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Histologic, immunologic and endocrine biomarkers indicate contaminant effects in fishes of the Ashtabula River

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Abstract The use of fish as sentinels of aquatic ecosystem health is a biologically relevant approach to environmental monitoring and assessment. We examined the health of the Ashtabula River using histologic, immunologic, and endocrine biomarkers in brown bullhead (BB; *Ameiurus nebulosus*) and largemouth bass (*Micropterus salmoides*) and compared fish collected from a reference site (Conneaut Creek). Seasonal analysis was necessary to distinguish differences in fish between the two rivers. Overall BB from the Ashtabula River had a lower condition factor and significantly more macrophage aggregates than those from the reference site. Reduced bactericidal and cytotoxic-cell activity was observed in anterior kidney leukocytes from both BB and largemouth bass from the Ashtabula River. Lower plasma thyroxine and triiodo-L-thyronine in both species in the Ashtabula River indicated disruption of the thyroid axis. Differences in physiological biomarker responses were supported by body burden chemical concentrations when data were analyzed on a seasonal basis. The use of two fish species added a level of rigor that demonstrated biological effects were not exclusive to a single species. The results provide strong evidence that contaminants have affected fish in the Ashtabula River, a Great Lakes Area of Concern, and provide a baseline by which to evaluate remediation activities.

Keywords Immunomodulation · Endocrine disruption · Bullhead · Bass · Great Lakes · Area of Concern · Ashtabula River · Northeastern Ohio

Introduction

The Ashtabula River is located in northeastern Ohio and drains a 350 km^2 watershed that empties into the central basin of Lake Erie (Imamoglu et al. 2002). Like many rivers that flow into Lake Erie, the Ashtabula River is contaminated with a multitude of industrial chemicals, and the lower two miles is listed as a Great Lakes Area of Concern (AOC). This AOC designation was issued by the International Joint Commission during 1988 based on numerous Beneficial Use Impairments (BUI) including, but not limited to (1) restrictions on fish and wildlife consumption, (2) degradation of fish and wildlife populations, and (3) fish tumors or other deformities. Contamination of the Ashtabula River is primarily the result of decades of industrial metals-fabrication, chemical production and mismanagement of waste disposal along Fields Brook, a United States Environmental Protection Agency (USEPA) Superfund site. Fields Brook is a tributary of the Ashtabula River with a confluence at approximately river kilometer 2.57, and was the major source of hazardous substances

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present in the lower Ashtabula River. Concentrations of dissolved phase polychlorinated biphenyls (PCBs) that exceed 89 ng L^{-1} have been measured in the Ashtabula River just downstream of the confluence of Fields Brook during 2001. Similarly, concentrations of dissolved phase hexochlorobenzene (HCB) that exceed 18 ng L^{-1} have been observed (USFWS 2004). Contaminants present in the Ashtabula River benthos and harbor include polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), organochlorine pesticides and industrial compounds (OCPs) and heavy metals.

Bioaccumulation of mercury and PCBs in resident fish populations of the Ashtabula River is a major concern in regards to human health in this geographical region. Consumption advisories have been issued for fishes in the Ashtabula River due to body burden concentrations of contaminants that exceed limits for human consumption (USFWS 2005). Indications of contaminant-associated detrimental impacts on fish health in the Ashtabula River include the observation of preneoplastic and neoplastic skin and liver lesions in brown bullhead (BB; Blazer et al. 2009a, b). Likewise, the Ashtabula River supports lower fish community diversity and biomass compared to the Ohio Environmental Protection Agency (EPA) designated reference area, Conneaut Creek. The difference in the fish communities between the two sites is attributed to contamination (Ohio EPA 2001). While there has been regular monitoring of contaminants in fish from the Ashtabula River and a handful of grey literature publications synthesized regarding water-borne and sediment associated contaminants; there has been little effort to assess the general health of Ashtabula River fishes using biological end-points other than cancer. The recent initiation of a natural resource damage assessment under the Comprehensive Environmental Response and Liability Act (CERCLA) by the U.S. Fish and Wildlife Service (USFWS) and the Ohio EPA has garnered interest in this specific issue.

The prevalence of neoplasia in BB is listed as a Beneficial Use Impairment (BUI) in Great Lakes Areas of Concern. Neo-plasia in BB from these locations is often attributed to PAH exposure, although other contaminants such as PCBs and other organochlorine contaminants are often present as well (Black 1983; Black et al. 1985; Baumann et al. 1990, 1991; Moore and Myers 1994; Rafferty et al. 2009). 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, neoplasms may take years to develop following relevant contaminant exposure. For this reason, neoplasia may not always be an appropriate end-point (in regards to sensitivity) of contaminant-associated biological insult in some locations. Physiological end-points that measure functional attributes of critical health related parameters such as immune and endocrine function are likely more sensitive biomarkers as they take less time to manifest. Used in tandem with less time sensitive biomarkers, a suite of physiological end-points could potentially yield a high resolution comparison of fish health between different, but geographically similar rivers.

In an attempt to assess the relative health of fish that inhabited the Ashtabula River prior to remediation a multi-tier, multi-season, multi-species field-study was designed. Brown bullhead (*Ameiurus nebulosus*) and largemouth bass (LMB; *Micropterus salmoides*) were selected as the target species and were collected during April, July and October to account for possible seasonal variability. Fish were collected from the Ashtabula River and from the less impacted reference river, Conneaut Creek, for comparison. General and specific biomarkers of fish health included measures of condition factor and organosomatic indices, gross necropsy-based observations, histopathological biomarkers, functional immune status, and plasma sex and thyroid hormone concentrations. Contaminant body burdens were also quantified and a comprehensive statistical analysis executed to infer relationships between chemical contamination and biomarkers. This comprehensive fish health assessment was sufficient to identify differences in fish health between the Ashtabula River and Conneaut Creek. Additionally, differences in biomarker responses were noted between species and across seasons that were associated with contaminant body burden.

Materials and methods

Species and sites

Brown bullhead inhabit the benthos of warm-water aquatic ecosystems and are an opportunistic benthivore that commonly burrow into soft sediment (Loeb 1964; Keast 1985). This benthic life history leads to chronic, intimate contact with potentially contaminated sediment and makes it an exemplary species for biological heath assessments (Baumann 1984; Pinkney et al. 2001). The LMB is a pelagic, top carnivore of commercial and recreational importance. The diet of the LMB leads to biomagnification of lipophilic contaminants including the organochlorine legacy compounds. Here, the BB and LMB were selected as target species due to their different life-history strategies and regional availability. Sampling was conducted in the Ashtabula River and the Ohio EPA designated reference site, Conneaut Creek. The Ashtabula River and Conneaut Creek are similar in regards to conventional water quality parameters, physical habitat and both are affected by seiches from

Lake Erie. While the physical habitat is somewhat better in the Ashtabula River, the most significant difference between these rivers is the level of contamination. Fish were collected from the Ashtabula River (near 41°53′59″N 80°47′38″W) between river kilometer (RK) 2.7 and 0.9 and between RK 3.2 and 1.0 of the reference site, Conneaut Creek (near 41°57′54″N 80°32′42 W). Fish were also sampled from the southwestern section of Conneaut Creek harbor. The vector distance between these sites is 22 km.

