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Transcriptional changes in innate immunity genes in head kidneys from *Aeromonas salmonicida*-challenged rainbow trout fed a mixture of polycyclic aromatic hydrocarbons

Lawrence R. Curtis[a], Claudia F. Bravo[a], Christopher J. Bayne[b], Fred Tilton[a], Mary R. Arkoosh[c], Elisabetta Lambertini[d], Frank J. Loge[d], Tracy K. Collier[e], James P. Meador[e], Susan C. Tilton[a,*]

[a] *Department of Environmental and Molecular Toxicology, Oregon State University, ALS 1007, Corvallis, OR 97331, USA*
[b] *Department of Integrative and Comparative Biology, Oregon State University, Cordley 3029, Corvallis, OR 97331, USA*
[c] *Environmental & Fisheries Sciences Division, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 2030 South Marine Science Drive, Newport, OR 97365, USA*
[d] *Department of Civil and Environmental Engineering, University of California Davis, 1 Shields Ave, Davis, CA 9561, USA*
[e] *Environmental & Fisheries Sciences Division, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 2727 Montlake Boulevard East, Seattle, WA 98112, USA*

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ABSTRACT

We previously observed that exposure to a complex mixture of high molecular weight polycyclic aromatic hydrocarbons (PAHs) increased sensitivity of rainbow trout (*Oncorhynchus mykiss*) to subsequent challenge with *Aeromonas salmonicida,* the causative agent of furunculosis. In this study, we evaluate potential mechanisms associated with disease susceptibility from combined environmental factors of dietary PAH exposure and pathogen challenge. Rainbow trout were fed a mixture of ten high molecular weight PAHs at an environmentally relevant concentration (7.82 μg PAH mixture/g fish/day) or control diet for 50 days. After 50 days of PAH exposure, fish were challenged with either *Aeromonas salmonicida* at a lethal concentration 30 (LC_{30}) or growth media without the pathogen (mock challenge). Head kidneys were collected 2, 4, 10 and 20 days after challenge and gene expression ($q < 0.05$) was evaluated among treatments. In animals fed the PAH contaminated diet, we observed down-regulation of expression for innate immune system genes in pathways ($p < 0.05$) for the terminal steps of the complement cascade (complement component C6) and other bacteriolytic processes (lysozyme type II) potentially underlying increased disease susceptibility after pathogen challenge. Increased expression of genes associated with hemorrhage/tissue remodeling/inflammation pathways ($p < 0.05$) was likely related to more severe head kidney damage due to infection in PAH-fed compared to control-fed fish. This study is the first to evaluate transcriptional signatures associated with the impact of chronic exposure to an environmentally relevant mixture of PAHs in disease susceptibility and immunity.

1. Introduction

Exposures of salmonid populations to degraded ecosystems often, if not typically, involve multiple stressors. The interaction of disease and contaminant exposure is of relevance to persistence of salmonid populations (Arkoosh et al., 2001). We previously reported an increase susceptibility for disease in rainbow trout (*Oncorhynchus mykiss*) upon exposure to a bacterial pathogen, *Aeromonas salmonicida,* after being fed a complex mixture of high molecular weight (HMW) polycyclic aromatic hydrocarbons (PAHs) (Bravo et al., 2011). The current study focuses on potential mechanisms for disease susceptibility by PAHs by global gene expression in rainbow trout after exposure to both the HMW PAH mixture and to the *A. salmonicida* pathogen. *A. salmonicida* produces the disease furunculosis in susceptible salmonid hosts. Signs of this disease are hemorrhage at the base of the fins, in the oral cavity and viscera (Cipriano and Bullock, 2001).

The primary source of environmental PAHs is incomplete combustion of carbonaceous materials: in the United State combustion of fuels and waste constitute almost 70% of atmospheric PAH releases (Zhang and Tao, 2009). In aquatic ecosystems, transport of these residues and fossil fuel spills are the major sources of PAH contamination. HMW PAHs strongly associate with sediments and particles in suspension in these systems (Collier et al., 2014). PAH concentrations in benthic invertebrates from salmonid stomachs (Varanasi et al., 1993) indicate a

* Corresponding author.
E-mail address: susan.tilton@oregonstate.edu (S.C. Tilton).

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key role for sediment contamination in their trophic transfer.

In fish, PAH exposures result in a broad spectrum of toxicities; including hepatic lesions and neoplasms, reproductive dysfunction, and immunotoxicity. Embryonic development appears to be a sensitive window for PAH exposure in fish with heart defects occurring after early life-stage exposure to PAHs (Collier et al., 2014; Incardona et al., 2004). Karrow et al. (1999) show PAH mixtures impair the innate immune system, particularly the oxidative burst in rainbow trout leukocytes. PAHs have been identified as immunotoxic agents in rodent models through suppression of humoral and cell mediated immunity, impaired T cell cytokine production, impaired B cell antibody production, and inhibition of functional differentiation and maturation of monocyte-derived dendritic cells (Kawabata and White, 1987; Laupeze et al., 2002). In fish models, the impact of oil exposure on immunological function has been reported in a variety of fish models resulting in increased mortality after pathogen infection; although the mechanisms associated with altered immunity are largely unknown (Bravo et al., 2011; Dussauze et al., 2015a). The work herein more broadly assesses changes in innate immunity gene networks.

