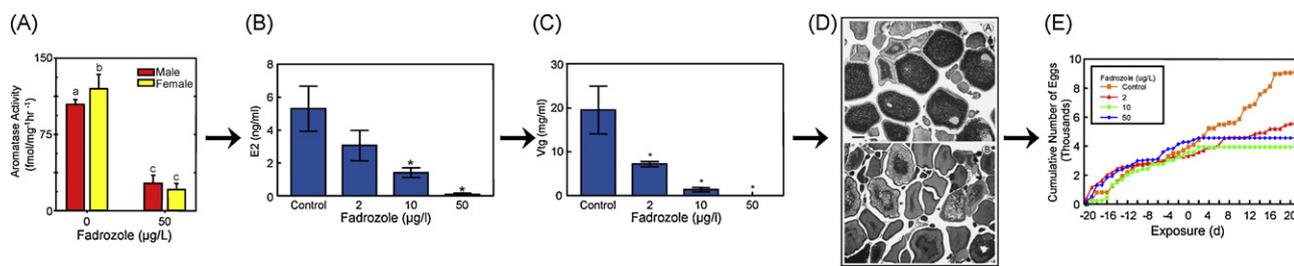


Fig. 3. Example of linkage of effects across biological levels of organization for a model endocrine-disrupting chemical, the aromatase inhibitor fadrozole (2, 10, 50 μg/L water), tested in a 21-d reproduction assay with the fathead minnow. Panels (from left to right) depict (A) inhibition of aromatase activity in male and female fish, (B) decrease in plasma estradiol (E2) concentrations in female fish, (C) depression in plasma vitellogenin (Vtg) concentrations in exposed females, (D) decreased Vtg deposition in the ovary (compare the amount of dark staining material in the top [control] versus bottom [treated] fish), and (E) reduction in cumulative egg production in the fish. Results from Ankley et al. (2002).

different ways (Ankley et al., 2007). For example, in males there was more than a two-fold increase in relative gonad weight, accompanied by a proliferation of testicular Leydig cells (responsible for steroid production), and up-regulation of genes coding for two key steroidogenic enzymes, CYP11A and CYP17, both of which could be specific targets of ketoconazole. The net result of these alterations was that circulating steroid concentrations in the fish did not differ from controls after 21 d of exposure to the fungicide. Understanding the basis of possible compensatory responses within the HPG axis clearly is needed to identify reliable exposure indicators and develop approaches to predict adverse effects of EDCs; achieving this understanding is a critical component of the Phase 3 studies of the overall project.

3.2. Phase 2

Phase 2 zebrafish exposures and subsequent microarray measurements have been completed for all the chemicals shown in Fig. 1. Initial analysis of the microarray data has been described for three of the chemicals: ethinylestradiol, trenbolone and fadrozole (Wang et al., 2008a,b). One goal of the Phase 2 research was to determine a flexible and efficient microarray experimental design to (1) characterize the zebrafish transcriptome, and (2) identify an optimal combination of gene feature selection/class prediction algorithms for evaluating gene expression changes caused by EDCs with different MOA. An unbalanced, incomplete block microarray experimental design was tested using various tissues of individual zebrafish exposed to fadrozole, trenbolone, or ethinylestradiol (Wang et al., 2008a). Based on the high microarray reproducibility/low variability, low gene-specific dye bias, and good similarity between microarray and PCR profiles observed, the design appears well suited to these and other ecotoxicogenomic studies. Hyperspectral imaging identified a cyanine 3-background contaminant, and correction of this fluorescence contamination reduced the variability of weakly expressed genes, which constitute a significant portion of the zebrafish transcriptome (Wang et al., 2008a). Evaluation of several methods for gene classifier (indicator) discovery determined that the optimal gene feature selection method (of those tested) for reducing the dimensionality of microarrays was via a genetic algorithm (GA), with the best prediction algorithm of those evaluated, support vector machine (SVM; Wang et al., 2008b). These algorithms are being applied in subsequent microarray experiments to identify multi-gene expression profiles (classifiers) capable of discriminating exposures to EDCs acting through varying MOA based on microarray responses. As an example, the preliminary analysis with the three chemicals identified classifiers that discriminated exposures to fadrozole, trenbolone, and ethinylestradiol, with the first two chemicals clustering more closely to one another as chemicals that depress rather than elevate plasma estrogen activity (Wang et al., 2008b).

