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Ecotoxicogenomics to Support Ecological Risk Assessment: A Case Study with Bisphenol A in Fish

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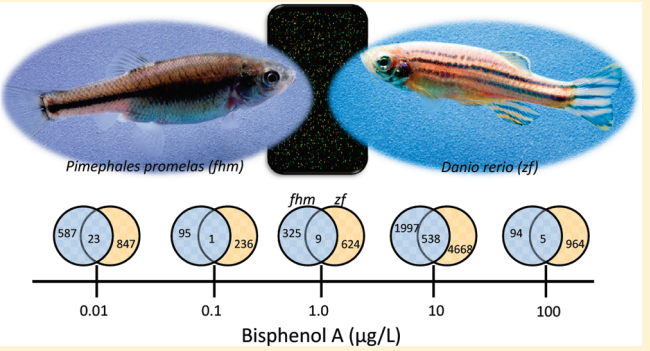
Supporting Information

ABSTRACT: Effects of bisphenol A (BPA) on ovarian transcript profiles as well as targeted end points with endocrine/reproductive relevance were examined in two fish species, fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*), exposed in parallel using matched experimental designs. Four days of waterborne exposure to 10 μg BPA/L caused significant vitellogenin induction in both species. However, zebrafish were less sensitive to effects on hepatic gene expression and steroid production than fathead minnow and the magnitude of vitellogenin induction was more modest (i.e., 3-fold compared to 13 000-fold in fathead minnow). The concentration−response at the ovarian transcriptome level was nonmonotonic and violated assumptions that underlie proposed methods for estimating hazard thresholds from transcriptomic results. However, the nonmonotonic profile was consistent among species and there were nominal similarities in the functions associated with the differentially expressed genes, suggesting potential activation of common pathway perturbation motifs in both species. Overall, the results provide an effective case study for considering the potential application of ecotoxicogenomics to ecological risk assessments and provide novel comparative data regarding effects of BPA in fish.



INTRODUCTION

DNA microarrays and other types of "global" analysis or 'omics' tools (e.g., metabolomics, proteomics, whole transcriptome shotgun sequencing) are now widely employed in ecotoxicological research. However, questions remain regarding how to best apply data generated using these techniques in support of ecological risk assessments. Three general applications have been proposed. First and perhaps foremost, it is expected that the application of "omics" tools will lead to improved understanding of chemical modes of action, cellular responses to stressors, and their linkage to traditional apical outcomes. Such understanding is viewed as critical to support a new approach to toxicity testing that would rely on detecting and characterizing a chemical's ability to initiate cellular perturbations that can ultimately manifest as toxicity, rather than directly measuring adverse apical outcomes.[1] A second proposed application centers on the development of libraries of ecotoxicogenomic fingerprints which would be either diagnostic of specific types of exposure or predictive of specific effects. The assumption underlying such fingerprint-based applications is that if the exposure is sufficiently severe to perturb an important cellular function, it should elicit a characteristic and recognizable profile of responses (pathway perturbation motif) that would be discernible from a background of stochastic responses that occur within the organism's normal homeostatic range. Finally, some have proposed that global analyses could be used to determine in vivo adverse effect levels (e.g., lowest/no observed adverse effect levels) more sensitively and/or rapidly than can be achieved using conventional apical end points such as mortality, reproductive failure, or developmental impairment. A prominent example of this is the no observed transcriptional effect level (NOTEL) concept.[2] The NOTEL proposal was based on observations that there was a threshold for eliciting estrogen-specific transcriptional responses in vitro.[2] In vivo studies conducted by Naciff et al.[3] reported that in rats exposed to three different estrogen receptor agonists

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(17α-ethynyl estradiol, bisphenol A, genistein) both numbers of genes significantly changed, and magnitude of change increased with dose, suggesting the NOTEL concept may be applied to in vivo studies. Assuming this type of threshold and dose-dependence for transcriptional (or other omic) response is a generalized phenomenon, and is more sensitive than targeted end points/analyses, omics data could have utility for determining hazard and exposure thresholds in risk assessments.

The present study reports on the effects of bisphenol A (BPA) on several targeted end points with reproductive/endocrine relevance and on ovarian transcriptional profiles in two cyprinid fish species, fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*). Bisphenol A is an industrial chemical widely used in the production of polycarbonate plastics, epoxy resins, thermal paper, coated papers, protective coatings, adhesives, and other products.[4] It has been detected in surface waters throughout in North America, Europe, and Japan with median concentrations around 0.01−0.081 μg/L and upper 95th percentile concentrations around 0.4 μg/L.[5] The occurrence of BPA in surface water is of concern as BPA has long been characterized as a xenoestrogen, and recent work suggests additional modes of endocrine action including potential antiandrogenic activity and aromatase inhibition.[6]

Using BPA as a case study, the present investigation was designed to explore a number of questions relevant to the use of ecotoxicogenomics to support risk assessments. First, we evaluated whether a threshold concentration, or NOTEL, for a transcriptional effect of BPA in the two fish species was evident, and whether the number and magnitude of transcriptional effects increased as a function of BPA concentration once that threshold was exceeded. Additionally, sensitivity of the transcriptional response was compared to that of more targeted endocrine end points examined in the fish, as well as effect concentrations reported in the literature. Second, we considered whether BPA elicited a similar profile of transcriptional responses in two common laboratory fish models exposed under highly uniform conditions. This permitted us to assess whether the assumption underlying transcriptional fingerprinting, that a chemical elicits a characteristic profile, extends across closely related species. This was evaluated at the level of the response of individual genes as well as at the functional level. Finally, BPA's potential mechanism(s) of action and associated toxicity pathways in fish in the context of key functions relevant to fish reproduction were evaluated using functional analysis of microarray results.