Fish collection and necrospy-based assessment

Largemouth bass and BB were sampled at each site over a 1- or 2-day sampling period using a pulsed DC electroshocking boat during October 2002, July 2003, October 2003 and April 2004. The target sample size was 12 fish of each sex for each species. A minimum length of 250 mm was targeted for both species to obtain sexually mature individuals. Fish were euthanized with a lethal dose of MS-222 prior to necropsy. Blood was drawn from the caudal vessels with a heparinized 5 cc syringe, transferred into a heparinized Vacutainer® tube, and stored on wet ice. Plasma was separated from the cellular fraction via centrifugation at 2000*g* for 10 min at 4°C within 4 h of collection. The plasma samples were frozen at −80°C and stored for analysis.

A complete necropsy-based fish health assessment as described by Goede and Barton (1990) was conducted. Briefly, total length (TL) and body mass were measured for all fish. The condition factor (K), hepatosomatic index (HSI) and gonadosomatic (GSI) were calculated by $K = 10^5 \times \text{weight/length}^3$, $HSI = 100 \times (\text{liver weight}/(\text{body weight} - \text{gonad weight}))$, $GSI = 100 \times (\text{gonad weight/body weight})$, respectively. External abnormalities including melanistic spots on body surfaces, raised lesions in the oral cavity and body surfaces, and missing, shortened and deformed nasal, maxillary, and chin barbels (for BB only) were recorded. See Yang et al. (2006) for synthesis of these data. Fish were aseptically necropsied and a portion of the anterior kidney was removed and placed into processing medium (PM; isotonic Leibovitz-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) at 4°C. Within an hour the tissue was homogenized with a sterile, hand-held Ten Broek homogenizer, stored on wet-ice and shipped to the Leetown Science Center, National Fish Health Research Laboratory, Leetown, WV, overnight for processing. Livers and gonads were removed and weighed to calculate organosomatic indices. Portions of liver, kidney, spleen, gonad and any lesioned tissue were removed and fixed in Z-Fix® (Anatech LTD, Battle Creek, MI) for histological analyses. Pectoral spines were removed from BB and scales (right side above the pectoral fin and lateral line) from LMB for age determination. During the October 2003 and May 2004 sampling carcasses of both species were wrapped in foil and frozen for chemical analyses.

Immunological analyses

Unless otherwise noted, all media components were obtained from Sigma Chemical Company (St. Louis, MO). All media was adjusted to the appropriate osmolality of each species (296 and 270 mOsm for LMB and BB, respectively).

Leukocyte isolation

Anterior kidney leukocytes were isolated and processed as described by Iwanowicz et al. (2009). Anterior kidney tissue from LMB and BB aseptically processed in the field were received within 24 h of excision. Simulations of this process in our laboratory have demonstrated that the impact on cell viability and function is negligible (data not shown). Tissue preparations were resuspended in 10 mL of PM and allowed to settle for 30 min on wet-ice to remove fragments. Supernatants containing single cell suspensions were transferred to a new sterile polypropylene conical tube and 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 and suspended in PM. Largemouth bass cell suspensions were then layered on 32% Percoll in Hanks Balanced Salt Solution without phenol red, pH 7.2 (HBSS) and BB cell suspensions were added to Histopaque® 1077. The cells were centrifuged at 500*g* for 40 min or 300*g* for 20 min at 4°C for LMB and BB, respectively, and the leukocyte fraction was removed from the interface of the discontinuous gradient. 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 culture medium (CM; L-15 media supplemented with 5% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin) or adherence medium (AM; L-15 supplemented with 0.1% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin). All tissues and cell suspensions were kept cold in an ice bath during processing.

Bactericidal activity

Functional assessment of adherent anterior kidney leukocytes 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 LMB or BB 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 cells 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 *Yersinia ruckeri* (Hagerman strain; NFHRL # 11.40) washed and suspended in HBSS ($OD_{600} = 1.5$) 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 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 − (CFU treated/CFU control) × 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.

Respiratory burst

The production of extracellular reactive oxygen species (ROS) was determined using the peroxidase luminol-enhanced chemiluminescence (PLCL) assay described by Coteur et al. (2002) as modified 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 as above, washed and subsequently incubated in CM to allow cells to reach a resting state. Culture medium was then removed and cells were gently washed with room temperature HBSS. Cells were then treated in quadruplicate with 25 µl of 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, and incubated for 1 min. The lectin LPS was selected as a stimulant rather than phorbol 12-myristate 13-acetate or zymosan to more closely simulate the response to a bacterial encounter. The PLCL reaction solution (500 µM luminol, 13.2 U ml^{-1} in HBSS was then added. Plates were immediately loaded into a SpectraFluorPlus microplate reader (Tecan, Austria; gain 180, integration 500 ms) and luminescence measured every 5 min for 20 min. Stimulation index (SI) values for LPS-induced ROS were calculated as the replicate mean luminescence for a given set of LPS-treated leukocytes divided by the replicate mean luminescence of the HBSS treated control. Stimulation index values were calculated for all time points and the maximum SI (mSI) value for a given treatment was defined as the highest SI value determined during the 20 min read.

Lymphocyte mitogenesis

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. 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 Vmax Kinetic Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA, USA).

Stimulation index (SI) 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.

Cytotoxic cell activity

The ability of LMB or BB anterior kidney leucocytes to lyse epithelioma papulosum cyprini (EPC) cells was determined using the calcein AM release-based cytotoxic cell assay as described by Iwanowicz et al. (2004). Fluorescence intensity (FI) was measured reading the plates from the bottom using a SpectraFluorPlus (Excitation = 485, Emission = 535 and gain = 60).

$$\text{Cytotoxic-cell activity } (\%) = 100 \times \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Total release} - \text{Spontaneous release}}$$

Histopathology

Tissue processing and staining

Tissues including liver, spleen, anterior and posterior kidney, and skin were processed for histology, embedded into paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E) or the Perl's method for iron (Luna 1992). The latter staining method was utilized to prepare tissues

for macrophage aggregate analysis. All tissues were examined for histological anomalies and to determine the cause of any observed gross lesions. Diagnostic criteria for neoplastic and preneoplastic liver and skin lesions were classified as described in Blazer et al. (2006, 2009a, b).

Macrophage aggregate quantification

Splenic macrophage aggregate (MA) parameters were evaluated in both LMB and BB (Fournie et al. 2001). Aggregates defined as three or more macrophages greater than 50 μm^2 were enumerated and measured in 10 fields at 25× using a microscope fitted with a video camera. Images were captured and analyzed with SigmaScan image analysis software (Jandel Scientific Software). Parameters including MA area, the number of aggregates × mm^2 of $tissue^{-1}$ and percent area of tissues occupied was quantified.

Hormone analysis

Plasma samples from BB and LMB were analyzed for the sex steroids 17β-estradiol (E2) and testosterone (T), and the thyroid hormones thyroxine (T_4) and triiodothyronine (T_3). All samples were quantified via radio immune assay (RIA). Antibodies were purchased from Accurate Scientific (T_4 and T_3), the lab of Gordon Niswender, University of Colorado (E2; #244 anti-estradiol-6-BSA) and Coralie Munroe, UC Davis (T; polyclonal R156/7). Extraction efficiencies were determined for the sex steroids, and all assays were validated and optimized for BB and LMB (data not shown). Plasma T_3 and T_4 were measured by direct RIA according to Dickhoff et al. (1978). The sex steroids E2 and T were determined using the RIA method of Sower and Schreck (1982).

Contaminant analysis

Whole body burden chemical analysis was performed on all BB and LMB collected during the October 2003 and April 2004 sampling. Analysis was performed by the Geochemical and Environmental Research Group (College Station, TX) according to standard methods. Contaminants analyzed and considered for the following analyses included total polychlorinated biphenyls (PCB), hexachlorobenzene (HCB), pentachloroanisole (PCA), lindane (β-HCB), α-chlordane, oxychlordane, heptachlor epoxide, aldrin, dieldrin, and the dichloro-diphenyl-trichloroethane related compounds (p,p-DDT, o,p-DDT, p,p-DDE and p,p-DDD). Concentrations of many contaminants were below the level of detection (LOD), particularly in fish collected from Conneaut Creek. For values < LOD, a value of one half the censored data was assigned for statistical analysis and graph construction.

