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Exposure to 17α-Ethinylestradiol Results in Differential Susceptibility of Largemouth Bass (*Micropterus salmoides*) to Bacterial Infection

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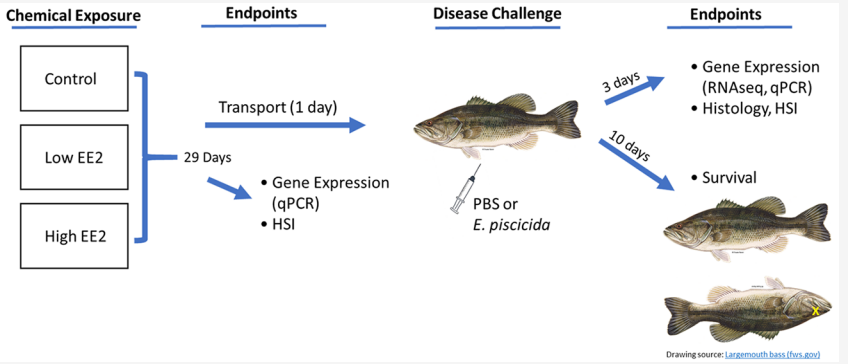

ABSTRACT: Disease outbreaks, skin lesions, mortality events, and reproductive abnormalities have been observed in wild populations of centrarchids. The presence of estrogenic endocrine disrupting compounds (EEDCs) has been implicated as a potential causal factor for these effects. The effects of prior EEDC exposure on immune response were examined in juvenile largemouth bass (*Micropterus salmoides*) exposed to a potent synthetic estrogen (17α-ethinylestradiol, EE2) at a low ($EE2_{Low}$, 0.87 ng/L) or high ($EE2_{High}$, 9.08 ng/L) dose for 4 weeks, followed by transfer to clean water and injection with an LD_{40} dose of the Gram-negative bacteria *Edwardsiella piscicida*. Unexpectedly, this prior exposure to $EE2_{High}$ significantly increased survivorship at 10 d post-infection compared to solvent control or $EE2_{Low}$-exposed, infected fish. Both prior exposure and infection with *E. piscicida* led to significantly reduced hepatic glycogen levels, indicating a stress response resulting in depletion of energy stores. Additionally, pathway analysis for liver and spleen indicated differentially expressed genes associated with immunometabolic processes in the mock-injected $EE2_{High}$ treatment that could underlie the observed protective effect and metabolic shift in $EE2_{High}$-infected fish. Our results demonstrate that exposure to a model EEDC alters metabolism and immune function in a fish species that is ecologically and economically important in North America.

KEYWORDS: *RNaseq, immunomodulation, immunometabolism, disease challenge, estrogenic endocrine disrupting compounds, Edwardsiella piscicida*

INTRODUCTION

Reproductive abnormalities have been observed in wild fish throughout the United States, with high prevalence in largemouth and smallmouth bass.[1] Additional health concerns for smallmouth bass (*Micropterus dolomieu*) and other species in the Chesapeake Bay Watershed (CBW) began in 2002 following a substantial multispecies fish mortality event in the South Branch of the Potomac River, West Virginia.[2] Since that time other centrarchid mortality events have been observed that are typically associated with skin lesions and opportunistic bacterial and parasitic pathogens.[3,4−7] The timing of mortality events and opportunistic nature of associated pathogens indicated a role for anthropogenic chemical inputs during high runoff events as potential contributors to immunosuppression. Numerous chemicals associated with harmful effects in aquatic organisms are detected in waterways and sediments in the CBW and throughout the United States, including pesticides, polycyclic aromatic hydrocarbons, antibiotics, phytoestrogens, and steroid hormones.[2,8−13]

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Several lines of evidence indicate a potential causal relationship between estrogenic endocrine disrupting compounds' (EEDCs) exposure and immunomodulation and declining fish health. In some regions of the CBW, for example, greater than 80% of male smallmouth bass and 60% of largemouth bass (*Micropterus salmoides*, LMB) display intersex characteristics, including testicular oocytes and increased expression of the female egg laying protein vitellogenin, indicating biologically relevant exposure to EEDCs.[3,9,14,15] Estrogen receptor agonists including estrone and estrogenicity assessed by bioassays are commonly detected in surface waters.[8,10,16−19]

Exposures to EEDCs are well-documented to modulate immunity in fish. EEDCs induce transcriptomic changes through binding and activation of estrogen receptors alpha (ERα) and beta (ERβ). These nuclear transcription factors regulate transcription of downstream genes and pathways involved in metabolism, immunity, and other processes.[20−23] Examples of EEDC gene targets include nuclear factor-κB (NF-κB) and toll-like receptors (TLRs) that are crucial for induction of the innate immune response and pathogen clearance.[24] The interaction of overlapping transcriptomic changes initiates a complex response that may either enhance or suppress the immune response depending on host, disease organism, and abiotic factors.[24−30] Moreover, exposure to EEDCs during early life stages and sexual differentiation may lead to permanent, organizational effects such as sex reversal, as well as temporary, activational effects such as expression of vitellogenin.[31−34] This presents adverse biological risks in agricultural areas, where the spring influx of estrogenic pesticides coincides with spawning and early development.[35]

The current study objective was to evaluate the developmental effects of exposure to a model estrogen representing environmentally observed total EEDC estrogenic activity on disease outcome in a fish species with economic and ecological importance in North America. Using a laboratory disease challenge model, we examined the effects of exposure to the potent synthetic estrogen 17α-ethinylestradiol (EE2) on gene expression and survivorship of juvenile (6 months old) LMB subsequently infected with the Gram-negative bacterium *Edwardsiella piscicida*. The disease challenge model with *E. piscicida* was established as a representative pathogen that LMB could contract in the wild.[36] The juvenile LMB were exposed to measured EE2 concentrations of 0.87 or 9.08 ng/L for 4 weeks, followed by a 10-day bacterial infection challenge. The effects of EE2 exposure, *E. piscicida* infection, and the combined effect of both were examined. Survival, liver histology, and whole-transcriptome sequencing (RNaseq) of liver and spleen tissues were assessed. This study examined the potential effects of a potent estrogen on disease outcome and associated molecular mechanisms in juvenile LMB.

MATERIALS AND METHODS

This study was conducted in two parts: (1) a 4-week EE2 exposure conducted at the U.S. Geological Survey Columbia Environmental Research Center (CERC, Columbia Missouri, USA) and (2) a bacterial disease challenge conducted at the U.S. Geological Survey Western Fisheries Research Center (WFRC, Seattle, Washington, USA). This was done to utilize the toxicity testing expertise and extensive laboratory resources at CERC to conduct the exposure portion of the experiment and to utilize the pathogen expertise and required biosafety at WFRC to conduct the disease challenge. The mock injection controls served as our control of the effect of transportation and injection stress. Survival was monitored as a direct measure of stress resulting from transport, handling, and injection procedures. A complete schematic of the experimental design can be found in Figure S1. This study followed all applicable sections of the Final Rules of the Animal Welfare Act regulations (9 CFR) and all CERC Institutional Animal Care and Use Committee (IACUC) guidelines for the humane treatment of the test organisms during culture and experimentation. Fish transport permit 7910-10-29-18 was obtained from the Washington Department of Fish and Wildlife. The disease challenge protocols for experimental use of LMB were approved by WFRC IACUC protocol 2008-34. LMB rearing and EE2 exposure were conducted at CERC as previously described (Text S1).[31,37]

Bacterial Cultivation. *Edwardsiella piscicida* isolate S11-285 was obtained from Matt Griffin (Mississippi State University) for this study. Bacteria were plated on tryptic soy agar supplemented with 5% sheep blood (Remel) and incubated at 20 °C overnight. Single colonies were picked and used to seed 3 mL of Brain Heart Infusion broth (Difco) that was then incubated at 20 °C for 18 h with shaking at 200 rotations per minute. Cultures were centrifuged at 2000*g*, media was removed, and the bacterial cell pellet was resuspended in 1X phosphate buffered saline (PBS). Bacteria were then diluted to OD_{600} (optical density at 600 nm) of 0.8 (approximately 5.6×10^8 colony-forming units (CFU)/mL) and further diluted with 1X PBS to determine the appropriate CFU/mL for injections. CFUs were determined using triplicate plate counting of 10-fold serial dilutions on blood agar plates.