Beyond identification of effective microarray experimental design and analysis approaches, the Phase 2 zebrafish experiments fulfill two critical roles. First, they provide a means to comprehensively interrogate the large number of transcripts that code for proteins known or hypothesized to play key roles in the regulation of the teleost HPG axis. As opposed to real-time PCR and similar approaches that target a single or relative handful of genes, microarrays can be used to survey hundreds or thousands of components of a biological system (represented by transcripts) and simultaneously evaluate their response to various stressors. This type of approach provides the basis for conducting a hypothesis-driven investigation of the response of an entire system and a means to test and refine biologically-based systems models that may ultimately be applied to predictive risk assessment (e.g., Villeneuve et al., 2007b).

The second critical role of the zebrafish experiments is discovery. Whereas Phase 1 studies examine only those endpoints selected

by the investigators based on some prior knowledge or hypotheses, the microarray and proteomic analyses conducted as part of the Phase 2 experiments are unsupervised. Because of the ability to screen hundreds (in the case of proteomics) or thousands (in the case of microarrays) of endpoints/targets, data from Phase 2 studies can be used to identify novel responses to the stressors examined. By examining gene ontologies and pathways associated with differentially expressed genes or proteins, it is possible to identify a broad spectrum of processes and/or targets that are impacted either directly or indirectly by the chemical stressor. Such knowledge can lead to an improved understanding of the overall biological impact of the stressor, and may also aid the identification of novel indicators (biomarkers) of exposure and/or effects. Hypotheses and putative indicators that emerge from the Phase 2 analyses are being tested in a supervised fashion in the subsequent Phase 3 experiments, examining the robustness of the observations both between experiments and among species. Through the combination of hypothesis- and discovery-driven analyses, Phase 2 experiments test our overall systems model, expand on the analyses conducted in Phase 1, and provide a foundation for novel hypothesis testing in Phase 3.

3.3. Phase 3

Phase 3 tests have been conducted with six chemicals to date: fadrozole, trenbolone, prochloraz, vinclozolin, flutamide and trilostane. In addition, a preliminary Phase 3 like exposure with ethinylestradiol using fewer sampling times and with a primary focus on metabolomic measurements has been completed (Ekman et al., 2008). Although much of the information associated with these studies (e.g., gene expression) is still being assembled and analyzed, some intriguing observations have already emerged.

Compared to transcriptomic measurements, metabolomic analyses have received less attention in the field of ecotoxicology (Lin et al., 2006). However, knowledge of profiles of endogenous metabolites can provide important information concerning chemical MOA, thereby helping to identify exposure indicators and define toxicity pathways. In addition, compared to transcriptomics and proteomics, metabolomic analyses are relatively inexpensive and amenable to high-throughput, which enables a comparatively large number of samples to be processed. This is an important attribute for time-course studies, such as the Phase 3 work. Initial metabolomic studies by Ekman et al. (2007) demonstrated the feasibility of NMR-based analyses of urine samples from the fathead minnow to assess impacts of the anti-androgen vinclozolin on metabolite profiles. The use of urine in such studies not only allows one to assess important metabolic endpoints, but also provides the potential for non-invasive and repeated sampling from individual fish over time. In more recent work, Ekman et al. (2008, in press) demonstrated the potential for metabolomic measurements to provide novel insights about responses of the fish HPG axis to chemical stressors. Adult fathead minnows of both sexes were exposed to two different concentrations of ethinylestradiol, and animals were sampled after 1, 4 and 8 d of exposure, and 8 d after termination of the exposure. NMR evaluation of polar metabolites in livers of the fish revealed a greater impact of the estrogen on males than females; in addition, the metabolite profile in exposed males reflected a “feminization” response, in that the profile assumed similarities to that of female fathead minnows (2008). Assessment of the metabolomic data using partial least-squares discriminant analysis revealed that response trajectories in the males showed evidence of compensation of the fish during the ethinylestradiol exposure, as well as a marked recovery after cessation of exposure to the estrogen (Fig. 4). Evaluation of other more traditional endpoints in the fish (changes in plasma Vtg concentrations and secondary sex characteristics) indicated fem-

