

A Graphical Systems Model to Facilitate Hypothesis-Driven Ecotoxicogenomics Research on the Teleost Brain—Pituitary—Gonadal Axis

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Graphical systems models are powerful tools that can help facilitate hypothesis-driven ecotoxicogenomic research and aid in mechanistic interpretation of results. This paper describes a novel graphical model of the teleost brain—pituitary—gonadal (BPG) axis designed for ecotoxicogenomics research on endocrine-disrupting chemicals using small fish models. The model incorporates six compartments representing the major organs involved in the fish reproductive axis and depicts the interactions of over 105 proteins and 40 simple molecules, transcriptional regulation of 25 genes, and over 300 different reactions/processes. Application of the model is illustrated in the context of a study examining effects of the competitive aromatase inhibitor, fadrozole, on gene expression in gonad, brain, and liver tissue of fathead minnows. Changes in mRNA transcript abundance were measured using a fathead minnow oligonucleotide microarray and quantitative real-time polymerase chain reaction. Gene expression changes observed in the ovaries of females exposed to 6.3 μg fadrozole/L for 7 d were functionally consistent with fadrozole's mechanism of action, and expected compensatory responses of the BPG axis to fadrozole's effects. Furthermore, microarray results helped identify additional elements (genes/proteins) that could be included in the model to potentially increase its predictive capacity. With proper recognition of

their utility and limitations, graphical models can serve as important tools for linking molecular and biochemical changes to whole organism outcomes.

Introduction

Genomic approaches offer great potential for advances in toxicological research (1). With these technologies, it has become practical to simultaneously measure hundreds to thousands of changes in gene expression (transcriptomics), protein levels (proteomics), and/or metabolic products (metabolomics) resulting from perturbation of a biological system after exposure to a chemical. Potential benefits and challenges associated with the application of "omics" technologies in various fields of research, including ecotoxicology, have been addressed in numerous articles (e.g., 2–6). A primary challenge to ecotoxicologists has been to determine how to apply these technologies, to their greatest effect, in order to understand both chemically induced toxicity and physiological compensatory responses to chemical stressors, and use that understanding to predict risk for adverse ecological effects (7).

While application of proteomic and metabolomic technologies in ecotoxicology has largely just begun, there have been several reports on effects of different chemical stressors on the transcriptome of multiple aquatic organisms in the recent literature (e.g., 8–15). To date, nearly all the transcriptomic aquatic/ecotoxicology studies published can be characterized as discovery-driven. Specifically, a common goal has been to identify profiles or patterns of differentially expressed genes that can serve as effective fingerprints, signatures, or biomarkers of exposure to a certain class of chemical, or that are indicative of specific toxicological effects (e.g., 8–12). These goals can be achieved without comprehensive understanding of the mechanistic or functional significance of the genes composing the profile. Results of discovery-driven transcriptomic studies generally consist of lists of differentially expressed genes, often clustered into functional groups based on function (usually from limited annotation) or temporally correlated patterns (in the case of time-course studies), accompanied by a post-hoc discussion of potential significance (e.g., 10–12, 14–16).

Discovery-driven studies clearly have an important role to play in ecotoxicology research. They are well suited for identification of the genes, proteins, and/or metabolites (i.e., biomolecules and chemical reactions) involved in an organism's physiological response to a chemical stressor. This is particularly important when evaluating chemicals or biological pathways for which there is little or no *a priori* information on which to base hypotheses. However, discovery-oriented experiments are not particularly well equipped to determine what the biomolecules do, how they interact, and/or which are most important (e.g., are rate-limiting, serve as key integrators, sensor molecules, initiator of pleiotropic responses, etc.). Another approach to the use of genomic data involves identification of individual genes or pathways relevant to the toxic mechanism of a chemical and/or compensation for its effects (e.g., 13, 14). Such experiments are typically used to generate hypotheses to be tested in future studies.

Systems biology can be thought of as the study of the integrated and interacting networks of genes, proteins, and biochemical reactions that regulate various processes in living organisms. Genomic technologies provide unparalleled ability to comprehensively examine biological systems. However,

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to truly develop an understanding of how biological systems function in response to chemical stressors it is necessary to conduct hypothesis-driven genomic research. Although ecotoxicologists are accustomed to developing and testing hypotheses for individual endpoints, development of hypotheses regarding the integrated function of tens, hundreds, or thousands of genes, proteins, and/or chemical reactions will require new approaches. We believe that graphical and computational biological systems models are critical tools for conducting hypothesis-driven ecotoxicogenomic research and, in turn, that such research will greatly enhance understanding of biological systems and the ability to predict risks associated with chemical exposure.

In this paper, we describe the development of a graphical biological systems model designed to facilitate hypothesis-based genomic research on endocrine-disrupting chemicals using small fish models. The model depicts key genes, proteins, and reactions known, or thought, to be associated with reproductive functions under control of the teleost brain-pituitary-gonadal (BPG; or BPG[L]-liver) axis. The model represents a graphical integration of information provided in numerous review articles and primary sources concerning various aspects of fish reproduction and endocrinology. Utility and application(s) of the model are examined in the context of a recent study that investigated the effects of exposure to the competitive aromatase inhibitor, fadrozole, on gene expression in the brains, gonad, and liver of fathead minnows using a fathead minnow oligonucleotide microarray (detailed elsewhere, 17). Although neither the fathead minnow microarrays nor the fadrozole experiment were designed specifically to test model-based predictions, the potential value, roles, and limitations of systems models in ecotoxicogenomics research are illustrated through the example.

Methods

Graphical Systems Model. A graphical systems model representing key genes, proteins, biochemical reactions, and phenotypic outcomes associated with reproductive functions of the teleost BPG axis was constructed based on published literature (Figure 1, Supporting Information parts 1–4). Information from nearly two dozen review articles (approximately half of which were fish-specific), over two dozen primary sources, as well as textbooks on both human and vertebrate endocrinology (Supporting Information part 2) was integrated into a graphical representation composed of seven integrated functional modules. These modules are (A) gonadotropin releasing hormone (GnRH) release from the GnRH neuronal system in the brain, (B) gonadotropin expression and secretion from the pituitary, (C) cholesterol transport, (D) steroidogenesis, (E) spermatogenesis/spermiation, (F) vitellogenesis/oocyte maturation in the gonads, and (G) steroid metabolism in the liver. The cholesterol transport, steroidogenesis, and steroid metabolism modules were primarily based on mammalian literature. In contrast, modules related to germ cell development relied almost exclusively on fish-specific sources, while development of modules related to GnRH and gonadotropin release was derived from both fish-specific and non-fish literature.

The functional modules were incorporated into an overall model structure consisting of six anatomical compartments representing the major organs involved in the fish reproductive axis: brain, pituitary, ovary, testis, liver, and blood (Figure 1). The brain, pituitary, and gonadal compartments were in turn divided into subcompartments representing major cell types within the organ that play a significant role in regulating reproduction (Figure 1). For the brain, subcompartments include two types of GnRH neurons and dopaminergic neurons (Figure 1 [a3–d9]). For the pituitary, subcompartments represent follicle-stimulating hormone (FSH) and

lutinizing hormone (LH) gonadotrophs (Figure 1 [f2–k19]). Finally, the gonad compartment incorporates three subcompartments representing leydig cells, sertoli cells, and spermatocytes for testes (Supporting Information part 4), or theca cells, granulosa cells, and oocytes for ovaries (Figure 1 [l2–q20]; only ovary shown). Overall, the model depicts the interactions of over 105 proteins and 40 simple molecules, transcriptional regulation of 25 genes, and over 300 different reactions/processes.