Depending on life history type, residence time in the lower reaches of rivers, including estuaries, varies substantially between different species and stocks of emigrating salmonid smolts (Healey, 1991; Thorpe, 1994). Acoustic tagging and tracking of steelhead smolts demonstrate rapid emigration in the Columbia River system (Harnish et al., 2012). Most of these fish move from river kilometer 153 into the ocean in less than a week. Subyearling (ocean-type) Chinook salmon exhibit another extreme of behavior within the Columbia River system. Smolts of this stock may reside in the lower river/estuary for several months to feed and grow before entering the ocean (Johnson et al., 2013; Healey, 1991). Lower reaches of rivers that pass through urban and industrialized areas often contain sediments with substantial contaminant concentrations. PAHs are a prototypical class of such contaminants. The work reported here employed juvenile rainbow trout as a surrogate for Chinook salmon, a PAH mixture representative of the invertebrate contaminant profile in an urban reach of a Pacific Northwest river and an exposure period relevant to emigrating ocean-type Chinook salmon (Meador et al., 2006; Varanasi et al., 1993).

This study applies oligonucleotide microarray technology (Tilton et al., 2005; Gerwick et al., 2007) to assess possible mechanisms for the observed increase in susceptibility of juvenile rainbow trout to *A. salmonicida* after dietary PAH exposure (Bravo et al., 2011). The head kidney of rainbow trout is the principal organ of the innate immune system for this species (Uribe et al., 2011) and thus critical to defense against a bacterial pathogen challenge. Therefore, transcriptional changes were evaluated in head kidneys of fish after exposure to multiple stressors of a chemical PAH mixture and *A. salmonicida* bacterial challenge.

2. Materials and methods

2.1. Animals

Subyearling juvenile rainbow trout (7–10 g wet weight) were obtained from the Sinnhuber Aquatic Research Laboratory at Oregon State University (Corvallis, OR) and transported to the Northwest Fisheries Science Center's Fish Disease Laboratory at the Hatfield Marine Science Center (Newport, OR) as described previously (Bravo et al., 2011). Water temperature and pH were maintained at 10 °C and pH 7.0, respectively. Fish were acclimated for 28 d and fed ad libitum a 1.5 mm pellet (Trout diet No. 4, Rangen) daily in the morning. The pellet contained 11% crude fat, 45% crude protein, 9–10% water, 30% fish meal protein, and 1% phosphorus. After the acclimation period, fish (10–15 g wet wt) were randomly distributed between the circular fiberglass experimental tanks (diameter 90 cm) for a total of 90 fish per tank. The experimental tanks contained 400 L of fresh water maintained at 7–8 °C. The flow-through fresh water system provided 3.8 L per min of charcoal-filtered water to each tank.

2.2. Experimental design

Fish were fed a diet that contained a PAH mixture (400 ppm dry weight in fish pellets, 7.82 μg PAH/g fish/day) constituted with pyrene (20%), fluoranthene (19.6%), benzo [b] fluoranthene (18%), chrysene (13.4%), benz [a] anthracene (7.3%), benzo [k] fluoranthene (6.1%), benzo [a] pyrene (6.0%), dibenzo [g, h, i] perylene (5.0%), indeno (1, 2, 3, c, d) pyrene, and dibenz (a, h) anthracene (1.1%) as previously described (Bravo et al., 2011). The PAH concentrations approximated individual PAHs previously measured in stomach contents of field-collected fish (Bravo et al., 2011; Varanasi et al., 1993). Pellets were immersed in a dichloromethane (DCM) HPLC grade stock solution that contained 10 high molecular weight PAHs (27 mg/ml). The control diet consisted of pellets that were treated with DCM vehicle control. Concentrations of the PAHs were confirmed in fish pellets by GC/MS and were equivalent to 390 ppm dry weight (Bravo et al., 2011). Fish were fed 2% body weight daily (six times a week) with experimental or control diets for a total of 50 days. Concentrations of fluorescent aromatic compounds in bile were analyzed by HPLC with fluorescent detection compared to standards for benzo[a]pyrene and phenanthrene confirming consumption of the fish pellets by fish (Bravo et al., 2011). After 50 days, feeding was stopped and the fish were challenged with waterborne *A. salmonicida* (5.0×10^5 cfu/ml) at the lethal concentration 30 (LC30) or media with no bacteria (mock challenge) for 24 h as described (Bravo et al., 2011). Trout exposed to a mixture of high molecular PAHs in the diet were more susceptible to infection by A. salmonicida and resulted in 38% mortality compared to animals on control diet at 28% mortality (Bravo et al., 2011).