Statistical data analysis

We used ordination techniques and nonparametric statistical tests to evaluate fish biomarker relationships to contaminant body burdens. We ordinated fish samples (170 individuals) by biomarkers using non-metric multidimensional scaling (NMS) and evaluated variation due to stream, species, season, and sex. Biomarker variables are listed in Table 1. To control for covariates, we further evaluated NMS biomarker ordinations between streams and within species × season × sex categories. For all ordinations, we used $\log(x + 1)$-transformations for ordinal data, arcsine transformations for percent data, and Bray−Curtis distances (Bray and Curtis 1957) to reduce effects of outliers (McCune and Grace 2002). We then represented individual contaminants with Kendall *tau* correlation coefficients $> |0.30|$ as vectors in biomarker-space. Ordinations thus evaluated biomarker–contaminant relationships within all sampled individuals and within species × season subsets.

All data were tested for normality with the Shapiro-Wilks *W* test and homogeneity of variance via Levene's test of homogeneity of variances. The Wilcoxon rank-sum test was used to analyze non-transformed data for inter-river comparisons at each sampling date. This non-parametric analysis was used regardless of the data distribution of immune biomarkers. Differences were considered statistically significant when $P \leq 0.05$. Spearman's rank correlation analysis was performed to evaluate relationships between immune function and contaminant body burdens. Statistical analyses were performed using SyStat 11 for Windows (SyStat Software Inc., San Jose, CA), Statistica 6 (StatSoft Inc., Tulsa, OK), and PC-Ord (MjM Software, Gleneden Beach, OR).

Table 1 Variation in immune and endocrine system biomarkers

Category	Variable	NMS I	NMS II
Immune	Corrected bactericidal activity	−0.008	−0.270
	Cytotoxic cell activity (10:1)	0.063	0.553
	% Adherent cells	0.046	0.100
	LPS stimulated ROS	−0.004	0.063
	Number of splenic MA/mm^2	−0.363	−0.004
	% Spleen occupied by MA	−0.256	0.026
Endocrine	17β-estradiol	−0.015	0.555
	Testosterone	−0.410	0.438
	T_4	0.559	0.090
	T_3	0.613	0.183

Values are Kendall *tau* correlation coefficients across NMS axes. NMS I primarily represents a gradient across species; NMS II primarily represents a gradient across seasons. The NMS ordination is plotted in Fig. 1

Results

A 2-dimensional NMS ordination explained 80.0% of the variation in biomarkers with 100% orthogonality and a final stress of 19.2 (Fig. 1). Scores along NMS I corresponded primarily to variation in endocrine biomarkers T_4 and T_3 whereas scores along NMS II corresponded to variation in immune and endocrine system biomarkers Cytotoxic cell activity (10:1) and 17β-estradiol, respectively (Table 1). Biomarker-space was organized more by species (LMB and BB) and season (fall and spring) than stream or sex (Fig. 1). However, biomarker differences between ASH and CON samples were identified within subsets defined by species and season (Fig. 2). Spring samples of LMB exhibited the weakest relationships to contaminant vectors (Fig. 2a) and fall samples of BB exhibited the strongest relationships in this regard (Fig. 2d; Table 2).

Non-metric multi dimensional scaling revealed that species and seasons show important differences in biomarker responses. When analysis controlled for the effects of species and season, differences in biomarker response were observed between rivers for all biomarker combinations except in LMB during the spring of 2004 (Fig. 2).

Necropsy and morphometrics: brown bullhead

Brown bullhead were unattainable from Conneaut Creek during October of 2002 thus inter-river comparisons were not possible. During July 2003, only five BB were collected from Conneaut Creek. Morphometric parameters between Ashtabula River and Conneaut Creek BB were not significantly different when combining both sexes. Females collected from the Ashtabula River had a significantly higher HSI than males ($P = 0.032$). This comparison was not possible for BB collected from Conneaut Creek as only one female was collected. Brown bullhead (8%) collected from the Ashtabula River were emaciated. Raised lip lesions were observed in 8.3% of BB from the Ashtabula River and were diagnosed as papillomas by histology. No skin neoplasia was observed in BB collected from Conneaut Creek in July (Suppl Table 1).

In October 2003 BB collected from Conneaut Creek were significantly longer ($P \leq 0.013$), heavier ($P \leq 0.003$) and had a higher condition factor ($P \leq 0.042$) than those from the Ashtabula River. The mean age of the two groups was not significantly different. The two fish with raised lesions from Conneaut Creek and one fish from Ashtabula

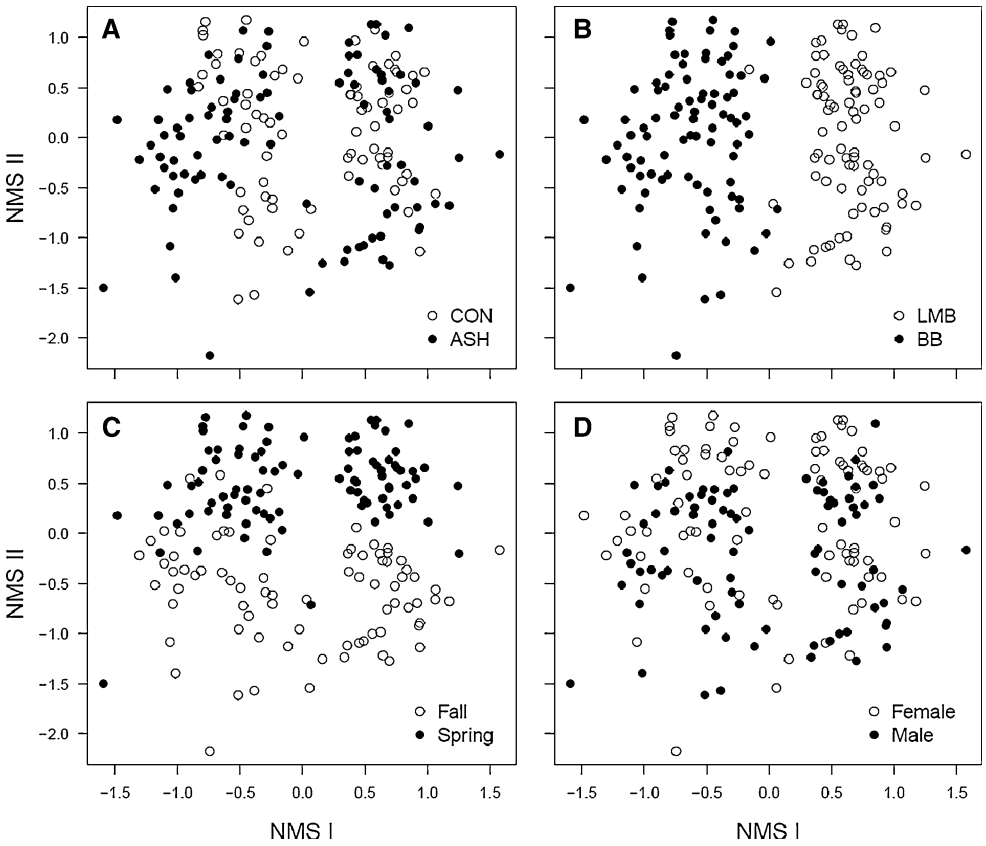

Fig. 1 Nonmetric multidimensional scaling (NMS) ordination of fish immune and endocrine system biomarkers across **a** streams, **b** species, **c** seasons, and **d** sex. Biomarker correlations to NMS axes are presented in Table 1