The model was constructed using CellDesigner 3.1 (www.systems-biology.org/002/) following notation described by Oda et al. (18), where feasible. Models constructed using CellDesigner's graphical interface can be stored as a systems biology markup language (SBML) file, a standardized format used by various graphical modeling and pathway analysis programs, as well as more sophisticated computational modeling software that can be used to simulate the dynamics of the biological system. Model elements can be annotated with notes regarding relevant references, gene ontology terms, relevant KEGG pathways, etc. Additionally within CellDesigner, connectivity to on-line databases allows searching for published articles, sequence information, chemical structures, etc., directly from the model's graphical user interface. Thus, the graphical model represents a useful, open source tool to aid systems-oriented research on fish reproduction.

It should be noted, however, that our graphical model is a hypothesis-based construct derived from a current understanding of the way the BPG(L) system functions. New developments/insights in the field of (fish) reproductive endocrinology emerge on a continual basis. It is not feasible for the model to incorporate information from all published reports concerning molecular endocrine control of fish reproduction, particularly since, in some cases, reports provide contradictory information. The model is simply a foundation upon which to conduct hypothesis-driven genomics research related to fish reproduction. By testing model-based predictions against empirical results we can begin to understand where the model does and does not predict observed biological function. This then allows for iterative model refinement toward an increasingly accurate representation and consequently greater predictive capacity.

Fadrozole Experiment. Male and female fathead minnows were exposed to measured concentrations of 0, 6, and 60 μg fadrozole/L for 24 h and 7 d using a continuous flow-through system (for details see Supporting Information part 5). Whole liver, gonad, and brain samples were collected for RNA extraction and microarray analysis (see ref 17 for detailed methods). Ovary samples from all treatments and both time points were analyzed. However, due to limitations in the number of arrays available for the experiment, liver and brain samples were only analyzed for females exposed to 60 μg fadrozole/L or control water for 7 days (i.e., single concentration, single time point). Three replicate arrays were run for each tissue/concentration/time-point combination analyzed. Each hybridization was performed using RNA pooled from two individual fish, with the same individuals being pooled for each tissue analyzed within a given treatment. Two-dye, Cy3 and Cy5, labeling was used and hybridizations were conducted using a pooled reference design (17). Statistical analysis of the microarray data was conducted as described by Larkin et al. (17).

Quantitative Real-Time PCR. Transcript levels of several genes were measured by quantitative real-time polymerase chain reaction (QPCR) using the same pooled RNA samples analyzed by microarray. QPCR methods were used to measure expression of several important BPG axis genes not represented on the 2000 gene fathead minnow microarray, including P450 cholesterol side chain cleavage (P450scc), steroidogenic acute regulatory protein (StAR), follicle-

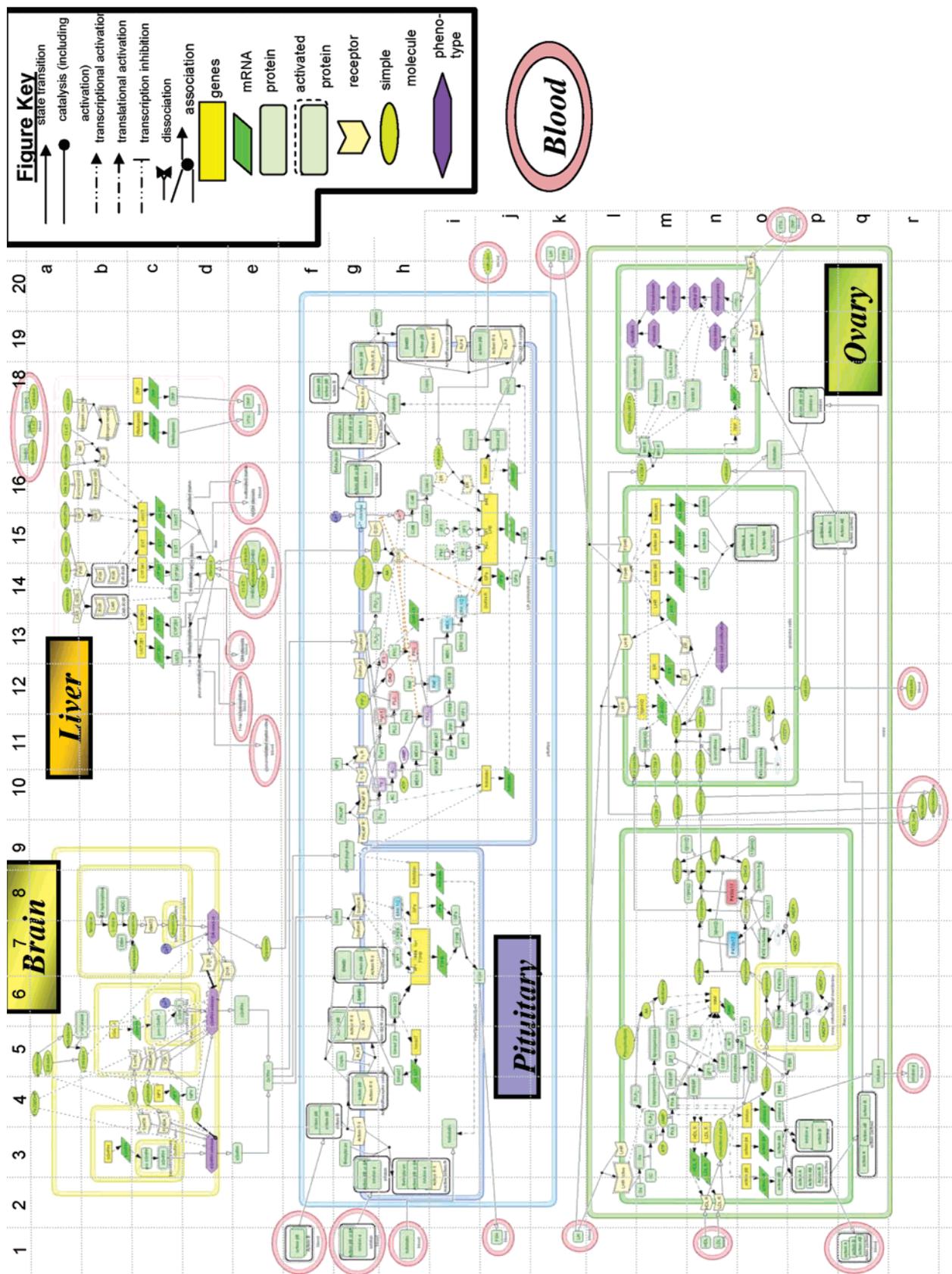


FIGURE 1. Conceptual model of the (female) teleost brain–pituitary–gonadal axis. The model was constructed in CellDesigner 3.1 (www.systems-biology.org/002/) using the notation described by Oda et al. (18). A grid system is provided to aid cross-reference between the model and descriptions in the text. Grid coordinates are provided in the text in [YX] format, where the Y-coordinate is represented by a letter and the X-coordinate is represented by a number. See Supporting Information part 1 for definition of all abbreviations used in the figure.