2.3. Sampling

There were four different treatment combinations based on PAH exposure and pathogen challenge: (1) control-fed, mock-challenged fish; (2) control-fed, *A. salmonicida*-challenged fish; (3) PAH-fed, mock-challenged fish; (4) PAH-fed, *A. salmonicida*-challenged fish. At 2, 4, 10, and 20 days after pathogen or mock challenge, fish (N = 6 replicates per condition) were euthanized with 200 mg tricaine methanesulfonate/L water, the head kidneys (0.5–1.0 g) were excised with a scalpel, then rinsed in ice-cold potassium chloride solution (1.15 M). Total RNA was extracted with Trizol solution as directed by Invitrogen (Carlsbad, CA). RNA concentration, purity and integrity were measured with a Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Based on these analyses, in which quality criteria were evaluated with RNA integrity numbers (RIN) between 9–10, four samples per condition were selected for microarray analysis. For use as a reference in the array studies, a pooled RNA sample was made by combining RNA extracts from 21 kidneys. The isolated RNA was aliquoted and stored at −80 °C until use. Total RNA (25 μg) was reverse transcribed with 200 units of Superscript III (Invitrogen) with primer tails that complemented Cy3 or Cy5 labeled flurophores (Genisphere 900™). In each reaction, 1 μL of Alien oligos® (Stratagene, La Jolla, CA) was used as a positive control to facilitate optimization of scanning parameters.

2.4. Microarray analysis and bioinformatics

Microarray analysis was performed using a custom rainbow trout 70-mer oligonucleotide array (Tilton et al., 2005). A standard reference design with dye swapping was utilized for individual fish. For each slide, 6.5 μL of sample cDNA labeled for CY5 was mixed with 6.5 μL of reference (pooled) cDNA labeled for CY3 and 17 μL of 2X formamide buffer for a total volume of 30 μL. There were 32 slides per time point. A Genisphere 900™ (Hatfield, PA) protocol was followed with modifications (Gerwick et al., 2007). To protect dyes from fading, slides were dipped for 5 s in Dye Saver™ 2 anti-fade coating (Genisphere 900™

Hatfield, PA) followed by centrifugation. Slides were air-dried for 15 min in the dark and immediately scanned. Slide images were acquired with an Axon 4200 A scanner (Molecular Devices, Union City, CA) at excitation wavelengths of 532 nm for CY3 and 635 nm for CY5. The photomultiplier tube settings and percent power of the laser for each fluorophore were set based on intensity of Alien control spots, so that the Alien controls had an overall ratio of means of the CY5 and CY3 signals close to one (0.995–1.290). This corrected for any differences in intensity between arrays and increased array comparability. The scanned array files were examined individually for spots that exceeded the acquisition maximum ("saturated"), yielded weak signals or were compromised by scratches or extraneous matter and these were flagged as "bad spots".

Raw intensity data were background subtracted and normalized by LOWESS. Complete results in the form of supplemental raw data files are available online through GEO ID: GSE87920. Differentially expressed transcripts were identified by ANOVA with FDR correction ($q < 0.05$) using GeneSpring v.11. These statistical criteria are have been determined to be robust for detection of significant gene expression on the trout oligonucleotide platform based on technical confirmation by quantitative qPCR (Tilton et al., 2005, 2006, 2007; Gerwick et al., 2007). Oligonucleotide sequences were queried against the NCBI database (BLAST) to acquire current annotations in rainbow trout or closely related species (e.g. *Salmo salar* or *Danio rerio*) for functional analysis. Functional enrichment statistics were determined using the DAVID functional annotation tool (Huang da et al., 2009), which utilizes the Fisher's Exact test to measure gene enrichment in biological process Gene Ontology (GO) category terms. All enrichment statistics were calculated against the custom trout array platform as background. Unsupervised hierarchical clustering of microarray data was performed using the Euclidean distance metric and centroid linkage clustering to group treatments and gene expression patterns by similarity. The clustering algorithms and centroid calculations were performed with Multi-Experiment Viewer (Saeed et al., 2003) software based on log2 transformed differential expression ratios.

3. Results

Significant transcriptional differences were observed in trout head kidneys after *A. salmonicida* challenge (compared to mock-challenged trout) in animals fed PAH- or control-diets for 50 days prior to infection (Fig. 1) indicating the consequences of chronic PAH exposure on the response to infection. The gene expression changes after pathogen challenge in control-diet fish were 2, 49, 1 and 1 differentially expressed genes on days 2, 4, 10 and 20 after challenge. Gene expression changes after pathogen challenge in PAH-diet fish were markedly different with 79, 35, 45 and 2 significant genes on days 2, 4, 10 and 20 after challenge indicating an earlier and stronger response to pathogens after PAH exposure. The differences in gene expression after challenge for animals fed control and PAH diets were examined in detail. Differences in expression were observed for two genes assayed from control-fed fish challenged with pathogen compared with control mock-challenged fish after two days (Fig. 1). Serum amyloid A exhibited a 17.1-fold induction in PAH-treated fish compared to control fish and a reduction in myeloperoxidase (−9.0-fold). The number of genes that were differentially expressed increased sharply to 49 after 4 days after pathogen challenge (Fig. 1, see Supplemental Table). Transcript levels decreased for 10 of the 49 genes. Many of the genes elevated at day 4 were associated with the innate immune response (e.g. chemotaxin, up 4.4 fold; complement component C3-4, up 14.5-fold; complement receptor-like protein, up 16-fold; GATA2 binding protein 2, up 24.9-fold; lysozyme-like 2 precursor, up 3.4-fold). Expression of genes associated with blood clotting (e.g. chondromodulin 1 up 4.4-fold; fibrinogen gamma chain, up 12.1-fold; plasminogen precursor, up 24-fold), tissue remodeling (e.g. endothelial collagen, up 17.6-fold) and apoptosis (e.g. caspase 10, up 9.7-fold) also increased.