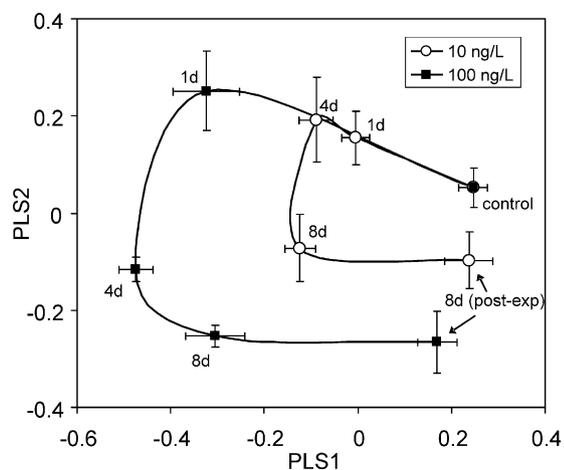


Fig. 4. Exposure response trajectory plots for male fathead minnows exposed to 17 α -ethinylestradiol for 1, 4, or 8 d, followed by 8 d of depuration (i.e., “post-exp”) during which the fish were maintained in water without test chemical. Exposures were conducted using two ethinylestradiol concentrations (either 10 or 100 ng/L) delivered via a continuous flow-through system. The scores plot shown was generated using the first two components (i.e., PLS1 and PLS2) of a validated partial-least squares discriminant analysis (PLS-DA) model built using NMR spectral data acquired from the livers of these fish. Each point represents the average score value for a given class ($n=7$ or 8), shown with its associated standard error. *Note:* the controls across all time points showed relatively little variation and thus were modeled as a single class. Results from Ekman et al. (2008).

inization of the males, as well as (in the case of secondary sex characteristics) recovery following termination of exposure, confirming that alterations observed in metabolite profiles are a robust indicator of the physiological state of the animals exposed to the estrogen. Ekman et al. (in press) also evaluated the non-polar fraction of hepatic metabolites from the ethinylestradiol study, and noted a number of alterations in lipid profiles associated with exposure to the estrogen. Ongoing MS-based metabolomic studies are focused on assessing changes in sex steroids and steroid precursors in fish exposed to EDCs for differing periods of time. Overall, the types of temporally-intensive data collected from NMR- and MS-based metabolomic analyses can be used to better understand how exposure parameters—such as chemical concentration, frequency, and duration—influence adverse outcomes. This new understanding can help regulators differentiate chemical exposures that have a lasting and detrimental biological effect from those that are either not effective, or those to which an organism can adapt (albeit with some potential cost to the organism).

Data from the fadrozole Phase 3 study also provide insights as to compensatory responses of the HPG axis (Villeneuve et al., in press). As would be expected based on the MOA of fadrozole (described above), exposure to the drug caused rapid (within 1 d), concentration-dependent reductions in estradiol production in ex vivo assays with ovary tissue held in culture (detailed methods for the ex vivo assay can be found in Ankley et al. (2007) and Martinović et al. (2008)), and plasma concentrations of both estradiol and Vtg in female fish (Fig. 5). However, by the eighth day of the exposure period, ex vivo estradiol production had returned to control levels, and plasma estradiol concentrations had also recovered to control levels in the fish exposed to 3 $\mu\text{g/L}$ fadrozole/L, albeit not in those exposed to 30 $\mu\text{g/L}$ (Fig. 5). This apparent compensation coincided with significant concentration-dependent increases in the abundance of mRNA transcripts coding for aromatase (CYP19A isoform), CYP11A, steroidogenic acute regulatory protein and follicle stimulating hormone receptor (Villeneuve et al., in press). Shortly after cessation of the fadrozole exposure, there was a rapid recovery of plasma estradiol concentrations in the fish, and a gradual recovery of plasma Vtg concentrations, even