EXPERIMENTAL SECTION

Waterborne Exposures. Bisphenol A (>99% pure) was obtained from Sigma-Aldrich (St. Louis, MO). Sexually mature adult fathead minnows (5−6 month old) and zebrafish (ab wild-type strain; 6−7 month old) were obtained from an on-site aquatic culture facility. Fish were exposed to 0, 0.01, 0.1, 1.0, 10, or 100 μg BPA/L delivered in a continuous flow (45 mL/min) of sand filtered, UV treated, Lake Superior water, without the use of carrier solvents. Following approximately 36 h of chemical (or control water) delivery to condition the tanks (20 L aquaria filled with 10 L of exposure water) and exposure apparatus, exposures were initiated by loading fish into the conditioned tanks. The design employed six replicate tanks per treatment. Three replicate tanks were loaded with three male and three female zebrafish each, while the remaining three were loaded with three male and three female fathead minnows each. Addition of fish was staggered by replicate such that all sampling could be completed within 60 min of the desired 96 h exposure duration. Zebrafish were loaded and sampled one day prior to loading and sampling the fathead minnows so that three of the four exposure days for each species directly overlapped. Both species were held under a 16:8 light:dark photoperiod and fed to satiation twice daily. Fathead minnows were fed frozen adult brine shrimp (San Francisco Bay Brand, Newark, CA) while zebrafish were fed newly hatched (<24 h old) brine shrimp nauplii (BioMarine Brand artemia cysts; Hawthorne, CA). Fish survival, water temperature (mean ± SD; 25.0 ± 0.3; both species), and dissolved oxygen (mean ± SD; zebrafish 6.7 ± 0.3 mg/L; fathead 7.0 ± 0.2) were monitored daily and did not vary significantly between treatment groups or species. Water from each tank was sampled daily for analytical verification of BPA exposure concentrations (see Supporting Information (SI)). After 96 h of exposure, fish were anesthetized in a buffered solution of tricaine methanesulfonate (MS-222; Finquel; Argent, Redmond, WA) and weighed. Blood was collected from the caudal vasculature using microhematocrit tubes, centrifuged to separate the plasma, and plasma was stored at −80 °C until analyzed. Liver, gonad, and brain tissues (including pituitary gland) were snap frozen in liquid nitrogen and stored at −80 °C until extracted. Prior to freezing, subsamples of gonad tissue [female fathead minnows 10.2 ± 4.3 mg; male fathead minnows 6.6 ± 2.3 mg; female zebrafish 12.0 ± 5.2 mg; male zebrafish 2.1 ± 1.3 (mean ± SD)] were collected and transferred to test-tubes containing 500 μL tissue culture medium for determination of ex vivo steroid production.

Targeted Analyses. Ex vivo steroid production assays were conducted using methods adapted from McMaster et al.[7] as described by Villeneuve et al.[8] Ex vivo testosterone (T) production was measured for males and females, whereas ex vivo production of 17β-estradiol (E2) was measured for females only. Fathead minnow plasma vitellogenin (VTG) concentrations were determined by enzyme linked immunosorbent assay (ELISA).[9] Zebrafish plasma VTG concentrations were determined using a commercial zebrafish VTG ELISA test kit (Biosense Laboratories, Bergen, Norway). Plasma steroid concentrations were measured by radioimmunoassay.[10] Targeted gene expression analyses used total RNA extracted from male liver. Total RNA was extracted with Tri Reagent (Sigma, St. Louis, MO) according to the manufacturer's protocol. RNA concentration and quality were evaluated using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Relative abundance of transcripts coding for vitellogenin (*vtg*) and estrogen receptor α (*esr1*) was determined by real-time quantitative PCR (QPCR) using Power SybrGreen RNA-to-Ct 1-step kits (Applied Biosystems, Foster City, CA; see SI).

Data from targeted end points were analyzed using similar statistical methods. Normality was evaluated using a Kolmogorov−Smirnov test and data were log transformed, where necessary, to meet assumptions of parametric statistics. Homogeneity of variance was evaluated using Levene's test. Differences among treatment groups were evaluated by one-way analysis of variance (ANOVA; $p < 0.05$) followed by Duncan's posthoc test ($p < 0.05$).

