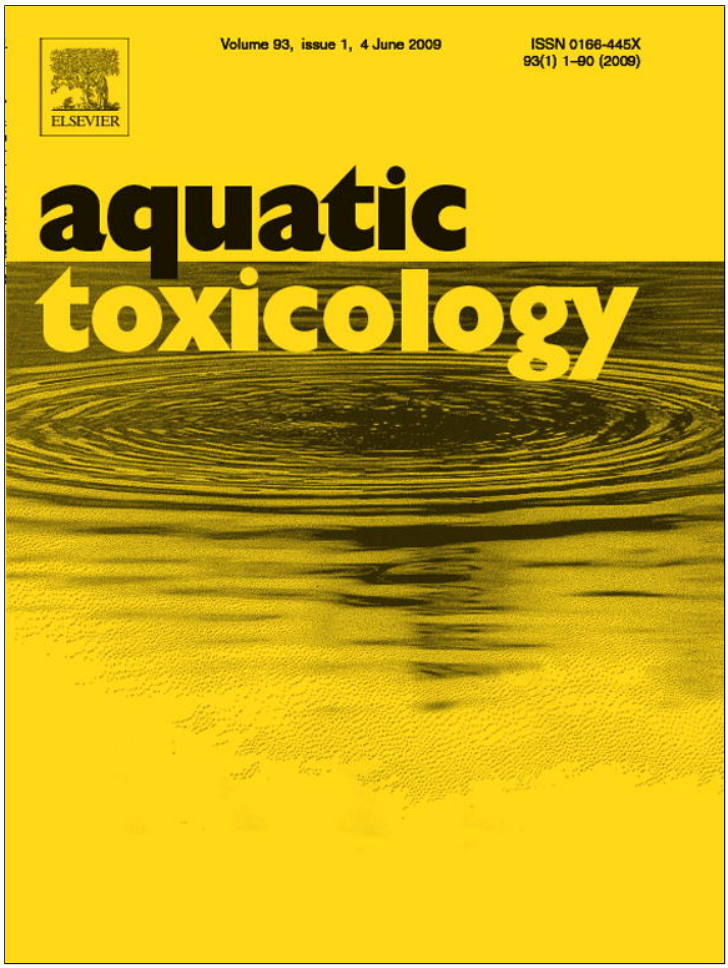

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Aroclor 1248 exposure leads to immunomodulation, decreased disease resistance and endocrine disruption in the brown bullhead, *Ameiurus nebulosus*

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ABSTRACT

The brown bullhead *Ameiurus nebulosus* is a species of the family Ictaluridae commonly used as a sentinel of environmental contamination. While these fish have been utilized for this purpose in areas contaminated with polychlorinated biphenyls (PCBs), few controlled, laboratory-based studies have been designed to document the effects of PCB mixtures in this species. Here, brown bullhead were exposed to the PCB mixture, Aroclor 1248, via intraperitoneal injection and the effects on immune function, plasma hormones and disease resistance were evaluated. Exposure to this mixture led to a decrease in bactericidal activity and circulating antibodies to *Edwardsiella ictaluri* present from a previous exposure to this pathogen. A subsequent *E. ictaluri* disease challenge led to significantly higher mortality in A1248 treated fish compared to vehicle-control fish. The mitogenic response to the T-cell mitogen, phytohemaglutinin-P, was increased compared to vehicle-control fish. The steroid hormone, cortisol, and the thyroid hormone, T3, were also significantly lower in A1248 exposed fish. In summary, we have validated a number of functional immune assays for application in brown bullhead immunotoxicity studies. Additionally, we have demonstrated that the PCB mixture (A1248) modulates both immune function and endocrine physiology in brown bullhead. Such data may compliment the interpretation of data yielded from applied field studies conducted in PCB contaminated aquatic ecosystems.

1. Introduction

Polychlorinated biphenyls (PCBs) are highly stable anthropogenic chemicals that were produced and most intensively used in the United States between 1929 and 1979. Their use was restricted in many countries during the 1970s in response to the associated impacts of PCBs on human health (Silberhorn et al., 1990). Theoretically there are 209 distinct congeners of PCBs based on the percent saturation and possible stereochemical arrangement of chlorine atoms on the biphenyl backbone. Of these congeners, those with a coplanar geometry are regarded as the most toxic as they bind and activate the aryl hydrocarbon receptor (AhR). The noncoplanar congeners can interfere with AhR signaling, but also affect cells via AhR independent pathways (Fischer et al., 1998). The fact that PCB congeners exert biological activity was clearly an unintentional outcome of chemical design, but the fact remains that exposure to these chemicals even at low concentrations leads to biological modulation. Confounding the issue is that the characteristics that made this class of chemicals exemplary candidates for commercial uses (i.e. coolants and insulating fluids for transformers and capacitors and flame retardants) are those that confer properties of environmental persistence. Due to widespread use, accidental spills and haphazard disposal, PCBs now exist as ubiquitous persistent organic pollutants (POPs) in numerous aquatic systems in the United States. They are prominent contaminants in aquatic environments such as the Hudson River and Great Lakes (Hanrahan et al., 1999; Butcher and Garvey, 2004; Heidtke et al., 2006). They are one of the many groups of organochlorine legacy compounds for which adverse biological effects including immunomodulation and endocrine disruption have been attributed.

Exposure to PCBs in controlled laboratory experiments has been reported to adversely modulate the normal immune response and

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immunocyte function in mammals and fishes (Tryphonas et al., 1991; Harper et al., 1993; Ganey et al., 1993; Luster and Rosenthal, 1993; Mayura et al., 1993; Tryphonas, 1994; Yoo et al., 1997; Regala et al., 2001; Smits et al., 2002; Sures and Knopf, 2004; Iwanowicz et al., 2005). Immunomodulation associated with PCB exposure has been observed in aquatic animals inhabiting natural ecosystems contaminated with this legacy compound (Arkoosh et al., 1994a,b; Grasman et al., 1996; Ross et al., 1996). As a functional manifestation of immunomodulation, PCB exposure is sometimes associated with decreased disease resistance (Arkoosh et al., 2001; Ekman et al., 2004; Maule et al., 2005).

Most laboratory studies investigating the effects of PCB exposure in teleosts focus on salmonid model systems. In some cases PCBs have been shown to modulate humoral responses (Arkoosh et al., 1994a,b; Stehr et al., 2000), induce thymocyte apoptosis *in vitro* (Miller et al., 2002; Sweet et al., 1998), affect T-cell mitogen responses (Iwanowicz et al., 2005) or increase disease susceptibility (Maule et al., 2005). Long-term effects on the immune response resulting from intermediate duration exposure during critical developmental stages have been noted as well (Iwanowicz et al., 2005). Despite the utility of the brown bullhead (*Ameiurus nebulosus*) as a sentinel of environmental health in locations of PCB contamination, fewer studies have comprehensively evaluated the effects of PCBs in ictalurids.

Brown bullhead have been utilized as a sentinel species of environmental health for a quarter century. Their benthic life-strategy and omnivorous benthic diet leads to chronic physical contact with sediment-associated contaminants as well as dietary exposure to these same compounds (Smith et al., 1989; Bowser et al., 1991; Poulet et al., 1994). Given a limited home range, their physiological status is considered a direct indicator of local ecological health (Maccubbin et al., 1985; Baumann et al., 1991; Smith et al., 1994). Historically they have been most often exploited as bioindicators in aquatic ecosystems where carcinogenic sediment-associated contaminants are a concern (Harshbarger and Clark, 1990; Baumann and Harshbarger, 1995; Pinkney et al., 2004; Blazer et al., 2006). In this regard the prevalence of neoplasia in bullhead is listed as a beneficial use impairment in Great Lakes Areas of Concern. Neoplasia in bullhead from these locations is often attributed to polynuclear aromatic hydrocarbon (PAH) exposure, although other contaminants such as PCBs and other organochlorine contaminants are often present as well (Sonstegard, 1977; Bowser et al., 1991). While neoplasia is a histologically apparent consequence of chronic exposure to contaminants, many biologically relevant physiological perturbations may be induced by contaminants at exposure concentrations too low or transient to cause cancer. To this effect, it may take years until neoplasms develop following relevant contaminant exposure. For this reason, neoplasia may not always be an appropriate end-point of contaminant-associated biological insult in some locations. End-points that measure health end-points of immune and endocrine status may be more sensitive and therefore more appropriate.

Despite the utility of the brown bullhead (*A. nebulosus*) as a sentinel of environmental health in locations of PCB contamination, few studies have comprehensively evaluated the effects of PCBs in ictalurids. Most laboratory studies investigating the effects of PCB exposure in teleosts focus on salmonid model systems. The following research was conducted to establish baseline data on the normal immune response of the brown bullhead, and better understand the effects of PCB exposure on the normal immune response and endocrine physiology in bullhead under laboratory-controlled conditions. Given the likelihood of observed PCB-associated immunmodulation, a disease challenge was integrated to examine the relevance of the putative effects on immune function. End-points of endocrine physiology were measured to investigate possible endocrine disruptive effects of PCB exposure and their relation to immunomodulation. A number of functional immune and endocrine end-points were assessed and functional immune assays validated for use with this species. Additionally, CYP1A quantification methods were evaluated to validate a suite of measurements that could be used for future field-applied research.