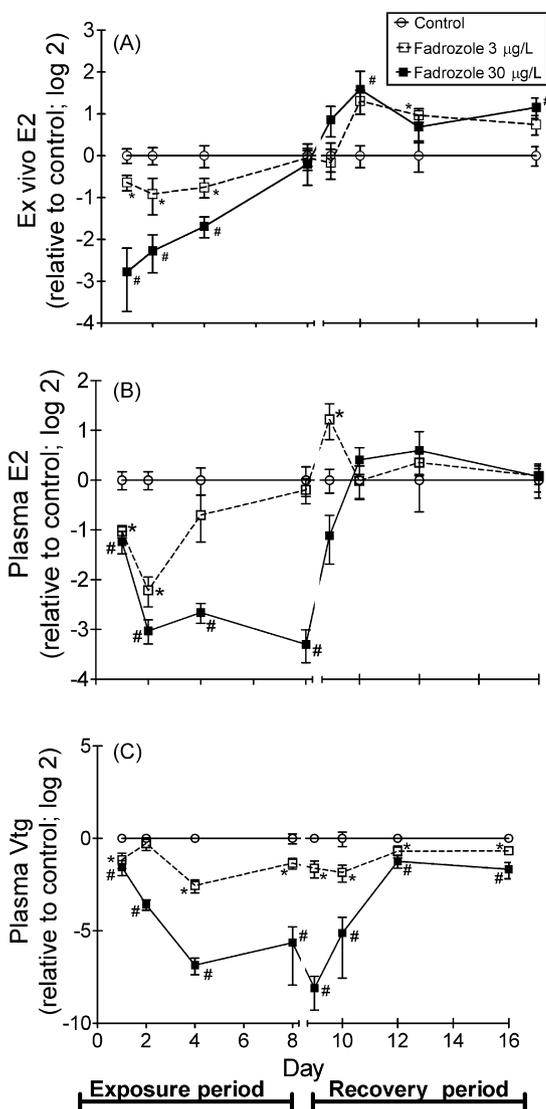


Fig. 5. (A) Ex vivo estradiol (E2) production, (B) plasma E2, and (C) plasma vitellogenin (Vtg) measured in female fathead minnows exposed to 0, 3, or 30 $\mu\text{g/L}$ fadrozole/L and sampled after 1, 2, 4, or 8 d of exposure or 1, 2, 4, or 8 d after cessation of exposure (days 9, 10, 12, 16, respectively; recovery period). Data are expressed as fold change (log 2) relative to the control mean measured on a given day. Error bars indicate standard error. The * and # indicate statistically significant difference from the control for the 3 and 30 $\mu\text{g/L}$ treatments, respectively ($p < 0.05$). Results from Villeneuve et al. (in press).

in the 30 $\mu\text{g/L}$ group. In fact, in the 3 $\mu\text{g/L}$ treatment, there was a brief period of elevated plasma estradiol accompanied by a seeming over-production of estradiol, ex vivo, relative to the control group (Fig. 5). These data are consistent with the idea that estradiol production rates had been increased as part of a compensatory response to the stressor. Of over a dozen transcript-level responses examined, expression of mRNAs coding for follicle-stimulating hormone receptor appeared to have the greatest potential utility as an indicator of reproductive dysfunction mediated through the estradiol synthesis-disruption toxicity pathway, based on the rapidity, persistence, and concentration-dependence of the response (Villeneuve et al., in press). Thus, based on the preliminary Phase 3 experiments substantively analyzed to date, the results have shown excellent promise for identifying potentially useful exposure indicators, detailing toxicity pathway characterization, and improving our understanding of compensatory responses of the HPG-axis to chemical stressors, all of which should enhance the