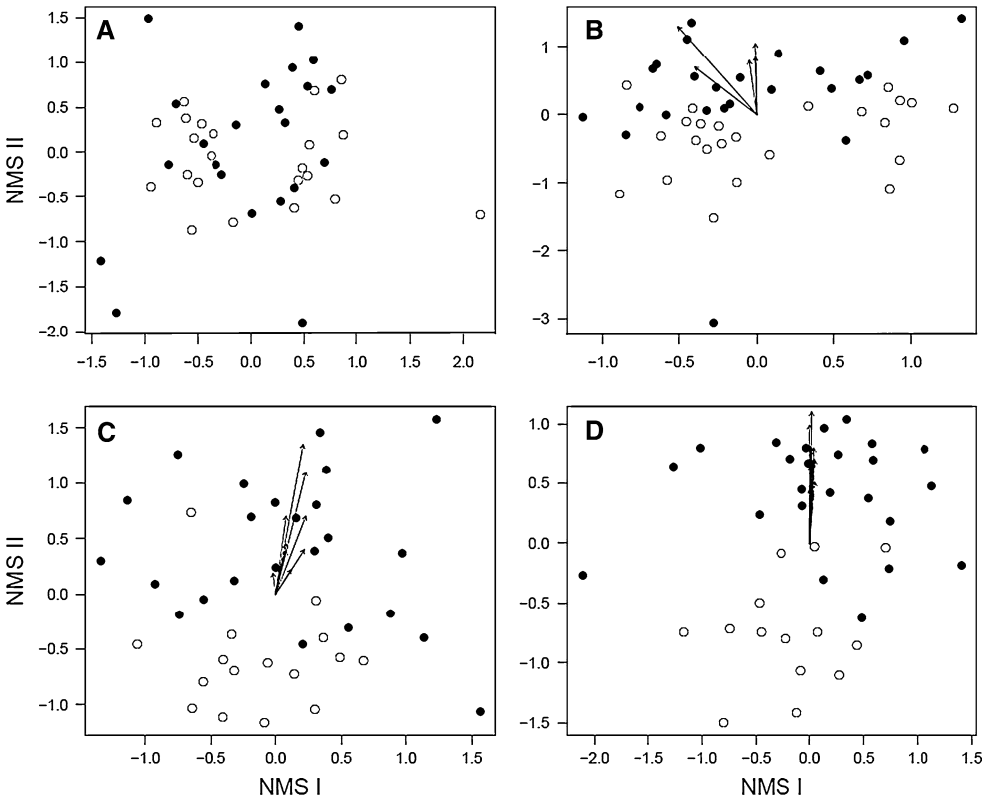

Fig. 2 Nonmetric multidimensional scaling (NMS) ordination of fish immune and endocrine system biomarkers for species × season combinations including **a** LMB-spring, **b** BB-spring, **c** LMB-fall, **d** BB-fall. *Closed circles* represent Ashtabula River sites; *open circles* represent Conneaut Creek sites. *Vectors* represent contaminants (Table 2)

Table 2 Contaminant body-burden relations to endocrine and immune system biomarkers

Variable	Largemouth bass		Brown bullhead	
	Spring	Fall	Spring	Fall
Total PCB (dry)	0.062	0.367	0.328	0.586
HCB (dry)	0.101	0.301	0.326	0.404
o,p′-DDE	−0.004	0.343	0.313	0.519
o,p′-DDT	0.024	0.390	0.287	0.559
Oxychlordane	−0.006	0.330	0.252	0.477
p,p′-DDD	−0.017	0.346	0.210	0.427
p,p′-DDE	−0.123	0.126	0.074	0.124
p,p′-DDT	0.071	0.225	0.188	0.471
Pentachloroanisole	−0.031	0.367	0.319	0.522
1234 TCB	−0.076	0.277	−0.279	0.309
Aldrin	0.017	0.316	0.248	0.460
Alpha chlordane	−0.115	0.294	0.283	0.548
Beta BHC	−0.015	0.232	0.233	0.643
Dieldrin	0.098	0.376	0.263	0.598
Heptachlor epoxide	0.148	0.272	0.302	0.331

Values are Kendall *tau* correlation coefficients for nonmetric multidimensional scaling (NMS) ordinations of fish biomarkers across streams (NMS axis II). Contaminants with *tau* coefficients > |0.30| are represented as vectors in Fig. 2

River were all diagnosed as papillomas microscopically. In addition, four of the BB from Ashtabula River had deformed fins (Suppl Table 1).

In April 2004 morphometric parameters of Ashtabula River and Conneaut Creek BB were not significantly different. Females had a significantly higher HSI than males ($P < 0.001$) regardless of the river of origin. Prevalence of raised lesions was statistically higher at Ashtabula River (Suppl Table 1). Histological evaluation of tissues collected from these lesions during April 2004 indicated that the raised lesions from Conneaut Creek BB consisted of inflammatory cells or hyperplastic epidermis with no characteristics of neoplasia. Of the 5 of BB with raised lesions collected at Ashtabula River, 2 were squamous cell carcinomas.

Necropsy and morphometrics: largemouth bass

Twenty-four LMB were collected from each site at each sampling event with the exception of October 2003 when only 16 LMB were collected from Conneaut Creek. During October of 2002 LMB collected at Conneaut Creek were qualitatively older than those collected at Ashtabula River, but this difference was not statistically significant. Largemouth bass from Conneaut Creek were significantly longer ($P \leq 0.001$) and heavier ($P < 0.001$). During the other sampling periods the mean age between sites was not significantly different and ranged between 3.3 and 3.6 years old. There were no significant differences in morphometrics between the two rivers in July 2003. During

the fall of 2003 LMB from Conneaut Creek were significantly heavier ($P \leq 0.026$) than those collected at Ashtabula River. In the spring of 2004 there were no significant differences between the sites (Suppl Table 2). The HSI of female LMB was, however, significantly higher ($P < 0.001$) than males regardless of river. Neoplasia was not observed in LMB from either the Ashtabula River or Conneaut Creek.

Immune function

Adherent cell (macrophage) function

Seasonal differences in bactericidal activity were observed in BB collected from Conneaut Creek (Fig. 3a). Bactericidal activity was significantly higher in October 2003 than either July 2003 ($P < 0.001$) or April of 2004 ($P = 0.032$). There were no significant differences in bactericidal activity between seasons in BB collected from the Ashtabula River. Bactericidal activity of adherent anterior kidney leukocytes from LMB collected from Conneaut Creek also differed by season (Fig. 3b). It was statistically higher during October 2003 compared to July 2003 ($P < 0.001$) and April 2004 ($P = 0.009$). While there appeared to be a similar season trend in LMB from the Ashtabula River, these seasonal differences were not statistically different. Bactericidal activity was generally lowest during July in both species from either river.

Bactericidal activity was significantly lower ($P < 0.001$, $P = 0.020$; Fig. 3a, b) in BB and LMB, respectively, from the Ashtabula River during October of 2003 compared to those from Conneaut Creek. During April 2004 bactericidal activity was significantly lower ($P = 0.040$) in Ashtabula River LMB when compared to the Conneaut Creek LMB. No differences were observed between rivers during the July sampling for either species.