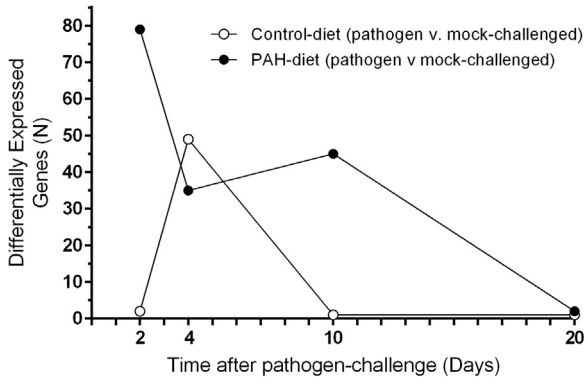

Fig. 1. Time-course for differentially expressed genes ($q < 0.05$) in rainbow trout head kidneys on days 2, 4, 10 or 20 after challenge with *A. salmonicida* (pathogen). Fish were fed a control diet or one that contained a mixture of 10 polycyclic aromatic hydrocarbons (PAH) for 50 days. On day 51 fish received waterborne exposures (challenges) with *A. salmonicida* or growth media without bacteria (mock). Differentially expressed genes were compared between pathogen and mock-challenged animals at each time point.

Overall, there were 79 changed gene features (33 up, 46 down) after 2 days in *A. salmonicida*-challenged compared to mock-challenged PAH-fed fish (Fig. 1, Supplemental Table). This declined to 35 (11 up, 24 down) after 4 days, slightly increased to 45 after 10 days, and markedly declined to 2 after 20 days. In order to focus on genes that were significantly different between PAH and control-fed diets after pathogen challenge, we directly compared these two groups for differential expression ($p < 0.05$). Microarray analyses that compared PAH-fed fish and control-fed fish at 2, 4, 10 and 20 days after pathogen challenge detected 126, 30, 49 and 3 differentially expressed genes, respectively (Fig. 2, Supplemental Table). Overall pathways significantly ($p < 0.05$) inhibited across the time course in pathogen-challenged fish fed PAH-diet include complement activation (GO:0006956), humoral immune response (GO:0006959) and defense response to bacterium (GO:0042742). Pathways significant for up-regulated genes in pathogen-challenged fish fed PAH diet include blood coagulation (GO:0007596), negative regulation of wound healing (GO:0061045), negative regulation of immune effector process (GO:0002698) and negative regulation of defense response (GO:0031348). Most transcriptional changes after pathogen challenge in PAH-fed animals were measured on day 2 prior to any mortality, which occurred after Day 4 (Bravo et al., 2011).

The changes on day 2 were evaluated in more detail and the functions of differentially expressed genes were organized in four general categories: (1) immune response-related (2) hemorrhage/inflammation/tissue remodeling, (3) lipid metabolism, and (4) housekeeping (Table 1). At 2 days after pathogen challenge there were substantial changes in mRNAs of 23 genes important in the immune response (Table 1_Immune Response), including genes of the complement cascade, host defense, T-cell mediated immune response and inflammation. Further, at 2 days after pathogen challenge there was substantial evidence for up-regulation of genes involved with coagulation: thrombin, hyaluronan binding protein 2, alpha and gamma chains of fibrinogen (Table 1_Hemorrhage, Inflammation and Tissue Remodeling). There were also changes that suggested decreased clot dissolution (plasminogen precursor down-regulation), and increased extracellular matrix remodeling (FKBP10 and tumor necrosis factor receptor associated factor 2 up-regulation). Up-regulation of hemoglobin alpha chain, haptoglobin 1 and ferritin suggested a response to hemolysis. Up-regulation of prostaglandin D synthase and down-regulation of angiotensin converting enzyme were consistent with vasodilation in head kidney, while up-regulation of cytochrome P4501A3, and down-regulation of peroxiredoxin 5 and superoxide dismutase suggested oxidative stress. There was up-regulation of head kidney genes associated with fatty acid oxidation, the estrogen synthesis pathway, export

