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Transcriptomic and Network Analyses Reveal Mechanistic-Based Biomarkers of Endocrine Disruption in the Marine Mussel, *Mytilus edulis*

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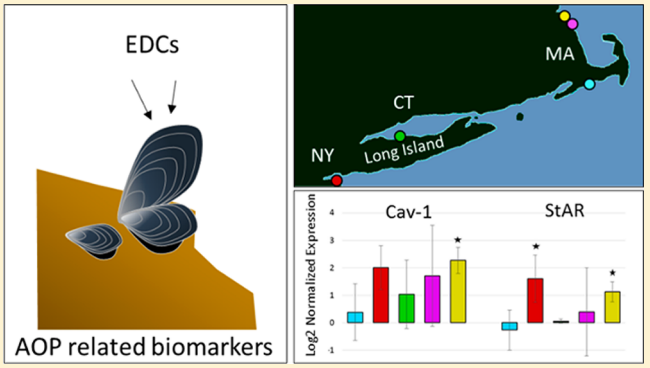
Supporting Information

ABSTRACT: Transcriptomics, high-throughput assays, and adverse outcome pathways (AOP) are promising approaches applied to toxicity monitoring in the 21st century, but development of these methods is challenging for nonmodel organisms and emerging contaminants. For example, Endocrine Disrupting Compounds (EDCs) may cause reproductive impairments and feminization of male bivalves; however, the mechanism linked to this adverse outcome is unknown. To develop mechanism-based biomarkers that may be linked through an AOP, we exposed *Mytilus edulis* to 17-alpha-ethinylestradiol (5 and 50 ng/L) and 4-nonylphenol (1 and 100 μg/L) for 32 and 39 days. When mussels were exposed to these EDCs, we found elevated female specific transcripts and significant female-skewed sex ratios using a RT-qPCR assay. We performed gene expression analysis on digestive gland tissue using an *M. edulis* microarray and through network and targeted analyses identified the nongenomic estrogen signaling pathway and steroidogenesis pathway as the likely mechanisms of action for a putative AOP. We also identified several homologues to genes within the vertebrate steroidogenesis pathway including the cholesterol side chain cleavage complex. From this AOP, we designed the Coastal Biosensor for Endocrine Disruption (C-BED) assay which was confirmed in the laboratory and tested in the field.



INTRODUCTION

Wastewater treatment plant (WWTP) and industrial discharges, nonpoint sources, and oil and chemical spills introduce a complex mixture of pollutants into marine systems. The numerous contaminants released presents monitoring challenges, as screening individual compounds or determining synergistic relationships within mixtures is difficult, time intensive, and costly. Endocrine Disrupting Compounds (EDCs) present unique obstacles as these compounds cause their effects at low levels (e.g., ng/L), are often below analytical detection limits, and are temporally and spatially variable.[1,2] One approach proposed to overcome these challenges is to develop mechanism-based biomarkers through an adverse outcome pathway (AOP) in which pollutant concentrations are correlated with responses in the organism at different levels of biological organization (e.g., cells, tissue, individual, population).[3] The AOP is a useful framework for monitoring and risk assessment as it assays biomarkers within the context of pathways that are expected to directly lead to an adverse outcome (AO). Therefore, genes responding in this pathway may be sensitive indicators of the AO or may represent the cumulative effects of contaminants which operate by the same mechanism of action.

Mussels are used globally as sentinel species in coastal monitoring programs due to their ability to accumulate pollutants in tissues over time as they filter feed. New assays developed to detect EDCs in mussels could more easily be integrated into existing marine monitoring programs without the need to develop a new framework. Currently, bivalves have been shown to be impacted by endocrine disruption as intersex may occur after EDC exposure (e.g., as shown in *Scrobicularia plana, Saccostrea glomerata,* and *Crassostrea gigas)* as well as in the field near polluted estuaries or WWTPs (e.g., as seen in *Scrobicularia plana, Mytilus edulis, Ruditaper decussatus,* and *Cerastoderma glaucum)*.[4−9] Additionally, development of an AOP in bivalves shows promise as changes in the expression of reproduction, cell cycle, and cell signaling genes have been identified in intersex bivalves.[8,10−13]

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However, the mechanism of endocrine disruption causing the AO still needs to be established in bivalves. In vertebrates, EDCs mimic estrogen and bind to estrogen receptors (ERs), ultimately causing gender reversal and impacts at the population and ecosystem level.[14,15] Although there has been an ER identified in mussels homologous to vertebrate ERs, the ER in bivalves does not bind EDCs and has been identified as a constitutive transcriptional activator.[16−19] Therefore, although vertebrates and bivalves may have a similar AO, they are likely occurring through different mechanisms and AOPs.

Omic methodologies may help overcome challenges working with nonmodel organisms that lack annotated biochemical pathways and regulatory mechanisms since it allows global examination of mRNAs, proteins, and metabolites, and several studies have supported the use of 'omics' to identify biomarkers of exposure in bivalves.[20] Omics can therefore provide the experimental methods for the development of predictive tools of EDC exposure, although exploratory analyses and computational tools are needed to further hypothesize links between molecular events and AOs. Network biology is one approach used to establish key events and key event relationships in an AOP, since it provides a macro view of the cascade of gene interactions and the functional state of the entire network without focusing on individual genes.[21] Hub genes in a network can also help target potential key events for candidate AOPs as they have many interaction partners and are essential to the responding pathway.[22,23] Therefore, using these approaches we can develop new biomarkers and AOPs which can later be tested and verified for use in regulatory toxicity testing, risk assessment, and monitoring.

To provide insight into the mechanism of action, we performed three independent analyses: 1.) A targeted approach investigating whether enzymes within the vertebrate steroidogenesis pathway are present in bivalves and impacted by EDCs; 2.) An enrichment analysis identifying overrepresented differentially expressed genes (DEGs); and 3.) Network analysis examining the interaction network of DEGs and the pathways linked with genes coding for reproduction and development. From these analyses we hypothesized that the nongenomic estrogen signaling pathway and the steroidogenesis pathway are likely the primary mechanisms of action of endocrine disruption in mussels. This enabled us to select robust and sensitive biomarkers of endocrine disruption that were validated in both the laboratory and field.

MATERIALS AND METHODS

Mussel Collection and Exposure. Gonadal monitoring was performed every 2 weeks for a year leading up to the initiation of the experiment. Using the circumference of a microcentrifuge tube (10.5 mm diameter) we dissected and weighed the gonadal tissues, took pictures, and noted the maturity of the gonadal tissues. When the mussels appeared to be developing based on these qualitative measurements, we started the exposure. Approximately 180 mussels, *M. edulis* between 45 and 55 mm, were collected on February 14, 2014 at low tide (35 PSU, 2.2 °C) at the Columbia Point peninsula, Boston Harbor (42.32°N, 71.04° W). Mussels were returned to the laboratory, cleaned, and acclimated for 18 days in 5-μm filtered seawater at 11 °C, 35 PSU, and a 10:14-h light dark cycle. After acclimation, *M. edulis* were placed in 4 replicate aquaria and exposed to nominal concentrations of 4-nonylphenol (4NP) (1 μg/L and 100 μg/L) and 17α-ethinylestradiol (EE2) (5 ng/L and 50 ng/L) in addition to an unexposed control (Supporting Information, SI). Due to sample loss during shipping, we were unable to analytically confirm exposure concentrations. However, based on the behavior of these compounds in seawater from an earlier study,[24] we expected EE2 and 4NP concentrations to be close to nominal at spiking and decrease to about 50% nominal after 24 h due to mussel uptake and remain close to this level until the next water change (every 3−4 days). 4NP is a nonionic surfactant commonly used in detergents, and EE2 is the synthetic estrogen used in birth control pills. EE2 and 4NP were selected as model EDCs as they represent the most potent or abundant xenoestrogens in coastal areas.[25−27] Each replicate consisted of eight individual mussels in 1 gallon aquaria with 3 L of seawater (375 mL seawater/mussel). At 32 and 39 days 4 mussels were collected from each replicate, and mantle tissue and digestive gland were dissected and stored at −20 °C in RNA later for less than 1 year before processing. The digestive gland was selected as the target tissue for global gene expression analysis as it has been identified as the target tissue for steroid hormone synthesis,[28] and our previous study identified this tissue as the one that accumulated the highest levels of EDCs.[24] Time points were selected based off a previous uptake kinetics experiment.[24]

Sex Identification and Expression of Female and Male Specific Transcripts. Total RNA was isolated and reverse transcribed from all *M. edulis* mantle tissues according to Poynton et al.[29] (additional details available in the SI methods). Samples were evaluated with a reverse transcription quantitative PCR (RT-qPCR) sex identification assay developed by Hines et al.[30] with slight modifications (SI methods). For 54 mussels, we also identified sex using digestive gland tissue after a 32 and 39 day EDC exposure. We found that digestive gland is also a reliable tissue for sex identification (Table S1), allowing for sex identification and selected biomarker analysis to be performed on the same tissue.

Microarray Construction and Hybridization. To construct an oligonucleotide microarray with broad representation of the *M. edulis* transcriptome, we performed de novo transcriptome assembly using 300 different mussel samples representing different developmental stages, tissues, chemical and stress treatments, and geographic locations (Table S2). RNA was isolated from each sample as described above, and a pooled cDNA library was created that contained equal amounts of RNA from each sample. RNA sample integrity was evaluated using an Agilent Bioanalyzer. Although RIN scores are not available because the Bioanalyzer software has difficulty identifying nonvertebrate rRNA bands, visual inspection of the electropherograms showed strong rRNA peaks and little to no RNA degradation. We also sequenced three larval stage libraries (Table S3) separately. Libraries were constructed using four different index adapters and the Illumina TruSeq Stranded RNA protocol (San Diego, CA) with resulting fragment sizes of approximately 400 bp (including the 122 adapter sequence). Libraries were sequenced using a PE100 protocol in a single lane on the Illumina HiSeq2000 at the UC Berkeley Functional Genomics Laboratory resulting in approximately 30 million reads per library. Raw reads were converted to FASTQ files using Illumina CASAVA_v1.8.0, then Illumina adapters were trimmed, and reads were QC-filtered using default parameters with Trimmomatic (v.0.32) software.[31] Filtered reads were