Establishment of *Edwardsiella piscicida* Challenge Methods. Juvenile LMB (obtained from Genoa National Fish Hatchery, U.S. Fish and Wildlife Service) with an average weight of 24 (±2.4) grams were injected with 3×10^7, 3×10^6, 3×10^5, and 7×10^4 CFU/fish of *E. piscicida*, resulting in 0% survival for the two high doses, 24% survival for 3×10^5 CFU/fish, and 41% survival for the lowest dose (Figure S2). Two weeks before the disease challenge of EE2 and solvent control-exposed fish with *E. piscicida*, cohorts of LMB from CERC were used to establish LD_{40} (lethal dose of 40%) values for the main experiment. Duplicate sets of tanks (n = 15/tank) were injected with 7.4×10^4 and 3.2×10^4 CFU/fish, resulting in 42% and 78% survival over 10 days, respectively.

EE2 Exposure and Chemical Analysis. Juvenile LMB were exposed to nominal EE2 concentrations of 2 ng/L ($EE2_{Low}$), 20 ng/L ($EE2_{High}$), or 0.0001% ethanol as the solvent control (SC; Sigma-Aldrich, St. Louis, Missouri, USA) for 28 days. Exposures were conducted in flow-through diluters with approximately four tank turnovers per day. Stock solution was prepared in a filtered well water at a nominal concentration of 20 μg EE2/mL from 1 mg EE2/mL EtOH superstock (Sigma-Aldrich E7023). This stock solution was used to make a 5 μg EE2/L dosing solution in the well water, and that was used to make a 0.5 μg EE2/L dosing solution in the well water. Thirty juvenile LMB were held in each of four randomized replicate tanks per exposure group (30 fish × 4 replicate tanks × 3 treatments = 360 fish total) to minimize tank effects. Dosing solutions were confirmed by liquid chromatography−mass spectrometry, and tank concentrations were monitored weekly (Text S2).

***Edwardsiella piscicida* Challenge Following EE2 Exposure.** On the 29th day, fish from each of the exposure tanks were placed into tank-associated plastic bags containing clean water. Bags were identified by codes to blind the disease challenge study. The bags of fish were placed into insulated

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Environ. Sci. Technol. 2022, 56, 14375−14386

coolers and immediately transported overnight by air freight to the Western Fisheries Research Center, Seattle, Washington. Total transport time was approximately 18 h. Upon arrival, fish were acclimated to 16−18 °C for 1 h prior to bacterial challenge. There were 4 bags of fish per exposure group (SC, $EE2_{Low}$, and $EE2_{High}$) and 26 fish/bag for a total of 312 fish (Figure S1A). Each bag was processed as follows: LMB were briefly anesthetized using buffered MS-222 (ethyl 3-aminobenzoate methanesulfonate, Argent); 20 fish were intraperitoneally injected with 50 μL of *E. piscicida* suspension containing 4.8×10^4 CFUs per fish, and 6 were mock injected with a matching volume of 1X PBS. CFUs for injection were confirmed with triplicate plate counts using 10-fold dilutions. Injected fish were distributed to 30 L tanks with aeration at 20−21 °C as follows: Eighteen of the 20 *E. piscicida*-injected fish were placed in a tank for assessment of mortality with the remaining two placed in a separate tank for transcriptomics, pooled by EE2 exposure group code. Five of the six mock-injected fish were placed into a separate tank for assessment of mortality with the remaining mock-injected fish being placed into a separate tank for transcriptomics, pooled by EE2 exposure group code. Therefore, at the end of injecting fish, to assess mortality, 18 fish were in each disease challenge tank and 5 fish in each mock-injected tank with 4 tanks per EE2 exposure group (24 tanks). For transcriptomics, 8 fish were in each *E. piscicida*-injected tank and 4 fish were in each mock-injected tank, pooled with 1 tank per EE2 exposure group (6 tanks). Tank placement was randomized to minimize potential tank effects (Figure S1B). Moribund fish displaying loss of buoyancy, hemorrhaging, and/or severe exophthalmia were euthanized and counted as an end point (mortality) for the survival analysis. Presence of *E. piscicida* in moribund and dead fish was confirmed by culturing brain tissue on blood agar plates and 16S sequencing (Table S1).[38]

Survival, Length, Weight, and Hepatosomatic Index. A subset of fish from each exposure group were euthanized with an overdose (300 mg/L) of buffered MS-222 (Sigma-Aldrich) for length, weight, and hepatosomatic index (HSI). Fish were blotted dry; then weight (±0.001 g) and total length (mm) were measured. Livers were weighed (±0.001 g) to calculate HSI (total liver weight/total fish weight × 100). Measurements were performed on fish at 0 d post-exposure to EE2 exposure and at 4 d post-exposure (3 d post mock injection). Survival was monitored for 10 d following *E. piscicida* exposure.

Histological Analysis. Livers from 33 fish sampled 3 d post-infection (4 d post-exposure) were preserved in 10% neutral buffered formalin and processed and embedded in paraffin wax according to standard protocols. Tissues were sectioned at 5 μm and stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), PAS-diastase (PAS-D), Lillie-Twort Gram, and May-Grünwald Giemsa and then examined at 200× magnification. Blinded histopathology evaluations of H&E-stained slides were scored for the distribution (0−3) and the degree (0−3) of host responses of inflammation, fibrosis, and necrosis[39,40] (Text S3). Periodic acid-Schiff- and PAS-D-stained slides were examined to estimate the amount of liver energy storage (as hepatocyte glycogen) and used as an index of relative nutritional status. Assessments were made by the appearance of hepatocyte cytoplasmic vacuoles and the staining characteristics of the cytoplasm. The estimated amount of glycogen was rated (0−3) as none, low, moderate, or high.[40] Bacteria were identified and visualized by Gram and Giemsa stains.[39]

Gene Expression Analysis. Tissue samples from the liver and spleen were obtained at two time points: (1) 0 d post EE2 exposure and (2) 3 d post-infection (4 d post EE2 exposure; Figure S1B). Tissues from individual fish were placed in RNAlater (Thermo Fisher Scientific) and stored at −20 °C until RNA extraction. Total RNA was extracted using the RNeasy mini kit according to the manufacturer's protocol (Qiagen). Tissues obtained 0 d post EE2 exposure were used for targeted gene expression analysis via qPCR (n = 10−16 per treatment),[41] and 3 d post-infection samples were assessed with RNaseq and verified targeted genes with qPCR. Complete methods for gene expression studies can be found in Text S4, and primer sequences are in Table S1.[41] All raw sequencing reads were submitted to the NCBI Sequence read archive (Bioproject Accession: PRJNA627494).