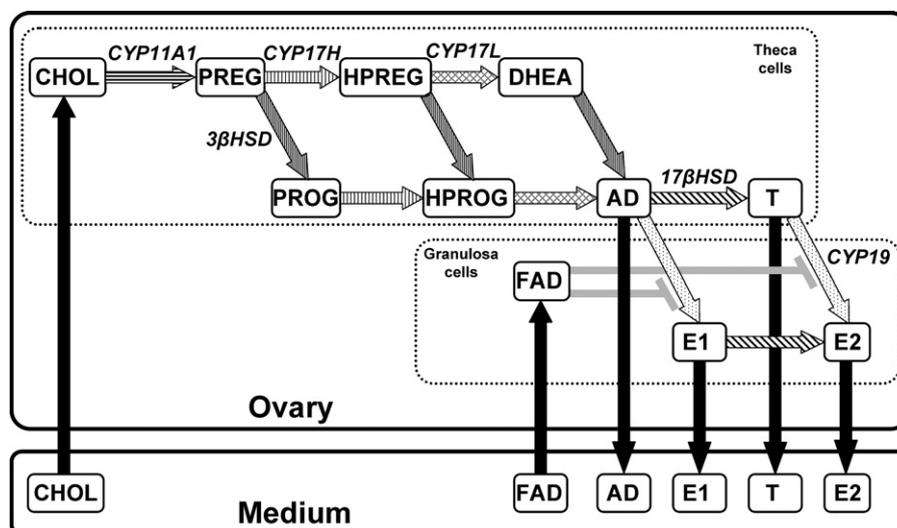


Fig. 6. Steroidogenesis model for the female fathead minnow gonad based on *in vitro* data from control and fadrozole-treated fish. The model consists of two compartments, medium and ovary tissue. Transport processes (black arrows) occur between the medium and ovary. Irreversible metabolic reactions (arrows with each pattern representing a unique enzyme) occur in the ovary. Six enzymes labeled in italics next to reactions they catalyze are: cytochrome P450c17 α hydroxylase (CYP11A1), cytochrome P450c17 α hydroxylase (CYP17H), cytochrome P450c17, 20-lyase (CYP17L), 3 β -hydroxy-dehydrogenase (3 β HSD), 17 β -hydroxy-dehydrogenase (17 β HSD), and cytochrome P450 aromatase (CYP19). Steroids and their precursors are: cholesterol (CHOL), pregnenolone (PREG), 17 α -hydroxypregnenolone (HPREG), dehydroepiandrosterone (DHEA), progesterone (PROG), 17 α -hydroxypregesterone (HPROG), androstenedione (AD), testosterone (T), estrone (E1) and 17 β -estradiol (E2). Fadroazole is depicted as an inhibitor of CYP19. The steroidogenic metabolic pathway encompasses two ovarian cell types; theca cells and granulosa cells. In theca cells, cholesterol is converted to AD and T. In granulosa cells, AD and T are converted to E1 and E2. Model from Breen et al. (2007).

ability to generate predictive models with utility for ecological risk assessment.

4. Integrating the data: predictive modeling

To help design the Phase 1, 2 and 3 studies and subsequently interpret and integrate the large amounts of data collected, we are using a systems biology/toxicology approach. Villeneuve et al. (2007b) described development of a graphical systems model focused on defining the HPG axis of teleost fish, which enables consideration of the interactive nature of the system at multiple levels of biological organization, ranging from changes in gene, protein and metabolite expression profiles to effects in cells/tissues that directly influence reproductive success. The model plays a role both in terms of designing our studies (e.g., deciding where to perturb the system), and interpreting the sometimes seemingly disparate biological responses observed (e.g., those associated with compensation), both from hypothesis- and discovery-driven perspectives. The model also enables consideration of the HPG axis in an integrated manner, such that effects of mixtures of chemicals with similar or dissimilar MOA can be more directly evaluated. The overall framework, which is written in open-source code (SBML; Systems Biology Markup Language) is not intended to be static but, rather, to evolve as this project (and the many other studies on EDCs and fish reproductive endocrinology being conducted throughout the world) generate mechanistic data to better inform the model. Although intended to support prediction of the effects of HPG-active chemicals with different MOA on reproductive function in fish, the model does not do so from a quantitative (“computational”) perspective. Rather, the model described by Villeneuve et al. (2007b) provides a framework for incorporation of more focused computational models into an integrated assessment of the potential ecological risk of EDCs. Several of these types of computational models associated with our current effort are discussed further below.