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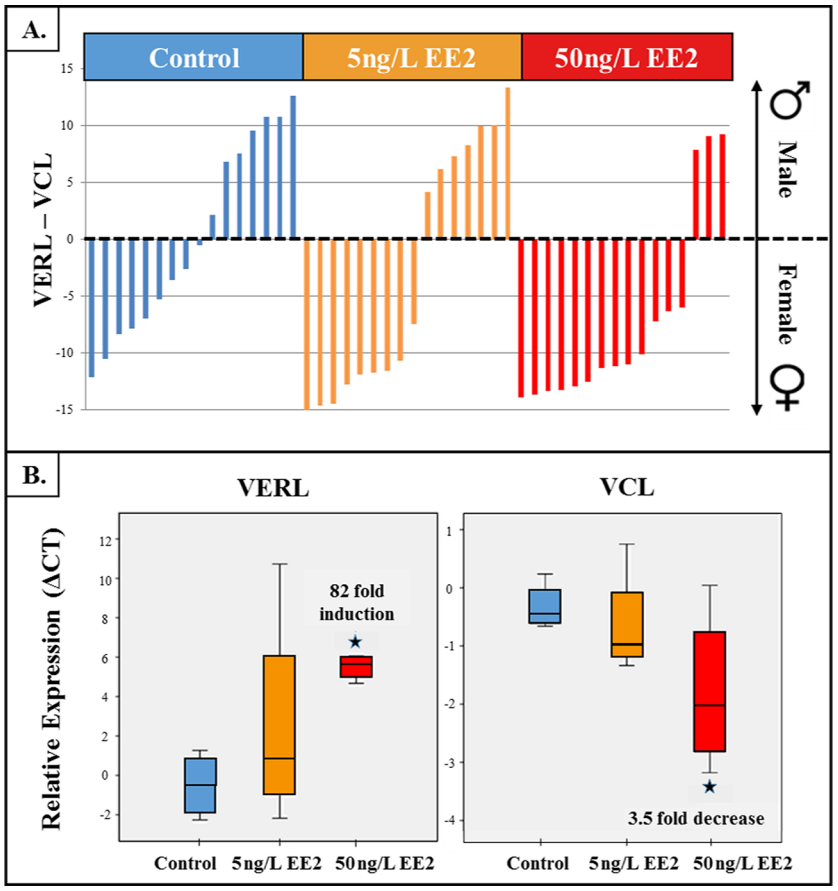

Figure 1. Results of the RT-qPCR sex identification assay for mussels exposed to 5 ng/L and 50 ng/L EE2 for 39 days. Sex of mussels were identified by using the difference of a female specific egg protein, the vitelline envelope receptor for lysin (VERL), and a male specific sperm protein, vitelline coat lysin (VCL).[39] If the difference between transcripts ΔCt(VERL-VCL) is positive the mussel is male, while negative values represent females. A: Individual lines represent one mussel with 16 mussels analyzed per treatment. The figure indicates significantly less males in 50 ng/L EE2 treatment compared to the control (p = 0.037). B: Normalized female specific (VERL) and male specific (VCL) transcripts analyzed across all mussels following exposure to 5 ng/L and 50 ng/L EE2. Relative expression was compared to the unexposed control, and significance (★) was determined with a student's t test ($p < 0.05$).

submitted to the NCBI Sequence Read Archive (SRA) and are available under BioProject PRJNA439300 with the following accession numbers: SRS3075123-SRS3075126. Transcriptomes were assembled using Velvet[32] and Oases[33] (K = 23, 31), and a cutoff of 300 bp was applied. The resulting transcriptome contained 39,955 contigs. Annotation was performed using Blast2GO.[34] The 39955 sequences were uploaded to Agilent Technologies (Santa Clara, CA) eArray custom oligonucleotide microarray service (https://earray.chem.agilent.com/earray/) with at least one oligonucleotide designed for each sequence (two oligonucleotides were designed for sequences that were functionally annotated with Blast2Go). The microarray was printed using 8 × 60,000 sequence format (AMADID no.066389). Details related to the design of the microarray and probe sequences are available at Gene Expression Omnibase (GEO; http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE111836.

Total RNA was isolated from the digestive gland of 4 male and 4 female mussels (1 mussel/sex/replicate) in each treatment at the 32 day time point as previously described. RNA was prepared for microarray analysis using Quick Amp One Color Labeling kit and One Color RNA Spike-in kit (Agilent Technologies). Labeled cRNA was hybridized to the custom 60,000 sequence microarray and scanned at five micron resolution using an Agilent DNA Microarray Scanner G2565CA at the University of Massachusetts Medical School in Worchester, MA. Feature extraction was performed using Feature Extraction Software 11.5 (Agilent Technologies), and intensity values were normalized by quantile normalization with median polish in Arraystar (DNAstar, Madison, Wisconsin). Each treatment was compared to the unexposed control of the corresponding sex, and DEGs were selected with a 99% confidence interval and 2-fold change.

Microarray Analysis. The microarray data was analyzed using three independent methods. First, we used a targeted approach to examine a specific metabolic pathway involved in sex steroid biosynthesis by identifying homologous enzymes with NCBI BLAST. Significant genes identified in the microarray analysis were evaluated to determine if steroidogenesis enzymes were altered by EDCs. Second, gene ontology (GO) terms were assigned to categorize gene function, and enrichment analysis was performed to highlight overrepresented gene categories. DEGs were separated by sex, up- or down-regulation, and EDC treatment, and enrichment analysis was performed using Blast2GO.[34] Comparisons were made ($p \leq 0.05$) with the entire set of annotated genes in the microarray and each DEG list. Finally, we used an exploratory network biology approach to investigate how pathways and genes interact and highlight the mechanisms related to reproduction and development. DEG sequences were

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compared to mouse and human using NCBI Blastx, and homologous proteins were recorded (Tables S4 and S5). Homologous proteins were uploaded into STRING (http://string-db.org/), and the protein−protein interaction networks were obtained for each treatment group. Each protein interaction network was uploaded into Cytoscape[35,36] (http://www.cytoscape.org/), and BINGO was run to assign GO terms to each protein node in the network. The male and female protein−protein interaction networks were simplified into subnetworks by examining GO terms and pathways merging with genes coding for reproduction and development.

Quantitative Reverse Transcription PCR of Candidate Biomarkers. Biomarkers were selected from the information provided by the three microarray analyses (see Results and Discussion). Primers were designed to selected biomarkers (Table S6) using Primer 3 software[37] following recommendations of the Mx3005P QPCR System (Agilent Technologies) and synthesized by Integrated DNA technologies (Skokie, Illinois, USA). Total RNA from the digestive gland was extracted and reverse transcribed into cDNA from 4 males and 4 females according to protocols described in the SI methods. If there were not 4 identifiable male or female mussels, 3 mussels were analyzed. Samples were analyzed at 32 and 39 days to ensure stable expression over time and to validate microarray results. Each sample and biomarker was normalized to 18S rRNA. 18S rRNA was used as the sole reference gene in this study as this gene was stably expressed in previous experiments when mussels are exposed to other stressors.[29] In addition, one-way ANOVA with Tukey test showed no statistical difference in 18S rRNA expression across all treatments and controls at 32 and 39 days. The normalized results were checked for equality of variances (Levene's test) and normality (Shapiro-Wilk test). Significance was determined with a $p < 0.05$ following analysis of a two-sample t test, Welch's test, or Mann−Whitney U test depending on whether the data met the assumptions of each test. The list of genes and primer sequences selected for RT-qPCR are available in Table S6. Additional MIQE essential information[38] can be found in the SI methods.

Field Evaluation of Candidate Biomarkers. Five sites throughout the Northeast including Jamaica Bay, NY; Port Jefferson NY; Boston Harbor, MA; Falmouth, MA; and Savin Hill Cove, MA (Table S7, Figure S1) were selected to validate the laboratory developed biomarkers in the field or to determine if sites may be susceptible to endocrine disruption. From each site 15 mussels were collected for sex identification in mantle tissue[39] and for biomarker analysis in the digestive gland. Each sample was dissected in the field, placed in RNA later on ice, and stored in RNA later at −20 °C when returned to the laboratory. All samples were processed within 6 months of collection. Sex identification was performed for all 15 mussels, and biomarker analysis was performed for 3 replicate male and 3 female mussels at each site as described above.

RESULTS AND DISCUSSION

EDC Exposure Causes Feminization of Mussels. The complete sex identification results for 32 and 39 days are found in Table S1. At both time points, the sex ratio in control treatments was close to equal (53% male at 32 days, 46% male at 39 days). In 5 ng/L EE2 and 4NP treatments, the percentage of male mussels ranged between 44 and 50%. In contrast, there were fewer males in the 50 ng/L EE2 treatments (36% at 32 days and 19% at 39 days), and at 39 days this ratio was significantly different from the control ($p = 0.037$) (Figure 1A). The normalized sex-specific transcripts showed a 63-fold induction of the female specific gene, Vitelline Envelope Receptor for Lysin (VERL) at the 50 ng/L EE2 treatment ($p = 0.0005$) (Figure 1B). The male-specific transcript, Vitelline Coat Lysin (VCL) was significantly down-regulated in the 50 ng/L EE2 treatment ($p = 0.025$). Therefore, the high fold induction of VERL is likely driving the female skewed sex ratio. Additionally, for both 5 ng/L and 50 ng/L EE2 the sex identification assay showed a feminization trend in which the female mussels had a greater difference in transcripts (ΔCT(VERL-VCL)).

Although the biological effects of xenoestrogens in bivalves are debated, there is accumulating evidence intersex and gender reversal can occur similarly as fish. For example, when the mussel *Choromytilus chorus* (family Mytildae) was exposed to estradiol there was a shift in the sex ratio toward females, but when mussels were exposed to dihydrotestosterone or fadrozole a male shift in the sex ratio occurred.[40] Similarly, *S. plana* was found to exhibit intersex after EDC exposure,[5] and intersex in male mussels was also found to be present at Mweeloon Bay, Galway, the Guadiana Estuary, Portugal, and 13 estuaries in NW France.[4−7] Additionally, oysters (*S. glomerata*, *C. gigas*) were found to show intersex in males, skewed sex ratios, and a dose-responsive increase in vitellogenin when exposed to EE2.[10,41−43] Studies with *R. decussatus* and *C. glaucum* from areas receiving effluent from wastewater treatment plants within the Gulf of Gabès (Tunisia) also showed incidents of hermaphrodite clams and EDCs accumulated in bivalve tissues.[9] Interestingly, a study performed by Ortiz-Zarragoitia et al. found intersex *M. edulis* (26%) collected from the Bay of Biscay had induction of VERL and VCL suggesting the results of our study may be reflective of intersex.[8] The induction of female-specific sex transcripts such as vitellogenin has been well documented in male and intersex fish after EDC exposure.[44] Our results, combined with data from other lab studies[41−43] and field studies,[5,6,8,46−48] suggest similar impacts to bivalves, which may lead to developmental impairments, intersex, or gender reversal.