2. Methods

2.1. Chemicals and reagents

Leibovitz-15 (L-15) was purchased from Invitrogen. Stock L-15 was adjusted to 270 mOsm. Aroclor 1248 (purity 99%, lot no. B8010193) was purchased from AccuStandard (New Haven, CT). Primers for *Edwardsiella ictaluri* detection (EictF: 5′-ACT TAT CGC CCT CGC AAC TC-3′ and EictR: 5′-CCT CTG ATA AGT GGT TCT CG-3′) were purchased from Integrated DNA Technologies (IDT Inc., Coralville, IA). GoTaq Green® was purchased from Promega (Promega Corporation, Madison, WI). Monoclonal antibody (mAb) 11A2 (IgG2b) is specific for constant region of channel catfish immunoglobulin light chain (IgL[G]), cross-reacts with brown bullhead IgL and was kindly provided by Dr. Norman Miller (University of Mississippi Medical Center, Jackson, MS). The mAbs C10-7 and 1-12-3 are specific for CYP1A in multiple teleosts and were gifts from Dr. Charles Rice, Clemson University (antibody now commercially available from Caymen) and Dr. John Stegeman, Woods Hole Oceanographic Institution, Woods Hole, MA (Rice et al., 1998). Other antibodies were purchased from Accurate Scientific (T4 and T3), the lab of Gordon Niswender, University of Colorado (17β-estradiol; #244 anti-estradiol-6-BSA) and Coralie Munroe, UC Davis (testosterone; polyclonal R156/7), and Esoterix, Inc. (cortisol; anti-cortisol F3-314 Lot# 345). Secondary antibodies included Cy2 AffiniPure goat anti-mouse IgG and peroxidase AffiniPure goat anti-mouse IgG purchased from Jackson ImmunoResearch (West Grove, PA), and alkaline phosphatase labeled goat anti-mouse IgG (BioRad, Hercules, CA). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were purchased from BioRad. The mitogens concanavalin A (Con A, Cat# C-0412), phytohemaglutinnin P (PHA-P, Cat# L-9132), pokeweed mitogen (PWM, Cat# L-8777), and lipopolysaccharide (LPS, Cat# L-4391) from *E. coli* 0111:B4 were purchased from Sigma–Aldrich (St. Louis, MO). Unless otherwise noted, all other reagents were obtained from Sigma–Aldrich.

2.2. Fish maintenance

During late September early October 2004, approximately 500 brown bullhead (120–250 mm total length) collected from South Creek (Aurora, NC) were transported to the National Fish Health Research Laboratory in Leetown, WV. Fish were held in a warmwater, recirculating system at 20 ± 3 °C with approximately 5–10% daily water replacement for 9 months under local (39°20′59″N, 77°55′58″W) natural photoperiod. During the first 3 months of captivity these fish experienced a spontaneous outbreak of *E. ictaluri* that led to approximately 35–40% mortality (Iwanowicz et al., 2006). Fish used for the following experiment were survivors of this outbreak. During December 2005 the bullhead were transferred to a 19 ± 1 °C, flow-through system. Twenty-four adult bullheads (297 ± 13.7 g and 290 ± 4.7 mm) were randomly stocked into each of six 190 L, black circular tanks. Fish were acclimatized to a spring photoperiod (14 h light:10 h dark) and new tank conditions for 21 days, and maintained on a diet of 1% body weight daily with FinFish Bronze (Zeigler Brothers, Gardners, PA).

2.3. Experiment 1

2.3.1. PCB exposure

To investigate the effects of intraperitoneal exposure to Aroclor 1248 on immune function and endocrine physiology, brown bullhead were exposed to a single concentration dose of Aroclor 1248. This mixture was selected as it is the congener fingerprint identified in the Ashtabula River of the Lake Erie drainage (Imamoglu et al., 2002a,b). The high concentration selected was similar to average body burden concentrations of this Aroclor measured in brown bullheads of the Ashtabula River (5.2 ± 0.4 mg kg^{-1}, unpublished data). On January 20, 2006 brown bullheads in replicate tanks received an intraperitoneal (i.p.) injection of 50 μg or 5 mg kg^{-1} of Aroclor 1248 or 500 μL kg^{-1} vehicle control (vegetable oil). Fish were maintained as above for 21 days and then sampled. Food was withheld 24 h prior to sampling.

All fish were euthanized in approximately 250 ppm tricaine methanesulfonate and bled via the caudal vessels with heparinized syringes within 5 min of capture. Blood was transferred to vacutainers containing 62 U of sodium heparin and stored on wet ice. Anterior kidney tissue was aseptically removed from six fish per replicate tank ($n = 12$) and placed in processing medium (PM; L-15 medium supplemented with 2% fetal bovine serum, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 10 U mL^{-1} sodium heparin) and stored on wet ice. Livers were removed and weighed. Portions of the liver were rinsed with cold Dulbecco's phosphate buffered saline (DPBS), wrapped in aluminum foil and snap frozen in liquid nitrogen for later CYP1A protein detection. The remaining liver and other organs including the spleen, posterior kidney and gonads were fixed in Z-fix (Anatech LTD, Battle Creek, MI) for histological analysis.

2.3.2. Leukocyte isolation

Anterior kidney leukocytes were processed as described by Harms et al. (2000). Briefly, anterior kidney tissue was aseptically removed and placed into PM. Following the fragmentation of the kidney tissue into a single-cell suspension and subsequent separation from settled tissue fragments, cells were pelleted by centrifugation at 500 × *g* for 10 min at 4 °C. Cells were washed by suspension in PM followed by centrifugation as above, suspended in PM, and then layered onto a Histopaque 1.077 cushion. Samples were then centrifuged at 300 × *g* for 30 min at 4 °C and the leukocyte fraction was removed from the medium/Histopaque interface. Leukocytes were pelleted and washed as described above and then suspended in PM for counting. The number of viable leukocytes isolated from each fish was determined by trypan blue exclusion (0.1% trypan blue in PM), and the cells were pelleted as described above. Leukocytes were suspended at 2×10^7 viable cells mL^{-1} in L-15 supplemented with 0.1% fetal bovine serum, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Adherence medium, AM) prior to plating for innate immune function assays.

Vacutainers containing whole blood were centrifuged at 1000 × *g* for 15 min at 4 °C. Plasma was removed and frozen at −80 °C for later analysis. The buffy coat was removed and transferred to PM. Cells were washed and isolated as above. Leukocytes were suspended at 1×10^7 cells mL^{-1} in culture medium (CM; L-15 supplemented with 5% fetal bovine serum, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin) and stored at 4 °C.

2.3.3. Bactericidal activity

Assessment of adherent anterior kidney leukocytes (AKLs) ability to kill the salmonid pathogen *Yersinia ruckeri* was determined using the method described by Harms et al. (2000). Briefly, 2×10^6 leukocytes suspended in AM were added in quadruplicate to the wells of a 96-well plate for bacterial challenge and in triplicate on the same plate for subsequent adherent cell enumeration. Leukocytes from bullheads for all treatments were included on all plates in this assay and those to follow to account for inter-plate variability. Plates were incubated at 20 °C for 2 h following cell plating. Media was then removed from all wells, replaced with CM and plates were cultured at 20 °C in a humidified chamber for 36 h to allow activated leukocytes to reach a resting state. Culture media was then removed from all wells, and wells were washed with antibiotic-free unsupplemented L-15 and replaced with 100 μL of L-15 supplemented with 5% FBS, but no antibiotics. A 48 h culture of *Y. ruckeri* (Hagerman strain; NFHRL # 11.40) washed and suspended in HBSS ($OD_{600} = 0.5$) was added to the treatment wells and a row of cell-free control wells in a volume of 25 μL. The plates were then incubated in the humidified chamber at 20 °C for 4 h. Media was subsequently removed from the treatment and control wells, cells were lysed with lysis buffer (0.2% Tween 20 in dH_2O) and the lysate was immediately serially diluted in serocluster plates containing tryptic soy broth. Diluted lysates were plated onto tryptic soy agar plates and colony forming units (CFUs) determined. Bactericidal activity was expressed as % CFU reduction $(1 - (\text{CFU treated}/\text{CFU control}) \times 100)$ where CFU treated = mean CFU value for replicate wells with adherent leukocytes and CFU control = mean CFU value for replicate wells with media only. A corrected % CFU reduction was defined as % CFU reduction × cell density correction where cell density correction = mean number of adherent cells from the same cell source used to determine bactericidal activity/1×10^6 cells.

2.3.4. Respiratory burst activity

The production of reactive oxygen species (ROS) was determined using the peroxidase luminol-enhanced chemiluminescence (PLCL) assay described by Ripley et al. (2008). In short, 1×10^6 leukocytes suspended in AM were added to the wells of a white 96-well plate, incubated for adherence, washed and subsequently incubated in CM as above to allow cells to reach a resting state. Culture medium was then removed and cells were washed with room temperature HBSS. Cells were then treated in quadruplicate with either LPS (100 μg mL^{-1} LPS in HBSS), LPS-SOD (100 μg mL^{-1} LPS, 352 U mL^{-1} SOD in HBSS) or HBSS alone. The PLCL reaction solution (250 μM luminol, 13.2 U mL^{-1} HRP in HBSS) was then added. Plates were incubated at RT in the dark for 5 min and luminescence was measured on SpectraFluorPlus microplate reader (Tecan, Austria; gain = 180, integration = 500 ms) at 5 min intervals for 15 min.

Stimulation index (SI) values were calculated as the replicate mean luminescence for a given set of LPS stimulated leukocytes divided by the replicate mean luminescence of the associated HBSS treated (control) leukocytes. Stimulation index values were calculated for all samples at 5, 10 and 15 min post-stimulation. The maximum SI (mSI) value for a given pair of LPS stimulated and control leukocytes was defined as the highest SI value determined for the three time points and used as the comparative value.