Microarray Analyses. Total RNA was isolated from ovary tissue of both fathead minnow and zebrafish using RNeasy kits (Qiagen, Valencia, CA). RNA quality was evaluated using an Agilent 2100 Bioanalyzer (Agilent, Wilmington, DE) and RNA was quantified spectrophotometrically (Nanodrop). Total RNA samples were stored at −80 °C until used for microarray analyses. Ovarian transcripts from 32 fathead minnows (6 treatments, $n = 5$

per treatment, except for control and 1.0 μg BPA/L, $n = 6$) were analyzed using a custom, 15 000 feature microarray (GEO Platform Accession GPL9248) purchased from Agilent Technologies (Palo Alto, CA). Ovarian transcripts from 34 zebrafish (6 treatments, $n = 6$ per treatment, except 0.01 and 0.1 μg BPA/L, $n = 5$) were analyzed using a 44 000 feature microarray (Agilent design 019161; GEO Platform Accession GPL6457). The same hybridization and scanning procedures were used for both platforms. Briefly, microarrays were hybridized following the manufacturer's guidelines for One-Color Microarray-Based Gene Expression Analysis (version 5.7; Agilent). Hybridizations were conducted with 1 μg of total RNA. Complementary DNA synthesis, cRNA labeling, amplification, and hybridizations were performed following the manufacturer's kits and protocols (Quick Amp labeling kit; Agilent). Scanning was conducted at 5 μm resolution using an Axon GenePix 4000B Microarray Scanner (Molecular Devices Inc., Concord, Ontario, Canada). Data were extracted from microarray array images using Agilent Feature Extraction Software. Text versions of the raw data and data matrices of Fastlo normalized intensity values have been deposited to the Gene Expression Omnibus (GEO: http://www.ncbi.nlm.nih.gov/geo/; Accession GSE28354).

Data sets for each species were analyzed independently. However, parallel approaches were employed in order to minimize the influence of the bioinformatic methods on the interspecies comparison. Microarray data sets for both species were normalized using Fastlo[11] implemented in R (http://www.r-project.org/). Basic determination of differential gene expression between each BPA treatment and the control was based on pairwise t tests implemented in MultiExperiment Viewer (MeV) v 4.5 (www.tm4.org; [12]) using a Welch approximation (assuming unequal group variances) and a critical p-value of 0.01, with no multiple test correction (SI Appendices S1, S2). One-way ANOVA ($p < 0.01$; no multiple test correction) was used to identify genes that were significantly differentially expressed among all treatments. That subset of genes was subjected to principal components analysis (implemented in MeV) using median centering. Over- or underrepresentation of functional annotations such as gene ontology (GO) categories or biological pathways within the various lists was evaluated using both eGOn v2.0[13] and DAVID[14] (see SI).

To facilitate direct comparison of differentially expressed genes between species, specific features (probes) from the fathead minnow microarray were mapped to homologous features on the zebrafish microarray. Mapping was based on annotation information available for each array (see SI). In total, 16 251 unique homologous pairings of fathead minnow and zebrafish microarray features were identified (see SI section E.3 and Table S2). The list of homologous pairings was used to create a database (Microsoft Access 2007) that was used to translate the lists of differentially expressed fathead minnow microarray features into their homologous zebrafish probe IDs. Translated lists were imported into MeV and compared to differentially expressed gene lists for zebrafish using MeV's Venn diagram tools. Significance of overlap between lists of differentially expressed genes for the two species was evaluated by comparing the number of overlaps observed, with the number that would be expected by chance given the relative size of each differentially expressed gene list (translated list in the case of fathead minnow) as a proportion of the total number of unique homologous pairings (see SI section E.4).

Gene set enrichment analysis (GSEA) was conducted for all treatment groups, compared with their respective controls (www.broad.mit.edu/gsea; v2.0). Two types of gene sets were included in the GSEA for each species (see SI Table S3). The first of these consisted of gene sets corresponding to features represented within the ovary compartment of a conceptual model of the teleost brain-pituitary−gonadal axis,[15] recently updated by the authors. This included both a compiled list for the entire ovary compartment, as well as sublists organized into key functional categories (e.g., steroid biosynthesis, oocyte growth, etc.). The second type of gene set was derived from zebrafish transcription factor networks described by Wang et al.[16] These corresponded to zebrafish transcription factor networks with either estrogen receptor α (esr1; DRTFovaryCy5Cy3_A_15_P100370) or androgen receptor (ar; DRTFovaryCy5Cy3_A_15_P113550) as their hub. Because the transcription factor networks reported by Wang et al. were derived using a different zebrafish microarray design (Agilent 015064), homologous features were matched to the zebrafish microarray design used in the present study (Agilent 019161), and subsequently translated to homologous fathead minnow probes using the homology database described above (see SI).

RESULTS AND DISCUSSION

Targeted Analyses. Measured concentrations of BPA were close to nominal over the duration of the test (see SI) and no mortalities occurred in either species. Consistent with its expected estrogenic mode of action, exposure to BPA induced *vtg* and *esr1* mRNA expression in livers of male fathead minnows (SI Figure S1) and significantly increased plasma VTG concentrations in both males and females (Figure 1). Ex vivo E2 production and plasma E2 concentrations were significantly decreased in females (SI Figure S2), while circulating T concentrations (but not ex vivo T production) were significantly reduced in males (SI Figure S3). Decreases in steroid concentrations are consistent with a negative feedback response to a physiologic excess of estrogenic signal. With the exception of hepatic *esr1* mRNA expression in males, significant effects on targeted end points in fathead minnows generally occurred at BPA concentrations ≥10 μg/L.