Bioinformatics. The quality and depth of the raw and trimmed reads for each sequencing library were assessed with FastQC (v0.11.9).[42] Trimmomatic (v0.39)[43] was used for trimming and quality control of raw sequencing reads, with low quality bases from each end removed (parameters: LEADING:5, TRAILING:5). Reads were then cut once the average phred quality score fell below 15 using a sliding window of length 4. Reads <25 bp after trimming were excluded from downstream analysis. Downstream mapping and quantification of trimmed reads was performed with Salmon[44] utilizing the *Micropterus salmoides* annotated genome and transcriptome obtained from the NCBI ftp database (assembly GCF_014851395.1_ASM1485139v1) with GC bias and selective alignment.

Quantification files were imported into R (v4.1.0) with tximport and aggregated to the gene level using transcript/gene relationships extracted from the genome assembly. Differential expression for each treatment was calculated using a Wald test in DESeq2 (v1.28.1).[45] Significance was defined as a minimum fold change of 1.5 and $p \leq 0.05$ after Benjamini-Hochberg false discovery rate correction. Gene expression patterns were also evaluated by principal component analysis (PCA). Statistical analysis of pathways and gene ontologies predicted to be altered in response to EE2 exposure were carried out with Advaita iPathwayGuide using annotated transcripts, their log-fold change values, and adjusted *p*-values. Pathway significance was defined as $p \leq 0.05$ following multiple testing correction. Specifically: Smallest Common Denominator Pruning (SCDP) for GO Terms and additional false discovery rate (FDR) correction for KEGG pathways.

Statistical Analysis. Kaplan−Meier survival analysis was used to compare survival rates between treatment groups using a log rank (Mantel-Cox) test in Graph Pad PRISM 9 where $p \leq 0.05$ was considered significant. To determine statistically significant differences in histological parameters, the normality of each parameter was assessed using a Shapiro test followed by a one-way ANOVA in R. Statistical differences in targeted analysis of the relative mRNA expression between experimental groups were assessed by one-way analysis of variance (ANOVA) followed by nonparametric comparisons using the Kruskal−Wallis test performed using JMP 14.2 (SAS Institute, Cary, NC). Differences were considered statistically significant at $p \leq 0.05$. Raw data can be found through the U.S. Geological Survey and are publicly available at 10.5066/P93OHUUS.[46]

RESULTS AND DISCUSSION

Establishment of a Bacterial Challenge Model for Largemouth Bass. Recent studies demonstrate that *Edwardsiella piscicida* is an emerging, virulent pathogen of warm water fish including catfish and LMB.[36,47] *E. piscicida* is an intracellular

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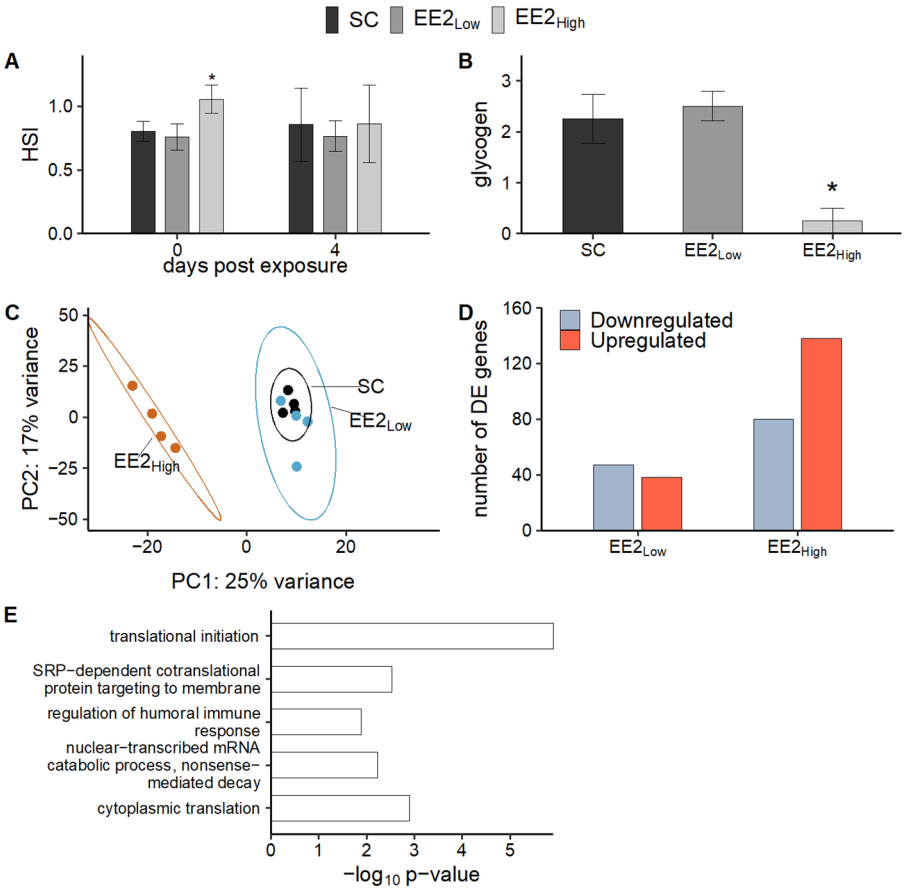

Figure 1. Changes in liver tissue and gene expression following a 4-week exposure to solvent control (SC), 0.87 ng EE2/L ($EE2_{Low}$), or 9.08 ng EE2/L ($EE2_{High}$) at 0 d or in mock-injected fish at 4 d post-exposure. (A) Hepatosomatic index (HSI) was measured at 0 d post-exposure (SC $n = 14$, $EE2_{Low}$ $n = 16$, $EE2_{High}$ $n = 10$) and 4 d post-exposure (SC $n = 4$, $EE2_{Low}$ $n = 8$, $EE2_{High}$ $n = 8$). (B) Glycogen levels were scored in histology sections at 4 d post-exposure. (C) Principal component analysis of overall gene expression patterns in EE2-exposed fish at 4 d post-exposure. (D) Number of differentially expressed genes at 4 d post-exposure at each EE2 exposure concentration in mock-injected fish. (E) Biological pathways enriched in $EE2_{High}$-exposed fish at 4 d postexposure. Error bars are one standard error; asterisks indicate significant difference from SC at $p < 0.05$.

bacterial pathogen that infects and replicates within host epithelial and phagocytic cells. Importantly, the pathogen can also infect human hosts post consumption of infected fish.[47]

To our knowledge, no published bacterial infection models for LMB are available to assess survival statistics post or during contaminant exposure. The present study established a novel, reproducible, acute disease challenge model in LMB. Based on the prevalence and importance of *E. piscicida* for LMB health, we used a well characterized strain of *E. piscicida* for the development of a LMB infection model.[48] We established dose-dependent mortality for *E. piscicida* infection in LMB using bacterial titrations where 7×10^4 CFU/fish resulted in ~40% survival over the course of 10 d (Figure S2). Importantly, *E. piscicida* has trophism for several tissues including the brain, and therefore, brain tissues were sampled and plated to confirm the presence or absence of *E. piscicida* in infected and mock-injected fish. All moribund and dead fish were positive for *E. piscicida*, whereas all control fish were negative.

Water Chemistry. The mean (±standard error) EE2 concentrations were 0.87 (±0.10) ng/L and 9.08 (±1.37) ng/L in the $EE2_{Low}$ and $EE2_{High}$ treatments over the 4-week exposure, respectively. The mean concentration of EE2 in solvent control tanks was 0.03 (±0.007) ng/L. Although water concentrations above 10 ng/L in waters receiving sewage discharge have been reported, the majority of freshwater measurements of EE2 in surface water are below 1.0 ng/L.[49] The concentrations of EE2 in the current study fall within the order of magnitude of environmentally observed concentrations.