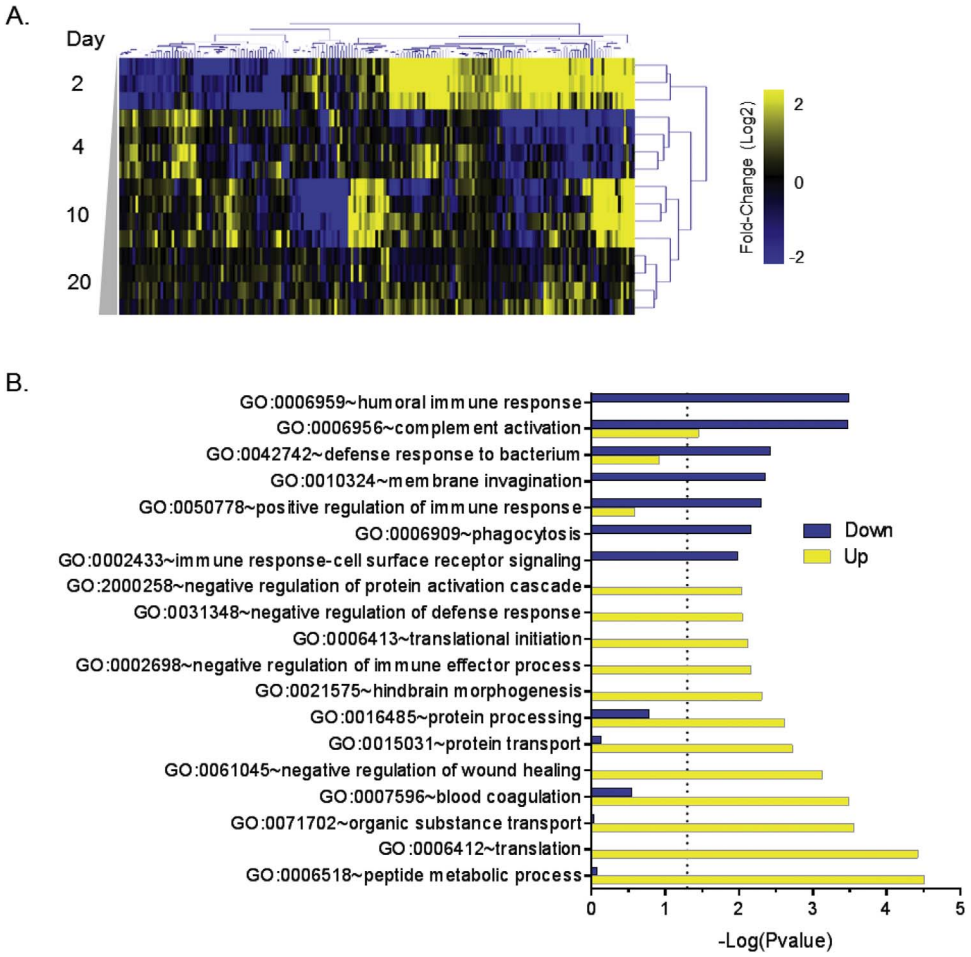

Fig. 2. Differentially expressed genes ($q < 0.05$) in PAH-fed trout compared to control-fed trout on days 2, 4, 10 and 20 after challenge with *A. salmonicida* (pathogen). (A) Bidirectional hierarchical clustering by Euclidean distance of differentially expressed genes. Heatmap reflects fold-change (Log2) in PAH-fed fish compared to control-fed fish. Yellow, blue and black coloring represents up-regulated, down-regulated and unchanged genes, respectively. (B) Functional enrichment of genes significantly ($q < 0.05$) increased (yellow bars) or decreased (blue bars) after pathogen challenge in trout fed a PAH-diet compared to a control-diet. The dashed line indicates the threshold for significance ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of cholesterol and perhaps other sterols, vitamin A storage, and oocyte maturation (Table 1_Lipid Metabolism and Transport).

4. Discussion

This study focused on differences in gene expression in head kidney cells of PAH-fed compared to control-fed rainbow trout after challenge with *A. salmonicida*. The work that provided phenotypic anchoring for the present study focused on the potential for PAH-increased disease susceptibility (Bravo et al., 2011). Previous work showed this PAH mixture increased the lethality of a waterborne dose of this bacterium (Bravo et al., 2011). These results are supported by other recent studies that have identified either increased sensitivity to viral challenge or reduced immune response in fish after exposure to heavy oil suggesting a role for PAH contaminants in reducing pathogen resistance and increasing disease susceptibility (Dussauze et al., 2015a, 2015b; Kim et al., 2014; McNeill et al., 2012; Nakayama et al., 2008). Increased mortality was observed in sea bass exposed to crude oil for 48 h and then challenged with Viral Nervous Necrosis Virus (VNNV) and in rockfish exposed to heavy crude oil for 4 days then challenged with Streptococcus iniae (Dussauze et al., 2015a; Kim et al., 2014). Further, evidence of reduced immune response after PAH exposure has been reported with down regulation of immune system genes in kidneys of flounder exposed to heavy oil (Nakayama et al., 2008).

Potential mechanisms associated with reduced pathogen resistance after PAH exposure were explored in this study. The microarray employed for this study was “targeted” in that it was designed with emphasis on immune system and toxicologically relevant genes (Karrow et al., 1999). It included 1672 features for approximately 1400 individual genes. While this limited the depth of pathway analyses, it did allow for focused analysis of the innate immune system in trout. Gene expression was evaluated in fish fed a mixture of PAHs in the diet (7.82 µg PAHs/g fish/day) for 50 days or control diet prior to challenge with *A. Salmonicida*. The absolute and relative concentrations of PAHs used in the present study reflect those detected in the stomach contents of field collected Chinook salmon from contaminated estuaries in Puget Sound, WA and other urban areas in the Pacific Northwest (Bravo et al., 2011; Johnson et al., 2007; Varanasi et al., 1993). The duration of PAH exposure and the life stage of trout at the time of exposure reflect a likely exposure scenario for some stocks of anadromous salmonids (sub-yearling ocean-type Chinook salmon) during emigration to the ocean (Meador et al., 2006; Johnson et al., 2013). Trout fed this diet were observed to have significant induction of CYP1A1 protein and elevated ethoxyresorufin (EROD) activity in liver after 3–50 days on diet and elevated biomarkers of oxidative stress after 14–28 days as measured by DNA strand breaks and lipid oxidation (Bravo et al., 2011). However, CYP1A1 protein levels in trout head kidney were back to normal levels after 14 days on diet suggesting that chronic exposure to PAHs did not result in long-term arylhydrocarbon-mediated signaling in head kidney (Bravo et al., 2011). These data are consistent with the gene expression observed in mock-challenged animals exposed to PAHs in which there was significant elevation of