Identification of a Nearly Complete Steroidogenesis Pathway in Mussels. The complete pathway of steroid biosynthesis in bivalves is still unclear. While some debate whether bivalves synthesize endogenous steroids,[49] others have proposed that a complete steroidogenesis pathway exists.[28] Initial examination of metazoan genomes suggested that the enzymes essential for steroidogenesis evolved independently within vertebrates and that mollusks were unlikely to contain a vertebrate-like steroidogenesis pathway.[50] However, when this analysis was conducted, only six, mostly partial, genome sequences were available for the superphylum lophotrochozoans including only one mollusk genome. More recent analysis concluded that several steroidogenesis enzymes are more broadly conserved.[51] Therefore, to re-examine whether steroidogenesis genes may be present in bivalves based on the pathway proposed by Janer et al.,[28] we explored the *M. edulis* transcriptome for gene homologues to steroidogenesis genes in vertebrates. Our analysis successfully identified the majority of genes present in this pathway; the only obvious omission was aromatase (Table S8). The first step of steroidogenesis involves conversion of cholesterol to pregnenolone catalyzed by an enzyme complex containing adrenodoxin (Adx), adrenodoxin reductase (AdR), and the cholesterol side chain cleavage enzyme (CYP11A).[52] We detected a partial protein

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Table 1. Enriched GO terms for *M. edulis* Exposed to EE2 (5 ng/L and 50 ng/L) and 4NP (1 μg/L and 100 μg/L) for 32 Days and then Analyzed with Microarrays[a]

Gene Function	5 ng/L EE2 Female	50 ng/L EE2 Female	5 ng/L EE2 Male	50 ng/L EE2 Male	1 ug/L 4NP Female	100 ug/L 4NP Female	1 ug/L 4NP Male	100 ug/L 4NP Male
Embryo Development (GO:0009790), Tissue Development (GO:0009888), Anatomical Structure Morphogenesis (GO:0009653)								
Cat eye syndrome critical region 2	3.18	2.45	0.49	0.43	2.89	2.02	0.44	0.24
Centromere F	1.02	0.93	2.74	1.09	1.27	1.01	1.25	1.04
cyclin-O	0.85	0.86	3.05	1.16	0.62	0.83	1.51	1.22
E3 ubiquitin- ligase SMURF2-like isoform X2	0.87	1.09	1.26	0.96	2.20	0.97	1.09	1.06
exostosin-1b	1.06	0.90	2.86	1.22	0.95	0.83	1.16	1.37
Kinesin KIF16B	1.91	1.49	0.95	0.75	1.18	2.54	1.10	0.85
leukotriene A-4 hydrolase	1.06	1.12	2.69	3.31	1.41	1.00	1.52	3.35
matrix metallo ase-14-like	1.88	1.12	0.96	2.94	0.75	1.00	1.52	2.09
Meckel syndrome type 1	2.66	1.85	0.83	0.77	2.43	2.15	0.73	1.17
mucin-19 isoform X1	1.45	1.40	3.96	1.53	1.98	1.94	1.47	1.10
notchless homolog 1	1.46	1.02	3.96	1.67	0.80	0.70	1.76	1.53
prolyl 4-hydroxylase subunit alpha-1-like	5.39	2.43	0.84	1.70	3.18	1.09	2.09	1.32
RNA polymerase-associated CTR9 homolog	1.12	0.84	5.39	1.79	0.84	0.87	1.65	1.19
serine threonine- kinase PLK4-like	1.79	2.54	5.47	2.18	1.13	1.21	2.14	1.21
serine threonine- kinase VRK1	1.02	1.66	1.36	0.70	1.50	2.52	0.88	1.05
SMARCB1	1.44	1.08	8.19	2.88	1.39	1.10	3.56	2.36
T-box transcription factor TBX20	7.00	1.28	0.89	1.22	2.89	2.16	2.14	0.88
TRAF3-interacting 1-like	3.56	3.16	0.26	0.31	2.97	2.78	0.33	0.22
transmembrane anterior posterior transformation 1	3.52	0.81	2.05	0.89	0.72	0.97	1.39	1.00
wntless homolog	0.84	1.07	1.36	1.90	0.95	1.33	1.40	1.20
zinc finger PLAGL1 isoform X1	0.87	0.86	2.97	1.65	0.71	1.16	1.10	0.88
G-protein Coupled Receptor Signaling Pathway (GO:0007186), Cell-Cell Signaling (GO:0007267), cGMP Biosynthetic Process (GO:0006182)								
atrial natriuretic peptide receptor 1-like	0.74	0.59	3.11	1.63	0.56	0.70	1.34	1.17
cardioacceleratory peptide receptor	4.16	2.89	0.55	1.04	2.76	3.05	1.36	1.25
cholecystokinin receptor type A-like	1.91	2.55	0.89	1.83	2.31	1.60	1.67	3.02
diacylglycerol kinase zeta-like isoform X10	0.57	0.41	2.61	1.79	0.64	2.03	1.07	2.55
dopamine receptor 2-like	0.82	1.47	1.72	3.76	2.46	1.12	3.24	2.82
dynamin-1-like isoform X2	1.50	3.07	2.42	3.25	3.35	2.19	1.21	1.10
Frizzled-4	2.06	1.52	0.39	0.47	3.16	2.33	0.51	0.26
glutamate receptor 1	1.17	0.66	3.17	1.45	0.92	0.81	1.51	1.11
guanylate cyclase 32E-like	1.47	1.03	9.89	4.55	1.17	0.85	2.74	1.24
LIM homeobox Lhx5	3.12	3.53	0.77	0.44	2.03	2.98	0.36	0.35
monoglyceride lipase	2.41	2.26	1.24	0.96	1.82	3.42	1.18	1.10
otoferlin-like isoform X3	1.07	0.66	7.65	2.50	1.05	0.56	1.92	1.68
Sodium- and chloride-dependent glycine transporter	2.80	2.28	1.10	0.84	8.32	2.34	1.18	0.85
substance-K receptor-like	1.58	1.82	0.52	1.12	2.17	1.10	1.48	1.66
transcription and mRNA export factor ENY2	0.82	0.66	6.29	1.94	0.67	0.90	2.56	1.22
Translational activator GCN1-like	2.08	2.61	1.12	1.01	2.6	2.93	0.97	0.84
Translational activator GCN1-like	1.8	3.19	1.59	0.88	1.6	2.99	1.9	1.67
ubiquitin carboxyl-terminal hydrolase 14-like	1.65	0.79	3.50	1.22	0.70	1.45	0.92	1.20
unc-13 homolog A-like isoform X13	1.02	1.05	7.41	1.57	1.37	0.77	2.05	1.34
Steroid Metabolic Process (GO:0008202), Peptide Hormone Processing (GO:0016486)								
furin-like protease 2	1.33	0.74	6.82	3.30	0.99	0.85	2.74	2.00
low-density lipo receptor-related 4	0.95	0.60	4.52	1.41	0.91	0.91	1.60	1.02
membrane-bound transcription factor site-2 protease	4.17	4.40	1.41	2.22	2.70	2.98	1.28	1.04
NF-kappa B	2.09	2.65	0.80	1.73	1.87	1.06	0.94	1.11
Mitotic Sister Chromatid Segregation (GO:0000070)								
cell division cycle 27 partial	0.81	0.96	1.33	2.84	1.01	0.90	4.42	3.06
chromosome transmission fidelity 8 homolog	0.60	2.29	1.22	3.62	0.83	0.76	2.01	2.10
Cellular Response to Stress (GO:0033554)								
acyl coenzyme a n acyltransferase	2.58	2.56	0.89	0.74	2.63	2.00	0.67	0.85
disulfide-isomerase A4	1.17	2.46	1.53	1.26	3.30	2.56	1.96	1.63
DNA repair and recombination partial	1.60	14.96	2.27	1.03	1.43	3.05	1.62	1.97
eukaryotic translation initiation factor 2 subunit 1	1.30	1.46	1.34	1.01	2.19	1.62	1.15	1.31
FAS-associated factor 2	1.66	2.16	0.90	0.88	2.80	1.55	1.55	0.97
mutS homolog 5-like isoform X1	1.93	1.51	0.68	0.63	1.46	2.36	0.69	0.77
Oligosaccharyl Transferase Subunit STT3B	1.05	1.95	0.81	0.67	1.69	2.02	0.87	0.96
structural maintenance of chromosomes 3-like	1.88	2.37	0.74	0.84	2.58	2.42	1.57	0.80
Translational activator GCN1-like	1.94	2.61	1.35	0.95	2.60	2.93	1.43	1.25
Sperm-Egg Recognition (GO:0035036)								
heat shock 70	0.75	0.46	0.94	0.68	0.36	0.26	1.30	3.16
T-complex 1 subunit epsilon	0.73	0.64	0.57	0.88	1.01	0.46	0.53	0.93
Positive Regulation of Signal Transduction (GO:0009967), Phosphatidylinositol Phosphorylation (GO:0046854)								
ankyrin repeat and FYVE domain-containing 1	0.98	0.47	1.87	2.50	0.80	0.41	1.84	1.72
calmodulin	0.64	0.56	0.61	1.18	0.65	0.41	0.99	0.86
PI3K-delta	0.49	0.90	0.52	0.86	0.56	0.36	1.05	0.54
radical S-adenosyl methionine domain-containing 2	1.36	2.18	1.42	0.85	0.31	0.84	1.73	1.01
ragulator complex LAMTOR5 homolog	0.50	0.57	0.73	0.77	0.53	0.44	1.21	0.66
type i inositol- -trisphosphate 5-phosphatase	0.24	0.42	0.84	0.61	0.23	0.36	0.46	0.38
Response to Oxidative Stress (GO:0006979)								
Eosinophil peroxidase	1.60	0.77	0.45	0.25	0.23	0.61	0.54	0.63
Eosinophil Peroxidase-like	2.61	0.91	0.46	0.44	0.37	0.92	0.75	1.55
microsomal glutathione S-transferase 1 isoform a	1.31	1.18	0.27	0.41	1.50	1.11	2.11	0.66
Embryonic Organ Morphogenesis (GO:0048562)								
brachyury isoform X2	0.90	0.69	0.45	0.68	1.09	0.71	0.98	0.53
coiled-coil domain-containing 40-like	0.99	1.46	0.04	0.14	0.61	1.21	0.80	0.71
Protein-DNA Complex Assembly (GO:0065004)								
DNA repair RAD52 homolog	0.45	0.45	1.91	1.50	0.44	0.71	1.00	1.70
Histone H4	0.28	1.09	1.00	0.52	0.39	1.18	1.10	0.99
Transcription initiation factor TFIID subunit 1	1.06	0.45	2.13	1.57	1.08	1.08	2.09	2.87

[a]Fold change is shown as the average of samples across each treatment divided by the unexposed control average. Ratios highlighted in red were significantly up-regulated, and ratios highlighted in green were significantly down-regulated compared to the unexposed control.