Survival, HSI, Histology, and Estrogen Biomarker Expression in EE2 Only Exposures. Differences in survivorship were not significant between groups during the 4-week EE2 exposure (Table S2), no mortality occurred during or after transport (prior to disease challenge), and no mortality was observed in mock-injected fish at 4 d post-exposure to EE2 (3 d post mock injection). Immediately following the 4-week EE2 exposure, the mean HSI (SC n = 14, $EE2_{Low}$ n = 16, $EE2_{High}$ n = 10) was significantly greater in $EE2_{High}$ fish than either SC or $EE2_{Low}$ (Figure 1A). At 4 d post-exposure to EE2 significant differences in HSI (SC n = 4, $EE2_{Low}$ n = 8, $EE2_{High}$ n = 8) between EE2 exposure groups were no longer observed (Figure 1A).

The histological effects of EE2 exposure on the liver were assessed in mock-injected fish at 4 d post EE2 exposure, timing that coincided with RNaseq sampling. PAS and PAS-D staining indicated that overall hepatic glycogen levels were significantly lower following $EE2_{High}$ exposure (Figure 1B), whereas livers from fish in the solvent control and $EE2_{Low}$ treatment groups exhibited glycogen presence in all fish, with 88% at moderate to high levels. Hepatocellular cytoplasmic vacuolization characteristic of glycogen presence was also observed in solvent control

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Environ. Sci. Technol. 2022, 56, 14375−14386

and $EE2_{Low}$ treatment groups, but not in the liver of any $EE2_{High}$ fish. Significant fibrosis, necrosis, and inflammation were not observed in any treatment group. Increased HSI and reduced hepatic glycogen stores observed in $EE2_{High}$ fish are commonly associated with increased metabolism due to vitellogenin synthesis, during both estrogen exposures[49−53] and normal vitellogenic development in female fish.[54] Moreover, suppression of glycogen synthesis during estrogenic exposure may also play a role in reduced glycogen, as has been observed in primary cultures of human endometrial cells.[55]

Estrogenic responses in $EE2_{High}$, but not $EE2_{Low}$, were confirmed through expression of *vtgl*, which was significantly elevated in both liver and spleen of the $EE2_{High}$ group (Figure S3). Expression of *vtgl* in liver is normal in adult female fish undergoing egg maturation but clearly represents a response to xenobiotic activation of the estrogen receptor signaling system in juveniles and in spleen tissue.[20] In liver, expression of *esr1* was also significantly elevated in the $EE2_{High}$ group relative to that observed in the $EE2_{Low}$ group, but not relative to the solvent control (Figure S3A). In spleen, there were no significant exposure group effects on *esr1* expression. Baseline expression of both *vtgl* and *esr1* genes was low in LMB tissues at this juvenile stage of development, and individual variation was high (Figure S3A). We attribute part of the variation observed to differential expression between sexes, which were not separated in our study due to logistical challenges of sex identification.

Hepatic and Splenic Transcriptome Responses to EE2 Exposure. Hepatic and splenic transcriptomes were assessed 4 d post EE2 exposure to evaluate potential latent EEDC effects that could contribute to altered disease outcomes. Patterns of hepatic gene expression in $EE2_{High}$ fish differed from solvent controls and $EE2_{Low}$ fish when evaluated by PCA (Figure 1C), corroborating differences observed among these treatments in HSI and histological findings.

Differential expression in the liver post EE2 exposure was dose-dependent, with 85 and 218 differentially expressed genes (DEGs) in $EE2_{Low}$ and $EE2_{High}$, respectively, compared to solvent control fish (Figure 1D). Of note, only 17 DEGs were common between $EE2_{Low}$ and $EE2_{High}$ treatments, indicating a nonuniform molecular response to varying EE2 concentrations. Most of the DEGs in the $EE2_{High}$ group were up-regulated (Figure 1D) and consisted of molecular biomarkers of exposure to EEDCs, including vitellogenin (*vtg2*, *vtg3*), estrogen receptor 1 (*esr1*), cytochrome p450 2k (*cyp21*), and zona pellucida (*zp3a.2*, *zp2l*) (Figure S4). Only *zp4* expression was significantly altered in response to $EE2_{Low}$. Targeted gene expression analyses of *vtgl* and *esr1* via qPCR in liver 4 d post-exposure (Figure S3) were consistent with the transcriptomic results from this time point. Alterations in these biomarker genes are consistent with previous EE2 exposure studies with juvenile LMB,[56] fathead minnow (*Pimephales promelas*),[57] and rainbow trout (*Oncorhynchus mykiss*).[30,58]

Conversion of the 85 ($EE2_{Low}$) and 218 ($EE2_{High}$) liver DEGs to human IDs for GO and KEGG pathway analysis resulted in 45 and 102 DEGs, respectively. Inspection of biological pathways altered in the liver indicated that prior exposure to $EE2_{High}$ significantly disrupted 5 biological processes, including 4 translational processes: translation initiation, cytoplasmic translation, SRP-dependent cotranslational protein targeting to membrane, the nuclear-transcribed mRNA catabolic process, and regulation of humoral immune response (Figure 1E). In the $EE2_{Low}$ group, negative regulation of intestinal cholesterol and phytoesterol was the only altered biological process, and no pathways were predicted to be significantly altered. Two pathways were predicted to be altered in the $EE2_{High}$ group: alterations in expression of ribosome-associated transcripts (KEGG 03010, $p = 0.004$) and complement and coagulation cascades (KEGG 04610), which agree with the associated GO terms in indicating that $EE2_{High}$ altered metabolic processes.

Perturbation analysis indicated that the membrane attack complex could be affected by $EE2_{High}$ exposure as five complement system genes were altered (Figure S5A). The complement and coagulation cascades are major components of the innate immune system, and the differential expression is indicative of inflammatory responses in the liver. However, these alterations must have been pro-inflammatory in nature, as no significant differences were observed in the inflammation response between the EE2 exposure groups among the infected fish. In fish, whole genome and local duplication events for complement genes have expanded the repertoire of the complement system, thus further highlighting the important role that complement components play for fish health.[50] A total of 15 translation initiation DEGs were observed in the $EE2_{High}$ exposure, all of which were significantly upregulated (Figure S5B). No perturbation was noted for the ribosome pathway, indicating that ribosomes associated with the liver are actively engaged or primed for protein translation. Importantly, ribosome priming increases disease resistance against microbial pathogens by contributing to enhanced innate immune sensing.[51]

Examination of the splenic transcriptome revealed a more robust response to EE2 compared to the liver as 448 and 401 DEGs observed in $EE2_{Low}$ and $EE2_{High}$ treatments, respectively. Only 88 of the DEGs were in common for the two groups, indicating a dose-dependent response, as observed in the liver. GO analysis also showed large differences as 12 biological processes were identified for the $EE2_{Low}$ group and only two for the $EE2_{High}$ group—and no common biological processes between exposures (Table S3). Platelet (thrombocytes) degranulation and aggregation were the top processes for the spleen, indicating acute thrombosis which could render fish more susceptible to disease. The B cell apoptotic process was the third most significant process for the $EE2_{Low}$ exposure group. Aside from antibody production, B cells are professional antigen presenting cells that are key for T-helper cell responses to infection. Unexpectedly, interleukin-1 beta production and neuroinflammatory response were the only processes affected in the $EE2_{High}$ group—both processes were indicative of pro-inflammatory responses. Pathway analysis identified six significant pathways for the $EE2_{Low}$ group and none for the $EE2_{High}$ group (Table S3). Rap-1 signaling, platelet activation, and transcriptional misregulation in cancer were the top pathways for $EE2_{low}$. Rap-1 is a GTPase that controls a variety of process, including cell-to-cell interactions, by regulating integrins and adhesion factors as well as controlling MAP kinase, which regulates responses to a variety of external stimuli. Together these data indicated that estrogen exposure had a substantial effect on inflammatory processes in the spleen, potentially indicating impairment of secondary immune tissues.