2.3.5. Cytotoxic-cell activity

The ability of brown bullhead anterior kidney leukocytes to lyse virally transformed epithelioma papulosum cyprini (EPC) cells was determined using the calcein AM release-based cytotoxic-cell assay as described by Iwanowicz et al. (2004). Briefly, an 18 h culture of EPC cells plated at a density of 1×10^5 cells well^{-1} in 96-well plates were incubated with 5 μM calcein AM in CM for 5 h. Cells were then washed four times with room temperature DPBS and cultured in CM for an additional 30 min. Media was removed and effector AKLs suspended in CM were added in quadruplicate at effector to target ratios of 10:1, 2:1 and 1:1. Lysis buffer (25 mM sodium borate, 0.1% Triton-X 100 in CM, pH 9.0) or CM alone was added in quadruplicate to another set of wells to determine total and spontaneous release, respectively. Plates were centrifuged at 50 × *g* for 5 min and incubated at 20 °C for 8 h in the dark. A volume of 50 μL supernatant was removed from all wells and added to a 96-well black plate pre-

loaded with 2× lysis buffer. Supernatant from the total release wells were added to 1× lysis buffer. Fluorescence intensity (FI) was measured reading the plates from the bottom using a SpectraFluorPlus (Ex = 485, Em = 535 and gain = 60).

$$\text{cytotoxic-cell activity (\%)} = \frac{100 \times \text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}}$$

2.3.6. Mitogen stimulation

Anterior kidney leukocytes suspended in CM were dispensed to 96-well tissue-culture plates at 1×10^6 cells well^{-1}. Anterior kidney leukocytes were plated such that each 96-well plate contained cells from all treatment groups to account for possible plate effect variability. A volume of 50 μL mL^{-1} of CM with or without mitogen (control) was added to the wells immediately after cells were plated. Mitogen concentrations used to stimulate the isolated leukocytes were as follows: 10 μg mL^{-1} CON A, 10 μg mL^{-1} PHA-P, 50 μg mL^{-1} PWM, or 100 μg mL^{-1} LPS from *E. coli* 0111:B4. Mitogen treated and control wells were replicated in triplicate and plating was performed with the plates on ice. Following plating, leukocytes were incubated in humidified chambers at 20 °C under atmospheric conditions. The mitogenic response was measured on the second day (48 h) of incubation post-mitogen stimulation. The mitogenic response was measured as described below. An additional plate of AKLs stimulated with LPS was measured 5 days post-stimulation as this kinetic time point was determined to optimal for this species using this assay format (data not shown).

2.3.7. Mitogenesis assay

The mitogen-induced proliferative response was evaluated using the BrdU-based ELISA at room temperature as described by Gauthier et al. (2003) with minor modifications. Briefly, 24 h prior to the ELISA, leukocytes were treated with 25 μL well^{-1} of sterile-filtered 65 μM BrdU in L-15 and incubated for an additional 24 h. Cells were washed with 100 μL well^{-1} DPBS and fixed for 15 min with 1% paraformaldehyde in DPBS (pH 7.2). Cells were washed three times as above and the cell membranes were porated by a 30 s exposure to 50 μL well^{-1} of 0.01% polyoxyethylene–sorbitan monolaurate (Tween-20) in DPBS. Cells were washed three times and the wells were then blocked with 250 μL well^{-1} of blocking buffer (DPBS containing 1% bovine serum albumin) for 1 h. Blocking buffer was removed and replaced with 50 μL well^{-1} of 0.2 U mL^{-1} anti-bromodeoxyuridine Fab fragments labeled with horseradish peroxidase (Roche Chemicals) in blocking buffer and incubated for 1 h. Cells were washed five times with 100 μL well^{-1} DPBS and 50 μL well^{-1} of an enzyme development solution (EDS; 10 mM sodium citrate buffer, 728 μM 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), 0.03% H_2O_2 and pH 4.0) as added according to Ottinger and Kaattari (1998). The optical density (405 nm) of the solution in each well was determined at 5 min increments over a period of 20 min using a Kinetic Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA).

Stimulation index values were calculated as the replicate mean optical density for a given set of mitogen treated leukocytes divided by the replicate mean optical density of the associated mitogen free (control) leukocytes. Stimulation index values were calculated for all time points and the maximum SI (mSI) value for a given pair of mitogen-treated and control leukocytes was defined as the highest SI value determined for the four time points.

2.3.8. Flow cytometric analysis

Peripheral blood leukocytes were suspended to 1×10^6 cells mL^{-1} in labeling buffer (ILB; L-15 containing 1% bovine serum albumin and 0.01% sodium azide) and incubated for 30 min. All incubations for this procedure were executed on wet ice. Cells were centrifuged at 500 × *g* for 10 min at 4 °C, suspended in ILB containing 11A2 hybridoma supernatant diluted 1:10 and incubated for 30 min. Hybridoma supernatant from the Warr's cell line was used as an immuno-irrelevant isotype control (DeLuca et al., 1983). Cells were washed three times in ILB, centrifuged as before, suspended in ILB containing donkey anti-mouse IgG-Cy2 diluted 1:500 and incubated for 60 min. Again cells were washed as before and resuspended in 1 mL of DPBS. Flow cytometry was performed using a FACS Calibur. The percent IgL(G) positive B-cells were determined using QuanitiCell software. Cell populations defined by forward and side scatter properties were compared.

2.3.9. Enzyme-linked immunosorbant assay (ELISA)

Brown bullhead plasma was screened for *E. ictaluri* reactive IgL(G) antibodies by indirect ELISA. In short, whole *E. ictaluri* bacteria from a 48 h culture were washed in DPBS and total protein determined via the bicinchoninic acid (BCA) method using a BCA kit (Pierce, Rockford, IL). The bacterial preparation was then washed and suspended in carbonate buffer (15 M sodium carbonate, 35 mM sodium bicarbonate, pH 9.6) added to the wells of a 96-well plate at 10 μg well^{-1} and incubated overnight at 4 °C. Wells were then washed three times with DPBS containing 0.05% Tween-20 (DPBS-T) using an automated plate washer (Skan Washer 400, Molecular Devices Corporation). Plasma samples were serial diluted (from 1:50 to 1:109,350 as 1:3 dilutions) in DPBS containing 0.02% sodium azide, added to the wells (50 μL well^{-1}), and incubated over night at 15 °C. Plasma from wild-caught brown bullheads (Aurora, NC) that failed to agglutinate *E. ictaluri* was used a control. Cells were washed three times with DPBS-T and wells were blocked with 300 μL of blocking buffer (BB; DPBS-T containing 1% BSA for 1 h at room temperature. Wells were aspirated and 50 μL well^{-1} of BB containing 11A2 hybridoma supernatant (working concentration 1:100) and plates were incubated for 1 h at room temperature. After wells were washed three more times, 100 μL of BB containing horseradish peroxidase conjugated goat anti-mouse IgG (diluted 1:1000) was added and incubated for an additional 30 min. Plates were washed an additional three times and 50 μL well^{-1} of EDS substrate was added. The optical density (405 nm) of the solution in each well was determined at 5 min increments over a period of 20 min using a Kinetic Microplate Reader (Molecular Devices Corporation). The ELISA titre was defined as the reciprocal of the OD_{50}.

2.3.10. Plasma lysozyme

Plasma was assayed for lysozyme (muriminidase) activity via the microplate, turbidimetric assay method as described by Parry et al. (1965). In short, plasma was diluted in 0.10 M sodium phosphate buffer containing 0.2 mg mL^{-1} of lyophilized *Micrococcus lysodeikticus* (ATCC # 4698, Sigma–Aldrich). Hen egg-white lysozyme (HEWL, Sigma–Aldrich) was used as a standard. Plates were incubated at 25 °C for 30 min and absorbance (450 nm) was measured every 5 min over a period of 20 min using a Kinetic Microplate Reader. Plasma lysozyme activity was interpolated from the HEWL standard curve and all data were expressed as relative units of HEWL.

2.3.11. Microsome preparation

Hepatic microsomes were isolated as previously described by Van Veld et al. (1990) with slight modifications. Briefly, partially thawed liver samples were added to a microcentrifuge tube containing 10 volumes of ice-cold stabilization buffer (SB; 100 mM potassium phosphate buffer containing 20% glycerol, 1 mM dithiothriotol, 1 mM ethylenediaminetetraacetic acid disodium, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.2). Tissue was homogenized at 30 Hz for 5 min using a Mixer Mill 300 and clarified twice by centrifugation at 12,000 × *g* for 11 min. The final supernatant was pelleted by centrifugation at 100,000 × *g* for 90 min, the resulting

pellet was resuspended in SB and centrifuged at 100,000 × *g* for and addition 60 min. Final pellets were suspended in SB and total protein was determined via the BCA as above.

2.3.12. CYP1A detection

Immunoblot analysis was performed to quantify CYP1A protein from microsome preparations (Van Veld et al., 1990). In short, microsomal proteins were separated by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 9% matrix. Samples were loaded at 10 μg of total protein well^{-1}. Each gel contained 0.0125, 0.025, 0.05, 0.10 and 0.20 pmol of mummichog (*Fundulus heteroclitis*) or spot (*Leistomus xanthurus*) CYP1A for standard curve development. Samples and standards were resolved at 200 V for 40 min and immunoblotted to a polyvinylidene fluoride membrane (Amersham Biosciences) at 130 V for 60 min at 4 °C. Membranes were blocked with TBS (50 mM Tris, 150 mM NaCl, 0.5%, pH 8.0) containing 5% skim milk (blocking buffer) for at 42 °C for 1 h. Membranes were then incubated with the C10-7 or 1-12-3 monoclonal antibodies diluted in blocking buffer at room temperature for 1 h. Following washes alkaline phosphatase (AP) labeled goat anti-mouse IgG was added and incubated for 1 h at RT. The membrane was thoroughly washed and specific binding detected with the AP substrate NBT/BCIP. Blots were digitally captured using a FluorChem SP Imaging System and volume integration of the bands determined AlphaEase FC StandAlone Software (Alpha Innotech Corporation, San Leandro, CA). Total CYP1A was determined relative to the appropriate standard as interpolated from a standard curve using curve fitting software (Prism 4.0, GraphPad Software Inc.).