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homologue for vertebrate CYP11A gene (Figure S2) and nearly complete sequences for Adx and AdR with high similarity to human (E-values < E-30). When the *M. edulis* CYP11A homologue amino acid sequence was aligned to human (E-value = 2×10^{-17}), common shrew (E-value = 1×10^{-12}), and hagfish (E-value = 2×10^{-10}), there appeared to be conserved residues in the active site that bind cholesterol and residues that form salt bridges and hydrogen bonds with Adx (Figure S2). Previous studies also suggested that a vertebrate CYP11A homologue exists in bivalves based on identification of CYP11A mRNA by Northern blot[53] and immunodetection of CYP11A-like proteins.[54] Together, these results strongly suggest that a CYP11A-like homologue exists in bivalves, although identification of the full length sequence and the biochemical characterization of its gene product are essential to establish its role within the steroidogenesis pathway in bivalves.

Our transcriptomic sequencing also identified homologues to enzymes in the steroid biosynthesis pathway required to convert pregnenolone to estradiol. These included 17α-hydroxylase (CYP17A1), 3β-hydroxysteroid dehydrogenase (3β-HSD), and 17β-hydroxysteroid dehydrogenases (17β-HSD) (Table S8), all with high similarity to vertebrate homologues (E-values ranging from 1×10^{-64} to 1×10^{-13}). These genes were previously suggested to exist in mollusks.[28,55−63] In addition, we identified multiple gene sequences homologous to steroidogenic acute regulatory proteins (StAR; lowest E-value = 5×10^{-53}), which control the rate-limiting step of steroidogenesis by transporting cholesterol into the mitochondria where the cholesterol side chain cleavage reaction occurs (Table S8). The only gene not detected in the *M. edulis* transcriptome was aromatase (CYP19), which catalyzes the conversion of testosterone to estradiol in the final step of the steroid biosynthesis pathway. Aromatase activity has been reported in other bivalve species, although at much lower rates compared with vertebrates,[59,64,65] and phylogenetic analysis suggests that CYP19 may be restricted to chordates.[51] Regardless of whether bivalves synthesize estrogens de novo or rely on exogenous estrogens taken up from their environment, they are clearly bioactive compounds in bivalves.[28,66] This is further illustrated by the substantial effects EE2 and 4NP have on gene expression described below.

Transcriptomic Analysis Indicates Disruption of Nongenomic Estrogen Pathway. We investigated potential mechanisms of action and global gene expression response through microarray analysis and identified 1,735 DEGs including 498 genes up-regulated in females, 703 genes up-regulated in males, 321 genes down-regulated in females, and 249 genes down-regulated in males (Figure S3). Many genes were differentially expressed in multiple treatments, while hierarchical clustering showed exposure samples grouped together across treatment conditions (Figures S4 and S5). This suggests that the pathways involved in endocrine disruption in bivalves are not distinct for EE2 and 4NP and that candidate biomarkers will likely respond similarly to multiple EDCs.

Enrichment analysis of GO terms converged upon two primary pathways that were affected by the treatments, the steroidogenesis pathway, and nongenomic estrogen signaling pathway. Enriched up-regulated GO terms included the steroid metabolic process and peptide hormone processing suggesting that the steroidogenesis pathway is a target of endocrine disruption in *M. edulis* (Table 1). StAR and Adx which are important genes in the first conversion of this pathway were up-regulated. Enriched up-regulated GO terms also included G-protein coupled receptor signaling pathway, cell−cell signaling, and cGMP biosynthetic process, while down-regulated terms included signal transduction and phosphatidylinositol phosphorylation. These enriched GO terms as well as specific DEGs (SI discussion) led us to hypothesize that the nongenomic estrogen signaling pathway and alteration of the steroidogenesis pathway serve as the mechanisms of endocrine disruption in bivalves.

The nongenomic pathway is activated through estrogen binding to ERs on plasma membranes, ERs in the cytosol, and other plasma receptors (e.g., G protein-coupled receptor 30). After these receptor−ligand interactions occur, rapid signaling cascades (MAPK/ERK or PI3-kinase pathway) and secondary messengers are activated which then can alter the transcription of genes related to reproduction, development, or the cell cycle.[67,68] ERs can also be located in caveolae, small invaginations in the plasma membrane, that provide a location for signaling molecules to aggregate and then activate kinase signaling pathways and endothelial nitric oxide synthase (eNOS) release.[69−72] Release of eNOS causes GTP to be converted to cGMP, a process that was found to be overrepresented in the enrichment analysis. cGMP is a secondary messenger involved in activating intracellular protein kinase cascades and regulates several processes including sperm function, sperm-egg recognition, and cell division.[73] Although the nuclear ER is not an active receptor in mollusks, our results suggest that EDC binding to a membrane ER could be an alternate molecular initiating event responsible for endocrine disruption in bivalves.

Downstream processes of the nongenomic pathway involved in reproductive and development were also overrepresented. For example, up-regulated GO terms included tissue development, embryo development, anatomical structure, and mitotic sister chromatid segregation, while sperm-egg recognition and embryonic organ morphogenesis were enriched down-regulated GO terms. Enrichment results also suggested a stress response. Cellular response to stress was an up-regulated enriched GO term, while response to oxidative stress was down-regulated. The DEGs seemed to span several functions of stress response including production of protective/stress proteins, DNA repair mechanisms, activation of cell cycle checkpoints, biotransformation, and cell death through apoptosis (SI discussion). In mussels, previous studies indicated an estrogen-induced stress response was regulated by kinase pathways (PI3-kinase, MAPK).[74−78] Therefore, players involved in the nongenomic estrogen signaling pathway may cross-talk with other pathways such as stress response.

We used network biology to examine pathways and genes that may be influencing reproduction and development. Network biology results were consistent with our previous analyses, providing another line of evidence to indicate impacts to the nongenomic estrogen signaling response and steroidogenesis pathway. The female and male down-regulated networks identified genes with GO terms of steroid metabolic process, cell cycle, and response to stress, and connected to genes coding for development (Figures S6 and S7). In the female and male up-regulated networks, genes involved in response to steroid hormone stimulus, steroid metabolic process, regulation of intracellular protein kinase cascade, cell cycle, and phosphorylation were identified (Figures 2 and 3).

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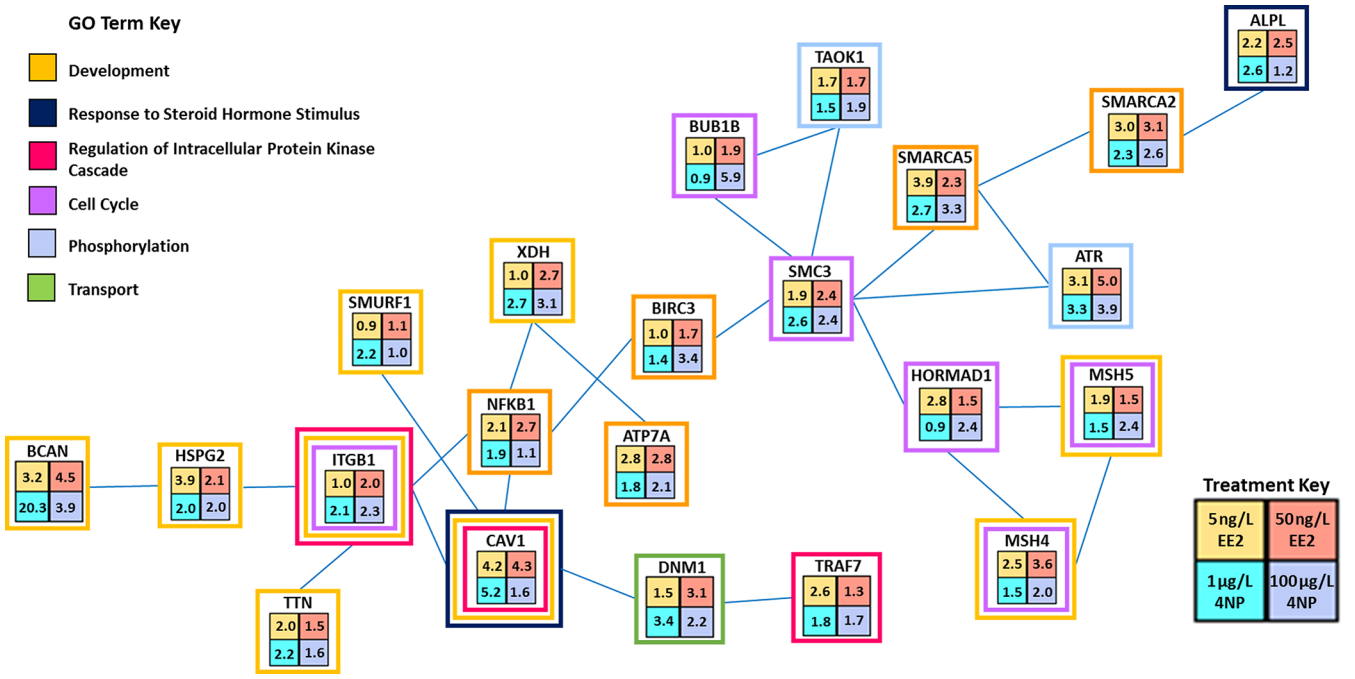

Figure 2. Female up-regulated protein−protein interaction network of differentially expressed genes that were homologous to mouse. Fold change is shown as the average of samples across each treatment divided by the unexposed control average. The 4 boxes within each square represent the fold change of each treatment (5 ng/L EE2, 50 ng/L EE2, 1 μg/L 4NP, and 100 μg/L 4NP). GO terms are shown as color coded boxes with some genes having multiple GO terms assigned.