Zebrafish were less sensitive to BPA than fathead minnows. In females, BPA had no significant effect on plasma VTG concentrations (Figure 1). In males, there was a statistically significant increase in plasma VTG concentrations (Figure 1). However, the 3-fold increase observed was modest compared to circulating VTG concentrations in female zebrafish, and relative to the magnitude of induction observed in male fathead minnows (Figure 1). Plasma volumes were too limited to facilitate evaluation of circulating steroid concentrations, but there were no significant effects of ex vivo steroid production in zebrafish (SI Figure S4). Additionally, in contrast to male fathead minnows, hepatic *esr1* and *vtg* transcripts were unaffected in male zebrafish (SI Figure S5). Thus, while plasma Vtg induction in males provided evidence for a weak estrogenic effect in zebrafish, the species did not appear nearly as sensitive or responsive as fathead minnows, based on the targeted analyses.

In the present study, the sensitivity of both fish species to the xenoestrogenic effects of BPA, after just 4 days of exposure, was comparable to or greater than that reported previously in longer term experiments. For example, 160 μg/L was the reported LOEC for VTG induction in adult male fathead minnows exposed to a continuous flow of BPA for 43, 71, or 164 days.[17] In the same study, VTG was significantly decreased in female fathead minnows exposed to 16 μg BPA/L for 43 days, but only

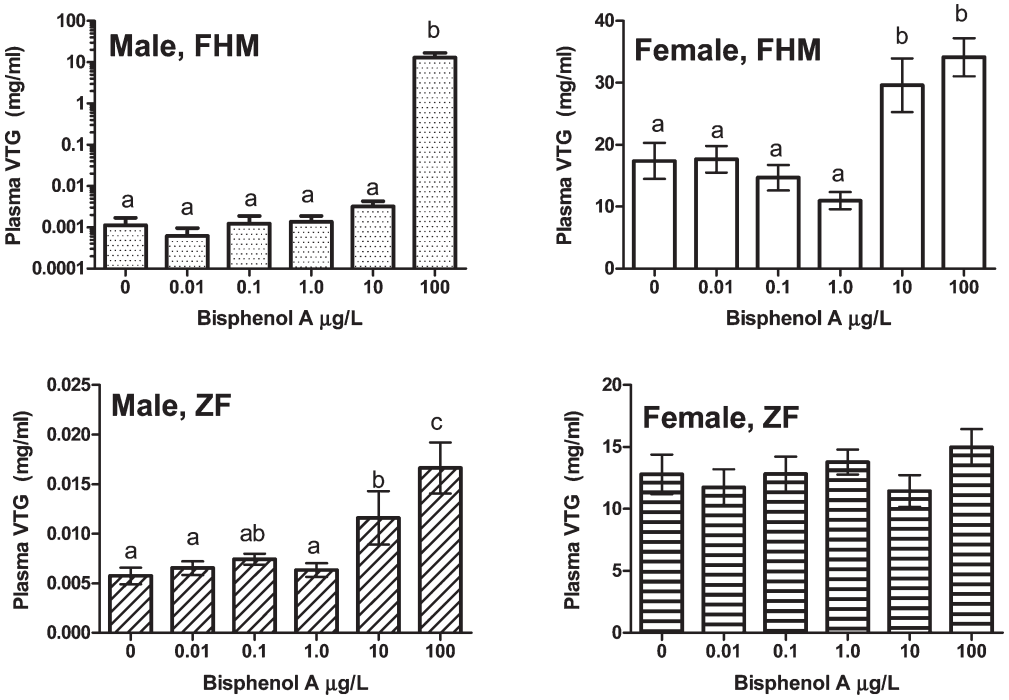

Figure 1. Plasma vitellogenin (VTG) concentrations (mean ± standard error) in male (stippled) and female (clear) fathead minnows (FHM) and male (hatched) and female (horizontal stripes) zebrafish (ZF) exposed to bisphenol A for 96 h. Different letters indicate statistically significant differences based on Duncan's posthoc test ($p < 0.05$). Note, *y*-axis for male FHM is on a log scale, all other *y*-axes are linear.

increased in those exposed to 640 μg BPA/L for 164 days. Similarly, in a three week static renewal experiment in which adult zebrafish were exposed to 40, 200, or 1000 μg BPA/L (nominal) only the 1000 μg/L concentration caused significant VTG induction in males,[18] whereas in the present study modest, statistically significant, effects on plasma VTG concentrations in male zebrafish were detected after just 4 days of exposure to 10 μg BPA/L. As noted above, decreased steroid production in the female fathead suggests a homeostatic response that would help keep effective estrogen concentrations in an appropriate range, despite the exogenous estrogenic signal. It is feasible that this type of homeostatic response could account for the apparent lesser sensitivity to BPA in the longer-term exposures. These results highlight the need to understand potential adaptive responses to stressors and their limits if short-term assays that evaluate the initiation of a toxicity pathway, rather than direct apical effects are to be used as a basis for quantitative risk assessment.[1]