2.3.13. Hormone assays

Plasma samples from brown bullheads were analyzed for the sex steroids 17β-estradiol (E2) and testosterone (T), and the thyroid hormones thyroxine (T4) and triiodothyronine (T3). All samples were quantified via radioimmunoassay (RIA). Plasma sex steroid samples were determined using the RIA method of Sower and Schreck (1982). Plasma T3 and T4 were measured by direct RIA according to Dickhoff et al. (1978). Plasma cortisol was measured according to by Redding et al. (1984) as modified by Feist et al. (1990). Plasma samples were heated to denature binding proteins for the cortisol RIA. All samples were run in duplicate and plasma estradiol values were extrapolated from a standard curve. Sample values were rejected and measured a second time if the coefficient of variation between duplicate tubes exceeded 10%. Plasma hormone concentrations were interpolated from the standard curve using curve fitting software (Prism 4.0).

2.4. Experiment 2

2.4.1. Bacterial challenge

Brown bullheads not sampled for the functional immune assays (24 fish per treatment) were exposed to *E. ictaluri* (NFHRL; E02-2004) by immersion 4-week post-Aroclor 1248 exposure. This isolate was responsible for mortality in this same group of bullheads approximately 1 year prior (Iwanowicz et al., 2006). Fish were exposed to 1×10^7 CFU mL^{-1} *E. ictaluri* for 1 h and returned to their respective tanks. Bullheads were monitored for 30 days post-bacterial exposure. Liver, spleen and anterior kidney were aseptically sampled for *E. ictaluri* from dead fish using classical bacteriological methods and cultured in brain heart infusion broth (BHIB; Bacto) at 30 °C for 5 days. Additionally, tissue was collected from these organs and stored in 95% ethanol for PCR. Isolates from the bacterial cultures were also fixed in 95% ethanol for PCR confirmation. The above procedures were conducted for bullheads surviving the 30 day challenge as well.

2.4.2. Polymerase chain reaction

Equal amounts (by weight) of liver, spleen and posterior kidney were pooled to obtain a total mass of 25 mg for DNA extraction. DNA was extracted using the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA) according to the manufacture's protocol for total DNA from animal tissues. A 129-base-pair fragment of a putative transposon located next to the phosphoserine transaminase (serC) gene of *E. ictaluri* (GenBank accession number AF110153) was targeted for amplification using the method described by Bilodeau et al. (2003). In short, 10 ng of template DNA (host and bacterial) was used for each reaction. Template DNA concentration was 100 pg for the bacterial samples. Reactions included 15 pmol of each primer, DNA template and GoTaq Green® master mix (Promega). The optimized amplification profile was as follows: hotstart for 10 min at 94 °C; 40 cycles of 30 s at 94 °C, 45 s at 60 °C, and 1 min at 72 °C; and 5 min at 72 °C. Each reaction was run on a Mastercycler® (Eppendorph International, Westbury, NY). Amplification of single products of the expected size was verified by electrophoresis through 2.0% agarose gels.

2.4.3. Soluble immune factors

Plasma lysozyme and *E. ictaluri* antibody titers from surviving fish were evaluated as described above.

2.5. Statistical analysis

All data were initially examined for differences between replicate tanks using a two sample *t* test. Significant differences were not found so data was statistically analyzed and reported based on combined tanks within treatment. Data were tested for normality using the Shapiro–Wilks *W* test and homogeneity of variance via the Brown–Forsythe test of homogeneity of variances. Data were analyzed using a one-way ANOVA and significant differences between group means were determined using the Tukey–Kramer post hoc test. Comparisons between sex within treatments and between individuals of the same sex were also analyzed to evaluate sex specific effects. ELISA data were square root transformed to meet the assumptions of parametric analysis. Pearson product-moment correlations were determined in an attempt to resolve the relationships between all immune end-points and all hormone concentrations using pooled data or treatment specific data. Statistical analyses of the bacterial challenge included the Kaplan–Meier survival curve analysis, and total % cumulative mortality was compared as a 2 × 2 contingency table using a two-tailed Fisher's Exact test. The influence of sex on survival was also investigated. Differences were considered statistically significant when $P < 0.05$. Statistical analyses were performed using Systat (Version 11; SyStat Software Inc., San Jose, CA, USA) or Prism (Version 4.03, GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Growth physiology

During the experiment fish from treatment groups increased in weight by 1.02–1.14%, but there were no significant differences between groups (Table 1). Similarly, there were no significant differences in condition factor among groups when sex was considered or when mean values from all fish within a group were pooled. Differences in mean HSI were not observed between groups when sex was not considered as a factor. The mean HSI of male bullhead exposed to 50 μg kg^{-1} of A1248 was significantly higher ($P = 0.040$) than that of the controls. While the mean HSI of males exposed to 5 mg kg^{-1} of A1248 was slightly higher than that of the controls, this increase was not significant ($P = 0.073$). There was however a

Table 1
Physiological parameters of male and female brown bullhead post-A1248 exposure.

	Treatment group		
	Vehicle control	50 μg kg^{-1} A1248	5 mg kg^{-1} A1248
Hepatosomatic index			
Female	2.52 ± 0.06^{A}	2.68 ± 0.07^{A}	2.55 ± 0.09
Male	2.28 ± 0.03^{B}	**2.47 ± 0.05^{B}**	**2.51 ± 0.07**
Condition factor			
Female	1.25 ± 1.15	1.24 ± 0.08	1.25 ± 0.03
Male	1.19 ± 0.03	1.22 ± 0.04	1.26 ± 0.07
Hematocrit			
Female	39 ± 2%	44 ± 2%^{A}	40 ± 4%
Male	42 ± 2%	40 ± 1%^{B}	40 ± 3%
CYP1A			
Female	0.93 ± 0.13	0.87 ± 0.11^{A}	1.06 ± 0.31^{A}
Male	0.91 ± 0.26	1.86 ± 0.57^{B}	2.18 ± 0.42^{B}

Values are means ± standard error. Values denoted with the different letters are significantly different between males and females within the same treatment group. Bold values indicate significant differences when compared to the control (one-way ANOVA followed by the Tukey–Kramer method; $P \leq 0.05$). Units for the CYP1A data are pmol CYP1A mg total protein^{-1}.

significant positive correlation ($r = 0.71$; $P = 0.022$, $n = 10$) between HSI and CYP1A in males, but not females ($r = 0.004$, $P = 0.99$, $n = 14$).

3.2. Functional activity of adherent cells

Approximately 56–59% of isolated AKLs were adherent. No significant differences in the proportion of adherent and non-adherent cells were noted among treatment groups. Aroclor 1248 exposure led to a significant decrease in bactericidal activity at both low and high doses ($P = 0.047$ and 0.033, respectively) compared to the vehicle control (Fig. 1). When individuals of a single sex were compared across treatment groups the only significantly differences were noted between females from the high dose group ($P = 0.030$) compared to the controls.

The LPS inducible respiratory burst response was not significantly different between treatment groups (Fig. 2A). However, the mean LPS inducible stimulation indices for control male and female bullheads were 4.24 ± 0.64 and 2.67 ± 0.34, respectively and significantly different ($P = 0.025$). Similar sex specific differences were not observed in treatment groups exposed to A1248. The amount of

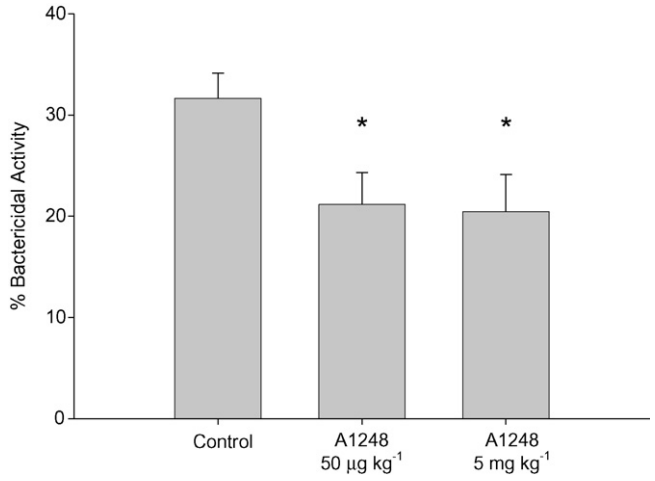

Fig. 1. Bactericidal activity of brown bullhead adherent anterior kidney leukocytes 3-week post-intraperitoneal exposure to 50 μg or 5 mg kg^{-1} of Aroclor 1248. Statistical differences from the vehicle control are indicated by an asterisk (*); one-way ANOVA followed by the Tukey–Kramer method; $P \leq 0.05$; $n = 12$ fish per treatment. Error bars represent the standard error from the mean.