stimulating hormone receptor (FSHR), 20 β -hydroxysteroid dehydrogenase (20 β HSD), aromatase A (the isoform predominantly expressed in ovary tissue), and cytochrome b5. Cytochrome P450c17 (CYP17) transcripts were measured both with the microarrays and QPCR. All QPCR assays were conducted as described elsewhere (19–21). When parametric assumptions were met, data were analyzed using one-way ANOVA. In some cases results were log transformed to meet parametric assumptions. Planned comparisons between controls and treatments were made using Dunnett's test. Post-hoc multiple comparisons were made using Duncan's multiple range test. When parametric assumptions were not met, a nonparametric Kruskal–Wallis test was used to evaluate differences among treatments, and Dunnett's test was used for post-hoc comparisons. All statistical analyses were performed using SAS 9.0 (SAS Institute, Cary, NC), except Dunn's test which was conducted using GraphPad InStat v. 3.01 (GraphPad Software, San Diego, CA).

Results and Discussion

Hypothesis Formulation. Fadrozole is a potent, nonsteroidal, competitive inhibitor of aromatase enzyme activity (22). On the basis of that anticipated mechanism of action one would expect system-wide effects consistent with a reduction in gonadal estrogen synthesis. This could result in considerable disruption of endocrine function in reproductive females, which are known to have significant gonadal aromatase activity (19). Reproductively mature male and female fathead minnows both have high levels of brain aromatase activity (e.g., 23–24), but, to date, the role brain aromatase plays (if any) in regulating reproduction is unknown and aromatase was not included in the brain compartment of our model. Based on the relationships represented in the graphical systems model, we manually formulated a series of hypotheses to predict fadrozole's potential effects on transcript levels of multiple genes involved in reproductive functions of the teleost BPG(L) axis for females (Table 1). Hypothesized responses to the chemical were expected to reflect both direct effects of the chemical and aspects of compensatory physiological adaptation of the BPG(L) axis to fadrozole exposure (Table 1).

Direct Effects. Based on the model, the initial direct effect of fadrozole would be to reduce the production of estradiol (E2) and/or estrone (E1) by the gonad resulting in declining plasma estrogen concentrations (Figure 1 [m11, n11]). Afonso et al. (25) reported significant decreases in plasma E2 concentrations in Coho salmon within 3 or 6 h of injection with fadrozole, suggesting that fadrozole's effects on plasma E2 can be fairly rapid. Within the liver compartment, one would expect a decrease in the expression of vitellogenin (Vtg) (Figure 1 [c17]). Decreased plasma E2 and Vtg concentrations have been observed previously following longer-term (21 d) exposures to fadrozole (26). Expression of zona radiata protein (ZRP) genes (e.g., ZP2, Zpg 3; Table 1) which, like Vtg, are regulated by E2 binding to the estrogen-receptor and expressed either in liver or in the gonad (depending on species), would also be expected to decrease (Figure 1 [c18, n17]). Over time, the reduced supply of Vtg, and ZRPs, needed to support proper follicle development could be expected to limit the overall population of mature follicles in the fathead minnow ovary (e.g., 26). As a result, expression of genes associated with final oocyte maturation and ovulation (e.g., membrane progesterone receptors [MIS-R], cyclin B, neprilysin, etc.; Figure 1 [m17–n18]) may be reduced. Within the pituitary, the promoter region of the luteinizing hormone-like β (LH β) subunit gene is thought to contain an estrogen response element (27; Figure 1 [j16]), suggesting that LH β transcripts may initially fall as plasma E2 concentrations decline.

Compensatory Responses. Biological systems are complex autoregulatory networks that have evolved to maintain homeostasis in the face of variable and uncertain environmental conditions (28–31). The overall robustness of biological systems to perturbation, stressors, and environmental change relies on interacting positive and negative feedback loops, oscillators, and a variety of biological “switches” (28–30). Feedback loops associated with the BPG axis and reproductive endocrine control have been well studied (see refs 31–33). Steroids produced in the gonad exert feedback regulation on GnRH release from hypothalamic cells of the brain, and gonadotropin release from the pituitary through both direct and indirect mechanisms (33). Thus, in the case of reproductively mature females, a normal physiological response to declining E2 levels would be increased GnRH secretion, which would serve to up-regulate pituitary gonadotropin release and ultimately E2 production in order to maintain appropriate endocrine homeostasis (33). More generally, in response to the direct effects of the stressor (fadrozole), the fish could be expected to mount a series of compensatory responses aimed at maintaining homeostatic conditions needed for survival, reproduction, and/or growth. Such responses can be viewed as indirect effects of the stressor that may be detected either transiently or for a sustained period, depending on the organism's success in compensating for the direct effects of the stressor.

At the level of the GnRH neuronal system in the brain, falling E2 levels would be expected to alleviate dopamine's inhibitory effect on GnRH expression (34; Figure 1 [b5, d7]), potentially concomitant with decreased expression of tyrosine hydroxylase and/or aromatic-L-amino acid decarboxylase (AADC) in dopaminergic neurons, resulting in a potential increase in (c)GnRH expression (Figure 1 [b8, d6]). That increase might be tempered somewhat by decreased expression of neuropeptide Y (Figure 1 [b5, c4]). Nonetheless, increased GnRH expression could be viewed as the initiation of homeostatic compensation. With an increase in GnRH expression and/or release, increased expression and activation of cell surface GnRH receptors in the pituitary gonadotropes would be expected to initiate signal transduction cascades that favor increased expression of LH β , follicle stimulating hormone β (FSH β), and glycoprotein α (GP α) subunit genes (Figure 1 [g7–h8, g12–j15]). As the increased levels of gonadotropins circulate back to the ovary, one could predict a compensatory up-regulation in the expression of genes coding for (i) cholesterol transport proteins including high-density lipoprotein (HDL) and low-density lipoprotein (LDL) receptors (Figure 1 [n2]) and StAR (Figure 1 [n6]); (ii) rate-limiting steroidogenic enzymes such as P450scc (Figure 1 [o6]) and aromatase A (Figure 1 [n11]); and (iii) transforming growth factor- β (TGF- β)-related proteins, including activin β A, activin β B, inhibin α , and follistatin (Figure 1 [o3, m14–16]).

Additional autoregulatory compensatory responses could be expected to occur in the liver. Steroids are involved in activation of the pregnane X receptor (PXR) which in turn regulates expression of steroid metabolizing enzymes such as estrogen- and/or hydroxysteroid sulfotransferases and cytochrome P450 3A (EST, HSST, CYP3A respectively; 35; Figure 1 [b14, c14–16]). Declining E2 levels could be expected to cause reduced activation of PXR, resulting in a decrease in EST, HSST, and CYP3A transcripts. This autoregulatory response would serve to reduce the rate of E2 metabolism, helping to maintain plasma E2 concentrations.