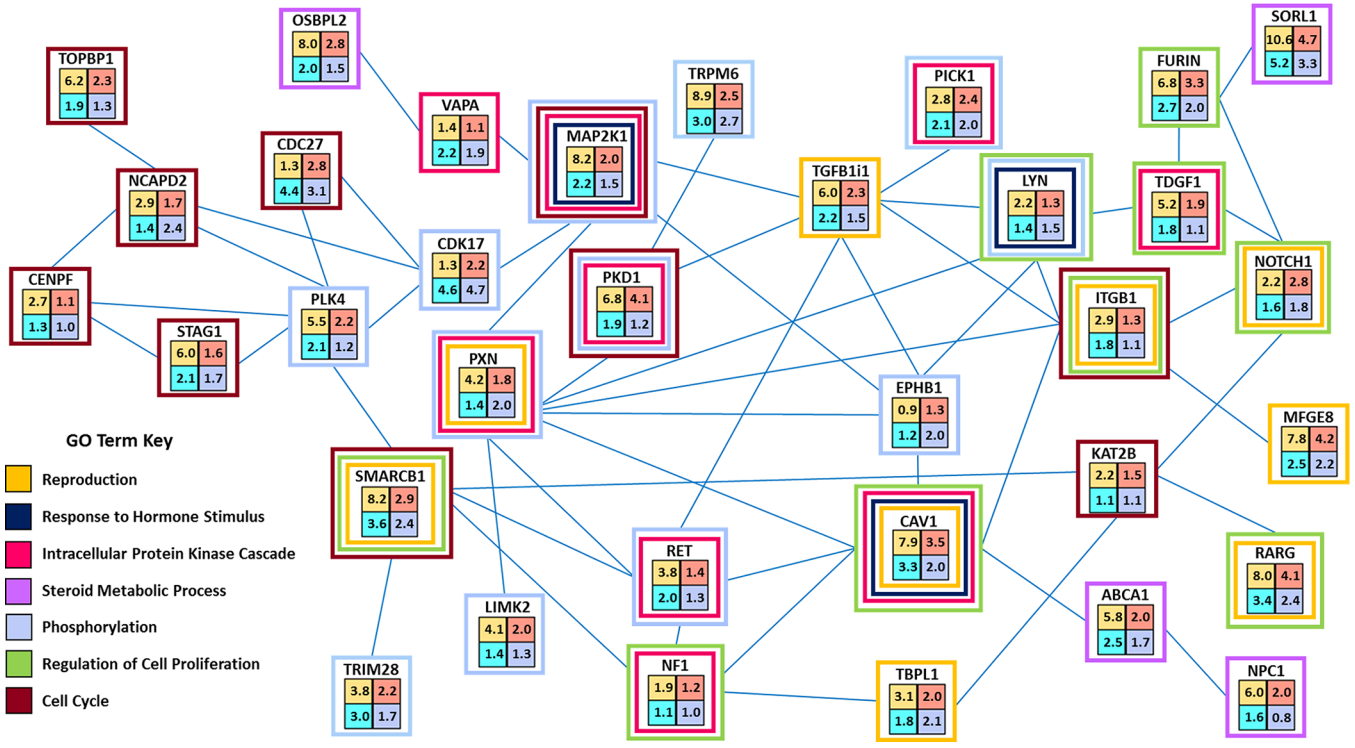

Figure 3. Male up-regulated protein−protein interaction network of differentially expressed genes that were homologous to mouse. Fold change is shown as the average of samples across each treatment divided by the unexposed control average. The 4 boxes within each square represent the fold change of each treatment (5 ng/L EE2, 50 ng/L EE2, 1 μg/L 4NP. and 100 μg/L 4NP). GO terms are shown as color coded boxes with some genes having multiple GO terms assigned.

Because these pathways and nodes are connected to genes coding for reproduction and development, they are likely associated with the observed AO. This relationship is further developed in the male up-regulated network created with human gene homologues (Figure S8). In this network, a gene homologous to the female egg protein, Vitelline Membrane Outer Layer 1 (VMO1), was highly up-regulated in male mussels across EE2 (13−14-fold) and 4NP (6−13-fold)

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treatments. The subnetwork surrounding VMO1 included genes of the nongenomic and steroidogenesis pathway, suggesting that these pathways may be predictive of feminization of male mussels (Figure S8).

Network analysis also allowed us to identify and investigate hub genes. Previous studies demonstrated that deletion of hub genes within networks leads to reproductive abnormalities and lethality.[22] We therefore hypothesized that hub genes represent key genes involved in the mechanism of endocrine disruption and are essential for progression toward the AO. Although many hub genes were identified in our networks (SI discussion), we focused on three hub genes Cav-1, mitogen-activated protein kinase kinase 1 (MAP2K1), and SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily B, Member 1 (SMARCB1) because they were highly differentially expressed and reflective of the suspected mechanism of action. MAP2K1 is an essential member of the MAPK cascade, and SMARCB1 is involved in repairing DNA damage and controlling cell growth. Caveolin-1 (Cav-1), the main structural component of caveolae in the plasma membrane, was also identified as a hub protein in both male and female up-regulated subnetworks. Cav-1 has been shown to interact with membrane receptors (including the ER) leading to the activation of signal transduction cascades and progression of the cell cycle.[79] The interaction of the plasma membrane ER with Cav-1 is thought to facilitate the formation of a "steroid receptor fast action complex" that can quickly respond and amplify the estrogen signal.[80] Although Cav-1 is involved in a number of cellular functions outside of ER signaling that may have been impacted by the exposures,[80] its interaction with several other upregulated genes within the ER signal transduction network supports its role here in ER signaling.

Overall, our results corroborate other studies that support the nongenomic pathway as a mechanism for estrogen signaling in bivalves. In *Mytilus galloprovincialis* estradiol and EDC exposures caused changes in the phosphorylation state of MAPK- and STAT-like proteins. These effects were prevented with a kinase inhibitor suggesting that kinase cascades were responsible for regulating estrogenic signaling.[81,82] Additionally, studies found a rapid increase of eNOS in bivalves exposed to estradiol, a response that is mediated by an ER located on the cell surface.[83]

Using transcriptomics and exploratory methods we identified potential key events for a putative AOP of endocrine disruption in bivalves. Our results suggest when 1.) EDCs interact with the ER on the plasma membrane it may cause a 2.) cell signaling cascade of secondary messengers which have impacts on the 3.) cell cycle, 4.) steroidogenesis pathway, and 5.) reproductive transcripts (e.g.,VERL, VCL). Ultimately these molecular interactions and cellular responses may be causing 6.) male mussel gonadal cells to differentiate into female reproductive tissue resulting in feminization of male mussels.[43,45,84] The changes in reproductive transcripts and gonadal tissue may also be causing 7.) reduced fertilization and production of viable eggs and 8.) decreased mussel populations. Although these key events and key event relationships need further validation, our analyses allowed hypotheses to be made for an AOP which can be tested in future experiments.

Development of the Coastal Biosensors of Endocrine Disruption Assay. Using a transcriptomic approach, we identified several candidate biomarkers that represent key events in the AOP of endocrine disruption in marine mussels. Criteria for the candidate biomarker selection included genes that were highly differentially expressed, specific to the suspected mechanism of action, or reflective of an AO (Table S9). Biomarker genes were selected from the steroidogenesis pathway or cholesterol transport (Oxysterol Binding Protein Like 2; OSBPL2, ATP-binding cassette transporter; ABCA1, Membrane Bound Transcription Factor Peptidase, Site 2; MBTPS2, StAR, Adx), the nongenomic signaling pathways (Cav-1, Breast carcinoma-amplified sequence 3; BCAS3, MAP2K1, Serotonin Receptor; SR), and the cell cycle (SMARCB1, cell cycle regulator Mat89bb). As the sex transcript analysis provided evidence of potential reproductive impairment, VERL and VCL were also included as biomarkers. Additionally, biomarkers were selected that responded in males (Cav-1, StAR, ABCA1, SMARCB1, BCAS3, OSBPL2, MAP2K1, Mat89bb, GLC32E, and SR) and females (Cav-1, Adx, and MBTPS2). However, because the microarray results showed that males and females had a unique set of DEGs, males were prioritized in selection of biomarkers. In male mussels, genes were generally more highly differentially expressed and more likely to be linked to reproductive impairment in the network analysis.

After biomarker genes were selected, RT-qPCR was used to 1.) validate microarray results and 2.) ensure stable expression at both time points. The biomarker genes that were validated using both techniques (microarray and RT-qPCR) in males at 32 days included Cav-1, StAR, BCAS3, Mat89bb, GLC32E, and SR (Table S10). Comparing the 32 and 39 day time points StAR, BCAS3, Adx, Mat89bb, GLC32E, and SR were all significantly up-regulated in males over time (Table S11). At 32 days RT-qPCR analysis showed Adx and MBTPS2 were also significantly up-regulated in males. In females, biomarkers Adx and MBTPS2 were significantly up-regulated using both microarray and RT-qPCR analysis at 32 days; however, the results were not consistent over time (Tables S10 and S11). When the results from male and female mussels were analyzed together, Cav-1, StAR, BCAS3, OSBPL2, Adx, GLC32E, and MBTPS2 were significantly up-regulated in at least one treatment at 32 days, while StAR was validated over both time points (Table S11).

Overall, it appeared that Cav-1 and StAR were the most highly differentially expressed biomarkers. Cav-1 was up-regulated up to 11-fold in males and females at 32 days and up to 8- and 12-fold in males and females respectively at 39 days. At 32 days StAR was also highly up-regulated in males (up to 15-fold) and females (up to 4-fold), while at 39 days StAR was up-regulated up to 9-fold in males. When all mussels were analyzed, these biomarkers remained highly expressed at 32 days (i.e., Cav1, 3−8-fold; StAR, 3−6-fold) and 39 days (i.e., Cav1, 1−9-fold; StAR, 1−3-fold). StAR represents the rate limiting step in the steroidogenesis pathway, and Cav-1 plays an important role in the nongenomic estrogen signaling pathway. Therefore, these genes not only are robust and sensitive biomarkers but also represent KEs in the two hypothesized mechanisms of endocrine disruption in the blue mussel.

In conclusion, our analysis validated key genes within the steroidogenesis pathway or cholesterol transport (Adx, StAR, MBTPS2), the nongenomic estrogen pathway and other cell signaling responses (Cav-1, GLC32E, BCAS3, and SR), and cell cycle (Mat89bb) as biomarkers of endocrine disruption. These genes were highly differentially expressed, significantly

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expressed over time, and confirmed with microarray and RT-qPCR. With the success of our biomarker validation, we combined these analyses into a suite of biomarkers: the Coastal Biosensor for Endocrine Disruption or C-BED assay. The C-BED assay consisted of 11 genes including the 8 biomarker genes validated in the RT-qPCR analysis, the two sex transcript biomarkers and a reference transcript. To evaluate if the C-BED assay may be a reliable field tool, we examined these biomarkers in the field at sites known to be contaminated with EDCs and also used the assay to determine potential endocrine disruption at unknown locations.