Table 1
Gene expression in trout head kidney after challenge in with *A. Salmonicida* in fish fed PAHs in diet for 50 days compared fish fed control diet ($q < 0.05$).

Name	Gene or Protein Symbol	Swissprot Accession	Function[1]	Fold Change
Immune Response				
MHC class I antigen	HLA-A	Q7YP10	Cytotoxic T-cell mediated inflammation.	−25.93
Lysozyme C II precursor	LYZ2	P11941	Bacteriolytic enzyme secreted into many body fluids: has important role in host defense.	−9.42
GATA-binding protein 2	Gata2	Q7T3G1	Transcription factor stimulates granulocyte proliferation and differentiation.	−9.27
Complement component C6	C6	Q6H964	Participates in lysis of a target cell as a member of the membrane attack complex [4].	−7.97
Natural killer cell enhancement factor	NKEF	Q9I886	Involved in mucosal anti-bacterial immune response in salmonids [3].	−7.90
C5a receptor	C5AR1	Q6UNA4	Lymphocyte receptor that signals enhanced immune and inflammatory responses.	−7.67
C-C chemokine receptor type 7 precursor	CCR7	UPI000065FD58	Cell receptor that triggers influx of calcium; immune integration.	−7.35
DEAD (Asp-Glu-Ala-Asp) box polypeptide 39b	ddx39ab	Q803W0	T-helper cell mediated inflammation.	−6.73
Chymotrypsinogen 2-like protein	CHTRII	Q4QY78	Precursor of protease with diverse roles including antigen processing.	−6.60
Cytokine receptor common gamma chain	IL2RG	Q9DEQ. 1	Signaling between immunocytes.	−4.79
MHC class I antigen	HLA-A	Q7YP06	Presents antigen peptide fragments to T cells responsible for cell-mediated immune responses.	−3.75
Complement factor Bf-2	BF2	Q9YGE8	Roles in both alternative and classical pathways of complement activation [2].	6.47
C-type mannose-binding lectin	MBL1	Q8JJ68	Carbohydrate binding; putatively opsonization of glycanated particles (e.g. bacteria).	10.05
Rhamnose binding lectin STL2	STL2	Q9IB52	Carbohydrate binding; putatively opsonization of glycanated particles (e.g. bacteria).	12.04
Pentraxin precursor	CRP	P79899	Soluble protein that binds and recruits complement; increases in serum during acute phase response.	12.41
Complement C3-1	C3	P98093	Central player in activation of both classical and alternative complement pathways.	12.96
C1 inhibitor precursor	SERPING1	Q70W32	Inactivates proteases in the C1 complex of the classical pathway of complement; this prevents proteolytic cleavage of later complement components C4 and C2 by C1 and mannose binding lectin.	13.10
C-type MBL-2 protein precursor	MBL2	Q4LAN6	Carbohydrate binding; putatively opsonization of glycanated particles (e.g. bacteria).	15.75
Rhamnose binding lectin STL3	STL3	Q9IB51	Carbohydrate binding; putatively opsonization of glycanated particles (e.g. bacteria).	16.54
Complement factor H1 protein	CFH	Q4QZ18	Regulates alternative complement pathway.	18.25
Complement component C3–4	C3	Q9DDV9	Central player in activation of both classical and alternative complement pathways.	18.47
Complement factor Bf−1	BF1	Q9YGE7	Cleavage yields 2 fragments: both influence B cell activation; one propagates the complement cascade.	20.07
Trout C-polysaccharide binding protein 1	CBLN1	Q9DFE5	Ca-dependent binding affinity for pneumococcal C-polysaccharide; inferred opsonic function.	20.61
Hemorrhage, Inflammation and Tissue Remodeling				
Catalase	CAT	Q7ZTT1	Conversion of hydrogen peroxide to water, antioxidant.	−10.45
Caspase 6	CASP6	Q9I8S9	One in a cascade of cytoplasmic proteases that initiate cell death.	−9.28
Superoxide dismutase	SOD2	Q6P980	Conversion of superoxide to hydrogen peroxide, antioxidant.	−7.73
Peroxiredoxin-5, mitochondrial precursor	PRDX5	UPI000065FD1E	Antioxidant through reduction of reactive oxygen species protects against inflammation.	−5.45
Angiotensin-converting enzyme, somatic isoform precursor	ACE	UPI000065CD0F	Angiotensin is a potent vasoconstrictor, repression increases blood flow.	−3.99
Ferritin, heavy subunit	FTH1	P49946	Intracellular iron storage	2.76
Ferritin-H subunit	FTH1	A9YVA6	Intracellular iron storage	3.87
Alpha-globin IV	HBA1	Q98974	Oxygen transport in blood	4.14
Focal adhesion kinase 1	PTK2	Q00944	Cell migration, adhesion formulation and disassembly	6.32
Hyaluronan-binding protein 2 precursor	HABP2	UPI000065E031	Activates coagulation factor VII	12.23
Thrombin	F2	Q91218	Cleaves fibrinogen to fibrin	14.13
Fibrinogen alpha chain	FGA	Q6DHS2	Cleaved to yield fibrin, blood clotting	14.83
Transferrin	TF	Q9PT13	Iron sequestration in blood	16.80
Prostaglandine D synthase	PTGDS	A9YVA4	Vasodilation	19.44
Gamma fibrinogen	FGG	O42309	Cleaved to fibrin, blood clotting	19.55
Haptoglobin 1	HAP1	Q9DFG1	Binds intracellular free iron	19.83
Fkbp10 protein	FKBP10	UPI00015A5C04	Stimulated collagen and elastin turnover	24.59
Lipid Metabolism and Transport				
20beta-hydroxysteroid dehydrogenase A	CBR1	Q9PT36	Role in metabolism and stress response	−9.63
Estrogen receptor	esr1	P16058	Transcription factor for estrogen	−6.59
Luteinizing hormone beta subunit	LHB	Q9DG92	Stimulates androstenediol production	5.38
Cytochrome P450 2K1	cyp2k1	Q92090	Fatty acid hydroxylation at omega or omega-1 position	6.16
Tumor necrosis factor receptor associated factor 2	TRAF2	Q7T2×2	Stimulated lipolysis, inhibits lipoprotein lipase	7.21
Zona radiata structural protein	VEPB	Q90XC3	Primary constituent of eggshell.	8.64
Breast cancer resistance protein	ABCG2	A8IJF9	Sterol efflux transporter	10.79