Microarray Analyses. The proportion (%) of microarray features identified as statistically differentially expressed ($p < 0.01$) compared to the controls, as a function of BPA concentration, was very similar for the two species (Figure 2). In females exposed to 0.01 μg BPA/L approximately 4% and 2% of microarray features had expression levels significantly different from controls in the fathead minnow and zebrafish, respectively. The level of impact on the ovarian transcriptome declined slightly in both species in the 0.1 and 1.0 μg BPA/L treatments, and then increased dramatically at the 10 μg BPA/L concentration before falling to less than 2.5% at 100 μg BPA/L (Figure 2). The marked increase in transcriptomic effect at 10 μg BPA/L was consistent with the targeted end points which generally showed significant effects at 10 μg BPA/L or greater. However, the dramatic reduction ovarian transcriptome effects at 100 μg BPA/L was in stark contrast with the increased impact on the targeted

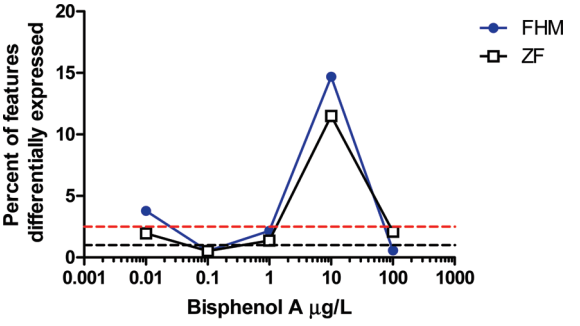

Figure 2. Percent microarray features (15 744 fathead minnow; 45 220 zebrafish) identified as differentially expressed compared to controls (*t* test; $p < 0.01$) in the ovary of fathead minnows (FHM) or zebrafish (ZF), as a function of bisphenol A concentration. Lower dashed line = 1%, upper dashed line = 2.5%.

end points. Furthermore, the apparent similarity in the sensitivity of the two species at the transcriptome level was inconsistent with sensitivity differences in targeted measurements such as plasma VTG.

Principal component analysis (PCA) was applied to the genes identified as differentially expressed based on a one-way ANOVA for each species (Figure 3). This data reduction approach provided a general impression of the degree of similarity/difference in the expression profiles among the various treatment groups. In both species, fish exposed to 10 μg BPA/L had ovarian gene expression profiles that were the most divergent from the controls (Figure 3). In the fathead minnow, the control class had the most variable expression profile and only the 0.01 and 10 μg BPA/L treatment classes were entirely separated from the control class along principal components 1 and 2, which captured