Similar seasonal observations were observed in both BB and LMB in regards to LPS induced respiratory burst activity (Fig. 3c, d). Reactive oxygen species (ROS) induced by LPS during October of 2003 were significantly lower ($P = 0.002$) than that measured in April of 2004 for BB from Conneaut Creek. While BB from the Ashtabula River exhibited a similar trend, the season difference was not significant ($P = 0.102$). A seasonal shift in LPS stimulated respiratory burst activity was similar in LMB to that in BB from the same river. Stimulation was significantly lower ($P = 0.013$) during October of 2003 than April of 2004. Largemouth bass collected from the Ashtabula River, however, did not exhibit a statistically significant difference ($P = 0.054$) between seasons (Fig. 3c, d).

During October of 2003, LPS stimulated respiratory burst activity was statistically higher in BB and LMB from the Ashtabula River compared to those from Conneaut Creek ($P < 0.001$; Fig. 3c, d) in October 2003. Differences were not observed during April 2004 and the assay was not run during July 2003.

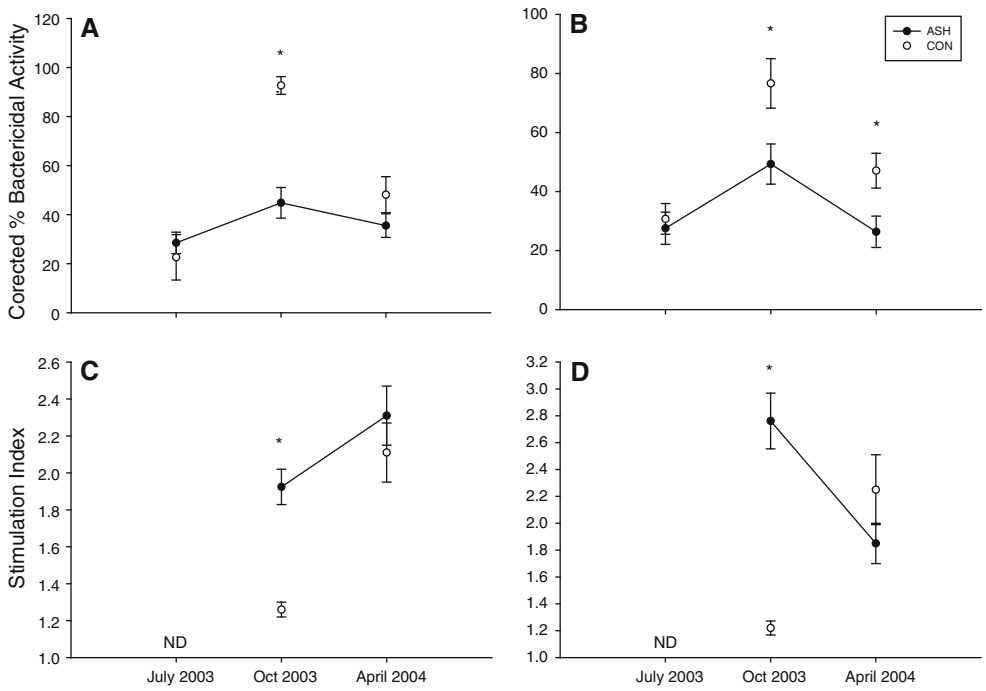

Fig. 3 Seasonal bactericidal and lipopolysaccharide-stimulated respiratory burst activity of adherent anterior kidney leukocytes from brown bullhead (**a**, **c**) and largemouth bass (**b**, **d**) collected from the Ashtabula River and Conneaut Creek. Data presented as mean ± standard error; * indicates a significant difference ($P \leq 0.05$)

Lymphocyte mitogenesis

Only five BB were collected form Conneaut Creek during July 2003. In general the stimulation index for all T-cell mitogens was higher in BB from the Ashtabula River when compared to Conneaut Creek. During October of 2003, the stimulation indices for all mitogens differed significantly, and were higher in BB collected from the Ashtabula River ($n = 24$); Con A ($P = 0.007$), PHA-P ($P < 0.001$) and PWM ($P < 0.001$) when compared to those from Conneaut Creek ($n = 14$; data not shown). Conversely stimulation indices were lower in the Ashtabula River during this sample period for LPS stimulated lymphocytes ($P = 0.006$). Significant differences ($P = 0.014$) in mitogenesis during April 2004 were observed in PWM sensitive lymphocytes (Suppl Fig. 1). Due to poor cell adherence of LMB leucocytes during this assay, data from these fish are not reported here.

Cytotoxic cell activity

Seasonal differences in anterior kidney-derived cytotoxic cell activity were observed in BB from both the Ashtabula River and Conneaut Creek. In the former, cytotoxic cell activity was greatest in April of 2004, and was significantly higher ($P = 0.031$) than that observed during October of 2003 (Fig. 4a). There were no statistical differences between the other seasons. Statistically significant differences were observed between all seasons in BB from Conneaut Creek, however. Again, activity of this cell population was highest in April of 2004, and statistically different than October of 2003 ($P = 0.007$) and July of 2003 ($P = 0.029$). In general cytotoxic cell activity was lowest in BB of both rivers during the fall.

Seasonal differences in cytotoxic cell activity were also noted in LMB. Mean cytotoxic cell activity was higher in April of 2004 in LMB from the Ashtabula River, and was significantly different than that measured in October of 2003 ($P = 0.010$; Fig. 4b). Cytotoxic cell activity was lowest during October of 2003 and significantly different ($P = 0.008$) from July of 2003. A similar seasonal pattern was noted in LMB from Conneaut Creek; however, statistically significant differences were on observed between April of 2004 and October of 2003 ($P = 0.002$) and July of 2003 ($P < 0.001$).

Cytotoxic cell activity was significantly lower ($P < 0.001$) in BB and LMB collected from the Ashtabula River during April 2004 compared to those from Conneaut Creek. Lower activity in this cell population was observed in LMB collected from the Ashtabula River October of 2003 season as well ($P = 0.010$). In general when differences were observed in cytotoxic-cell activity between rivers, lower activity was always observed in fish from the Ashtabula River. Similar trends in activity were observed between both species (Fig. 4).

Macrophage aggregate parameters

Seasonal comparisons: brown bullhead and largemouth bass

The microscopic appearance of macrophage aggregates differed between the different species. Brown bullhead MAs primarily contained ceroid and lipofuscin, and little hemosiderin. Conversely, LMB MAs stained an intense blue with Perl's prussian blue stain indicating the presence of the iron containing hemosiderin (Suppl Fig. 2). The number of MAs per mm^2 was evaluated in spleen and liver of BB and spleen of LMB. When tissues were surveyed for the presence of MAs, only BB had an appreciable quantity observed in the liver. For this reason, MAs were not quantified in LMB livers.