In $EE2_{High}$-exposed mock-injected fish the observations of upregulation of estrogen biomarkers, altered global gene expression in the liver and spleen, and reduction in glycogen storage indicated a stress response and metabolic shift that persisted for at least several days.

Survival, Hepatosomatic Index, and Histology from EE2-Exposed and *Edwardsiella piscicida*-Challenged

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Fish. Maintaining homeostasis by adjusting metabolic and immunological processes in response to chemicals, nutrition, and infectious disease is required for basic animal health. Prior to conducting this study, our central hypothesis was that exposure to EE2 would render fish more susceptible to infection, as has been observed in previous studies.[23,59] Unexpectedly, LMB in the $EE2_{High}$ group showed significantly increased survivorship at 10 d post-infection compared to both SC ($p < 0.0161$) and the $EE2_{Low}$ group ($p < 0.0004$, Figure 2). Although unexpected, this

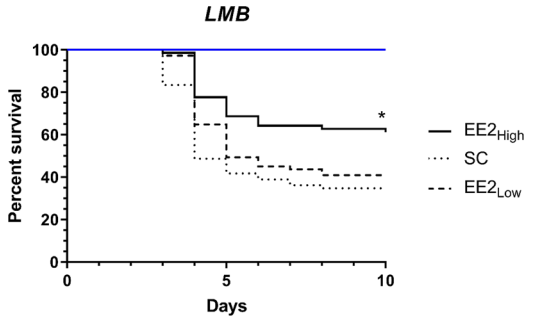

Figure 2. Survival of largemouth bass following a 4-week exposure to solvent control (SC), 0.87 ng EE2/L ($EE2_{Low}$), or 9.08 ng EE2/L ($EE2_{High}$) at indicated time postinfection with *Edwardsiella piscicida*. For mock-injected fish $n = 60$; 5 fish × 4 replicates per EE2 exposure group. For fish infected with *E. piscicida* $n = 216$; 18 fish × 4 replicates per EE2 exposure group. Blue line denotes survival of mock-injected fish. Asterisk indicates significantly higher survival in $EE2_{High}$ compared to SC or $EE2_{Low}$ ($p < 0.02$).

finding is congruent with studies conducted in rainbow trout, where parasite intensity and disease severity were reduced in EE2-exposed fish.[30] These studies highlight the complexity of estrogenic endocrine disruption in fish and the variability in response. No mortality was observed in the mock-injected fish during the 10-day infection trial, regardless of the chemical exposure group prior to the disease challenge. This survival in the control fish indicated that any stress induced by the transport or injection process did not directly induce mortality.

Histologically, all groups infected with *E. piscicida* exhibited an inflammatory response at 3 d post-infection. The mean HSI was significantly greater in all infected treatments compared to mock-injected fish and significantly higher in $EE2_{High}$ compared to other infected treatment groups (one-way ANOVA, Tukey's post-hoc, $p \leq 0.05$, Figure 3A). The total severity of the host response (Figure 3B) and inflammation (Figures 3C, 4A, and 4B) were significantly greater in infected fish from all EE2 exposures relative to mock-injected fish (Figure S6). Hepatic glycogen was significantly reduced by infection (Figures 3D, 4C, and 4D), as was seen in $EE2_{High}$ mock-injected fish, indicating increased metabolic stress in fish infected with *E. piscicida*.

Molecular Effects *of Edwardsiella piscicida* Infection in the Liver and Spleen. Day 3 post-infection corresponds with the first mortalities for infected fish in this study as well as mid-onset of the proinflammatory response in fish.[60−63] As such, we performed a thorough examination of the RNaseq data for day 3 post-infection to address potential protective mechanisms that might have increased survival in $EE2_{High}$-infected fish. Because survival to acute infection is largely dependent upon innate immunity, our focus was on protective innate immune responses.

Two approaches for examining RNaseq results were used to address potential protective mechanisms induced by EE2

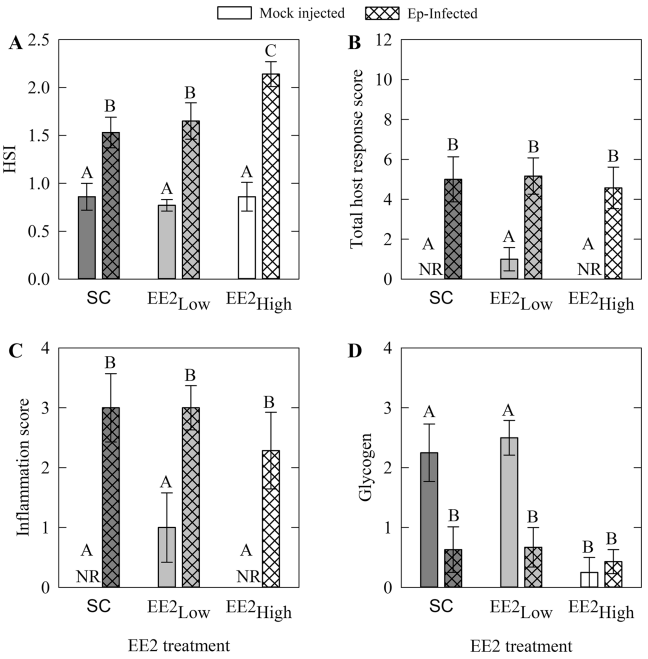

Figure 3. Changes in liver tissue histology following a 4-week exposure to solvent control (SC), 0.87 ng EE2/L ($EE2_{Low}$), or 9.08 ng EE2/L ($EE2_{High}$) at 3 d post-infection with *Edwardsiella piscicida* (Ep-infected) or mock injection. (A) Hepatosomatic index (HSI). (B) Glycogen levels were scored in histology sections. (C) Inflammation score. (D) Total host response score, including inflammation, fibrosis, and necrosis. For mock-injected fish $n = 4$ fish per EE2 exposure group. For Ep-infected fish $n = 8$ per EE2 exposure group. Error bars are one standard error; treatment groups with different letters are significantly different at $p < 0.05$. NR = no response.

exposure. First, we used a standard approach (Approach 1) determining significant GO terms and KEGG pathways associated with the three treatment groups—SC-infected, $EE2_{Low}$-infected, and $EE2_{High}$-infected—to examine EE2 exposure-related effects. However, transcriptomic responses to the EE2 exposure were not discernible against the background of the robust immune response to the infection. As an alternative approach, we assessed differential expression and pathway analysis using PCA groupings based on disease progression and histological identification of bacteria (Approach 2).

EE2 Exposure-Related Alteration in Gene Expression. Both infection plus EE2 were responsible for increased hepatic DEGs in the low and high dose EE2 exposures. RNaseq analysis of SC-infected, $EE2_{Low}$-infected, and $EE2_{High}$-infected hepatic tissue using Approach 1 resulted in 1375, 2969, and 2655 DEGs with corresponding human IDs, respectively. Importantly, ∼1100 DEGs were in common for all three groups. As expected, genes governing innate immunity such as *il1b*, *tnfa*, *il11*, *ifng*, and their receptors, antigen presentation (e.g., PSMB8-10), as well as chemokines (CC and CXC) and signaling molecules (e.g., JAK1/STAT1/IRF9/Nf-κB) were significantly induced to similar levels for all three groups in the liver, indicating an overall pro-inflammatory response to infection (Figure S7). Several innate immune genes (*tnf*, *il10*, *il11*, and *ifng*) identified by RNaseq were verified by targeted qPCR analysis (Figure S8).