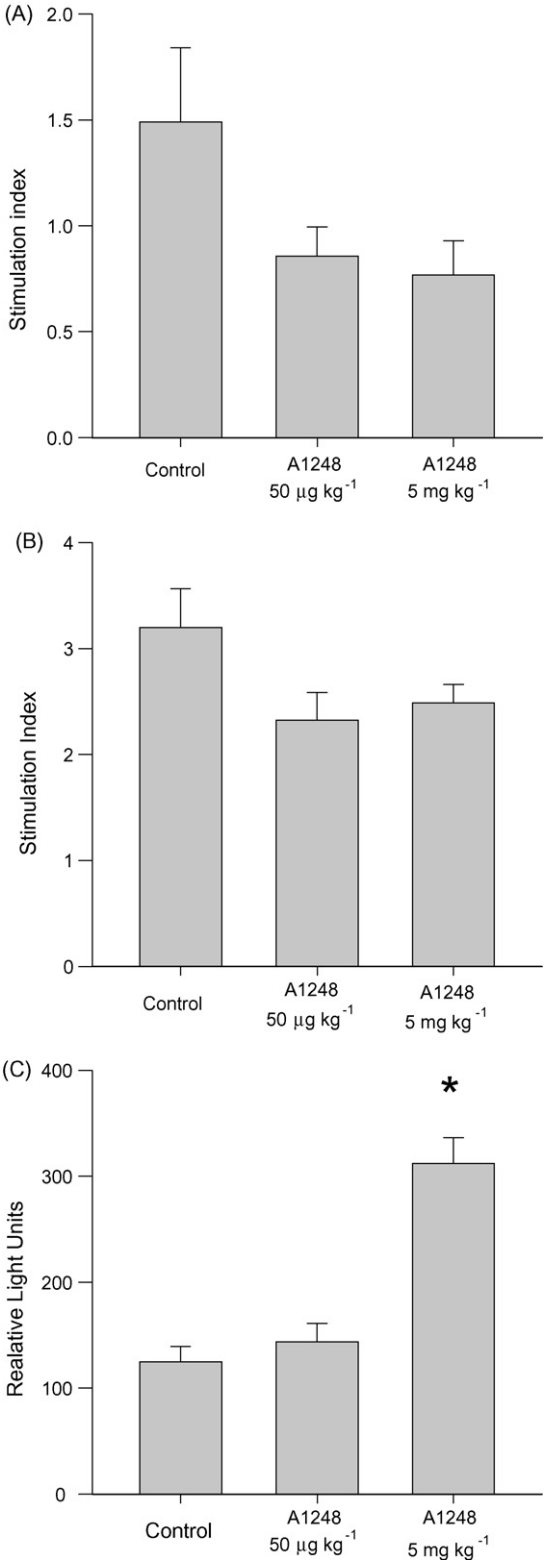

Fig. 2. Basal levels of reactive oxygen species indicated by relative light units of brown bullhead anterior kidney adherent leukocytes 3-week post-i.p. exposure to 50 μg or 5 mg kg^{-1} of Aroclor 1248. Statistical differences from the vehicle control are indicated by an asterisk (*); one-way ANOVA followed by the Tukey–Kramer method; $P \leq 0.05$; $n = 12$ fish per treatment. Error bars represent the standard error from the mean.

SOD-insensitive reactive species produced in response to LPS stimulation were not significantly different between A1248 exposed fish and control when sex was not considered as a factor (Fig. 2B). Males exposed to 50 μg kg^{-1} or 5 mg kg^{-1} of A1248 produced significantly more SOD-insensitive reactive species ($P = 0.007$ and 0.012, respectively) compared to controls when individuals of a single sex were compared. The basal level of reactive species was significantly higher in fish exposed to 5 mg kg^{-1} of A1248 than control fish ($P < 0.001$) when sex was not considered as a factor (Fig. 2C). Similarly, when individuals of the same sex were compared, both males and females had significantly higher basal ROS than control fish ($P \leq 0.001$ and 0.002, respectively). Significant differences in basal ROS production between the low dose treatment group and controls or high dose A1248 were not observed (Fig. 2C).

3.3. Cytotoxic-cell activity

Anterior kidney leukocytes (10:1) collected from bullhead exposed to 5 mg kg^{-1} had a higher mean level of lytic activity towards EPC cells (20.0% ± 3.2) compared to those exposed to 50 μg kg^{-1} (12.4% ± 3.4) or control (11.3% ± 2.4), but this difference was not significant (Fig. 3). No significant differences were noted at the other effector-to-target ratios. When males and females were compared within treatment groups females exposed to 5 mg kg^{-1} of A1248 exhibited a mean cytotoxic-cell activity twice that of males, but this difference was not significant ($P = 0.079$).

3.4. Mitogen stimulation

The proliferative response to the T-cell mitogen PHA-P was significantly higher in AKLs isolated from bullheads treated with 50 μg ($P = 0.045$) or 5 mg kg^{-1} ($P = 0.011$) compared to the vehicle only control. Stimulation indexes resulting from stimulation by the macrophage-dependant T-cell mitogen Con A treated cells were generally higher in A1248 treated fish, but these differences were not significant. No significant differences were observed in leukocyte proliferation between treatments following PWM or LPS stimulation 48 h post-stimulation (Fig. 4). The mean stimulation index of AKLs 5 days post-LPS stimulation were 1.97 ± 0.28 (vehicle controls), 1.29 ± 0.10 (50 μg kg^{-1} A1248) and 1.28 ± 0.31 (5 mg kg^{-1} A1248). Stimulation indices were not significantly different for the 50 μg ($P = 0.098$) or 5 mg kg^{-1} ($P = 0.092$) treated groups compared to the vehicle only control. Mitogen responses were independent of sex.

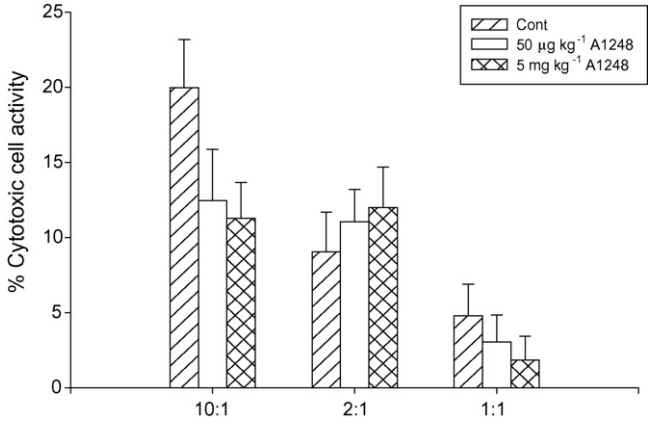

Fig. 3. Cytotoxic activity of brown bullhead anterior kidney leukocytes 3-week post-i.p. exposure to 50 μg or 5 mg kg^{-1} of Aroclor 1248. Statistical differences from the vehicle control are indicated by an asterisk (*); one-way ANOVA followed by the Tukey–Kramer method; $P \leq 0.05$; $n = 12$ fish per treatment. Error bars represent the standard error from the mean.

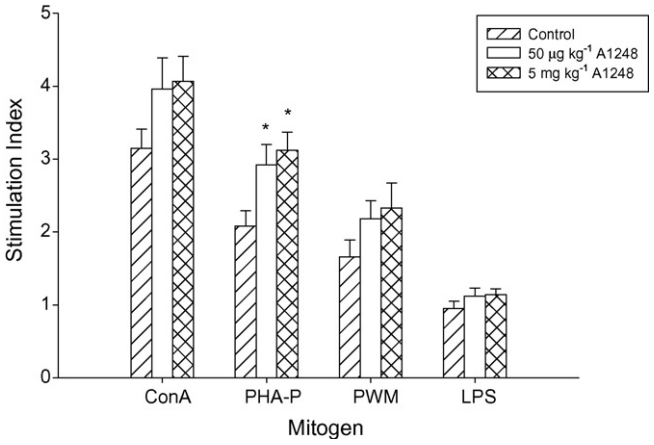

Fig. 4. Mitogenic responses brown bullhead anterior kidney leukocytes 3-week post-i.p. exposure to 50 μg or 5 mg kg^{-1} of Aroclor 1248. Statistical differences from the vehicle control are indicated by an asterisk (*); one-way ANOVA followed by the Tukey–Kramer method; $P \leq 0.05$; $n = 12$ fish per treatment. Error bars represent the standard error from the mean.

3.5. Flow cytometry

The proportion of 11A2 positive B-cells from PBL or AKL populations did not differ between treatment groups. This cell population represented 9.1 ± 0.5%, 9.4 ± 0.7%, and 10.8 ± 0.9% of total PBLs in fish exposed to 5 mg or 50 μg kg^{-1} of A1248 or vehicle controls, respectively. Cell populations defined simply by forward and side scatter properties were defined as lymphocytes (R1; small low-complexity), neutrophils (R2; large-complex), and macrophages (R3; large-low-complexity; Fig. 5A). Differences in AKL subpopulations based on forward and side scatter properties were observed between treatment groups. The percent of lymphocytes (R1) was significantly higher ($P = 0.026$) in fish treated with 50 μg kg^{-1} of A1248 than those from vehicle controls (Fig. 5B). A similar difference was not observed between 50 μg kg^{-1} of A1248 treated and controls ($P = 0.121$). Changes in this lymphocyte population are not ascribed to a shift in 11A2 positive cells (B cells expressing IgL[G]). Anterior kidney associated neutrophils were significantly lower ($P = 0.022$ and $P = 0.037$) in 5 mg or 50 μg kg^{-1} of A1248 treated groups, respectively compared to vehicle controls.