When macrophage aggregate data was analyzed independent of season there were no significant differences observed between rivers for LMB. Statistically more MAs per area ($P < 0.001$) were present resulting in a greater

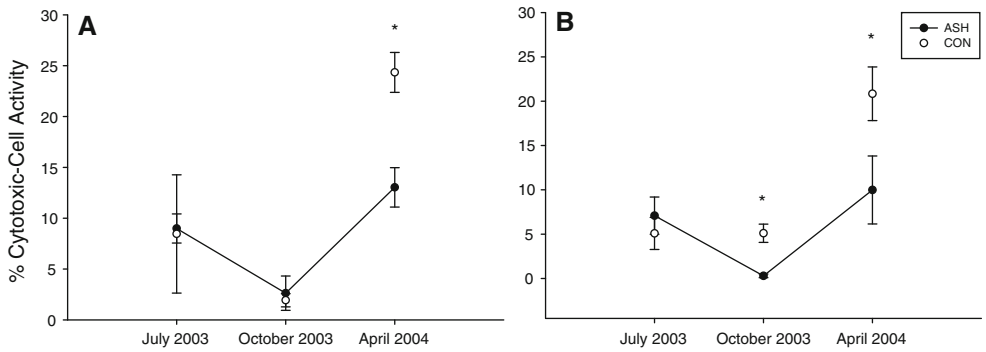

Fig. 4 Seasonal cytotoxic-cell activity of anterior kidney leukocytes from brown bullhead (**a**) and largemouth bass (**b**) collected from the Ashtabula River and Conneaut Creek. Data presented as mean ± standard error; * indicates a significant difference ($P \leq 0.05$)

Table 3 Comparison of macrophage aggregate parameters in largemouth bass and brown bullhead collected from the Ashtabula River and Conneaut Creek, 2002–2004

Parameter Largemouth Bass Spleen	Ashtabula $n = 96$	Conneaut $n = 88$
Mean age	3.3 ± 0.09	3.5 ± 0.10
Mean number	2.8 ± 0.23	2.7 ± 0.27
Percent tissue occupied	1.0 ± 0.11	1.0 ± 0.10
Bullhead spleen	$n = 99$	$n = 43$
Mean age	4.8 ± 0.16	4.0 ± 0.27
Mean number	6.6 ± 0.40*	3.6 ± 0.15
Percent tissue occupied	2.4 ± 0.20*	1.1 ± 0.07
Bullhead liver	$n = 99$	$n = 43$
Mean Number	1.9 ± 0.19*	0.8 ± 0.15
Percent Tissue Occupied	0.3 ± 0.04	0.2 ± 0.14

* Indicates a significant difference between sites at $P \leq 0.05$

percent area occupied ($P < 0.001$) were observed in the spleen of BB collected from the Ashtabula River compared to those from Conneaut Creek (Table 3). Correlation analysis revealed that age was significantly correlated ($P < 0.001$) with the number of MAs per area of tissue in all tissues examined for both species from both rivers; however, there was no difference in mean age in BB collected from the two rivers.

Histopathology

A number of microscopic lesions including bile duct proliferation, bile duct fibrosis, altered foci, and cholangioma (Suppl Fig. 3) were noted in the liver of BB collected from both rivers in the present study. The prevalence of bile duct proliferation was significantly higher ($P = 0.015$) in BB from the Ashtabula River compared to those from Conneaut Creek (Suppl Table 3). The frequency of focal necrosis, bile duct fibrosis, altered foci, and cholangioma was not statistically different between rivers. Only one fish (4 years old) from Conneaut Creek was observed with neoplastic biliary changes (cholangioma). Cholangiosarcoma was never observed in fish from this river. Of the Ashtabula River samples, seven fish (6–9 years old) were observed with cholangiomas and six (2–8 years old) had cholangiocarcinomas.

Reproductive health: brown bullhead

Gonadosomatic index

Seasonal differences in GSI were observed in female BB that coincided with the known reproductive cycle of this species (Suppl Fig. 4). Peak GSI was observed during April of 2004. While the difference in GSI was significantly higher ($P < 0.001$) during April of 2004 than October of 2003 in BB from the Ashtabula River, this was not the case for BB from Conneaut Creek ($P = 0.081$). In the case of males, again there was a significantly higher ($P = 0.015$) GSI in fish from the Ashtabula River between April of 2004 and October of 2003. Although there appeared to be a marked increase in GSI in male BB from the Conneaut Creek during this seasonal interval, it was not statistically significant ($P = 0.051$).

No statistical differences between sites were observed during any season in female BB. In general male BB from the Ashtabula had a higher GSI than those from Conneaut Creek; however, statistical differences were only observed during July 2003 ($P = 0.039$). Microscopic evaluation of the gonads of both male and female BB did not reveal any significant differences between those collected in the Ashtabula River and those collected in Conneaut Creek.

Sex steroids

Extraction efficiencies of BB plasma were 91 ± 2.4 and 85 ± 3.5% for E2 and T, respectively. Seasonal changes in E2 were similar in female BB from both rivers (Suppl Fig. 4D). The highest concentrations of E2 were measured during the spring collection season. The plasma E2 concentrations measured during this season were statistically higher than that during the fall of 2003 in females from the Ashtabula River ($P = 0.042$) and Conneaut Creek ($P = 0.043$). Plasma testosterone concentrations followed a similar seasonal trend as E2 and were highest during the spring sample season (Suppl Fig. 4E, F). This value was only statistically higher ($P < 0.001$) than that in October of 2003 in females from the Ashtabula River. There were no significant differences in E2 concentrations of female BB between sites during any collection season. Plasma testosterone, however, was significantly higher in females from the Ashtabula River during October of 2003 ($P = 0.033$) and April of 2004 ($P = 0.012$) compared to fish from Conneaut Creek.

There were no differences in plasma E2 in males collected from the Ashtabula River between seasons except for October 2002 (Suppl Fig. 4C). Concentrations during this sampling period were significantly higher than all other sample seasons ($P \leq 0.001$). Plasma E2 was significantly higher ($P = 0.034$) in April of 2004 than October of 2003 in male BB collected from Conneaut Creek. Seasonal profiles of plasma T were similar between rivers. The lowest concentrations of T were measured during July of 2003 in male BB from both rivers, and were significantly different than all other seasons ($P < 0.001$). Plasma E2 was significantly higher ($P = 0.029$) in males collected from

the Ashtabula River during October of 2003 compared to those from Conneaut Creek.

Statistically significant differences were observed in sex hormone concentrations of BB. In general male BB from the Ashtabula River had higher concentrations of E2 than males from Conneaut Creek. Statistical differences were observed during October 2003 ($P = 0.016$). Similarly, female BB from the Ashtabula River had higher plasma T than females from Conneaut Creek. Statistically significant differences were observed during October 2003 and April 2004 ($P = 0.041$ and 0.022, respectively). Male BB from the Ashtabula River also had significantly higher T during October 2003 ($P \leq 0.001$).

Pearson correlation statistics were executed to investigate the relationship of GSI and sex steroid concentrations independent of river. The GSI of female BB was positively correlated with E2 ($r = 0.55$) and T ($r = 0.56$); Bonferroni adjusted Pearson $P < 0.001$; $n = 59$). Significant correlations were not observed in males.

Reproductive health: largemouth bass

Gonadosomatic index

The highest GSI was observed during April of 2004 in female LMB from both rivers, and this difference was statistically different than that observed during October of 2003 ($P < 0.001$; Suppl Fig. 5). Male GSI appeared to reach a maximum during October. In both rivers GSI was significantly higher in female ($P = 0.041$) and male ($P = 0.036$) in LMB from Conneaut Creek compared to the Ashtabula River.

Sex steroids

Extraction efficiencies were 86 ± 3.1 and 81 ± 2.55% for LMB plasma E2 and T, respectively. Statistical differences were not observed in plasma sex hormone concentration in male or female LMB at any season.

Pearson correlation statistics were executed to investigate the relationship of GSI and sex steroid concentrations independent of river. The GSI of female LMB was positively correlated with E2 ($r = 0.75$) and T ($r = 0.48$); Bonferroni adjusted Pearson $P < 0.001$; $n = 66$). Significant correlations were not observed in males.