Splenic transcriptome responses were similar to hepatic responses with 1206, 2064, and 1999 DEGs for the SC-infected, $EE2_{Low}$-infected, and $EE2_{High}$-infected groups, respectively, again demonstrating additional effects of EE2 on gene transcription during infection. Notable exceptions included a

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Environ. Sci. Technol. 2022, 56, 14375−14386

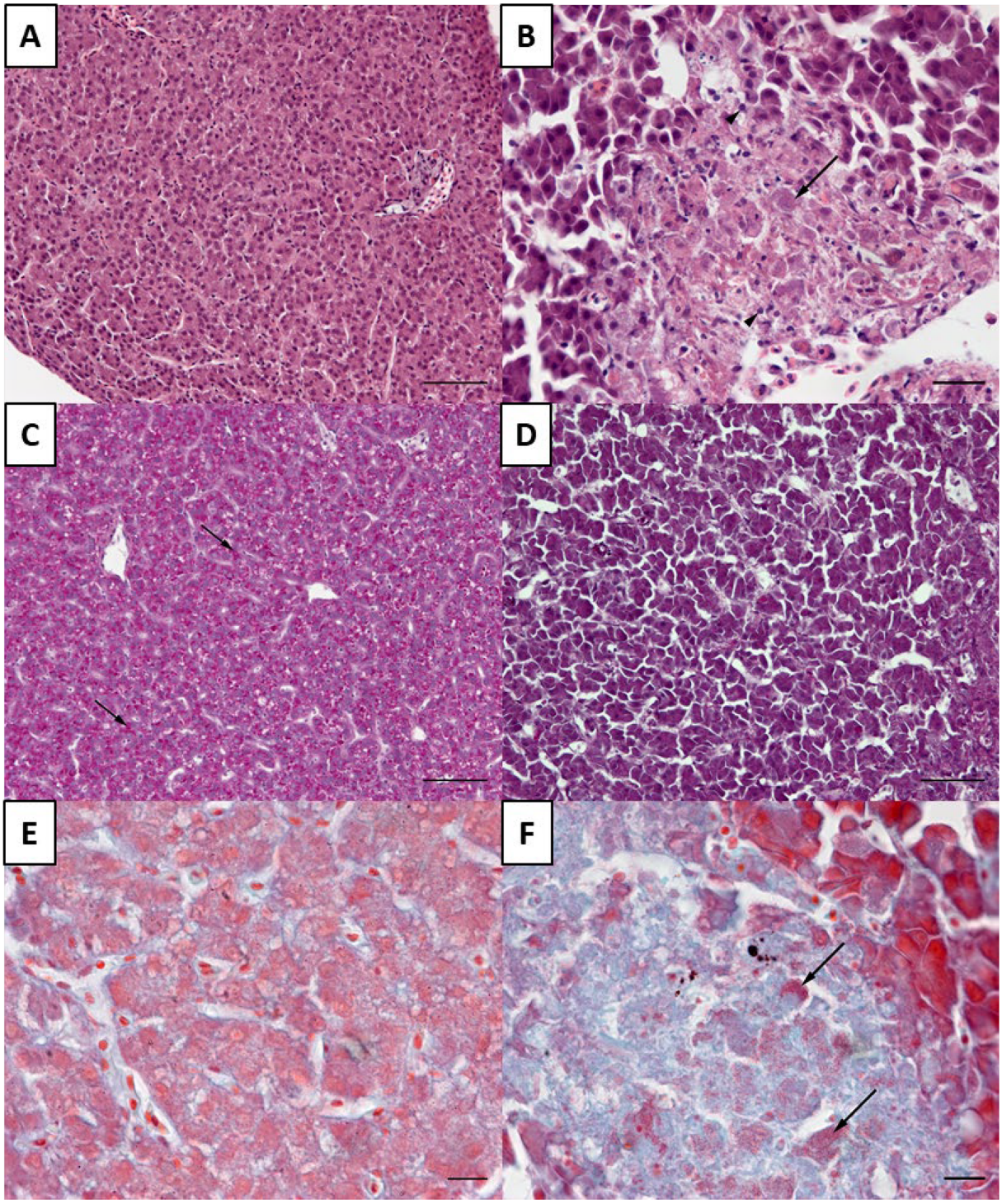

Figure 4. Representative images of largemouth bass liver tissue histology following a 4-week exposure to solvent control (SC), 0.87 ng EE2/L ($EE2_{Low}$), or 9.08 ng EE2/L ($EE2_{High}$) at 4 d post-exposure and 3 d post-infection with *Edwardsiella piscicida* (Ep) or mock injection (Mock). (A) SC + Mock, hematoxylin and eosin (H+E) stain, normal liver (50 μm scale). (B) $EE2_{High}$ + Ep, H+E stain, inflammation as evident by the presence of inflammatory cells indicated by the arrow (macrophage) and arrowheads (lymphocytes) (20 μm scale). (C) $EE2_{Low}$ + Mock, periodic acid−Schiff (PAS) stain, presence of glycogen stores indicated by the arrows (50 μm scale). (D) $EE2_{High}$ + Ep, PAS stain, absence of glycogen stores (50 μm scale). (E) SC + Mock, Gram stain, absence of bacteria (50 μm scale). (F) $EE2_{High}$ + Ep, Gram stain, presence of Gram-negative bacteria within apparent macrophages indicated by the arrows (50 μm scale).

lack of differential expression for *il10* and induction of chemokines in the spleen. IL10 is an anti-inflammatory cytokine;[64] thus, expression in the liver is likely involved in controlling inflammation mediated by *E. piscicida*. Overall, both hepatic and splenic responses with and without EE2 were consistent with Th1 cell immunity—typical for combating intracellular pathogens such as *Edwardsiella*.[65] The spleen also had 4 unique molecular processes that were all associated with acyl-CoA ligase including long-chain fatty acid-CoA ligase activity, and only 1 pathway, phagosome, was unique to the