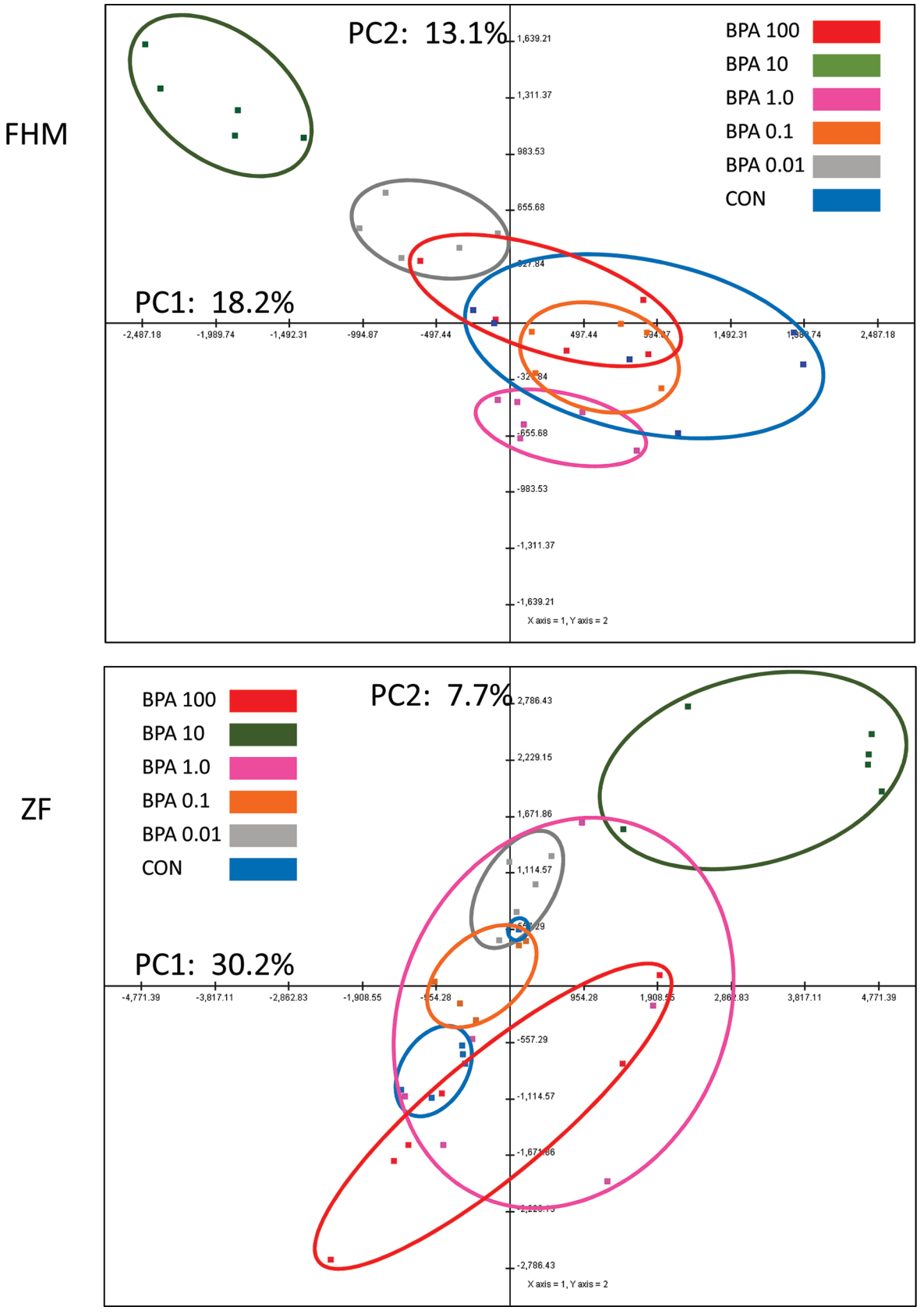

Figure 3. Principal components (PC) scores plots showing the relative similarity of ovarian transcription profiles for fathead minnows (FHM) or zebrafish (ZF) exposed to 0, 0.01, 0.1, 1.0, 10, or 100 μg bisphenol A/L for 96 h. Points represent PC scores for individual fish along PCs 1 and 2. Circles are intended as a general (nonstatistical) characterization of the PC space occupied by the treatment class.

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about 31% of the data set's variability. In contrast, zebrafish expression profiles were quite consistent for five of the six control samples, with one control sample grouping near the intersection of the 0.01 and 0.1 μg BPA/L treatment classes. With the exception of one outlying control sample, as in the fathead minnow, the 0.01 μg BPA/L zebrafish class separated from the control class, while the 100 μg BPA/L class was one of the more variable classes and overlapped the control class. Overall the PCA results support the conclusion that overall divergence of the ovarian gene expression profile from that of the controls did not exhibit a conventional monotonic concentration−response profile.

At the functional level, the bulk of the statistically enriched functional terms associated with genes differentially expressed in BPA-exposed fish were specific to concentration and species (see SI Tables 5−22). However, there were a few noteworthy trends. For example, in zebrafish there was statistical enrichment of differentially expressed genes associated with cell cycle control and mitosis in fish exposed to 0.01, 0.1, or 10 μg BPA/L (SI Tables S10−S12; S19, S21). A similar functional enrichment was not observed in the fathead minnow. However, in the case of the fathead minnow, four of the five BPA concentrations were associated with enrichment of genes annotated with functions related to intracellular signaling processes (SI Tables S5, S7−S9). Enrichment of genes related to intracellular signaling was rare for zebrafish; in fact at the two greatest BPA concentrations, annotations related to G-protein coupled signaling were statistically under-represented (SI Tables S12, S13). Differential expression of a rather small number of genes in both species led to multiple observations of enriched terms associated with camera-type eye development (SI Tables S5, S8−S10, S14, S21). This was notable as one of the functional enrichments common to both species at the lowest exposure concentration tested. However, the greatest cross-species similarities in functional enrichment were observed between fathead minnows exposed to 10 μg BPA/L and zebrafish exposed to either 10 or 100 μg BPA/L. Among these treatments, terms related to WD40 repeat domains, protein catabolism, DNA repair/response to DNA damage, chromatin modification and organization, RNA recognition motifs, and SANT/Myb DNA binding were common to both species (SI Tables S16, S21, S22). The greater extent of common enriched functional terms between the fathead minnow 10 μg/L group and the zebrafish 100 μg/L group, as compared to the zebrafish 10 μg/L group suggests that the similarity was likely the result of the organism response to BPA, rather than solely an artifact of the larger number of differentially expressed genes detected at the 10 μg BPA/L concentration.