Thyroid hormones

There appeared to be seasonal differences in plasma T_4 and T_3 of BB from Conneaut Creek; however, there were no statistically significant differences between sample seasons (Fig. 5a, c). The absolute mean of plasma T_4 and T_3 concentrations were lowest during July of 2003 in both rivers. Inter-river comparisons identified that T_4 was significantly lower in BB from the Ashtabula River compared to those from Conneaut Creek during October of 2003 ($P = 0.025$)

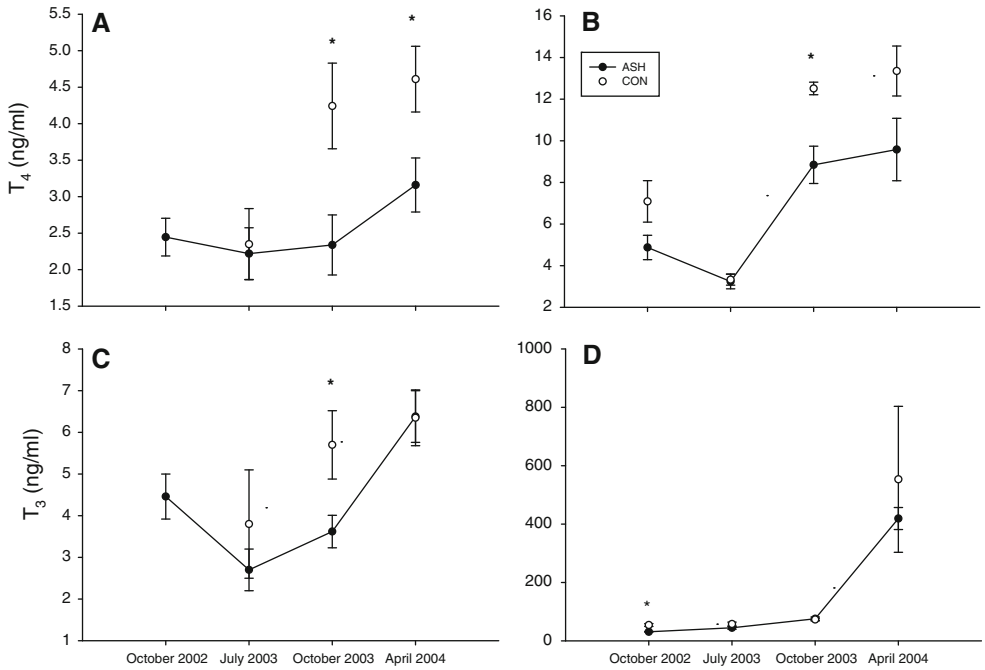

Fig. 5 Seasonal plasma thyroid hormone concentrations from brown bullhead (**a**, **c**) and largemouth bass (**b**, **d**) collected from the Ashtabula River and Conneaut Creek. Data presented as mean ± standard error; *indicates a significant difference ($P \leq 0.05$)

and April of 2004 ($P = 0.021$). Plasma T_3 was also lower in Ashtabula River BB during October 2003 ($P = 0.012$).

Seasonal changes in plasma T_4 were statistically supported in the case of LMB (Fig. 5). Plasma T_4-was statistically higher ($P < 0.001$) during October of 2003 compared to that in July of 2003 in LMB collected from both the Ashtabula River and Conneaut Creek. During October of 2003 mean plasma T_4 was statistically higher than concentration during April of 2004 ($P = 0.041$), but only in LMB collected from Conneaut Creek. Plasma T_3 was significantly higher ($P < 0.001$) in April of 2004 compared to October of 2003 in LMB from both rivers. Inter-river differences were observed during October of 2003 when plasma T_4 was significantly higher in LMB from Conneaut Creek ($P = 0.016$), and in October of 2002 when plasma T_3 was again statistically higher ($P < 0.001$) in LMB from Conneaut Creek.

Contaminant analysis

Total PCB body burdens in both BB and LMB were significantly higher in fish collected in the Ashtabula River when compared to those collected from Conneaut Creek (Fig. 6). Numerous other contaminants including HCB, chlordane, dieldrin, DDT, DDE and DDD were found at significantly higher ($P < 0.001$) concentrations in BB (Suppl Fig. 6A) and LMB (Suppl Fig. 6B) collected in the Ashtabula River. In fact, the only measured contaminant that was not significantly different between rivers was p,p DDE, and this was only the case for BB.

A number of contaminants were correlated with immune function. In general, contaminant body burdens significantly negatively correlated with bactericidal activity and cytotoxic-cell activity in both species (Suppl Tables 4 and 5). Significant positive correlations were observed with PHA-P mitogenesis and LPS stimulated respiratory burst (LPS ROS) in BB (Suppl Table 6). Similar relationships were observed in LMB. Concentrations of testosterone positively correlated with body burdens of certain contaminants in BB, while T_3 and T_4 were negatively correlated (Suppl Table 7).

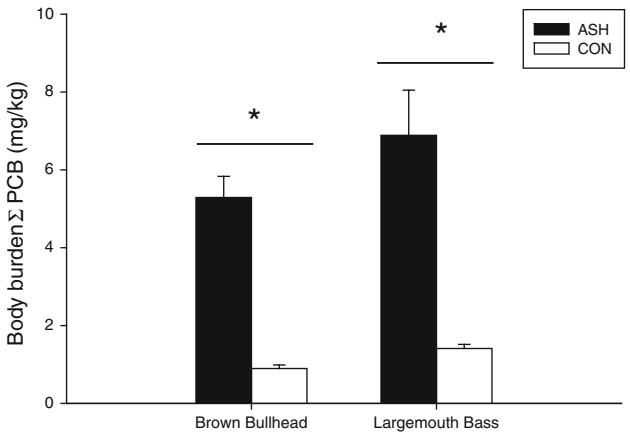

Fig. 6 Seasonal composite total PCB body burden concentrations in brown bullhead and largemouth bass collected from the Ashtabula River and Conneaut Creek during October 2003 and April 2004. Data are presented as *box* (25th and 75th), *whiskers* (10th and 90th percentile) and median plot. *Solid circles* indicate outliers

Discussion

Seasonality is an aspect of environmental monitoring that is poorly addressed and often dismissed during the stages of data interpretation. Snapshot (single time-point) sampling strategies are most common in regards to such fish health assessments due to logistical and financial hurdles associated with these projects. While these snapshot collections generate valid and useful data, this approach risks missing biological differences that are sensitive to seasonal influence. This was evident in the present field study. In fact, seasonal differences in biomarker responses masked the underlying effects of contaminants. Aspects of the immune response in fish are temporally dynamic as they are influenced by circulating hormones and factors including photoperiod and water temperature (Vainikka et al. 2005; Bowden 2008). Similarly, endocrine physiology is very often seasonal. This research emphasizes the value of non-metric multidimensional scaling as an exploratory analysis of ectotoxicological relationships. In addition to statistically identifying seasonal differences in biomarker response, differences in biomarkers across species were identified. Quantitative body burden chemical analysis demonstrated clear differences in tissue concentrations of all chemicals reported here. Differences in tissue contaminant concentration, however, do not in singularity support evidence of a biological effect. The inclusion of multi-tiered biomarker responses including immune and endocrine end-points was necessary to identify associations among contaminant body burden and biological responses. The current research provided strong evidence supporting a conclusion of biological disruption in the Ashtabula River after for accounting for seasonality and species differences in biomarker responses.