Validation of C-BED Assay Suggests Endocrine Disruption in Field Collected Marine Mussels. Five sites throughout the Northeast including Jamaica Bay, NY; Port Jefferson NY; Boston Harbor, MA; Falmouth, MA; and Savin Hill Cove, MA were analyzed with the candidate C-BED biomarkers to determine if sites may be susceptible to endocrine disruption. Jamaica Bay was selected as the positive control site because four WWTPs discharge into the bay, and EDC concentrations and intersex have been documented in the estuary.[85−87] Nobska Point Lighthouse in Falmouth, MA, served as a reference site because the location was less populated, well flushed by the Atlantic Ocean, and had similar characteristics (i.e., temperature) as the other sites. There was little data available on EDC exposure and effects in Port Jefferson (NY), Boston Inner Harbor (MA), and Savin Hill cove (MA). Therefore, for these sites the objective was to investigate potential risk of endocrine disruption at these locations.

Results from Jamaica Bay, NY, and Falmouth, MA, were consistent with our expectations for positive control and reference field sites, respectively. The sex identification analysis revealed 27% males at the Jamaica Bay site in comparison to 53% males at Falmouth (Figure S9) and suggested a female skewed sex-ratio at Jamaica Bay. This is consistent with previous studies that identified skewed intersex, sex ratios, or induction of vitellogenin in fish at this site.[85,88] Similar to the EE2 exposure, the sex identification assay revealed the Jamaica Bay female mussels had greater difference of ΔCt(VERL-VCL) compared to the Falmouth, MA mussels (ΔCt(VERL-VCL) averages: −11.1 vs −8.6). StAR was found to be up-regulated 3.4-fold in male mussels at the Jamaica Bay location and was significant compared to the reference site (p = 0.045) (Figure 4, Table S12). Additionally, Cav-1 was up-regulated 4.4-fold in males but not quite as significant (p = 0.098) compared to the Falmouth, MA site (Figure 4, Table S12). This was due to the variation in the Cav-1 transcripts at Falmouth, while Jamaica Bay still showed up-regulation 2−6-fold across samples and suggests the need to increase the number of replicates in this analysis.

The unknown sites showed mixed results from the C-BED assay. The sex identification assay did not detect skewed sex ratios at Boston Inner Harbor, Port Jefferson, or Savin Hill Cove. However, the biomarkers suggested potential endocrine disruption at a couple of these locations. At Boston Inner Harbor StAR was up-regulated 2.2-fold in males and was significantly different from the Falmouth control site (p = 0.04). Cav-1 was also up-regulated 5-fold in males at the Inner Harbor location (p-value = 0.032). At Savin Hill Cove, Cav-1 (4.2 fold) and StAR (2.0-fold) were up-regulated in male mussels although not significantly. Mussels from Port Jefferson did not show a response with any of the analyzed biomarkers (Table S12), and the sex identification assay suggested a slight

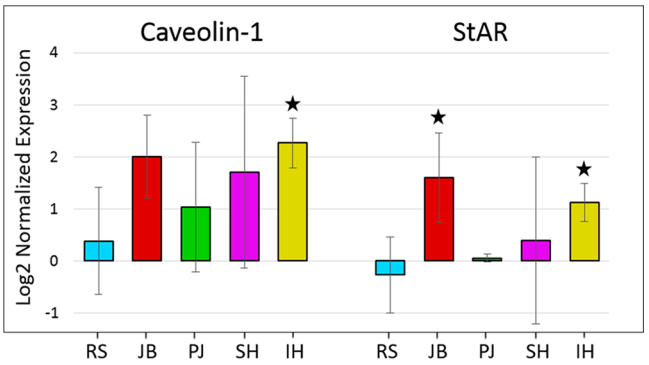

Figure 4. Caveolin-1 and Steroidogenic Acute Regulatory Protein (StAR) log2 normalized relative expression for male mussels (n = 3) collected from the Falmouth, MA reference site (RS); Jamaica Bay, NY (JB); Port Jefferson, NY (PJ); Savin Hill Cove, MA (SH); and Boston Inner Harbor, MA (IH). Relative expression of each site was compared to the reference site, and significance (★) was determined with a student's t test (p < 0.05).

skewed sex ratio toward males (67%; Figure S9). Similar to the lab validation study, males displayed a stronger response than females and remain the recommended sex to analyze biomarkers.

Although the C-BED assay was responsive at Jamaica Bay, an area contaminated with EDCs, moving the assay out of the lab and into the field showed additional variation likely due to confounding environmental variables (e.g., temperature, salinity, tides). Indeed, transcriptomic studies have highlighted different environmental factors and physiological conditions that impact gene expression including temperature,[89] salinity,[90] hypoxia,[91] nutritional status, and reproductive stage[11] in mussels and may mask the pollutant response.[92] Other studies have revealed that transcriptomic profiles are specific when faced with multiple stressors and contaminants,[29,89,93] provide a reproducible cellular stress response,[94] and are robust in field studies when faced with confounding variables.[95] As a first test of the C-BED assay in the field, Cav-1 and StAR confirmed our laboratory results at the control site and indicated its potential utility for marine monitoring for EDCs. In future validation studies, we recommend increasing the number of mussels assayed to increase the statistical power of the assay. In addition, the assay should be tested under different environmental conditions to identify additional factors that influence the expression of the biomarker genes and determine threshold expression levels for distinguishing between EDC exposures and background variation in gene expression.

Using a transcriptomic and network biology approach, we effectively developed sensitive and robust biomarkers that could detect EDC exposure in mussels in both the lab and field. In particular, we identified two sensitive biomarkers, Cav-1 and StAR, that were effective in monitoring endocrine disruption in the marine mussel, *M. edulis*. These results also suggest that other sites throughout the Northeast may be contaminated with EDCs and that endocrine disruption in bivalves may be a widespread problem. In conclusion, our study illustrates the power of transcriptomic approaches and exploratory analyses for identifying biomarkers that represent potential KEs within an AOP. This approach provides a method for linking pathways to genes representative of the AO which can help develop AOPs for contaminants or organisms that have an unknown mechanism of action.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b01604.

Additional materials and methods, results and discussion, Tables S2, S3, S6, and S7, and Figures S1−S9 (PDF)

Tables S1, S4, S5, and S8−S12 (XLSX)

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B.J.B., H.C.P., and W.E.R. designed the study, B.J.B., A.L., C.D.V., K.S.K., H.C.P., and W.E.R. carried out the research, B.J.B. performed the data analysis, and B.J.B. and H.C.P. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Filali-Meknassi, Y.; Auriol, M.; Adams, C. D.; Surampalli, R. Y. Quantification of steroid sex hormones using solid-phase extraction followed by liquid chromatography−mass spectrometry. *Water Environ. Res.* **2007**, *79* (6), 687−696.

(2) Vandenberg, L. N.; Colborn, T.; Hayes, T. B.; Heindel, J. J.; Jacobs, D. R., Jr.; Lee, D.-H.; Shioda, T.; Soto, A. M.; vom Saal, F. S.; Welshons, W. V. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. *Endocr. Rev.* **2012**, *33* (3), 378−455.

(3) Ankley, G. T.; Bennett, R. S.; Erickson, R. J.; Hoff, D. J.; Hornung, M. W.; Johnson, R. D.; Mount, D. R.; Nichols, J. W.; Russom, C. L.; Schmieder, P. K. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ. Toxicol. Chem.* **2010**, *29* (3), 730−741.

(4) Raleigh, J.; Keegan, B. F. The gametogenic cycle of *Scrobicularia plana* (Mollusca: Bivalvia) in Mweeloon Bay (Galway, west coast of Ireland). *J. Mar. Biol. Assoc. U. K.* **2006**, *86* (5), 1157−1162.

(5) Langston, W.; Burt, G.; Chesman, B. Feminisation of male clams *Scrobicularia plana* from estuaries in Southwest UK and its induction by endocrine-disrupting chemicals. *Mar. Ecol.: Prog. Ser.* **2007**, *333*, 173.

(6) Gomes, T.; Gonzalez-Rey, M.; Bebianno, M. Incidence of intersex in male clams *Scrobicularia plana* in the Guadiana Estuary (Portugal). *Ecotoxicology* **2009**, *18* (8), 1104−1109.

(7) Tankoua, O. F.; Amiard-Triquet, C.; Denis, F.; Minier, C.; Mouneyrac, C.; Berthet, B. Physiological status and intersex in the endobenthic bivalve *Scrobicularia plana* from thirteen estuaries in northwest France. *Environ. Pollut.* **2012**, *167*, 70−77.

(8) Ortiz-Zarragoitia, M.; Cajaraville, M. P. Intersex and oocyte atresia in a mussel population from the Biosphere's Reserve of Urdaibai (Bay of Biscay). *Ecotoxicol. Environ. Saf.* **2010**, *73* (5), 693−701.

(9) Hamza-Chaffai, A. Estrogenic endocrine disruptors and their possible deleterious effects on marine organisms: use of a novel monitoring bioassay. *Int. J. Biotechnol. Wellness Ind.* **2013**, *2* (1), 1−9.

(10) Andrew, M.; O'Connor, W.; Dunstan, R.; MacFarlane, G. Exposure to 17α-ethynylestradiol causes dose and temporally dependent changes in intersex, females and vitellogenin production in the Sydney rock oyster. *Ecotoxicology* **2010**, *19* (8), 1440−1451.

(11) Ciocan, C. M.; Cubero-Leon, E.; Puinean, A. M.; Hill, E. M.; Minier, C.; Osada, M.; Fenlon, K.; Rotchell, J. M. Effects of estrogen exposure in mussels, *Mytilus edulis*, at different stages of gametogenesis. *Environ. Pollut.* **2010**, *158* (9), 2977−2984.

(12) Ciocan, C. M.; Cubero-Leon, E.; Minier, C.; Rotchell, J. M. Identification of Reproduction-Specific Genes Associated with Maturation and Estrogen Exposure in a Marine Bivalve *Mytilus edulis*. *PLoS One* **2011**, *6* (7), e22326.

(13) Ciocan, C. M.; Cubero-Leon, E.; Peck, M. R.; Langston, W. J.; Pope, N.; Minier, C.; Rotchell, J. M. Intersex in *Scrobicularia plana*: transcriptomic analysis reveals novel genes involved in endocrine disruption. *Environ. Sci. Technol.* **2012**, *46* (23), 12936−12942.

(14) Colborn, T.; vom Saal, F. S.; Soto, A. M. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect.* **1993**, *101* (5), 378.

(15) Kidd, K. A.; Blanchfield, P. J.; Mills, K. H.; Palace, V. P.; Evans, R. E.; Lazorchak, J. M.; Flick, R. W. Collapse of a fish population after exposure to a synthetic estrogen. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104* (21), 8897−8901.