(continued on next page)

Table 1 *(continued)*

Name	Gene or Protein Symbol	Swissprot Accession	Function[1]	Fold Change
Retinol-binding protein 1	RBP1	A9Z0L9	Intracellular storage and transport of vitamin in blood.	11.67
Acetyl-CoA acetyltransferase 2	ACAT2	Q5SPA3	Formulation of cholesterol esters	15.19
Apolipoprotein A-I-1 precursor	APOA1	O57523	Major protein in high denisty lipoprotein	15.26
Novel protein similar to cytochrome P450 family 2 subfamily J	cyp2p8	Q5TZ86	Fatty acid mono-oxygenase	15.86
Hydroxysteroid (17-beta) dehydrogenase 10	hsd17b10	Q5XJS8	Conversion of androstenedione to testosterone	16.43
Serum albumin 1 precursor	ALB	P21848	Blood binding protein for long chain fatty acids, billirubin	20.54
P450aromB-I	cyp19	Q8JG19	Conversion of adrostenedione to estrone.	26.91

stress response and heat shock response related genes, but no changes in cyp1a1 gene expression (Supplemental Table).

The rainbow trout head kidney is a lymphoid organ that contains high numbers of white blood cells. Population assessment of white blood cells of this tissue (Braune-Nesje et al., 1981) yielded an estimate of relative cell type numbers for lymphocytes (55%), macrophages (35%), and neutrophils plus melanocytes (10%). Relative to the innate immune system, nonspecific cytotoxic cells are a granular lymphocytes, while neutrophils and macrophages are the primary phagocytic cells (Uribe et al., 2011). The innate immune system plays the key role in resistance to pathogens for the first week or two after infection in rainbow trout (Bayne et al., 2001). Therefore, interpretation of a reduction in survival of rainbow trout fed a mixture of PAHs and challenged with *A. salmonicida* (Bravo et al., 2011) focuses on genes of the innate immune system in the key lymphoid tissue, head kidney. Regulation of the immune response is dependent on tissue type as well as the pathogen (Bessede et al., 2014). Therefore, the pattern of gene expression changes which this paper reports is likely particular to head kidney and *A. salmonicida*. There is good agreement between differential expression and gene families in the current work and those in turbot head kidney during *A. salmonicida* infection (Millan et al., 2011). Among innate immune system response pathways, lectins and early events in the complement system cascade increase in both studies due to pathogen. This is also true for genes in inflammatory, oxidative stress, response to hemorrhage, lipid transport and protein synthesis pathways. The key difference is that this study demonstrates amplification of some responses to *A. salmonicida* in PAH-fed relative to control-fed fish.