For the purposes of this paper, hypothesis formulation focused on predicted changes in mRNA transcript levels. However, the model could be applied similarly to predict effects on protein levels and/or modifications, as well as changes in simple molecules including steroids, their metabolites, amino acid neurotransmitters, etc. Thus, graphical

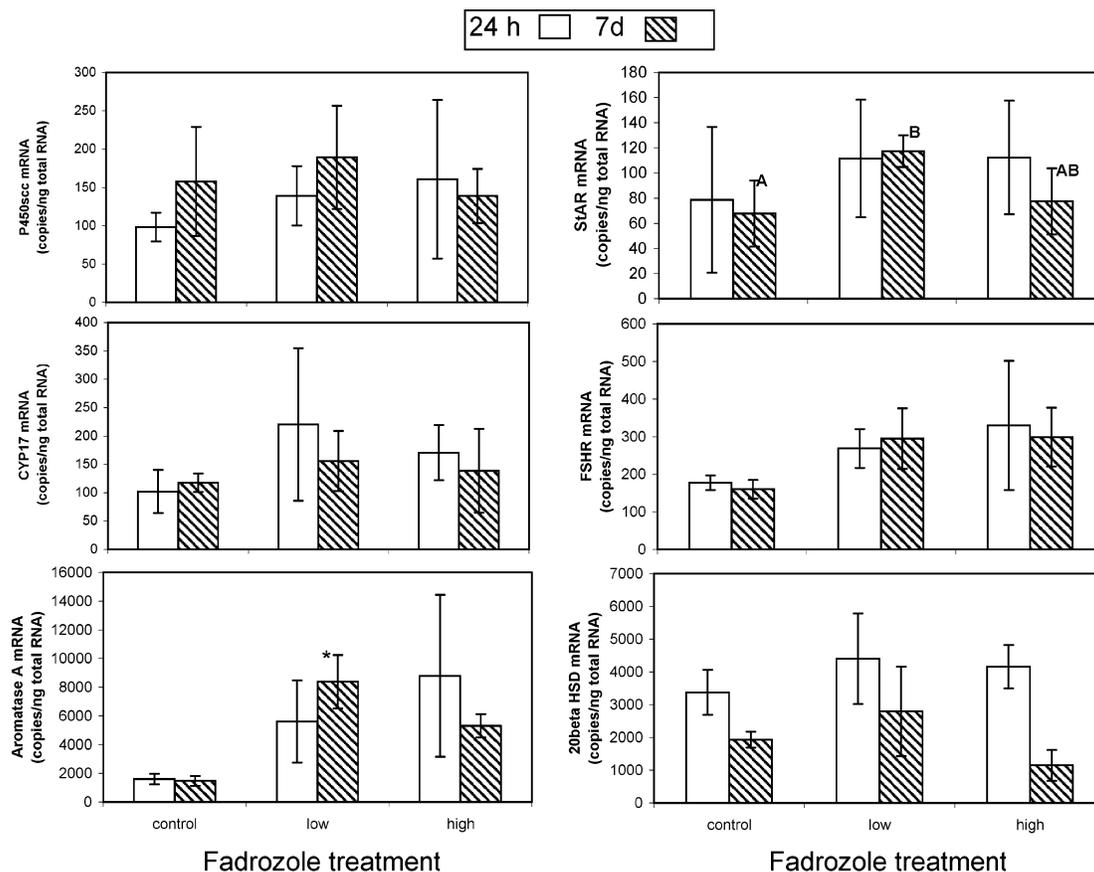


FIGURE 2. Transcript abundance in ovary tissue from female fathead minnows exposed to Lake Superior water (control), 6.3–6.5 μg fadrozole/L (low), or 60 μg fadrozole/L (high) for 24 h (white bars) or 7 d (hatched bars) measured for six genes relevant to teleost brain–pituitary–gonadal axis function. Bars = mean ($n = 3$ samples); ovary RNA pooled from $n = 2$ fish per sample. Error bars = 1 standard deviation. P450scc = cytochrome P450 cholesterol side chain cleavage (CYP11A); CYP17 = cytochrome P450 c17 α -hydroxylase/17,20 lyase (P450c17); aromatase A = aromatase (A isoform predominantly expressed in ovary); StAR = steroidogenic acute regulatory protein; FSHR = follicle-stimulating hormone receptor; 20beta HSD = 20 β hydroxysteroid dehydrogenase.

genes represented on the microarrays, 13 corresponded with proteins incorporated into the BPG(L) axis model (Table 1). Five additional genes were evaluated by QPCR, and CYP17 was evaluated by both microarray and QPCR (Table 1; Figure 2).

Liver. After 7 d of exposure to 60 μg fadrozole/L, there was significant down-regulation of Vtg precursors, ER α , and CYP3A in the liver (Table 1). This was consistent with the model-based expectations of the direct endocrine-modulating effect of fadrozole on Vtg and ER α expression, and the hypothesized compensatory reduction in the expression of genes coding for steroid hormone metabolizing enzymes (Table 1). Given that both direct and compensatory responses were observed at the 7 d time point, the results suggest that any potential compensatory response along the BPG(L) axis was not sufficient to completely overcome the continuous competitive inhibition of aromatase associated with the flow-through exposure to fadrozole. The liver response profile following a transitory exposure to similar concentrations of fadrozole may look quite different.

Brain. Within the brain, there was a significant decrease in aromatase B transcript abundance in females exposed to 60 μg fadrozole/L for 7 d (Table 2). This was consistent with an overall reduction in circulating estrogen, given that the promoter region of the aromatase B gene contains estrogen response elements and is known to be up-regulated by estrogens (36). In a previous study using QPCR we also observed a significant reduction in aromatase B transcripts in the brain of female fathead minnows after 7 d exposure

TABLE 2. Effects of Fadrozole Exposure on Transcript Levels of Several Genes Not Included in the Brain–Pituitary–Gonadal (BPG) Axis Model (Figure 1) but Functionally Linked to BPG Axis Function (i.e., Candidates for Future Integration into the Model) and Analyzed Using a Fathead Minnow 2000 Gene Microarray

gene ^a	60 $\mu\text{g}/\text{L}$ 24 h ^b	6.3 $\mu\text{g}/\text{L}$ 7 d ^b	60 $\mu\text{g}/\text{L}$ 7 d ^b
Ovary			
Aromatase B	=	(9.6) [↑]	=
GAD 65	=	(1.2–2.6) [↑]	=
GAD 67	=	(1.8–2.6) [↑]	=
HMG-CoA reductase	=	(2.8–5.4) [↑]	=
LRP	=	(1–16) [↑]	=
Brain			
Aromatase B			(2.5–2.9) [↓]

^a Abbreviations used are as follows: aromatase B = cytochrome P450 aromatase (isoform B); GAD 65 = glutamate decarboxylase 65 kDa isoform; GAD 67 = glutamate decarboxylase 67 kDa isoform; HMG-CoA reductase = 3-hydroxy-3-methylglutaryl coenzyme A reductase; LRP = low-density lipoprotein receptor associated protein. ^b ↑ indicates significant increase in transcript levels relative to controls; ↓ indicates significant decrease in transcript levels relative to controls; = no significant difference relative to controls; empty entries indicate transcripts that were not analyzed.

to 50 μg fadrozole/L (19). Thus, the 7 d brain results were consistent with known direct effects of fadrozole.