(16) Bridgham, J. T.; Keay, J.; Ortlund, E. A.; Thornton, J. W. Vestigialization of an allosteric switch: genetic and structural mechanisms for the evolution of constitutive activity in a steroid hormone receptor. *PLoS Genet.* **2014**, *10* (1), e1004058.

(17) Keay, J.; Bridgham, J. T.; Thornton, J. W. The Octopus vulgaris estrogen receptor is a constitutive transcriptional activator: evolutionary and functional implications. *Endocrinology* **2006**, *147* (8), 3861−3869.

(18) Keay, J.; Thornton, J. W. Hormone-activated estrogen receptors in annelid invertebrates: implications for evolution and endocrine disruption. *Endocrinology* **2009**, *150* (4), 1731−1738.

(19) Nagasawa, K.; Treen, N.; Kondo, R.; Otoki, Y.; Itoh, N.; Rotchell, J. M.; Osada, M. Molecular characterization of an estrogen receptor and estrogen-related receptor and their autoregulatory capabilities in two Mytilus species. *Gene* **2015**, *564* (2), 153−159.

(20) Suárez-Ulloa, V.; Fernández-Tajes, J.; Manfrin, C.; Gerdol, M.; Venier, P.; Eirín-López, J. Bivalve Omics: State of the Art and Potential Applications for the Biomonitoring of Harmful Marine Compounds. *Mar. Drugs* **2013**, *11* (11), 4370.

(21) Junker, B. H.; Schreiber, F. *Analysis of biological networks*; John Wiley & Sons: 2011; Vol. 2.

(22) He, X.; Zhang, J. Why do hubs tend to be essential in protein networks? *PLoS Genet.* **2006**, *2* (6), e88.

(23) Villeneuve, D. L.; Crump, D.; Garcia-Reyero, N.; Hecker, M.; Hutchinson, T. H.; LaLone, C. A.; Landesmann, B.; Lettieri, T.; Munn, S.; Nepelska, M. Adverse outcome pathway (AOP) development I: strategies and principles. *Toxicol. Sci.* **2014**, *142* (2), 312−320.

(24) Ricciardi, K. L.; Poynton, H. C.; Duphily, B. J.; Blalock, B. J.; Robinson, W. E. Bioconcentration and depuration of 14C−labeled 17α-ethinyl estradiol and 4-nonylphenol in individual organs of the marine bivalve *Mytilus edulis* L. *Environ. Toxicol. Chem.* **2016**, *35* (4), 863−873.

(25) Aris, A. Z.; Shamsuddin, A. S.; Praveena, S. M. Occurrence of 17α-ethynylestradiol (EE2) in the environment and effect on exposed biota: a review. *Environ. Int.* **2014**, *69*, 104−119.

DOI: 10.1021/acs.est.8b01604
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

(26) Giger, W.; Brunner, P. H.; Schaffner, C. 4-Nonylphenol in sewage sludge: accumulation of toxic metabolites from nonionic surfactants. *Science* **1984**, *225* (4662), 623−625.
(27) Gutendorf, B.; Westendorf, J. Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. *Toxicology* **2001**, *166* (1−2), 79−89.
(28) Janer, G.; Porte, C. Sex steroids and potential mechanisms of non-genomic endocrine disruption in invertebrates. *Ecotoxicology* **2007**, *16* (1), 145−160.
(29) Poynton, H. C.; Robinson, W. E.; Blalock, B. J.; Hannigan, R. E. Correlation of transcriptomic responses and metal bioaccumulation in *Mytilus edulis* L. reveals early indicators of stress. *Aquat. Toxicol.* **2014**, *155*, 129−41.
(30) Hines, A.; Yeung, W. H.; Craft, J.; Brown, M.; Kennedy, J.; Bignell, J.; Stentiford, G. D.; Viant, M. R. Comparison of histological, genetic, metabolomics, and lipid-based methods for sex determination in marine mussels. *Anal. Biochem.* **2007**, *369* (2), 175−186.
(31) Bolger, A. M.; Lohse, M.; Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **2014**, *30* (15), 2114−2120.
(32) Zerbino, D. R.; Birney, E. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res.* **2008**, *18* (5), 821−829.
(33) Schulz, M. H.; Zerbino, D. R.; Vingron, M.; Birney, E. Oases: robust de novo RNA-seq assembly across the dynamic range of expression levels. *Bioinformatics* **2012**, *28* (8), 1086−1092.
(34) Conesa, A.; Götz, S.; García-Gómez, J. M.; Terol, J.; Talón, M.; Robles, M. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **2005**, *21* (18), 3674−3676.
(35) Maere, S.; Heymans, K.; Kuiper, M. BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. *Bioinformatics* **2005**, *21* (16), 3448−3449.
(36) Shannon, P.; Markiel, A.; Ozier, O.; Baliga, N. S.; Wang, J. T.; Ramage, D.; Amin, N.; Schwikowski, B.; Ideker, T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* **2003**, *13* (11), 2498−2504.
(37) Koressaar, T.; Remm, M. Enhancements and modifications of primer design program Primer3. *Bioinformatics* **2007**, *23* (10), 1289−1291.
(38) Bustin, S. A.; Benes, V.; Garson, J. A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M. W.; Shipley, G. L. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **2009**, *55* (4), 611−622.
(39) Anantharaman, S.; Craft, J. A. Annual variation in the levels of transcripts of sex-specific genes in the mantle of the common mussel, *Mytilus edulis*. *PLoS One* **2012**, *7* (11), e50861.
(40) Ruiz-Velásquez, M.; Zapata, M.; Gonzalez, M. T.; Escalona, M.; Fajardo, M.; Tarifeño, E.; Morales, J. Role of sex steroids in gonadal differentiation of the mussel Choromytilus chorus (Bivalvia Mytilidae) (Molina 1782). *Aquacult. Res.* **2018**, *49* (1), 404−414.
(41) Nice, H. E.; Thorndyke, M.; Morritt, D.; Steele, S.; Crane, M. Development of *Crassostrea gigas* Larvae is Affected by 4-nonylphenol. *Mar. Pollut. Bull.* **2000**, *40* (6), 491−496.
(42) Nice, H. E.; Morritt, D.; Crane, M.; Thorndyke, M. Long-term and transgeneration effects of nonylphenol exposure at a key stage in the development of *Crassostrea gigas*. Possible endocrine disruption? *Mar. Ecol.: Prog. Ser.* **2003**, *256*, 293−300.
(43) Nice, H. E. Sperm motility in the Pacific oyster (*Crassostrea gigas*) is affected by nonylphenol. *Mar. Pollut. Bull.* **2005**, *50* (12), 1668−74.
(44) Jobling, S.; Nolan, M.; Tyler, C. R.; Brighty, G.; Sumpter, J. P. Widespread sexual disruption in wild fish. *Environ. Sci. Technol.* **1998**, *32* (17), 2498−2506.
(45) Andrew, M.; Dunstan, R.; O'connor, W.; Van Zwieten, L.; Nixon, B.; MacFarlane, G. Effects of 4-nonylphenol and 17α-ethynylestradiol exposure in the Sydney rock oyster, *Saccostrea glomerata*: Vitellogenin induction and gonadal development. *Aquat. Toxicol.* **2008**, *88* (1), 39−47.
(46) Dublinowska, M.; Smolarz, K.; Zabrzańska, S.; Larsson, J.; Czerniawska, N. Intersexuality in the blue mussel *Mytilus edulis* complex (Mytilidae) from the Baltic Sea and the Danish strait. *American Malacological Bulletin* **2016**, *34* (1), 28−39.
(47) Jobling, S.; Casey, D.; Rodgers-Gray, T.; Oehlmann, J.; Schulte-Oehlmann, U.; Pawlowski, S.; Baunbeck, T.; Turner, A.; Tyler, C. Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. *Aquat. Toxicol.* **2003**, *65* (2), 205−220.
(48) Tankoua, O. F.; Amiard-Triquet, C.; Denis, F.; Minier, C.; Mouneyrac, C.; Berthet, B. Physiological status and intersex in the endobenthic bivalve Scrobicularia plana from thirteen estuaries in northwest France. *Environ. Pollut.* **2012**, *167*, 70−77.
(49) Scott, A. P. Do mollusks use vertebrate sex steroids as reproductive hormones? Part I: Critical appraisal of the evidence for the presence, biosynthesis and uptake of steroids. *Steroids* **2012**, *77* (13), 1450−1468.
(50) Markov, G. V.; Tavares, R.; Dauphin-Villemant, C.; Demeneix, B. A.; Baker, M. E.; Laudet, V. Independent elaboration of steroid hormone signaling pathways in metazoans. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106* (29), 11913−11918.
(51) Goldstone, J. V.; Sundaramoorthy, M.; Zhao, B.; Waterman, M. R.; Stegeman, J. J.; Lamb, D. C. Genetic and structural analyses of cytochrome P450 hydroxylases in sex hormone biosynthesis: Sequential origin and subsequent coevolution. *Mol. Phylogenet. Evol.* **2016**, *94*, 676−687.
(52) Strushkevich, N.; MacKenzie, F.; Cherkesova, T.; Grabovec, I.; Usanov, S.; Park, H.-W. Structural basis for pregnenolone biosynthesis by the mitochondrial monooxygenase system. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108* (25), 10139−10143.
(53) Wootton, A. N.; Herring, C.; Spry, J. A.; Wiseman, A.; Livingstone, D. R.; Goldfarb, P. S. Evidence for the existence of cytochrome P450 gene families (CYP1A, 3A, 4A, 11A) and modulation of gene expression (CYP1A) in the mussel *Mytilus* spp. *Mar. Environ. Res.* **1995**, *39* (1−4), 21−26.
(54) Martínez, A. A.; Muñoz, Y. R.; Serrano, F. S. J.; García, P. M. Immunolocalization of cholesterol side chain cleavage enzyme (P450scc) in *Mytilus galloprovincialis* and its induction by nutritional levels. *J. Comp. Physiol., B* **2008**, *178* (5), 647−654.
(55) De Longcamp, D.; Lubet, P.; Drosdowsky, M. The in vitro biosynthesis of steroids by the gonad of the mussel (*Mytilus edulis*). *Gen. Comp. Endocrinol.* **1974**, *22* (1), 116−127.
(56) Lupo Di Prisco, C.; Dessi'Fulgheri, F. Alternative pathways of steroid biosynthesis in gonads and hepatopancreas of *Aplysia depilans*. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry* **1975**, *50* (1), 191−195.
(57) Gottfried, H.; Dorfman, R. I. Steroids of invertebrates V. The in vitro biosynthesis of steroids by the male-phase ovotestis of the slug (*Ariolimax californicus*). *Gen. Comp. Endocrinol.* **1970**, *15* (1), 120−138.
(58) Krusch, B.; Schoenmakers, H.; Voogt, P.; Nolte, A. Steroid synthesizing capacity of the dorsal body of *Helix pomatia* L. (Gastropoda)—an in vitro study. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry* **1979**, *64* (1), 101−104.
(59) Le Curieux-Belfond, O.; Moslemi, S.; Mathieu, M.; Séralini, G. Androgen metabolism in oyster *Crassostrea gigas*: evidence for 17β-HSD activities and characterization of an aromatase-like activity inhibited by pharmacological compounds and a marine pollutant. *J. Steroid Biochem. Mol. Biol.* **2001**, *78* (4), 359−366.
(60) Le Guellec, D.; Thiard, M.-C.; Remy-Martin, J. P.; Deray, A.; Gomot, L.; Adessi, G. L. In vitro metabolism of androstenedione and identification of endogenous steroids in *Helix aspersa*. *Gen. Comp. Endocrinol.* **1987**, *66* (3), 425−433.
(61) Matsumoto, T.; Osada, M.; Osawa, Y.; Mori, K. Gonadal estrogen profile and immunohistochemical localization of steroidogenic enzymes in the oyster and scallop during sexual maturation.