Prior to onset of mortality after 4 days in pathogen-challenged fish (Bravo et al., 2011), expression of 126 genes in head kidney were altered in PAH-fed fish compared to control-fed fish (Fig. 2). There were two reasons interpretation of the array data focused on fish sampled two days after pathogen challenge. First, assessment of the immune system status soon after pathogen challenge likely reflected capacity for resistance to infection. Onset of mortality at 4 days after pathogen challenge was nearly identical (about 3%) in control and PAH-fed fish (Bravo et al., 2011). Marked differences in gene expression were detected at 2 days after pathogen challenge (126 genes, Fig. 2) when PAH and control-fed fish were compared. This difference declined to 30, 49 and 3 differences after 4, 10, and 20 days, respectively. Expression of only 2 genes differed between pathogen and mock challenged fish fed control diet. This difference increased to 49 genes after 4 days and nearly disappeared to a single gene after 10 and 20 days. This demonstrated that markedly altered gene expression occurred earlier in PAH-fed than in control-fed fish after pathogen challenge. It seemed highly probable that increased susceptibility to *A. salmonicida* in PAH-fed fish was related to some of the gene expression responses that preceded onset of mortality. Second, most *A. salmonicida*-induced mortality occurred by 10 days after pathogen challenge. Fish collected after 10 and 20 days were selected for resistance to this pathogen and were therefore not representative of the population as a whole.

Multiple gene expression networks relevant to innate immunity were altered in the head kidney of PAH-fed fish after pathogen challenge (Table 1). Genes of the complement cascade were broadly modulated in PAH-fed compared to control-fed fish after pathogen challenge. There were strong signals for up-regulation of the complement cascade via two activation pathways (both lectin MBL and alternative). Many genes of the lectin pathway were up-regulated. This suggests increased capacity for opsonization (coating) of pathogens by lectins with potential for enhanced phagocytosis. A number of early genes in the activation sequence for the complement pathway were upregulated (e.g. pentraxin precursor), as well as was C-1 inhibitor (protein product inactivates proteases that repress the complement cascade). However, expression of CXC chemokine and several lymphocyte receptor or transcription factor genes important in immune signaling for integration of function/proliferation/differentiation were repressed. Expression of genes involved in the terminal steps of bacterial killing by phagocytes were also repressed. Complement component C6 (membrane attack complex) and C5a receptor (oxidative burst trigger) gene expression were repressed which suggested the potential for impaired phagocytic bacterial killing capacity. Repression of lysozyme CII precursor and chymotrypsinogen 2-like protein expression indicated the potential for depressed lysosomal capacity for degradation of debris in phagocytes. Reduced phagocytes in head kidney is an alternative explanation, while this seemed unlikely in the face of up-regulation of other genes involved in the complement cascade. Suppression of key pathway steps in bacterial killing by phagocytes at least partially explained increased susceptibility to *A. salmonicida*-induced mortality in PAH-fed rainbow trout (Bravo et al., 2011).

Infection of salmonid fish with *A. salmonicida* produces dermal ulcers with or without subsequent septicemia (Cipriano and Bullock, 2001). Two days after exposure to this pathogen, up-regulation of multiple genes in head kidneys from PAH-fed compared to control-fed rainbow trout suggest increased hemorrhage with hemolysis due to contaminant exposure (Table 1). Bacteria scavenge iron from the host for growth and vertebrate transferrin plays an important role in innate immunity by sequestering iron and keeping it away from microbial pathogens (Barber and Elde, 2014). Upregulation of genes associated with hemorrhage/inflammation and tissue remodeling (Table 1) in PAH-fed compared to control-fed fish 2 days after pathogen challenge is consistent with more severe infection.

Up-regulation of genes in multiple lipid/steroid metabolism pathways was apparent in PAH-fed relative to control-fed fish after pathogen challenge (Table 1). Alteration of the sex steroid production pathway is consistent with elevated expression of the egg shell zona radiata structural protein. Liver was identified as the dominant organ in production of protein and lipid required in maturation of oocytes to eggs (Arukwe and Goksoyr, 2003). It is uncertain if expression of genes in this pathway in head kidney was vestigial or functional. Increased expression of genes related to fatty acid oxidation and export of cholesterol and perhaps other sterols from head kidney may influence generalized lipid mobilization in response to stress (Table 1) (Sheridan, 1994).

There were numerous changes in “housekeeping genes” two days after pathogen challenge in PAH-fed compared to control-fed fish (Supplemental Table). Up-regulated genes for cyclins, ribosomal proteins, and peptide lengthening and transport suggested cell proliferation. Down-regulated genes were associated with diverse functions but multiple steps in particular pathways were infrequent. There was evidence for down-regulation of genes associated with apoptosis and protein degradation. Three genes for heat shock/chaperonin proteins were also repressed. The functional significance of lower expression of genes for proteins that protect tertiary structure of other proteins was unclear. The overall pattern for up-regulated and down-regulated genes of head kidney in the time-treatment combination was for cell proliferation. This perhaps was in compensation for exacerbated tissue damage.

5. Conclusion

Transcriptional changes in head kidneys in PAH-fed compared to control-fed rainbow trout after *A. salmonicida* challenge provide a basis for better understanding increased disease susceptibility of the PAH-fed fish. Repressed expression of key genes of the innate immune system and up-regulation of many genes associated with hemorrhage, inflammation, and tissue remodeling is consistent with more severe head kidney damage by *A. salmonicida* in PAH-fed fish. Evidence for lipid mobilization and increased cell proliferation also supported exacerbated stress and compensation to necrosis in head kidney in PAH-fed fish after bacterial challenge. These studies provide the first insight into the potential mechanisms associated with increased disease susceptibility and reduced resistance to pathogen challenge in fish after chronic exposure to an environmentally relevant mixture of high molecular weight PAHs.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2017.03.044.

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