3.6. CYP1A

Prior to quantitative analysis western blots were probed with the C10-7 or 1-12-3 mAbs to determine which antibodies best cross-reacted with brown bullhead CYP1A. The C10-7 cross-reacted best with brown bullhead CYP1A for this detection format (data not shown), and was used for quantitative analysis. In general the amount of CYP1A protein was low in all samples. Vehicle-control fish expressed 0.92 ± 0.06 pmol CYP1A mg total protein^{-1}. While the mean values of CYP1A were higher in livers of A1248 treated fish (1.24 ± 0.21 and 1.76 ± 0.27 pmol CYP1A mg total protein^{-1} for low and high doses, respectfully), they were not statistically different. When CYP1A expression was compared between males and females within a treatment, statistical differences were noted. Male bullheads exposed to 50 μg kg^{-1} of Aroclor 1248 expressed significantly more CYP1A protein (1.86 ± 0.58 pmol CYP1A mg total protein^{-1}) than females (0.87 ± 0.11 pmol CYP1A mg total protein^{-1}; $P = 0.037$). Males exposed to 5 mg kg^{-1} of Aroclor 1248 expressed 2.18 ± 0.43 pmol CYP1A mg total protein^{-1} and females expressed 1.06 ± 0.3 pmol CYP1A mg total protein^{-1}, but these mean values were not significantly different ($P = 0.083$). Expression was similar between vehicle treated males and female (Table 1). Additionally, there was a significant negative correlation ($r = -0.72$,

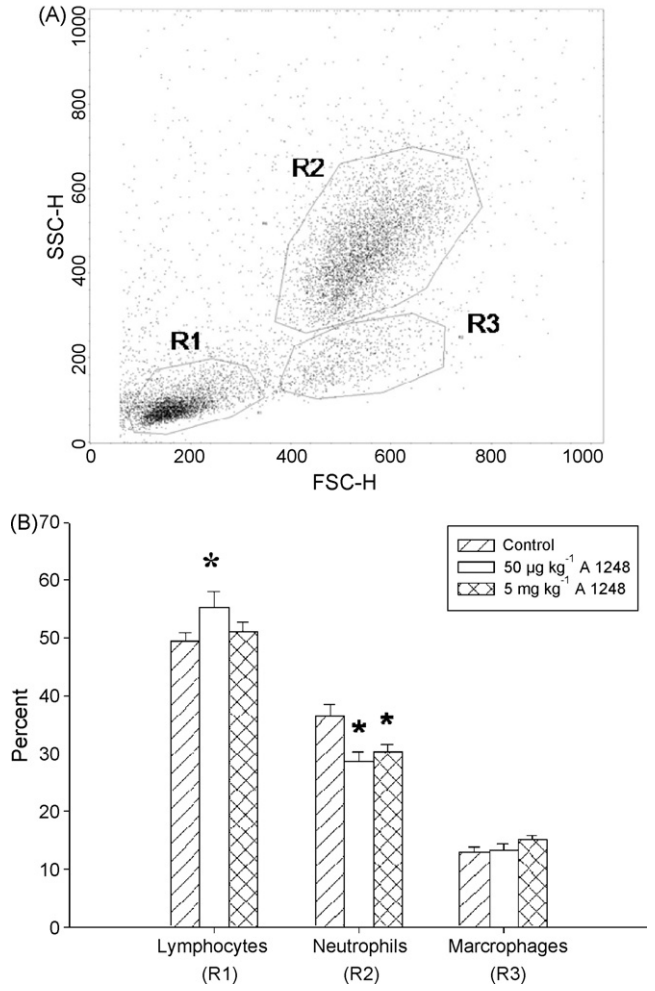

Fig. 5. Size and light scattering characteristics of brown bullhead anterior kidney leukocytes. Representative dot plot and defined gates (A) and histogram depicting the proportion of gated populations of anterior kidney leukocytes 3-week post-i.p. exposure to 50 μg or 5 mg kg^{-1} of Aroclor 1248. Statistical differences from the vehicle control are indicated by an asterisk (*); one-way ANOVA followed by the Tukey–Kramer method; $P \leq 0.05$; $n = 12$ fish per treatment. Error bars represent the standard error from the mean.

$P = 0.004$, $n = 14$) observed between CYP1A and plasma E2 in female fish.

3.7. Hormones

Concentrations of plasma sex steroids were not significantly different between fish of the same sex across treatment groups. Plasma 17β-estradiol concentrations were significantly higher in females than males in the vehicle control ($P = 0.001$), low ($P \leq 0.001$), and high ($P \leq 0.001$) treatment groups. While mean 17β-estradiol concentrations appeared to increase with increasing A1248 concentration this difference was not significant (Table 2). Average testosterone concentrations were not significantly different between fish of the same sex across treatment groups. Circulating concentrations of T4 did not differ between males and females within a treatment group or across groups when compared as pooled values limited by sex. Plasma T3 was significantly lower in fish exposed to 50 μg kg^{-1} ($P < 0.001$) or 5 mg kg^{-1} ($P = 0.001$) of Aroclor 1248 compared to vehicle controls (Fig. 6A). Plasma cortisol was also significantly lower in fish exposed to 5 mg kg^{-1} ($P = 0.004$) of Aroclor 1248 compared to controls. While there was a reduction in plasma cortisol in the fish exposed to 50 μg kg^{-1} of Aroclor 1248

Table 2
Plasma hormone concentrations of male and female brown bullhead post-A1248 exposure.

	Treatment group		
	Vehicle control	50 μg kg^{-1} A1248	5 mg kg^{-1} A1248
E2 (pg mL^{-1})			
Female	2361.4 ± 378.4[a]	2582.8 ± 600.9[a]	2429.6 ± 482.0[a]
Male	88.3 ± 17.7[b]	94.0 ± 13.0[b]	92.1 ± 12.6[b]
T (pg mL^{-1})			
Female	961.1 ± 119.8	1172.6 ± 238.2	1346.8 ± 264.2
Male	1239.9 ± 197.2	1297.8 ± 170.1	1261.4 ± 150.9
Cortisol (ng mL^{-1})			
Female	11.0 ± 2.3	7.0 ± 1.5	**4.9 ± 1.6**
Male	9.7 ± 2.3	7.0 ± 1.4	5.1 ± 1.1
T4 (ng mL^{-1})			
Female	2.0 ± 0.2	2.6 ± 0.2	2.4 ± 0.3
Male	2.0 ± 0.2	2.2 ± 0.2	2.3 ± 0.3
T3 (ng mL^{-1})			
Female	6.6 ± 0.5	**4.4 ± 0.5**	**4.6 ± 0.6**
Male	6.6 ± 1.2	**4.1 ± 0.4**	4.5 ± 0.6

Values are means ± standard error. Values denoted with the different letters are significantly different between males and females within the same treatment group. Bold values indicate significant differences when compared to the control (one-way ANOVA followed by the Tukey–Kramer method; $P \leq 0.05$).

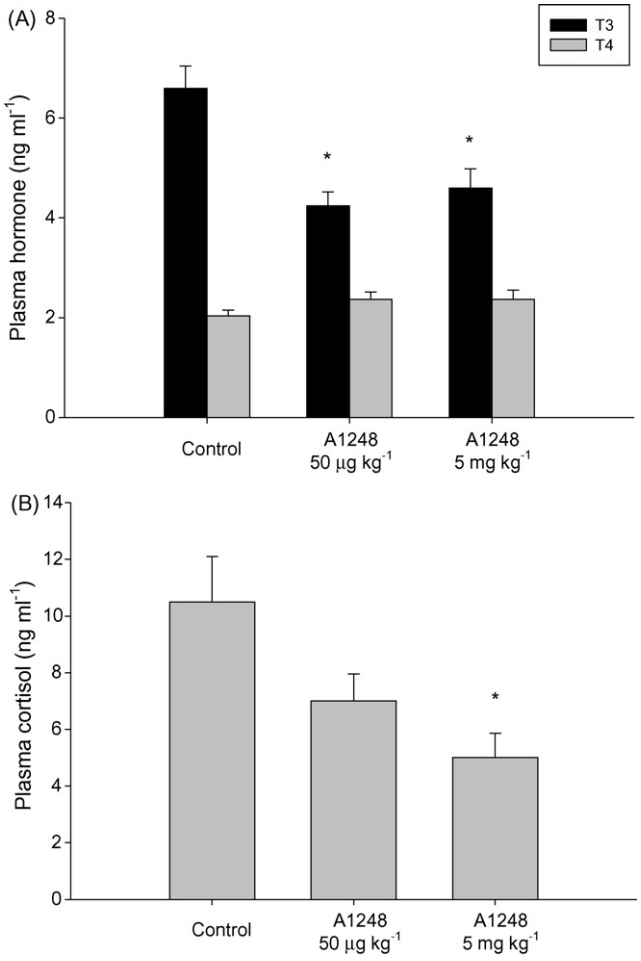

Fig. 6. Plasma concentrations of (A) thyroxine (T4) and triiodothyronine (T3), and (B) cortisol 3-week post-i.p. exposure to 50 μg or 5 mg kg^{-1} of Aroclor 1248. Statistical differences from the vehicle control are indicated by an asterisk (*); one-way ANOVA followed by the Tukey–Kramer method; $P \leq 0.05$; $n = 12$ fish per treatment. Error bars represent the standard error from the mean.

Table 3
Soluble immune parameters of brown bullhead post-A1248 exposure, but pre- and post-*E. ictaluri* challenge.

Treatment	Average titer (pre-challenge)	Cumulative % mortality	Average titer (post-challenge)	Lysozyme (pre-challenge)	Lysozyme (post-challenge)
Wild-caught controls	30 ± 16^{A}	NA	NA	NA	NA
No-injection control	$1567 \pm 477^{B,C}$	$0\%^{A}$	1663 ± 446^{A}	16.1 ± 2.7	20.3 ± 4.9^{A}
Vehicle control	1925 ± 490^{B}	$37.5\%^{B}$	$\mathbf{12{,}986 \pm 3768}^{B}$	18.8 ± 3.2	$\mathbf{99.0 \pm 12.3}^{B}$
50 μg kg^{-1} A1248	425 ± 140^{A}	$87.5\%^{C}$	$\mathbf{8550 \pm 4409}^{B}$	11.8 ± 2.1	$\mathbf{89.2 \pm 13.3}^{B}$
5 mg kg^{-1} A1248	$400 \pm 142^{A,C}$	$79\%^{C}$	$\mathbf{6030 \pm 2871}^{B}$	12.4 ± 1.9	$\mathbf{86.4 \pm 14.5}^{B}$

Values are means ± standard error. Values denoted with the different letters are significantly different between treatment groups. The no-injection controls were survivors of the 2006 *E. ictaluri* outbreak, but were not exposed to A1248 or the *E. ictaluri* challenge. They were included to provide baseline values of the above parameters. Units of the lysozyme values are U mL^{-1} (relative to HEWL). Bold values indicate significant differences when compared to the pre-challenge condition (one-way ANOVA followed by the Tukey–Kramer method; $P \leq 0.05$); NA = not applicable.

compared to controls ($P = 0.083$), the difference was not significant (Fig. 6B). The changes in T3 or cortisol were independent of sex (Table 2). There were no significant correlations between hormone concentrations and any of the immune function end-points based on Pearson product-moment correlation analysis ($P > 0.05$).