Ovary. Exposure to 6.5 or 60 μg fadrozole/L for 24 h (Table 1) did not significantly affect transcript abundance of BPG-related genes in the ovaries of female fathead minnows. We had expected competitive inhibition of E2 synthesis by fadrozole to cause a relatively rapid down-regulation of estrogen receptor α (ER α) and compensatory up-regulation of aromatase A in the ovary (Table 1). However, based on the 24 h microarray results, ER α transcripts were unchanged (Table 1). Based on QPCR analysis, mean aromatase A transcript abundance in ovarian tissue from females exposed to 6.5 $\mu\text{g}/\text{L}$ for 24 h was approximately 3.5-fold greater than that in controls, while aromatase A mRNA expression in females exposed to 60 μg fadrozole/L was approximately 5.5-fold greater than that in controls (Figure 2). However, there was also a substantial increase in the variability of transcript abundance among the three RNA pools from the exposed females (CVs of 72% and 91% for 6.5 and 60 $\mu\text{g}/\text{L}$, respectively) versus those of controls (CV = 32%), such that the difference in means was not statistically significant (Figure 2). The increased variability in aromatase A transcript abundance in the ovaries of fadrozole-treated females suggests that fadrozole was affecting transcript levels after as little as 24 h of exposure, but that the sensitivity and/or time-course of the response to fadrozole varied significantly among individuals.

The molecular responses observed in the ovaries of females exposed to 6.3 $\mu\text{g}/\text{L}$ for 7 d were consistent with the direct mechanism of action of fadrozole and anticipated compensatory responses as predicted based on the graphical systems model. A number of the changes in transcript abundance could be viewed as responses that would act to elevate/restore estrogen production. Most obviously, aromatase A, the aromatase isoform predominantly expressed in ovary and regulated through gonadotropin-activated signal transduction (36), was significantly up-regulated (Figure 2). Aromatase B expression was also up-regulated in the ovaries of fathead minnows exposed to 6.3 μg fadrozole/L (Table 2). Expression of aromatase B in the ovary is generally on the order of 75-fold lower than that of aromatase A (19) and thought to be less influenced by gonadotropin-activated signal transduction pathways (36); thus a change in aromatase B was not anticipated, but nonetheless would be consistent with a homeostatic need to increase gonadal estrogen synthesis.

Microarray and QPCR results did not support model-based predictions that cytochrome P450 side-chain cleavage (P450 scc ; CYP11A) and cytochrome P450 c17 α -hydroxylase/17,20-lyase (CYP17; P450c17) expression might be up-regulated and that expression of 20 β -hydroxysteroid dehydrogenase would decrease as part of a compensatory response to the effects of fadrozole (Figure 2). However, there were expression changes that could favor increased transport of cholesterol, the initial precursor for overall steroid hormone synthesis (37). QPCR results demonstrated a significant up-regulation in StAR expression (Figure 2). StAR plays a critical, rate-limiting, role in facilitating transfer of cholesterol from the outer to inner mitochondrial membrane where P450 scc initiates the first step of steroid synthesis (Figure 1 [n6, o5]; 37, 38). These results suggest that even though there was not necessarily a change in the expression of multiple genes coding for enzymes involved in androgen/estrogen synthesis, the genes coding for proteins that would be considered most rate limiting for production of estrogens (i.e., StAR and aromatase) were significantly up-regulated as part of a possible compensatory response after 7 d exposure to 6.3 $\mu\text{g}/\text{L}$ fadrozole.

Maturation inducing steroid (MIS) receptors are thought to play a critical role in triggering oocyte maturation (39; Figure 1 [m17]). Progesterone receptor membrane component 2 (PRMC 2) is a member of one of the two classes of membrane-bound MIS receptors thought to participate in

progesterone-induced oocyte maturation in fish (40). Another gene found on the array, cyclin B, codes for a protein thought to be induced by 17 α ,20 β -dihydroxy-4-pregnen-3-one (17,20P) binding to the MIS receptor (Figure 1 [n18]; 41). As hypothesized, both PRMC 2 (a MIS-R) and cyclin B expression were down-regulated in the ovaries of females exposed to 6.3 $\mu\text{g}/\text{L}$ fadrozole for 7 d (Table 1). This likely reflects an overall reduction in follicle development and maturation associated with impaired vitellogenesis, which would be consistent with the inhibitory effect of fadrozole on E2 synthesis. Thus, as a whole, the gene expression changes detected using the microarrays and QPCR were generally consistent with model-based predictions.

Model Refinement. One of the primary goals of hypothesis-driven ecotoxicogenomics research is to improve our understanding of biological systems and their responses to chemical stressors in order to enhance the ability to conduct predictive (as opposed to strictly empirical) risk assessments. Thus, an important part of hypothesis-driven ecotoxicogenomics research involves iterative refinement of biological models toward increasingly accurate representations and consequently greater predictive capacity. In evaluating the microarray results from the fadrozole experiment, we identified several differentially expressed genes with functional relations to elements depicted in the model that were not directly incorporated into our original depiction of the system (Table 2).

For example, HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase, a rate-limiting enzyme for cholesterol synthesis in steroidogenic cells, also known to stimulate the LDL receptor and uptake of LDL cholesterol (37; Figure 1 [n1-2]), was significantly up-regulated after 7d exposure to 6.3 μg fadrozole/L (Table 2). Increased expression of HMG-CoA reductase could serve to increase the local synthesis of cholesterol in order to increase the rate of steroid production or to serve as membrane components for proliferating steroidogenic cells (42). A probe for the LDL receptor gene was not present on the arrays, but an LDL receptor associated protein (LRP) represented on the microarray was shown to be up-regulated (Table 2). The exact identity and function of the LRP included on the fathead minnow array was not clear from the array annotation, but a BLAST (Basic Local Alignment Search Tool, used to find regions of similarity in nucleotide and amino acid sequences; 43) search revealed close homology to a LRP studied in birds that was reported to play a role in vitellogenesis (44). Either function, facilitating vitellogenesis or increasing cellular cholesterol supply, could reflect a compensatory response to decreased circulating E2 levels (and consequent decreased Vtg production in the liver).

The glutamate decarboxylases GAD 65 and GAD 67 are involved in the synthesis of the neurotransmitter GABA (gamma amino butyric acid; 45). GABA was included in the brain compartment of our model due to its role in regulating GnRH release (45; Figure 1 [d4]). However, the microarray results suggested up-regulation of GAD 65 and GAD 67 in the ovary of fadrozole-exposed fish (Table 2), which was unexpected. A literature search revealed that GABA is also synthesized in non-neural tissues (including gonadal tissue) and may play a role in regulating the proliferation of steroidogenic cells and/or regulating steroid synthesis (46). Thus, the array results suggest a possible role for GAD 65, 67, and GABA-mediated pathways within the BPG axis.