Gene set enrichment analysis provided another approach to link changes in the ovary transcription profile with function. However, unlike the analyses conducted using eGoN and DAVID, GSEA considers all genes in the data set, not just the genes identified as statistically differentially expressed. As a result GSEA can facilitate statistical detection of either small changes in many genes within a gene set defined a priori or large changes in just a few of those genes.[19] In the fathead minnow, only two significant effects on functional gene sets derived from the ovary compartment of a graphical model of the teleost brain-pituitary−gonadal axis[15] were detected. There was enriched expression of prostaglandin-related genes in fish exposed to 1.0 μg BPA/L, while controls showed enriched expression of oocyte growth-related genes, compared to fish exposed to 100 μg BPA/L (SI Table S1). Generally speaking, graphical model derived gene sets were much more impacted in zebrafish. Specifically, expression of the set of 11 genes associated with cholesterol uptake (e.g., apolipoproteins, steroidogenic acute regulatory protein, cholesterol esterase, lipoprotein lipase, sterol carrier protein 2) was significantly enriched in the control group compared to all other treatments. Additionally, expression of genes coding for various matrix metalloproteinases, which play a role in ovulation, was also more highly enriched in the control zebrafish than in those exposed to 0.01, 0.1, or 10 μg BPA/L. In contrast, expression of genes related to steroid biosynthesis was significantly enriched in fish exposed to the lowest three concentrations of BPA (0.01, 0.1, and 1.0 μg/L) compared to controls. In general, the zebrafish results suggest that BPA exposure was significantly influencing expression of genes known to play important roles in ovarian function.

The two gene sets based on reverse engineered zebrafish transcription factor networks for *ar* and *esr1* were not significantly impacted in the fathead minnow. This was not unexpected, as genes in the mutual information network are those whose expression profiles were highly correlated in zebrafish over a range of experimental conditions. They would not necessarily be as well conserved among species as the genes directly regulated by the cognate receptors. Nonetheless, significant impacts on both the *ar* and *esr1* transcription factor network gene sets were detected in zebrafish (SI Table S1). Most notably, the *esr1* transcription factor network was impacted by exposure to 1.0 μg BPA/L or greater. Expression was enriched in fish exposed to 1.0 or 100 μg BPA/L, where overall numbers of differentially expressed genes were low. In contrast, expression of the *esr1* transcription factor network genes was enriched in the controls compared to the 10 μg BPA/L group, where overall numbers of differentially expressed genes were highest. At present, it is unclear whether the opposing GSEA profiles and the dramatic differences in overall differential gene expression are linked, but a connection is at least plausible. Based on DAVID functional enrichment analysis, the zf_esr1_TF_network gene set was enriched with features associated with VTG and lipid transport, cytoskeleton, WD40 repeat domains, ubiquitin/protein catabolism, ribosomes, response to estrogen stimulus, tRNA metabolism, and coagulation/wound-repair related proteins (SI Table S23). Effects on such genes are both consistent with BPA's estrogenic mode of action and show significant similarity to some enriched functions associated with zebrafish exposure to 10 or 100 μg BPA/L or fathead minnows exposed to 10 μg BPA/L. The zf_ar_TF_network was only impacted in the zebrafish exposed to 10 μg BPA/L and expression was enriched in the controls compared with the BPA-exposed fish. Functional annotations associated with the zf_ar_TF_network included serine/threonine kinase activity, amino acid transport, notch signaling, pleckstrin homology, and TGFβ signaling. The impact on the zf_ar_TF_network gene set could be viewed as broadly consistent with potential antiandrogenic activity of BPA.[20,21] However, unlike the zf_esr1_TF_network gene set, there were no corroborating effects at other BPA concentrations. While the present study focused solely on two reverse engineered transcription factor networks, similar approaches could be applied to screen for effects on any one of the 550 transcription factor networks developed by Wang et al.[16]

Implications for Ecological Risk Assessment. *NOTEL.* The present study provided an opportunity to explore a number of questions underlying the potential use of ecotoxicogenomic data to support ecological risk assessments. First, we considered our transcriptomic results in light of the potential of the NOTEL concept as a means for setting hazard thresholds. Given the

number of end points considered in the typical transcriptomic analysis, defining a no effect concentration is complicated by the high background of false positive discovery that can be expected using traditional statistical methods. However, application of stringent multiple testing corrections can lead to very high false negative rates,[22] which is equally undesirable from the standpoint of hazard assessment.[2] No standardized methods or guidelines for deriving NOTEL's have been published. Therefore, at present differentiation of a "significant" transcriptional effect from "no transcriptional effect" is rather arbitrary. For example in the present study, applying a statistical cutoff of $p < 0.01$, one could theoretically expect false discovery of 1% of the features as differentially expressed. Using a 1% threshold, one could define the NOTEL for both species as <0.01 μg BPA/L (Figure 2). Being a bit more stringent, one could arbitrarily require that at least 2.5% of the features be detected as differentially expressed. In this case, the NOTEL for fathead minnow would be <0.01 μg BPA/L, while that for zebrafish would be 10 μg BPA/L. Based on a qualitative evaluation of the concentration−response, the NOTEL, viewed as a response threshold, might be best defined as 10 μg BPA/L for both species. In terms of sensitivity, the 10 μg BPA/L threshold was in good agreement with the sensitivity of most of the targeted end points evaluated here. In general, the transcriptional effect was also more sensitive than apical responses (e.g., impacts on survival, reproduction, development) reported in a variety of fish, amphibian, and invertebrate models.[23] This suggests that if specific motifs within the transcriptional response profile could be adequately linked to adverse outcomes, an omics-based approach, like the NOTEL, may be viable as a more sensitive method for setting hazard thresholds.