3.8. Pathogen challenge

E. ictaluri was isolated from all bullheads challenged with *E. ictaluri* regardless of the mortality outcome. While *E. ictaluri* was not cultured from control bullheads (those not exposed to this pathogen), it was detected via PCR in some tissue homogenates. The cumulative percent mortality following *E. ictaluri* exposure was significantly lower in the vehicle-control group compared to those exposed to 50 μg kg^{-1} ($P < 0.001$) or 5 mg kg^{-1} ($P = 0.008$), respectively. Similarly, the vehicle-control fish exposed to *E. ictaluri* had a significantly higher cumulative percent mortality than non-challenged fish ($P = 0.002$, Table 3). Kaplan–Meijer survival analysis revealed that in addition to differences in total survival, the kinetics of mortality differed. However, this was only the case when the controls and low dose exposure groups were compared ($P = 0.024$), but not controls and high dose groups ($P = 0.083$, Fig. 7). There was no effect of sex on total mortality or mortality rate.

3.9. Plasma based immune factors

Brown bullheads exposed to 50 μg kg^{-1} or 5 mg kg^{-1} of A1254 had significantly lower titers of circulating IgL(G) antibodies that bind epitopes of *E. ictaluri* ($P = 0.005$ and $P = 0.004$, respectively) than vehicle-control bullheads prior to bacterial challenge. Antibody titers were significantly higher in survivors of the control

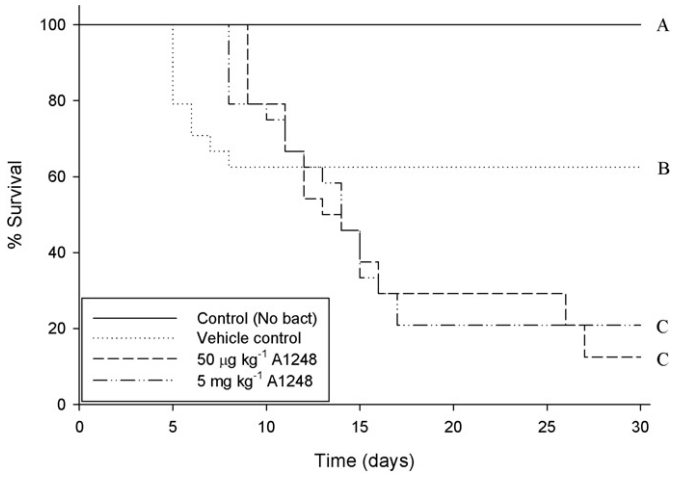

Fig. 7. Survival curves of brown bullheads challenged with *Edwardsiella ictaluri* following a 3-week i.p. exposure to 50 μg or 5 mg kg^{-1} of Aroclor 1248. Day 30 cumulative mortality values denoted with different letters are significantly different; Fisher's exact test; $P \leq 0.05$; $n = 24$ fish per treatment.

($P = 0.004$), 50 μg kg^{-1} ($P < 0.001$) or 5 mg kg^{-1} ($P = 0.001$) groups challenged with *E. ictaluri* when compared to pre-challenge titers. The percent increase in titers was 675%, 2010%, and 1510% in vehicle control, 50 μg kg^{-1} or 5 mg kg^{-1} exposed fish, respectively. Post-challenge *E. ictaluri* IgL(G) Ab titers of surviving bullheads were 34% and 54% lower in the 50 μg kg^{-1} and 5 mg kg^{-1} groups, respectively compared to controls, but were not significantly different (Table 3).

Plasma lysozyme was similar between vehicle-control bullheads and A1248 exposed fish prior to pathogen challenge and in surviving fish. Lysozyme activity was not correlated with sex or other immune parameters. Fish that survived the *E. ictaluri* challenge had significantly increased plasma lysozyme compared to pre-challenge values in all treatment groups ($P < 0.001$), but there were not significantly differences among groups (Table 3).

4. Discussion

Numerous studies have demonstrated the immunotoxic effects of various PCB congeners in fish; however, such research is rarely performed in the context of endocrine physiology or to demonstrate direct impacts on disease resistance. The current research investigated the effects of A1248 exposure in brown bullheads under controlled laboratory conditions in an attempt to better understand the impact of this PCB mixture in this sentinel species. This work demonstrates that A1248 modulates aspects of innate and adaptive immunity, and decreases disease resistance to *E. ictaluri* in fish that previously survived an outbreak of the same pathogen. In addition, A1248 exposure modulated plasma cortisol and T3 in a fashion similar to that observed previously in salmonids following A1254 exposure (Lerner et al., 2007). Other physiological parameters including hepatic CYP1A expression and the hepatic somatic index were affected as well. Thus it appears that A1248 perturbs numerous physiological systems in brown bullhead likely via AhR and non-AhR mediated pathways. Given that the fish used for this work were naturally selected for resistance to *E. ictaluri*, it is expected that some immune responses exhibited less variation than would be expected in non-selected population. This, however, provided an opportunity to examine aspects of immunological memory and responses to A1248 in individuals with less variable physiological and immunologic responses due to such selection. While the data collected here may not be representative of the average observed responses in wild bullhead populations natural population, it certainly reflects that of subpopulations in the wild.

An increase in hepatosomatic index due to A1248 exposure was observed in males, but not females. Hepatomegaly is a common response to contaminant exposure and is often the result of cellular hypertrophy of parenchymal cells. This hypertrophy is a direct result of increased production of metabolic enzymes that facilitate the modification of toxic substances into less toxic forms that have increased water solubility and are more easily excreted. One such protein that is induced by coplanar PCBs and other AhR agonists is cytochrome P4501A (CYP1A). Here, hepatic CYP1A was increased in males in a dose-dependant manner, but not in females. This obser-

vation is not unprecedented as 17β-estradiol is known to down regulate CYP1A expression (Navas and Segner, 2001; Elskus, 2004). Females in this study had significantly higher plasma concentrations of 17β-estradiol than males, and plasma E2 was significantly negatively correlated with CYP1A protein expression in females. Thus, it is likely that the elevated levels of this female sex steroid led to a reduction in CYP1A protein and perhaps other hepatic-derived toxic response proteins. Females in the control groups had a higher HSI than males likely due to hepatic production of vitellogenin in response to the elevated E2. The increase in CYP1A while significantly different in some cases was not as high as that measured by others (Elskus and Stegeman, 1989). Based on a pilot trial, this specific lot of A1248 induced hepatic CYP1A expression to 11.43 ± 3.68 and 27.21 ± 4.45 pmol CYP1A mg total protein^{-1} in bullheads injected with 0.05 and 5 mg kg^{-1} A1248, respectively 1-week post-exposure. Thus, it is likely that levels of CYP1A protein were higher shortly after exposure initiation in the 3-week study, but waned over time. Our pilot studies also indicate that this lot of A1248 induces CYP1A to a lesser extent than A1254 (Lot# LB58885) or similar concentrations (5 mg kg^{-1}) of the coplanar PCB-126. Thus it appears that perhaps the A1248 used here has a lower concentration of coplanar PCBs, and that the biological effects are not exclusive to AhR mediated mechanisms.

Aspects of functional immune responses were clearly affected by A1248 exposure. For instance, bactericidal activity of adherent anterior kidney leukocytes (primarily macrophages) was significantly reduced. Other studies have also demonstrated that macrophage function is negatively impacted by PCB exposure. For instance, phagocytic activity of macrophages or their functional homologues is reduced in other species of fish, mammals and some invertebrates (Jones et al., 1979; Goven et al., 1993; Lacroix et al., 2001; Canesi et al., 2003; Levin et al., 2005). Likewise respiratory burst activity is increased or decreased (Rice and Schlenk, 1995; Regala et al., 2001). The production of reactive ions (superoxide, hypochlorite, hydrogen peroxide, or nitric oxide) play an integral role in antibacterial activities of macrophages; however, they are not the only factors involved. Enzymes and peptides (i.e. lysozyme, defensins, hepcidin) and pH driven changes during phagosome–lysosome fusion play a critical role in intracellular killing while other peptides facilitate intracellular and extracellular killing. In the current study, ability of bullhead macrophages to produce reactive gas species in response to LPS stimulation was not impacted. It is unknown if there was a specific effect on phagocytosis, however, bactericidal activity of this cell population was clearly negatively impacted.

Non-specific cytotoxic cells are known to have a pivotal role in innate immune responses (Graves et al., 1985; Jaso-Friedmann and Evans, 1999; Jaso-Friedmann et al., 2000). Previously, intraperitoneal exposure of the coplanar PCB-126 has been shown to effect natural cytotoxic cell (NCC) activity in channel catfish (Rice and Schlenk, 1995). That work demonstrated that NCC activity was reduced by 1 mg kg^{-1} PCB-126 at 3 and 7 days post-exposure. While there appeared to be a qualitative decrease in NCC activity, this change was not statistically significant. Exposure also lessened activity of NCC activity in females to a level more similar to that of males. The lack of statistical significance may reflect the high variability of activity of this cell type and low sample size in the case of the comparisons between sexes.