Steroid hormones are critical to maintenance of HPG axis function, and feedback controls on the system are achieved largely through alterations in steroid production. In addition, several estab-

lished EDCs exert adverse effects through their ability to directly modulate (generally inhibit) different enzymes involved in steroid synthesis (Figs. 1 and 6). Despite the importance of steroid production, until recently there had been no mechanistic computational models for describing baseline and/or chemically-perturbed conditions in vertebrates. As part of our effort, Breen et al. (2007) developed a steady-state model to predict synthesis and release of testosterone and estradiol by ovarian tissue, and evaluated the model using data generated from the fathead minnow (Fig. 6). Model-predicted concentrations of the two steroids over time corresponded well with both baseline (control) data, and information from experiments in which estradiol synthesis was blocked by fadrozole. A sensitivity analysis of the model identified specific processes that most influenced production of testosterone and estradiol, thereby lending insights as to potential points of control in the HPG axis. We have further developed predictive capabilities for understanding steroidogenesis by integration of the graphical model of Villeneuve et al. (2007b), with the steady state model of Breen et al. (2007), and the Hao et al. (2006) model of G protein, protein kinase A, and steroid acute regulatory protein activation, to examine effects of chemicals on steroid production and regulation (Shoemaker et al., 2008). In this expanded model, we examined the role of local regulation (within the ovary) and global regulation (between components of the HPG axis) in maintaining control of steroid synthesis. Incorporation of gene expression data into the Shoemaker et al. (2008) model suggests that local regulation reacts to fadrozole to increase gene expression of steroidogenesis enzymes. Higher enzymatic capability is then coupled with increased cholesterol transport due to testosterone and estradiol feedback regulation via the pituitary and hypothalamus. The fathead minnow appears to react locally in the ovary to increase steroidogenic enzymes and inter-organ signaling reacts to increase cholesterol pools available to the enzymes. From this model we can formulate additional, testable hypotheses by which feedback regulation, combined with local gene expression, could compensate for low-dose chemical exposure. In addition, these mechanism-based models should facilitate the study of the effects of mixtures of EDCs with different MOA, by predicting inhibition constants of each

steroidogenic enzyme for the individual chemicals. As information from additional EDCs are used for parameterization and validation, the computational models will serve as an effective “module” within the broader systems framework for making quantitative predictions of the impacts on endocrine function of either direct or indirect chemical effects on steroidogenesis.

Documenting alterations in function of the HPG axis at molecular and biochemical levels is useful to regulatory decision-making only if these changes can be translated into effects at higher biological levels of organization (Fig. 2). To achieve this we are developing two predictive models: one for male and another for female fathead minnows. Both models are physiologically-based computational models that link exposure to EDCs with changes in measured reproductive endpoints such as plasma concentrations of sex steroids and Vtg. The models are parameterized using information both from large-scale “control” datasets (Watanabe et al., 2007), and data from Phase 1 and 3 studies with EDCs with differing MOA. In males, our model currently accounts for endocrine responses to estradiol or ethinylestradiol based on their relative binding affinities to ERs and salient downstream effects of ER activation, such as induction of Vtg production (Watanabe et al., *in press*). The model is formulated in such a way that other estrogenic chemicals (or mixtures of chemicals) can be simulated with only minor modifications. Our model for female fathead minnows incorporates biological processes analogous to the male model, but extends the male model by including the AR, and interactions of the receptor with agonists and antagonists (K.H.W., unpublished data). Both models yield predictions of plasma concentrations of sex steroids and Vtg which fit measured data from unexposed and exposed fathead minnows. To connect the physiologically-based model at the individual organism level with a population model for predictions at a higher level of biological organization, predictions of changes in plasma Vtg concentrations can be used as input into a dynamic population model (Murphy et al., 2005).