DOI: 10.1021/acs.est.8b01604
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol. **1997**, *118* (4), 811−817.
(62) Ronis, M.; Mason, A. The metabolism of testosterone by the periwinkle (*Littorina littorea*) in vitro and in vivo: effects of tributyl tin. *Mar. Environ. Res.* **1996**, *42* (1−4), 161−166.
(63) Zanette, J.; Goldstone, J. V.; Bainy, A. C.; Stegeman, J. J. Identification of CYP genes in *Mytilus* (mussel) and *Crassostrea* (oyster) species: first approach to the full complement of cytochrome P450 genes in bivalves. *Mar. Environ. Res.* **2010**, *69*, S1−S3.
(64) Morcillo, Y.; Porte, C. Monitoring of organotin compounds and their effects in marine molluscs. *TrAC, Trends Anal. Chem.* **1998**, *17* (2), 109−116.
(65) Osada, M.; Tawarayama, H.; Mori, K. Estrogen synthesis in relation to gonadal development of Japanese scallop, *Patinopecten yessoensis*: gonadal profile and immunolocalization of P450 aromatase and estrogen. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **2004**, *139* (1), 123−128.
(66) Matthiessen, P. An assessment of endocrine disruption in mollusks and the potential for developing internationally standardized mollusk life cycle test guidelines. *Integr. Environ. Assess. Manage.* **2008**, *4* (3), 274−84.
(67) Lösel, R.; Wehling, M. Nongenomic actions of steroid hormones. *Nat. Rev. Mol. Cell Biol.* **2003**, *4* (1), 46.
(68) Norman, A. W.; Mizwicki, M. T.; Norman, D. P. Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. *Nat. Rev. Drug Discovery* **2004**, *3* (1), 27.
(69) Bjornstrom, L.; Sjoberg, M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. *Mol. Endocrinol.* **2005**, *19* (4), 833−842.
(70) Evinger, A. J., III; Levin, E. R. Requirements for estrogen receptor α membrane localization and function. *Steroids* **2005**, *70* (5−7), 361−363.
(71) Levin, E. R. Cellular functions of plasma membrane estrogen receptors. *Steroids* **2002**, *67* (6), 471−475.
(72) Razandi, M.; Oh, P.; Pedram, A.; Schnitzer, J.; Levin, E. R. ERs associate with and regulate the production of caveolin: implications for signaling and cellular actions. *Mol. Endocrinol.* **2002**, *16* (1), 100−115.
(73) Revelli, A.; Ghigo, D.; Moffa, F.; Massobrio, M.; Tur-Kaspa, I. Guanylate cyclase activity and sperm function. *Endocr. Rev.* **2002**, *23* (4), 484−494.
(74) Canesi, L.; Borghi, C.; Ciacci, C.; Fabbri, R.; Lorusso, L. C.; Vergani, L.; Marcomini, A.; Poiana, G. Short-term effects of environmentally relevant concentrations of EDC mixtures on *Mytilus galloprovincialis* digestive gland. *Aquat. Toxicol.* **2008**, *87* (4), 272−279.
(75) Canesi, L.; Borghi, C.; Ciacci, C.; Fabbri, R.; Vergani, L.; Gallo, G. Bisphenol-A alters gene expression and functional parameters in molluscan hepatopancreas. *Mol. Cell. Endocrinol.* **2007**, *276* (1−2), 36−44.
(76) Canesi, L.; Lorusso, L. C.; Ciacci, C.; Betti, M.; Rocchi, M.; Pojana, G.; Marcomini, A. Immunomodulation of *Mytilus* hemocytes by individual estrogenic chemicals and environmentally relevant mixtures of estrogens: in vitro and in vivo studies. *Aquat. Toxicol.* **2007**, *81* (1), 36−44.
(77) Franco, R.; Sánchez-Olea, R.; Reyes-Reyes, E. M.; Panayiotidis, M. I. Environmental toxicity, oxidative stress and apoptosis: menage a trois. *Mutat. Res., Genet. Toxicol. Environ. Mutagen.* **2009**, *674* (1), 3−22.
(78) Koutsogiannaki, S.; Franzellitti, S.; Fabbri, E.; Kaloyianni, M. Oxidative stress parameters induced by exposure to either cadmium or 17β-estradiol on *Mytilus galloprovincialis* hemocytes. The role of signaling molecules. *Aquat. Toxicol.* **2014**, *146*, 186−195.
(79) Razandi, M.; Alton, G.; Pedram, A.; Ghonshani, S.; Webb, P.; Levin, E. R. Identification of a structural determinant necessary for the localization and function of estrogen receptor α at the plasma membrane. *Molecular and cellular biology* **2003**, *23* (5), 1633−1646.
(80) Patel, H. H.; Murray, F.; Insel, P. A. Caveolae as organizers of pharmacologically relevant signal transduction molecules. *Annu. Rev. Pharmacol. Toxicol.* **2008**, *48*, 359−391.
(81) Canesi, L.; Ciacci, C.; Betti, M.; Lorusso, L. C.; Marchi, B.; Burattini, S.; Falcieri, E.; Gallo, G. Rapid effects of 17β-estradiol on cell signaling and function of *Mytilus* hemocytes. *Gen. Comp. Endocrinol.* **2004**, *136* (1), 58−71.
(82) Canesi, L.; Lorusso, L. C.; Ciacci, C.; Betti, M.; Zampini, M.; Gallo, G. Environmental estrogens can affect the function of mussel hemocytes through rapid modulation of kinase pathways. *Gen. Comp. Endocrinol.* **2004**, *138* (1), 58−69.
(83) Stefano, G. B.; Zhu, W.; Mantione, K.; Jones, D.; Salamon, E.; Cho, J. J.; Cadet, P. 17-beta estradiol down regulates ganglionic microglial cells via nitric oxide release: Presence of an estrogen receptor beta transcript. *Neuroendocrinology letters* **2003**, *24* (3-4), 130−136.
(84) Anderson, K.; Burnell, F.; Roiko, A.; Andrew, M.; O'Connor, W.; Elizur, A. Development of a method for identifying elevated vitellogenin gene expression in the Sydney Rock Oyster (*Saccostrea glomerata*) as an indicator of endocrine disruption on the Sunshine Coast. *Ecological Management & Restoration* **2010**, *11* (2), 143−146.
(85) Duffy, T. A.; McElroy, A. E.; Conover, D. O. Variable susceptibility and response to estrogenic chemicals in Menidia menidia. *Mar. Ecol.: Prog. Ser.* **2009**, *380*, 245−254.
(86) Reddy, S.; Brownawell, B. J. Analysis of estrogens in sediment from a sewage-impacted urban estuary using high-performance liquid chromatography/time-of-flight mass spectrometry. *Environ. Toxicol. Chem.* **2005**, *24* (5), 1041−1047.
(87) Zulkosky, A.; Ferguson, P.; McElroy, A. Effects of sewage-impacted sediment on reproduction in the benthic crustacean *Leptocheirus plumulosus*. *Mar. Environ. Res.* **2002**, *54* (3−5), 615−619.
(88) Mena, L.; Arukwe, A.; Denslow, N.; McElroy, A. Endocrine disruption in winter flounder (*Pseudopleuronectes americanus*) from an urban esturary, Jamaica Bay, NY, USA. *Marine Environmental Research* **2006**, *62*, S233−S234.
(89) Mohamed, B.; Hajer, A.; Susanna, S.; Caterina, O.; Flavio, M.; Hamadi, B.; Aldo, V. Transcriptomic responses to heat stress and nickel in the mussel *Mytilus galloprovincialis*. *Aquat. Toxicol.* **2014**, *148*, 104−112.
(90) Lockwood, B. L.; Somero, G. N. Transcriptomic responses to salinity stress in invasive and native blue mussels (genus *Mytilus*). *Mol. Ecol.* **2011**, *20* (3), 517−29.
(91) Giannetto, A.; Maisano, M.; Cappello, T.; Oliva, S.; Parrino, V.; Natalotto, A.; De Marco, G.; Fasulo, S. Effects of Oxygen Availability on Oxidative Stress Biomarkers in the Mediterranean Mussel *Mytilus galloprovincialis*. *Mar. Biotechnol.* **2017**, *19* (6), 614−626.
(92) González-Fernández, C.; Albentosa, M.; Sokolova, I. Interactive effects of nutrition, reproductive state and pollution on molecular stress responses of mussels, *Mytilus galloprovincialis* Lamarck, 1819. *Mar. Environ. Res.* **2017**, *131*, 103−115.
(93) Mezzelani, M.; Gorbi, S.; Fattorini, D.; d'Errico, G.; Benedetti, M.; Milan, M.; Bargelloni, L.; Regoli, F. Transcriptional and cellular effects of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) in experimentally exposed mussels. *Aquat. Toxicol.* **2016**, *180*, 306−319.
(94) Miao, J.; Chi, L.; Pan, L.; Song, Y. Generally detected genes in comparative transcriptomics in bivalves: Toward the identification of molecular markers of cellular stress response. *Environ. Toxicol. Pharmacol.* **2015**, *39* (1), 475−481.
(95) Rossi, F.; Palombella, S.; Pirrone, C.; Mancini, G.; Bernardini, G.; Gornati, R. Evaluation of tissue morphology and gene expression as biomarkers of pollution in mussel *Mytilus galloprovincialis* caging experiment. *Aquat. Toxicol.* **2016**, *181*, 57−66.

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