In mammals PCB exposure is associated with thymic atrophy (Grasman and Whitacre, 2001), leukopenia, shifts in T-cell populations (Lai et al., 1995), decreased antibody production (Tryphonas et al., 1991), and differential sensitivity of lymphocytes to lectin stimulation (Segre et al., 2002). In the present study, there was a slight but apparent shift in anterior kidney leukocyte populations based on size (forward scatter) complexity (side scatter) properties of leukocytes as determined by flow cytometry. These changes did not reflect a change in IgL(G)$^{+}$ B-cells, but this population may not be reflective of the entire B-cell repertoire. Additionally, long-lived plasma cells that purportedly reside in this tissue are not dressed with membrane-associated IgM as found in circulating naïve B-cells (Bromage et al., 2004). A lack of appropriate cell-surface markers for bullhead leukocytes makes it impossible to conclusively determine specifically which leukocyte population(s) contributed to this shift. Neutrophila as observed by Rice and Schlenk (1995) was not observed here.

Significant modulation in T-cell responsiveness to PHA-P was observed at both exposure concentrations in the current study. This is in agreement with similar work in other species of fish in which PCB exposure is associated with an increased mitogenic response of T-cells (Arkoosh et al., 1996; Iwanowicz et al., 2005). Exposure to PCB mixtures has been shown to increase the mitogenic response to the T-cell mitogens concanavalin A and phytohemagluttinin in rats (Smialowicz et al., 1989), mice (Wu et al., 1999; Segre et al., 2002) and birds (Smits et al., 2002). The toxicity of PCBs to lymphocytes has been well established in a number of animal models and T-cells appear to be most sensitive (Sweet et al., 1998; Miller et al., 2002). While an increase in T-cell mitogenesis seems contrary to expectations of a toxic response it has been suggested that PCBs may (1) lead to the selection of T-cell subpopulations more responsive to mitogen stimulation; (2) target regulatory T-cell-like populations that down-regulate the proliferative response (Arkoosh and Kaattari, 1987) or (3) alter cytokine and cytokine receptor expression thus resulting in a modulated proliferative response (Kwon et al., 2002; Segre et al., 2002). Likewise, tyrosine kinase-mediated cell signaling may be affected (Canesi et al., 2003). Certainly a combination of these explanations is possible as well.

While the present research with brown bullheads clearly demonstrates that immune function as measured by *in vitro* functional assays is modulated by PCB exposure suggesting that these immune-associated endpoints may be useful for assessing immune health in wild bullhead populations, results of the *in vivo* challenge experiment and pathogen-specific antibody titers bear more profound implications. At the commencement of the PCB exposure experiments, bullheads had moderate titers of specific antibodies against *E. ictaluri* due to exposure to this pathogen approximately 1 year prior (Iwanowicz et al., 2006). Previous experiments with channel catfish have demonstrated that *E. ictaluri* agglutinating antibody titers confer some protection to subsequent exposure (Vinitnantharat and Plumb, 1993). Exposure to A1248 in the current work led to a reduction in these antibody titers by 453% and 481% in low or high dose exposed fish respectively compared to the control group. Work by Bromage et al. (2004) has demonstrated the presence of long-lived plasma cells that reside in the anterior kidney and maintain serum antibodies from previous exposure to pathogens. It is unknown if this cell type is specifically sensitive to PCB exposure, but a depletion of this cell type would explain the observed decrease in Ab titers. A qualitative decrease in anterior kidney leukocytes was not observed in the current work at the time point evaluated; however, a depopulation of AKLs following PCB exposure is likely and has been observed previously (Iwanowicz et al., 2005). Depopulation of regulatory T-cell populations that may maintain a tissue-specific environment conducive to the maintenance of function of long-lived plasma cells would also lead to a similar observation.

While exposure to A1248 led to a decrease in *E. ictaluri* reactive antibodies it did not ablate the amnestic response upon subsequent exposure. Previous work by Stolen (1985) demonstrated that PCB exposure leads to a delayed humoral response to specific antigen in fish immunized with *E. coli*. A shift in response kinetics would explain our observations. Correspondingly, Chinook salmon exposed to sublethal doses of Aroclor 1254 exhibit a suppressed primary and secondary plaque forming-cell (B cell) response to the T-cell-independent antigen TNP-LPS which indicates a direct

impairment of B-cells (Arkoosh et al., 1994a,b). Others have demonstrated no effect on the amnestic response following PBC exposure (Jones et al., 1979). In the present work it is not known if the non-survivors mounted similar antibody responses, or if an attenuated response was associated with an inability to survive infection.

Exposure to A1248 was also associated with a concomitant reduction in disease resistance (increased lethality) to *E. ictaluri*. Previous studies regarding the effects of PCB exposure on disease resistance have yielded inconsistent results. For instance, Arkoosh et al. (2001) demonstrated that Aroclor 1254 exposure leads to a higher predisposition to infection and subsequent disease in Chinook salmon at environmentally relevant concentrations. Similarly, reduced disease resistance to *Aeromonas salmonicida* has been observed in fasting Arctic charr (*Salvelinus alpinus*) with laboratory controlled A1254 body burdens (Maule et al., 2005). Conversely, Mayer et al. (1985) reported increased disease resistance to *Y. ruckeri* in rainbow trout exposed to A1254 and 1260 when challenged with this pathogen via bath exposure. Interestingly, fish injected i.p. with *Y. ruckeri* died more quickly than controls. Others have found that PCB exposure has no effect on disease resistance in salmonids challenged with infectious hemorrhagic septicemia virus (IHNV) or *Listonella anguillarum* (Spitsbergen et al., 1988; Powell et al., 2003). Based on the available data it is likely that factors including dose and route of exposure are important determinants of whether PCB exposed fish will succumb to microbial pathogens. The specific pathogen is likely a critical variable as well.

The pathogenesis of *E. ictaluri* in channel catfish is complex and not fully understood. While increased antibody titers to *E. ictaluri* are sometimes associated with increased disease resistance (Vinitnantharat and Plumb, 1993; Klesius and Sealey, 1995), often increased titers resulting from vaccination offer little or no protection (Saeed and Plumb, 1986; Thune et al., 1997). Thus it is suspected that cell mediated immunity (CMI) involving T-cells is a significant component of a protective host response to this pathogen (Lawrence et al., 1997). An important aspect of *E. ictaluri* pathogenesis is the reliance of phagocytic cells for dissemination (Shotts et al., 1986; Janda et al., 1991; Baldwin and Newton, 1993; Reger et al., 1993). This pathogen also exploits the immunoprivileged site of the macrophage phagosome to replicate (Booth et al., 2006). In the current study aspects of macrophage function, T-cell and the amnestic response were clearly modulated by A1248 exposure. Given that numerous components of immune function were affected by A1248 exposure it is not possible to speculate which altered function is associated with the increased mortality. It is likely that the altered macrophage function affected the kinetics of pathogenesis, and the cumulative immunotoxicity of A1248 contributed to enhanced pathogenesis. Regardless of the mechanism, A1248 exposure clearly increased disease susceptibility to this organism.

The current work provides evidence that A1248 leads to a significant reduction in circulating T3 in bullheads at environmentally relevant concentrations. This is consistent with the findings in other teleosts including American plaice, Arctic charr, coho salmon and Atlantic salmon exposed to PCBs (Leatherland and Sonstegard, 1980; Adams et al., 2000; Jorgensen et al., 2004; Lerner et al., 2007). Mechanisms proposed for this decrease include a reduction in outer ring deiodinase activity required for the conversion of T4 to T3 (Adams et al., 2000). Although it is unknown which deiodinases are present in bullheads, a decrease in T3 could also be explained by an increase in type III deiodinase that effectively cleaves the inner ring of thyroid hormones (T4 and T3) or type I deiodinase that deiodinates both rings of T4 and T3. This mechanism seems unlikely given that plasma T4 concentrations were unaffected. Increased sulfonation and subsequent excretion of T3 which is a natural nondeiodinative process of T3 clearance is also possible; however, hydroxylated-PCBs are known to decrease sulfotransferase activity (LoPresti and Nicoloff, 1994; Wang and James, 2007).

While sex steroids were not significantly affected by A1248 exposure there was a significant reduction in plasma cortisol at the high dose. Previous studies in fish have demonstrated that PCB exposure affects the stress response likely via exhaustion of the hypothalomus–pituitary–interrenal (HPI) axis (Sivarajah et al., 1978; Quabius et al., 1997; Hontela et al., 1992; Aluru et al., 2004). Others have suggested that the down-regulation of cortisol by PCBs is the result of reduced steroidogenic acute regulatory protein and CYP11A (20,22-desmolase) expression that are required for steroidogenesis (Aluru et al., 2005). The relationship between cortisol and immune function is complex. High physiological concentrations of this glucocorticoid are immunosuppressive; however, cortisol is immunostimulatory as well. In the current study there were no clear correlations between hormone concentrations and immune function. Thus it is unlikely that the decrease in cortisol concentration observed here is directly associated with the immunomodulatory effects of A1248.

Degradation of aquatic ecosystems has been an unfortunate consequence of anthropogenic activity during the last century. Although myriad factors influence the health of these systems, the introduction of chemical contaminants has been shown to exert biologically meaningful effects on aquatic communities. Given the need by agencies with custodial responsibilities to monitor for such effects, fish have been advocated as sentinels of water quality and aquatic environmental health for decades. Suitable, validated biomarkers and methods must be established on a species-by-species basic to legitimately perform such monitoring. The current research provides data from laboratory controlled conditions and documents the effects of biologically relevant concentrations of A1248 on bullhead immune and endocrine parameters. While it is unlikely that the exposure duration here satisfactorily simulates exposures experienced in contaminated aquatic ecosystems, the data provide evidence that this mixture of PCBs is capable of modulating immune and endocrine parameters in bullheads. Our data also suggest that while this mixture affects some immune parameters in a sex-independent fashion, aspects of macrophage function are affected in a sex-dependant manner. Perhaps of greatest organismal significance, exposure to Aroclor 1248 increases the susceptibility of bullheads to morbidity or mortality upon exposure to the ictalurid enteric pathogen *E. ictaluri*. Therefore, exposure of fish to A1248 and other PCB mixtures in nature may make them more susceptible to disease following encounters with pathogens.

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