Nonetheless, in the present study, regardless of where the threshold is set, the number of transcriptional effects observed did not increase monotonically as a function of BPA concentration. Similarly, the overall profile of transcriptional response, as characterized by PCA, did not show predictable concentration-dependence. Thus, the data violate fundamental assumptions underlying the NOTEL approach. This lack of conformity with NOTEL assumptions may be due in part to unsupervised application of the approach. Others have based their NOTEL determination on subsets of genes known to respond to a certain class of chemicals, or to generalized cellular stress.[3,24] In considering application of the NOTEL concept in ecological risk assessment, participants of an expert workshop suggested that the scope would need to be restricted to groups or families of genes known, a priori, to be associated with specific toxicity pathways of regulatory concern.[25] With that in mind, we restricted the scope of our analysis to a subset of gene expression end points with known roles in fish reproduction (e.g., those in the gonad_all gene sets used for GSEA; SI Table S3). However, that did not produce a significantly different concentration-dependent response profile (SI Figure S6). The approach of Naciff et al.[3] was to restrict their analysis to a subset of genes whose expression was consistently altered, in the same direction, by three chemicals with a (putative) common mode of action. However, broad implementation of this approach would make the NOTEL concept heavily dependent on the development of libraries of robust and reproducible omic fingerprints or profiles linked to specific adverse outcomes. In the near term, selection of gene subsets to consider in a NOTEL determination would seem so arbitrary that the use of such supervised NOTEL determination in risk assessment could be highly contentious. It is notable that at least some studies employing an unsupervised approach have provided support that the NOTEL concept could be useful.[26,27] However, based on the results of the present study, that is clearly not universally true. Rather, results of the present study suggest that a critical review of the conformity of microarray concentration−response data with NOTEL assumptions and development of standard methods/guidelines for NOTEL determination are critical, if this approach is to be used to support ecological risk assessment.

Reproducible Transcriptomic Fingerprints. The broad applicability of transcriptomic fingerprinting approaches in either exposure or hazard assessment is dependent on the ability to induce consistent profiles of response under different experimental conditions and, ideally, in different species. In a previous ecotoxicogenomic study with zebrafish and fathead minnows exposed to the dopamine 2 receptor antagonist, haloperidol, we observed very little similarity in the ovarian transcriptional response of the two species.[28] In that study, haloperidol generally did not appear to cause significant disruption of reproductive end points in either species. Therefore, it was hypothesized that transcript changes reflected relatively stochastic responses associated with modest or negligible effects on the ovary tissue and/or reproductive status. The assumption was that a chemical exposure that elicited a significant apical response would be more likely to trigger a pathway perturbation motif that would be conserved across species and discernible from a background of stochastic transcriptional response. The present study was designed, in part, to test this. Overall, the functional analyses provided some support for the hypothesis, as there were notable similarities in the functions impacted by BPA exposure, albeit at different concentrations for the two species. Overlaps in the individual genes differentially expressed in the two species were not markedly different from that expected by chance alone. Consequently, it is difficult to evaluate which overlapping genes reflect a potential pathway perturbation motif, and which may represent random overlaps. Unfortunately, our analysis was also confounded by the apparent difference in the sensitivity of the two species to BPA. Consequently, while providing some tentative suggestion of a conserved response at the functional level, further investigation ideally at chemical concentrations that elicit similar magnitudes of apical effect in both species will be needed to evaluate the hypothesis further. To date, studies of this nature are rare, but they are critical to define the bounds and conditions within which various libraries of transcriptomic fingerprints can be reliably applied.

Overall, the results of our case study with BPA support both hope and caution regarding the application of ecotoxicogenomics in risk assessment. Our data suggest that transcriptomic responses in a short-term study do have sensitivity comparable to or greater than that of apical responses in longer-term experiments. Additionally, at the functional level there was notable similarity in the response among species suggesting that identification of reproducible pathway perturbation motifs meaningful to risk assessment may be feasible. We were able to leverage the comparison of responses in two species exposed under very comparable conditions to focus our attention, and future investigation, on subset of the putative functional responses. Additionally, using GSEA and custom gene sets anchored to a graphical systems model or to reverse engineered transcription factor networks we were able to link the broader profile of expression changes to a more readily interpreted biological context. Nonetheless, a great deal of additional study and development is needed to critically evaluate the use of omics data to define hazard thresholds, to identify informative motifs,

and enhance the ability to infer the biological significance of results through linkage to adverse outcome pathways.

■ ASSOCIATED CONTENT

Supporting Information. Additional methodological details (sections E.1−E.5; Tables S1−S3); results figures for targeted analyses (Figure S1−S5) and supervised NOTEL estimation (Figure S6); functional analysis results (Tables S5−S24); and appendices with full lists of differentially expressed genes (Appendix S1, S2) and lists of probes for each gene set used for GSEA (Appendix S3) in Excel format. This material is available free of charge via the Internet at http://pubs.acs.org.

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