Concentration-Dependence. Overall, the pattern of differential ovarian gene expression observed following 7 d of exposure to 6.3 μg fadrozole/L was functionally consistent with model-based predictions of probable biological response to fadrozole's competitive inhibition of aromatase activity. Given the observed gene expression patterns at the 6.3 $\mu\text{g}/\text{L}$ fadrozole exposure, we expected changes of greater magnitude in the ovaries of fish exposed to 60 $\mu\text{g}/\text{L}$ for 7 d.

However, this was not the case (Table 1). Both fecundity and plasma Vtg results suggested a concentration-dependent effect on apical endpoints (unpublished data), and exposure concentrations were verified. However, for the differentially expressed genes detected either by microarray (Table 1) or QPCR (Figure 2; StAR and aromatase A), fish exposed to 6.3 $\mu\text{g}/\text{L}$ were significantly different from controls while those exposed to 60 $\mu\text{g}/\text{L}$ were not.

The QPCR data and consideration of some of the limitations of transcript measurements in the context of the experimental design provide some possible explanations for the apparent discrepancy between the 6.3 and 60 $\mu\text{g}/\text{L}$ treatments. Although concentration-dependence (typically monotonic concentration–response relationships) is widely recognized as a central principle in toxicology research, responses to a chemical stressor are, more accurately, a function of both concentration and time. In this study, the analyses conducted capture two snapshots in time at two different concentrations. Furthermore, although transcripts can be translated into protein, transcript abundance and protein levels are not necessarily correlated at any one point in time. A rise in transcripts would be expected to precede a significant increase in protein levels, and conversely, elevated protein levels can be sustained long after transcript abundance falls back to normal levels. An example of this disconnect was provided by Korte et al. (47) where significant increases in Vtg transcript abundance in male fathead minnows could be measured 8 h after exposure to E2, while Vtg protein levels were not increased until 16 h after exposure. In that study, Vtg transcripts fell back to control levels by 144 h posttreatment, while protein levels remained elevated through at least 432 h posttreatment (47). In assessing system-wide effects of a stressor that encompass both direct actions of that stressor as well as indirect compensatory responses (and potential cross-talk among biological pathways), it is likely critical to consider both the dimensions of dose (concentration) and time.

Model Evaluation. Given the somewhat limited representation of BPG-related genes on the fathead minnow array used in this study, a comprehensive empirical test of model-based hypotheses was not possible. However, this exercise provided a clear example of how a graphical systems model can be applied to enhance the ability to translate large toxicogenomic data sets into meaningful mechanistic information that enables testing of *a priori* hypotheses regarding responses of a biological system to a stressor. The microarray and QPCR results were relatively consistent with model predictions based on the known mechanism of action for fadrozole. Further, the model provided a useful framework that readily allowed some of the other differentially expressed genes (i.e., those not included in the current model) to be, potentially, functionally linked to BPG axis function. In combination with efforts such as the gene ontology project (48), which will facilitate greater automation in linking genomic data points with biological function, and biological pathway databases (e.g., KEGG; <http://www.genome.jp/kegg/pathway.html>), that can provide curated sources for additional modules that can be incorporated into project/question-specific models, graphical models should improve the ability of ecotoxicologists to derive useful information from large genomic data sets.

The array results suggested a number of possible elements to add to our graphical model, and to consider when selecting components to include in a computational BPG axis model. Potentially significant roles for locally produced GABA in the gonads and local cholesterol synthesis via HMG-CoA reductase were suggested by the array results. Further literature research into the roles of each in regulating gonad cell proliferation and steroid synthesis could improve our understanding of the function of the axis, as well as aid in

the identification of other compounds with the potential to disrupt BPG-mediated processes. Overall, based on examination of just a few genes, it is clear how iterative rounds of model revision (i.e., identifying elements that should be added, determining which can be removed or ignored), hypothesis formulation, and empirical testing, can facilitate refinement of the model toward greater predictive sophistication.

Model Limitations. Based on the handful of genes evaluated, it was also apparent that there are limitations relative to the utility of graphical systems models. First, the graphical model only provides a static representation of the system. Similarly, array, proteomic, or metabolomic data can only capture snapshot observations of the system. In order to formulate hypotheses based on the model, it is necessary to make time-course assumptions concerning effects of a stressor on the system. This is problematic in two respects. First, if the time-course assumptions are incorrect, one may conclude there are inaccuracies or deficiencies in the model when, instead, the snapshot provided by the data simply did not match the assumed time-course. Conversely, there may be a tendency to assume the model is correct and attribute any deviations from model predictions to incorrect time-course assumptions. This limitation can be off-set by collecting data at multiple time points when testing model-based hypotheses, but given costs and logistics, extensive time-course sampling is often not feasible. Time-course issues will continue to be problematic in the use of graphical systems models for hypothesis-based toxicogenomic research.

Similar limitations exist relative to concentration-dependence. Most ecotoxicology testing involves exposure of organisms to various concentrations or doses of the test chemical/sample of interest. Inherent in most toxicological hypothesis testing is the concept that concentration X will lead to an effect on endpoint Y. However, in the case of toxicogenomic experiments, the sheer number of endpoints dictates that their sensitivity to direct effects of the stressor being examined is likely to vary widely. Furthermore, changes resulting from secondary effects such as compensatory responses, feedback, cross-talk among biochemical pathways, etc., are dependent on dynamic concentrations of endogenous compounds and/or structural changes in biomolecules, many of which cannot be readily quantified. Though the model depicts the basic types of interactions among biomolecules and reactions, it cannot adequately capture the concentration–response relationships governing those interactions.

In the context of this study, an additional limitation was that measurements were made on a tissue-specific basis, while function, and the ability to model function, may require a finer degree of resolution or compartmentalization (for example, the ability to differentiate transcript abundance in theca cells versus granulosa cells within the ovary). Thus, the hypotheses that can be generated and tested are limited by the degree of analytical resolution. Failure to account for this could lead to misunderstanding and/or confusion regarding system function, regulation, or response to stressors. This limitation is a factor that should be considered in choosing candidate biomarkers. Elements whose function depends strongly on relative expression of the same gene within different cell types within a tissue are not likely to be useful biomarkers if measurements will (or can) only be made at the tissue level. While differences in the scale of resolution can be problematic in formulating model-based hypotheses, careful application of the model can also help avoid erroneous conclusions and/or pursuit of confounded endpoints as potential biomarkers.

Overall Assessment. Despite the limitations, we feel that graphical systems models, such as the one presented here, are critical tools for ecotoxicology research in the era of

genomic analyses. The initial act of constructing and/or analyzing the graphical model evokes an important shift in thinking, away from that of individual endpoints in isolation, and toward interdependent endpoints organized into interacting functional networks. In cases where genomic tools are still being developed for the species of interest (e.g., a second-generation fathead minnow microarray), the model can help direct sequencing and bioinformatic efforts to populate and annotate microarrays with features likely to be informative and relevant to specific research questions. As illustrated, the functional relationships depicted in the model make it possible to formulate reasonable hypotheses regarding potential changes in large numbers of genes, proteins, or reaction products, based on the anticipated mechanism of action for a chemical of interest. They also facilitate the task of identifying functional linkages between the profile of responses at the molecular level and apical effects relevant to survival, growth, and reproduction, a critical need if biomarkers and toxicogenomic data are to be successfully used for risk assessment or regulatory applications (7). Though their application requires that assumptions be made, iterative testing and refinement of graphical models should aid in the effective use of toxicogenomic approaches to understand chemical-induced toxicity and physiological compensatory responses to chemical stressors, and predict risk.