The inclusion of microarray, proteomics, and metabolomics measurements in these studies also allows the reverse engineering of molecular networks (Schadt and Lum, 2006) which can then be compared with the measured endpoints. The use of this approach for disease characterization (Loscalzo et al., 2007) and subsequent target discovery for drug development (Chen et al., 2008) has been described, and application to environmental risk assessment has been proposed (Edwards and Preston, 2008). In this project, the molecular networks derived from genome-wide measurements provide an unbiased assessment of the physiologically-based models discussed above. This aids in the interpretation of the existing models since the completeness of each model can be estimated based on the percentage of variation in the molecular network explained by the descriptive model. It also aids in further development of the physiologically-based models by providing clues as to the missing components of each model.

The final component of the project involves development of tools for the prediction of population-level effects of EDCs. Except for instances in which threatened or endangered species are involved, most ecological assessments of the risk of contaminants ultimately are concerned with potential population-level responses. Kidd et al. (2007) evaluated the effects of ethinylestradiol on fish populations in dosing studies with a whole-lake ecosystem; however, the opportunity to conduct a controlled study of this magnitude is rare, so the only practical way to routinely link the effects of EDCs in individuals to population-level impacts is via modeling (Gleason and Nacci, 2001; Brown et al., 2003; Hurley et al., 2004; Gurney, 2006). Miller and Ankley (2004) describe a modeling approach for predicting the status of fathead minnow populations exposed to trenbolone, based on fecundity data from the 21-d Phase 1 study design. The basic model employs a Leslie matrix in conjunction with the logistic equation (to account for density dependence) to trans-

late laboratory toxicity information into prediction of population trajectories. Miller et al. (2007) expanded on this effort by first relating changes in Vtg to fecundity in female fathead minnows, and then using this relationship in the population model to predict population status in fish exposed to EDCs which inhibit production of the egg yolk protein, most notably compounds that depress steroid synthesis (e.g., fadrozole, prochloraz, trenbolone; Fig. 1). That analysis is unique in that it focuses on a biochemical endpoint, female Vtg, that reflects both toxic MOA of EDCs and has a functional relationship to reproductive success (formation of eggs). As such, within the overall systems framework for the project, the computational model described by Miller et al. (2007) and Miller and Ankley (2004) can serve as the basis via which genomic information can be quantitatively translated to responses in populations.

5. Prospectus

In this paper we describe a MOA/systems-based research effort with HPG-active chemicals that will help provide the technical basis for development of predictive toxicology tools (models, *in vitro* and short-term *in vivo* assays) which could improve the efficiency of current testing and monitoring programs for EDCs. As we contemplate informational needs for chemical risk assessments in the coming years, it is clear that historical toxicology approaches which focus mostly on generating empirical data cannot solely suffice. Toxicologists and risk assessors are being asked to do more with fewer resources, in a sociopolitical environment that emphasizes reduced animal testing. Examples of new testing mandates that promise to require additional toxicity data for a large number of chemicals include the REACH (registration, evaluation, authorization and restriction of chemicals) program in Europe, and the high production volume challenge program in the US, in addition to a variety of EDC testing efforts throughout the world. In recognition of these informational needs, the NRC (2007) proposed a greater emphasis on predictive toxicology tools to support human health assessments. There is an analogous requirement for advanced predictive methods in ecotoxicology, and many of the tools discussed in the NRC report are applicable to ecological risk assessments. However, there are added challenges in ecological assessments; for example, in contrast to human health toxicology, ecological assessments need to extrapolate toxicity from a few (sometimes one) species to many (sometimes thousands), and require an understanding of impacts of chemicals at the population (rather than individual) level. We feel that the research approach presented herein provides a broad conceptual framework for developing mechanism-based, predictive approaches for effectively assessing the ecological risk of chemicals with a variety MOA, in addition to EDCs.

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