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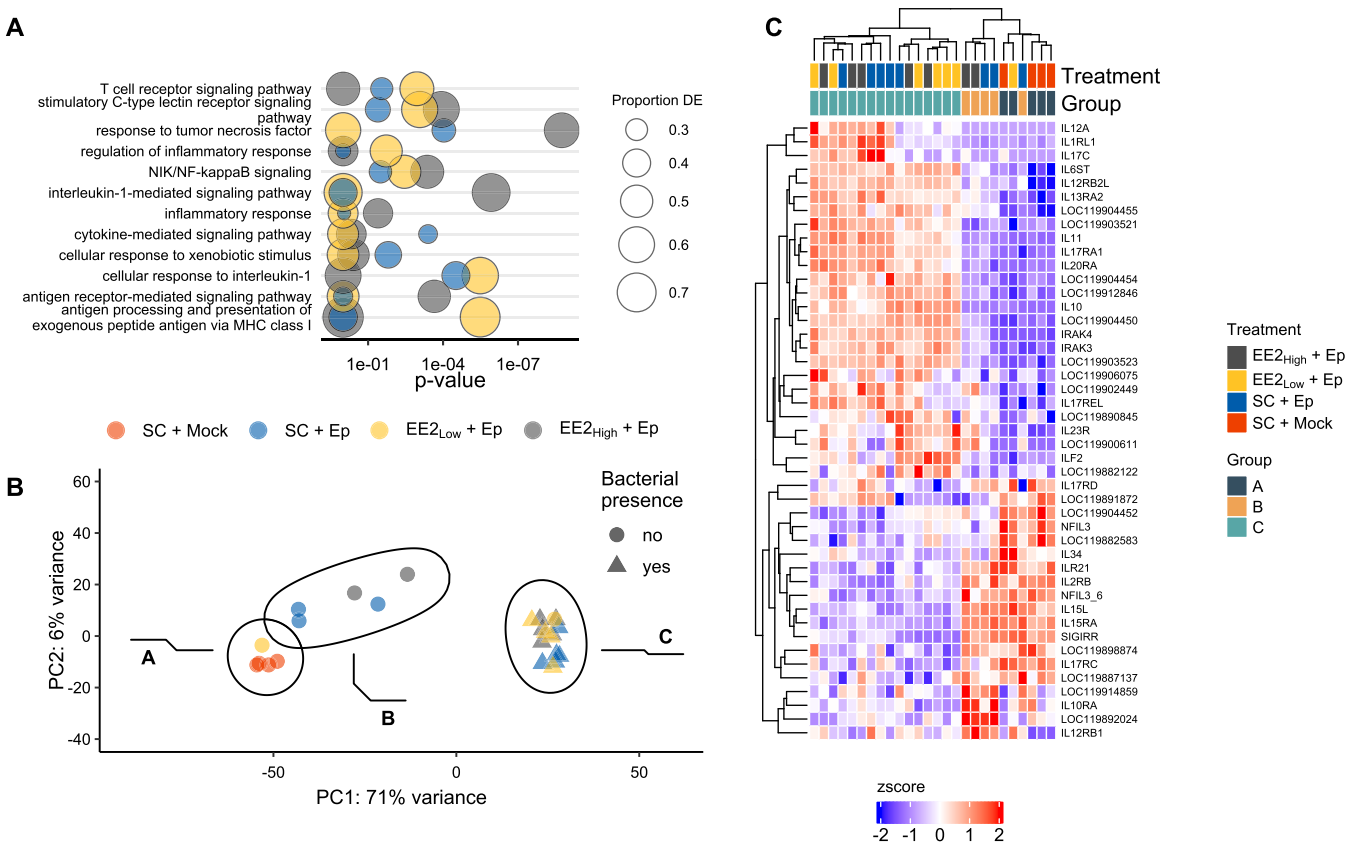

Figure 5. Transcriptomic changes in largemouth bass liver tissue following a 4-week exposure to solvent control (SC), 0.87 ng EE2/L ($EE2_{Low}$), or 9.08 ng EE2/L ($EE2_{High}$) at 3 d post-infection with *Edwardsiella piscicida* (Ep) or mock injection (Mock). (A) Enriched GO biological processes related to immune function that were altered in fish infected with Ep. Proportion DE represents the ratio of genes differentially expressed (DE) compared to all genes in the pathway. Treatment groups are shown by color; figure legend is shared with panel B. (B) Principal component analysis of gene expression patterns, labeled by treatment group (marker color) and bacterial presence (marker shape). Presence or absence of Gram-negative bacteria was identified via histology. Overall transcriptomic patterns were correlated with histological presence or absence of Gram-negative bacteria and not correlated with previous EE2 exposure concentration. Treatment figure legend is shared with panel A. (C) Cluster analysis of expression of interleukin transcripts. Dendrogram of expression correlations between samples (top) was assessed using a Pearson correlation.

splenic $EE2_{High}$-infected response—indicating that this higher concentration of EE2 may enhance phagocytic activity for bacterial infection.

Numerous GO biological processes related to immune function were enriched in the liver of all *E. piscicida*-infected treatments (Figure 5A). For the $EE2_{High}$-infected group 13 unique biological processes were identified, including IL1-mediated signaling, antigen receptor-mediated signaling, regulation of RNA stability, and fatty acid oxidation. Furthermore, immune-related pathways, including response to TNF, NIK/Nf-κB signaling, inflammatory response, and IL1-mediated signaling, were most significantly enriched in the $EE2_{High}$-infected group compared to other infected treatments (Figure 5A). Fish and mammalian studies have shown that estrogens can act through interaction with the pro-inflammatory cytokine (e.g., TNF) and regulatory factor (e.g., Nf-κB) pathways to induce immunomodulation, in addition to acting through estrogen receptor pathways.[66] KEGG pathway analysis identified 11 unique pathways for the liver in the $EE2_{High}$-infected group, 7 of which corresponded to metabolic pathways including glycolysis/gluconeogenesis and Butanoate metabolism (short-chain fatty acids) where both down- and up-regulation of DEGs were observed (Table S4). These data could indicate that reduced mortality in the $EE2_{High}$-infected group could be associated with innate immune pathways and increased metabolic processes—particularly those associated with oxidation of fatty acids as an energy source to contend with infection.

EE2-mediated immunomodulation, resulting in positive outcomes for infection, has also been observed in previous studies in rainbow trout infected with the myxozoan parasite *Tetracapsuloides bryosalmonae*.[28,30] Co-exposure of infected fish with 5.5 ng/L EE2 reduced both parasite intensity and pathological alterations in the posterior kidney. Reduced infection intensity was correlated with a more pronounced induction of pro-inflammatory genes and greater investment in infection resolution. In the present study, immune responses to the disease challenge, as measured by RNaseq, were much stronger than that observed in the previous rainbow trout studies[28,30] and likely obscured our ability to observe the more subtle effects of EE2 treatment at the molecular level after infection. However, the response observed in EE2-exposed mock-injected fish is consistent with the previous observation of estrogen stimulation of autoimmune processes.[67−69] Fish typically do not eat during the first few days of acute infection, and therefore, induction of innate effectors requires a significant metabolic shift to fuel the immune response. Stimulation of metabolic activity elicited by previous $EE2_{High}$ (Figure 1E) exposure, including depleted glycogen and enrichment of metabolic pathways, indicates that these fish may have experienced a metabolic shift similar to an immune response[52]

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Environ. Sci. Technol. 2022, 56, 14375−14386

prior to the infection challenge. The preemptive increase in metabolic activity induced in fish in the $EE2_{High}$ group may have primed some individuals to survive the bacterial challenge by early activation of shared metabolic pathways and increased translational processes. Additionally, increased HSI has also been linked to transient, beneficial metabolic and performance outcomes for seabream when faced with "winter disease". Although increased metabolic activity and glycogen usage may elicit transient positive outcomes,[52] they are likely not sustainable and can exacerbate organisms' metabolic stress long-term.[53]

Disease Progression-Based Comparisons of Gene Expression Patterns. Alternatively, differential expression in fish liver was evaluated at different stages of disease progression (Approach 2), based on PCA analysis that showed expression patterns correlated with histological presence or absence of Gram-negative bacteria and immune response (Figures 4E, 4F, and 5B). Bacteria were not identified in fish displaying a moderate immune response (Group B) similar to mock-injected fish (Group A). However, bacteria were identified in all fish showing a more robust immune response (Group C), indicating that Group B could represent fish that have either cleared infection or are in the process of clearing infection and are likely to survive. Group C fish showed a robust immune response compared to Group A fish, with 9423 total hepatic DEGs for which 4508 could be assigned human IDs. Group B fish also showed a robust immune response in the liver, with 2291 DEGs corresponding to 944 DEGs with human IDs. Both Groups A and B contained DEG alterations in interferon, interleukin, and tumor necrosis factor transcripts as expected during innate immune stimulation. However, a dendrogram assessing the expression of all interleukin-related gene expressions in LMB identified a distinct expression profile for fish in Group C that was unrelated to pre-exposures to EE2 (Figure 5C), implicating a greater degree of response compared to Group B and implying higher levels of infection at the time of sampling.