Acknowledgments

This work was funded in part through the Computational Toxicology Program of the U.S. EPA Office of Research and Development and the U.S. EPA Office of Science Council Policy. Initial support for D.L.V. was provided by a National Research Council Postdoctoral Research Associateship. The work was also supported through a cooperative research and development agreement (CRADA) between the U.S. EPA and EcoArray (CRADA 301-03). We thank Dr. Michael Breen and Dr. John Nichols for review comments on an earlier version of the manuscript. Thanks also to Michael Breen and Dr. Rory Conolly for helpful suggestions relative to the preparation of Figure 1. The information in this document has been subjected to review by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Supporting Information Available

(SI-1) Abbreviations used for features depicted in the graphical model of the teleost BPG(L) axis; (SI-2) references used in developing the graphical model; (SI-3) the complete model shown in Figure 1, provided as a SBML file; (SI-4) a graphical model of the testes compartment provided as a SBML file; and (SI-5) additional details regarding the fathead minnow exposures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Literature Cited

- (1) Balbus, J. M. Ushering in the new toxicology: toxicogenomics and the public interest. *Environ. Health Perspect.* **2005**, *113*, 818–822.
- (2) Miracle, A. L.; Ankley, G. T. Ecotoxicogenomics: linkages between exposure and effects in assessing risks of aquatic contaminants in fish. *Reprod. Toxicol.* **2005**, *19*, 321–326.
- (3) Snape, J. R.; Manud, S. J.; Pickford, D. B.; Hutchinson, T. H. Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. *Aquat. Toxicol.* **2004**, *67*, 143–154.
- (4) Neumann, N. F.; Galvez, F. DNA microarrays and toxicogenomics: applications for ecotoxicology? *Biotechnol. Adv.* **2002**, *20*, 391–419.

- (5) Robertson, D. G. Metabonomics in toxicology: a review. *Toxicol. Sci.* **2005**, *85*, 809–822.
- (6) Viant, M. R.; Rosenblum, E. S.; Tjeerdema, R. S. NMR-based metabolomics: a powerful approach for characterizing the effects of environmental stressors on organism health. *Environ. Sci. Technol.* **2003**, *37*, 4982–4989.
- (7) Ankley, G. T.; Daston, G. P.; Degitz, S. J.; Denslow, N. D.; Hoke, R. A.; Kennedy, S. W.; Miracle, A. L.; Perkins, E. J.; Snape, J.; Tillitt, D. E.; Tyler, C. R.; Versteeg, D. Toxicogenomics in regulatory ecotoxicology. *Environ. Sci. Technol.* **2006**, *40*, 4055–4065.
- (8) Larkin, P.; Folmar, L. C.; Hemmer, M. J.; Poston, A. J.; Denslow, N. D. Expression profiling of estrogenic compounds using a sheephead minnow cDNA microarray. *Environ. Health Perspect.* **2003**, *111*, 839–846.
- (9) Larkin, P.; Sabo-Attwood, T.; Kelso, T.; Denslow, N. D. Gene expression analysis of largemouth bass exposed to estradiol, nonylphenol, and *p,p'*-DDE. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **2002**, *133*, 543–557.
- (10) Tilton, S. C.; Gerwick, L. G.; Hendricks, J. D.; Rosato, C. S.; Corley-Smith, G.; Givan, S. A.; Bailey, G. S.; Bayne, C. J.; Williams, D. E. Use of a rainbow trout oligonucleotide microarray to determine transcriptional patterns in aflatoxin B₁-induced hepatocellular carcinoma compared to adjacent liver. *Toxicol. Sci.* **2005**, *88*, 319–330.
- (11) Krasnov, A.; Koskinen, H.; Rexroad, C.; Afanasyev, S.; Mölsa, H.; Oikari, A. Transcriptome responses to carbon tetrachloride and pyrene in the kidney and liver of juvenile rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* **2005**, *74*, 70–81.
- (12) Volz, D. C.; Bencic, D. C.; Hinton, D. E.; Law, J. M.; Kullman, S. W. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induces organ-specific differential gene expression in male Japanese medaka (*Oryzias latipes*). *Toxicol. Sci.* **2005**, *85*, 572–584.
- (13) Reichert, K.; Menzel, R. Expression profiling of five different xenobiotics using a *Caenorhabditis elegans* whole genome microarray. *Chemosphere* **2005**, *61*, 229–237.
- (14) Meyer, J. N.; Volz, D. C.; Freedman, J. H.; Di Giulio, R. T. Differential display of hepatic mRNA from killifish (*Fundulus heteroclitus*) inhabiting a Superfund estuary. *Aquat. Toxicol.* **2005**, *73*, 327–341.
- (15) Soetaert, A.; Moens, L. N.; Van der Ven, K.; Van Leemput, K.; Naudts, B.; Blust, R.; De Coen, W. Molecular impact of propiconazole on *Daphnia magna* using a reproduction-related cDNA array. *Comp. Biochem. Physiol., C: Toxicol. Pharmacol.* **2006**, *142*, 66–76.
- (16) Koskinen, H.; Pehkonen, P.; Vehniäinen, E.; Krasnov, A.; Rexroad, C.; Afanasyev, S.; Mölsa, H.; Oikari, A. Response of rainbow trout transcriptome to model chemical contaminants. *Biochem. Biophys. Res. Commun.* **2004**, *320*, 745–753.
- (17) Larkin, P.; Villeneuve, D. L.; Knoebl, I.; Miracle, A. L.; Carter, B. J.; Denslow, N. D.; Ankley, G. T. Development and validation of a 2,000 gene microarray in fathead minnow, *Pimephales promelas*. Submitted for publication.
- (18) Oda, K.; Matsuoka, Y.; Funahashi, A.; Kitano, H. A comprehensive pathway map of epidermal growth factor receptor signaling. *Molec. Syst. Biol.* **2005**, *msb4100014*, 17 pp.
- (19) Villeneuve, D. L.; Knoebl, I.; Kahl, M. D.; Jensen, K. M.; Hammermeister, D. E.; Greene, K. J.; Blake, L. S.; Ankley, G. T. Relationship between brain and ovary aromatase activity and isoform-specific mRNA expression in the fathead minnow (*Pimephales promelas*). *Aquat. Toxicol.* **2006**, *76*, 353–368.
- (20) Villeneuve, D. L.; Orlando, E. F.; Greene, K. J.; Blake, L. S.; Jensen, K. M.; Kahl, M. D.; Makynen, E. A.; Durhan, E. J.; Linnun, A. L.; Miracle, A. L.; Ankley, G. T. Quantitative RT-PCR assays for fathead minnow StAR and CYP11A and effects of ketoconazole on their expression in vivo. *The Toxicologist* **2006**, *90*, 320.
- (21) Ankley, G. T.; Jensen, K. M.; Kahl, M. D.; Makynen, E. A.; Blake, L. S.; Greene, K. J.; Villeneuve, D. L. Ketoconazole in the fathead minnow: reproductive toxicity and biological compensation. *Environ. Toxicol. Chem.* In press.
- (22) Steele, R. E.; Mellor, L. B.; Sawyer, W. K.; Wasvary, J. M.; Browne, L. J. In vitro and in vivo studies demonstrating potent and selective inhibition with the non-steroidal aromatase inhibitor CGS 16949A. *Steroids* **1987**, *50*, 147–161.
- (23) Ankley, G. T.; Jensen, K. M.; Durhan, E. J.; Makynen, E. A.; Butterworth, B. C.; Kahl, M. D.; Villeneuve, D. L.; Linnun, A.; Gray, L. E.; Cardon, M.; Wilson, V. S. Effects of two fungicides with multiple modes of action on reproductive endocrine function in the fathead minnow (*Pimephales promelas*). *Toxicol. Sci.* **2005**, *86*, 300–308.

