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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

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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

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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™