Those fish with identified bacteria (Group C) contained DEGs associated with 12 significantly enriched biological processes, including response to TNF and IL1B, antigen presentation, and fatty acid and amino acid metabolism (Table S5). Group B DEGs were largely limited to biological processes (5 total) associated with host defense, including complement activation and humoral immunity. For the complement cascade, this includes the induction of C5 through C9—which are responsible for the membrane attack complex required for lysing pathogenic bacteria. In addition, upon FDR correction, Group B had only one unique KEGG pathway, Cell Cycle (KEGG 04110, $p = 5.1 \times 10^{-4}$). In contrast, Group C had 16 unique pathways including >500 DEGs associated with metabolic pathways (KEGG 01100, $p = 5.96 \times 10^{-4}$), PPAR-signaling (lipid metabolism) as well as immune pathways including cytokine−cytokine receptor interactions (KEGG 04060, $p = 3.7 \times 10^{-4}$). Complement and coagulation cascades was the only common pathway, but perturbation analysis indicated both groups would have impaired complement activation, indicating complement may not be a critical factor for the apparent protective response found in the $EE2_{High}$ group.

Lastly, our analysis of the splenic response for Groups B (1199 DEGs with human IDs) and C (4767 DEGs with human IDs) was complicated by the fact that Group B had over 56 unique biological process, whereas Group C had 3 unique processes, including antigen processing and presentation for the MHC class I pathway ($p = 1.5 \times 10^{-5}$). Most of the processes unique to Group B were involved in cell division and DNA replication. Pathway analysis did not offer any additional insight as to potential protective mechanisms, but DNA replication, excision repair, and other cellular pathways found for Group B could be involved in cell division during growth as well as repair of tissues damaged by *E. piscicida*.

Overall, our study demonstrated measurable effects of 9.08 ng/L EE2 in juvenile largemouth bass at multiple levels of biological organization. By using a novel bacterial challenge model that incorporated the emerging fish pathogen *E. piscicida*, we showed that previous exposure to $EE2_{High}$ significantly increased survivorship. Our results indicate stimulation of metabolic pathways evoked by EE2 improved infection outcomes by providing metabolic resources to augment host defenses against microbial infection. At 3 d post-infection, the overwhelming nature of the transcriptomic immune response to this highly virulent pathogen likely prevented detection of subtle differences that could lead to protection. However, pathways related to innate immunity were more significantly enriched (number of DEGs) in $EE2_{High}$-infected fish compared to the other two groups and, therefore, could have played a role in the outcomes observed.

CONCLUSIONS

Endocrine disrupting chemicals have been implicated as causal factors for disease outbreaks and related centrarchid mortality events. In this study we established a reproducible disease challenge model in LMB and assessed the interaction of prior exposure to a model estrogen on disease susceptibility. This work presents a reproducible bacterial infection model for LMB that can be used to assess potential effects of environmental stressors on disease susceptibility. The finding that exposure to EE2 at 9.08 ng/L resulted in enhanced disease resistance was not anticipated given the known reproductive and developmental adverse effects of exposure to EEDCs. Our histological and molecular observations at a critical point in the innate immune response to *E. piscicida* infection post-EE2 exposure indicate that alterations in metabolism coupled with immune function (immunometabolism) likely explain the differential survival observed in this study but also indicate a stress response resulting in depletion of energy stores. Infection of LMB with *E. piscicida* resulted in differential regulation of thousands of genes. A more focused approach in future studies is warranted that includes time course analysis to capture temporal kinetics of both metabolic and immune genes and pathways with particular attention on regulatory controls as they relate to adverse immune outcomes from EEDC exposure. Future studies could also investigate responses to complex environmental mixtures, as mixture effects may differ from responses to the model estrogen tested in the present study. This study demonstrates the potential role EEDCs can play in modulating disease susceptibility in a fish species that is ecologically and economically important in North American watersheds.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c02250.

Text S1: Largemouth bass (LMB; *Micropterus salmoides*) animal care and feeding. Text S2: Chemical analysis. Text S3: Histopathology host response scoring criteria. Text S4: RNA extraction, qPCR, and RNaseq. Figure S1: (A)

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Environ. Sci. Technol. 2022, 56, 14375−14386

Experimental design. (B) Schematic of tank randomization. Figure S2. Establishment of *Edwardsiella piscicida* (Ep) infection model for largemouth bass (LMB). Figure S3. Targeted qPCR measurements of gene expression following a 4-week exposure to solvent control (SC), 0.87 ng EE2/L ($EE2_{Low}$), or 9.08 ng EE2/L ($EE2_{High}$) at 0 d post-exposure. (A) largemouth bass *estrogen receptor 1* (*esr1*, GenBank XM_038726319.1) in liver, (B) largemouth bass *vitellogenin-like* (*vtgl*, LOC119900642, GenBank XM_038715782.1) in liver, (C) *esr1* in spleen, and (D) *vtgl* in spleen immediately following estrogen exposures. Figure S4. Changes in gene expression in biomarkers of estrogen exposure measured by RNaseq following a 4-week exposure to solvent control (SC), 0.87 ng EE2/L ($EE2_{Low}$), or 9.08 ng EE2/L ($EE2_{High}$) at 4 d post-exposure. Figure S5. Differentially expressed genes responsive to $EE2_{High}$ exposure in the complement and coagulation cascades (KEGG: 04610) following a 4-week exposure to 9.08 ng EE2/L ($EE2_{High}$) relative to solvent control (SC) in mock-injected fish at 4 d post-exposure. (A) Differentially expressed complement pathway genes from the $EE2_{High}$ treatment relative to solvent control (SC). (B) Upregulation of ribosomal genes in $EE2_{High}$-exposed fish. Figure S6. (A) Example of fibrosis. Area of minimal fibrosis in a low EE2-exposed, *Edwardsiella piscicida*-challenged fish showing fibrocytes (arrowheads). (B) Example of necrosis. Area of necrosis in a low EE2-exposed, *Edwardsiella piscicida*-challenged fish showing karyorrhexis (arrowhead) and cellular debris (asterisks). Figure S7. Post-infection RNaseq measurements of liver gene expression following a 4-week exposure to solvent control (SC), 0.87 ng EE2/L ($EE2_{Low}$), or 9.08 ng EE2/L ($EE2_{High}$) at 4 d post-exposure and 3 d post-injection in mock-injected (mock) and *E. piscicida*-infected (Ep) largemouth bass of (A) infg-like, (B) il10, (C) il11, (D) il1b, and (E) tnf. Figure S8. Post-infection targeted qPCR measurements of liver gene expression following a 4-week exposure to solvent control (SC), 0.87 ng EE2/L ($EE2_{Low}$), or 9.08 ng EE2/L ($EE2_{High}$) at 4 d post-exposure and 3 d post-injection in mock-injected (mock) and *Edwardsiella piscicida*-infected (Ep) largemouth bass (*Micropterus salmoides*) of (A) infg-like, (B) tnf, (C) il1b, (D) il10, (E) il11a, and (F) il11b. Table S1. Primer and probe sequences for qPCR analysis of gene expression and 16S sequencing for bacteria identification. Table S2. EE2 exposure fish metrics. Table S3. Biological processes (BP) and KEGG pathways (KP) altered in the spleen following EE2 exposure. Table S4. KEGG pathways that were significantly enriched in liver only in fish previously exposed to $EE2_{High}$ and after *E. piscicida* infection. Table S5. Biological processes enriched in Group C-infected fish. (PDF)

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†The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. J.K.L. and J.B.G. contributed equally as colead authors.

Notes

The authors declare no competing financial interest.

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