- (24) Ankley, G. T.; Kuehl, D. W.; Kahl, M. D.; Jensen, K. M.; Linnum, A.; Leino, R. L.; Villeneuve, D. A. Reproductive and developmental toxicity and bioconcentration of perfluorooctanesulfonate in a partial life-cycle test with the fathead minnow (*Pimephales promelas*). *Environ. Toxicol. Chem.* **2005**, *24*, 2316–2324.
- (25) Afonso, L. O. B.; Iwama, G. K.; Smith, J.; Donaldson, E. M. Effects of the aromatase inhibitor fadrozole on plasma sex steroid secretion and ovulation rate in female coho salmon, *Oncorhynchus kisutch*, close to final maturation. *Gen. Comp. Endocrinol.* **1999**, *113*, 221–229.
- (26) Ankley, G. T.; Kahl, M. D.; Jensen, K. M.; Hornung, M. W.; Korte, J. J.; Makynen, E. A.; Leino, R. L. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (*Pimephales promelas*). *Toxicol. Sci.* **2002**, *67*, 121–130.
- (27) Ando, H.; Hew, C. L.; Urano, A. Signal transduction pathways and transcription factors involved in the gonadotropin-releasing hormone-stimulated gonadotropin subunit gene expression. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **2001**, *129*, 525–532.
- (28) Hastay, J.; McMillen, D.; Collins, J. J. Engineered gene circuits. *Nature* **2002**, *420*, 224–230.
- (29) Csete, M. E.; Doyle, J. C. Reverse engineering of biological complexity. *Science* **2002**, *295*, 1664–1668.
- (30) Tyson, J. J.; Chen, K. C.; Novak, B. Sniffers, buzzers, toggles, and blinkers: dynamics of regulatory and signalling pathways in the cell. *Curr. Opin. Cell Biol.* **2003**, *15*, 221–231.
- (31) Costa, D. P.; Sinervo, B. Field physiology: physiological insights from animals in nature. *Annu. Rev. Physiol.* **2004**, *66*, 209–238.
- (32) Kime, D. E. *Endocrine Disruption in Fish*; Kluwer Academic Publishers: Boston, MA, 1998.
- (33) Norris, D. O. *Vertebrate Endocrinology*, 3rd ed.; Academic Press: San Diego, CA, 1997.
- (34) Trudeau, V. L. Neuroendocrine regulation of gonadotrophin II release and gonadal growth in the goldfish, *Carassius auratus*. *Rev. Reprod.* **1997**, *2*, 55–68.
- (35) You, L. Steroid hormone biotransformation and xenobiotic induction of hepatic steroid metabolizing enzymes. *Chem.-Biol. Interact.* **2004**, *147*, 233–246.
- (36) Callard, G. V.; Tchoudakova, A. V.; Kishida, M.; Wood, E. Differential tissue distribution, developmental programming, estrogen regulation, and promoter characteristics of *cyp19* genes in teleost fish. *J. Steroid Biochem. Mol. Biol.* **2001**, *79*, 305–314.
- (37) Miller, W. L. Molecular biology of steroid hormone synthesis. *Endocr. Rev.* **1988**, *9*, 295–318.
- (38) Stocco, D. M.; Clark, B. J. Regulation of the acute production of steroids in steroidogenic cells. *Endocr. Rev.* **1996**, *17*, 221–244.
- (39) Thomas, P.; Zhu, Y.; Pace, M. Progesterone membrane receptors involved in the meiotic maturation of teleost oocytes: a review and some new findings. *Steroids* **2002**, *67*, 511–517.
- (40) Mourout, B.; Nguyen, T.; Fostier, A.; Bobe, J. Two unrelated putative membrane-bound progesterone receptors, progesterone membrane receptor component I (PGMRCI) and membrane progesterone receptor (mPR) beta, are expressed in the rainbow trout oocyte and exhibit similar ovarian expression patterns. *Reprod. Biol. Endocrinol.* **2006**, *4*, 6; <http://www.rbej.com/content/4/1/6>.
- (41) Yaron, Z. Endocrine control of gametogenesis and spawning induction in the carp. *Aquaculture* **1995**, *129*, 49–73.
- (42) Chang, T.-Y.; Chang, C. C. Y.; Ohgami, N.; Yamauchi, Y. Cholesterol sensing, trafficking, and esterification. *Annu. Rev. Cell. Dev. Biol.* **2006**, *22*, 129–157.
- (43) Altschul, S. F.; Madden, T. L.; Schäffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acid Res.* **1997**, *25*, 3389–3402.
- (44) Nimpf, J.; Stifani, S.; Bilous, P. T.; Schneider, W. J. The somatic cell-specific low density lipoprotein receptor-related protein of the chicken. Close kinship to mammalian low density lipoprotein receptor gene family members. *J. Biol. Chem.* **1994**, *269*, 212–219.
- (45) Trudeau, V. L.; Spanswick, D.; Fraser, E. J.; Larivière, K.; Crump, D.; Chiu, S.; MacMillan, M.; Schulz, R. W. The role of amino acid neurotransmitters in the regulation of pituitary gonadotropin release in fish. *Biochem. Cell Biol.* **2000**, *78*, 241–259.
- (46) Geigerseder, C.; Doepner, R. F. G.; Thalhammer, A.; Krieger, A.; Mayerhofer, A. Stimulation of TM3 leydig cell proliferation via GABA_A receptors: a new role for testicular GABA. *Reprod. Biol. Endocrinol.* **2004**, *2*, 13; article available from <http://www.rbej.com/content/2/1/13>.
- (47) Korte, J. J.; Kahl, M. D.; Jensen, K. M.; Pasha, M. S.; Parks, L. G.; LeBlanc, G. A.; Ankley, G. T. Fathead minnow vitellogenin: complementary DNA sequence and messenger RNA and protein expression after 17 β -estradiol treatment. *Environ. Toxicol. Chem.* **2000**, *19*, 972–981.
- (48) Gene Ontology Consortium. The gene ontology (GO) project in 2006. *Nucleic Acid Res.* **2006**, *34*, D322–D326.

Received for review July 21, 2006. Revised manuscript received September 26, 2006. Accepted October 4, 2006.

ES061739X