Allometric end-points including condition factor (Ktl) and hepatosomatic indices are commonly utilized in ecotoxicological-based field studies (Goede and Barton 1990; Schmitt et al. 2005; Hinck et al. 2006). In the current investigation differences in HSI were only observed between males and females on a seasonal basis. Such an observation is not unexpected and most likely reflects normal shifts in reproductive physiology, and has been reported previously (Yang et al. 2006). While increased HSI has been used as an indicator of contaminant-associated

environmental stress in previous studies (Fabacher and Baumann 1985; Pinkney et al. 2001), this biomarker did not support evidence of differences between rivers in the present study. Reduced condition factor (Ktl) in wild species inhabiting ecosystems contaminated with organochlorines have been previously reported (Rocha and Monteiro 1999; Anderson et al. 2003). Condition factor of brown bullhead collected from the Ashtabula River during October of 2003 was significantly lower than that from Conneaut Creek. Interestingly this was the only season that differences were observed in BB between the two rivers. Inter-river differences in Ktl were not observed in largemouth bass.

Immune function has been advocated as a sensitive biomarker of moderate ecological significance (Mathews et al. 1990; Fournier et al. 2000; Bols et al. 2001). In agreement with this assertion, measures of cellular innate immune function including phagocytic cell activity (phagocytosis, bactericidal activity, reactive oxygen or nitrogen species production) and cytotoxic-cells have been successfully utilized as markers of immunomodulation by a number of investigators (Faisal et al. 1991; Kelly-Reay and Weeks-Perkins 1994; Arkoosh et al. 1996; Rice and Schlenk. 1995; Roszell and Anderson 1997; Clemons et al. 1999; Carlson et al. 2002; Ripley et al. 2008). In the current investigation immune function of anterior kidney leukocytes was clearly influenced by the river of origin. This was the case for both brown bullhead and largemouth bass. Notably, differences were consistent across species but differed in the magnitude and absolute values of the biomarker response. In the case of macrophage mediated innate immune function, bactericidal activity was depressed and respiratory burst activity was elevated in fish from the Ashtabula River. Decreased function of fish phagocytes is a well-documented consequence of contaminant exposure (Weeks and Warinner 1986; Rice and Schlenk 1995; Bols et al. 2001; Lacroix et al. 2001; Iwanowicz et al. 2009). Respiratory burst activity is modulated by contaminants as well, but has been shown to increase or decrease depending on the chemical stimulus (Rice and Schlenk 1995; Rice et al. 1996; Bols et al. 2001; Regala et al. 2001). Additionally, respiratory burst activity was elevated in both brown bullhead and largemouth bass from the Ashtabula River compared to those from the reference site. Such an increase has been documented is fish exposed to PAHs (Reynaud and Deschaux 2006). This class of contaminants is certainly present in the Ashtabula River, and PAH metabolites have been identified in the bile of BB from this river (Yang et al. 2006). Similar observations have been made in mummichogs, *Fundulus heteroclitis*, collected from PAH contaminated sites (Kelly-Reay and Weeks-Perkins 1994) and medaka in laboratory experiments (Carlson et al. 2002). PAH contamination is documented to lessen the ability of macrophages to kill bacteria (bactericidal activity) while altering the respiratory burst physiology such that more free radicals (ROS) are produced in response to stimulation. Heavy metals have also been shown to increase respiratory burst activity (Robohm 1986; Rice and Weeks 1991; Bols et al. 2001). For instance, arsenic exposure leads to a significant increase in respiratory burst but a decrease in bactericidal activity (Datta et al. 2009).

Similar to data yielded by the bactericidal and respiratory burst assays, October 2003 samples appeared to be most informative for the mitogenesis assay. Increased proliferative responses of T-cell populations have been documented in rats, mice, birds, turtles and fishes collected from sites contaminated with PCBs or in controlled contaminant PCB experiments, and the PHA-P sensitive lymphocyte population seems to be most sensitive to PCB associated immunomodulation (Arkoosh et al. 1996; Segre et al. 2002; Iwanowicz et al. 2005; Keller et al. 2006; Iwanowicz et al. 2009). In general the PHA-P response of brown bullhead from the Ashtabula River was higher than that of fish from Conneaut Creek, and this response is significantly positively correlated with a number of contaminants measured in this study, including PCBs. This increase is consistent with the effects of PCB on immune function. Similar increases are seen in the Con A sensitive T-cell population further suggesting a T-cell dysfunction.

Cytotoxic cell activity for brown bullhead and largemouth bass was generally lower in the Ashtabula River than Conneaut Creek. This cell population is most known for activity against parasites and virally infected cells (Jaso-Freidmann et al. 2000; Somamoto et al. 2002). However, they are also tumoricidal in fish (Cuesta et al. 2003). Disruption of this cell population, therefore may have serious ramifications including reduced resistance to parasites and viruses and a lessened ability to remove neoplastic cells. PAH contamination has been reported to affect cytotoxic cell activity in environmental studies, and the effect is suppressive (Faisal et al. 1991; Seeley and Weeks-Perkins 1997). Likewise, PCB exposure is known to affect this cell population (Regala et al. 2001).

Contemporary understanding of macrophage aggregates (MAs) in fish is that these structures are the functional homologues of germinal centers of the spleen found in higher vertebrates (Ellis 1980). In this capacity they are thought to actively process endogenous and exogenous waste products, store and facilitate iron recycling, and possibly serve as antigen presenting centers of the immune response. A number studies have demonstrated an increase in the number, size or amount of hemosiderin in MAs in fish collected from contaminated aquatic ecosystems. Given their role in physiological, immunologic maintenance and

sensitivity of contaminants they have been exploited as biomarkers of environmental stress (Blazer et al. 1987; Wolke 1992; Blazer et al. 1997; Fournie et al. 2001; Agius and Roberts 2003; Facey et al. 2005).

In general, fish collected from the Ashtabula River, regardless of species, had more macrophage aggregates per mm^2 than fish collected from Conneaut Creek. Elevated prevalence or size of macrophage aggregates have previously been used as an indicator of environmental degradation in a number of monitoring programs (Zdanowicz et al. 1987; Bucke et al. 1992; Long et al. 1995; Fournie et al. 2001). Exposure to heavy metals such as mercury (Meinelt et al. 1997), pulp mill effluent (Couillard and Hodson 1996), dioxins (van der Weiden et al. 1994), crude oil (Khan and Kiceniuk 1984), polybrominated flame retardants (Raldúa et al. 2008) and PCBs (Pierce et al. 1980; Anderson et al. 2003; Fisher et al. 2008) leads to an increase in MAs. Bowser et al. (1990) observed increased hepatic macrophage aggregates in brown bullhead collected at a site contaminated with PCBs and metals when compared to a reference site. A previous study in Lake Champlain compared brown bullhead collected from a site with high sediment concentrations of PAHs and PCBs to a reference with undetectable levels. Significantly more and larger macrophage aggregates were found in fish from the contaminated site (Blazer et al. 1994). A subsequent study at those same sites following remediation showed a decrease in spleninc tissue occupied by MA which exemplifies the utility of MAs in monitoring improvement of environmental quality (Facey et al. 2005).

The brown bullhead is a key indicator species for Great Lakes Areas of Concern (AOC) due to their benthic life-history, limited home range and propensity to absorb contaminants from food and sediments (Maccubbin et al. 1985; Smith et al. 1994). An increased incidence of hepatic neoplasms is associated with PAH and other contaminant exposure in this species (Harshbarger and Clark 1990; Baumann et al. 1991; Blazer et al. 2009b). There is also convincing evidence that exposure to PAHs and PCBs increase the likelihood of hepatic neoplasm and other microscopic liver lesions in other fish species, including English sole *Paraphyrs vetulus* (Myers et al. 1990, 1998), mummichog *Fundulus heteroclitus* (Vogelbein et al.1990), marine flatfish *Platichthys flesus* (Grinwis et al. 2001; Kohler et al. 2002) and walleye *Stizostedium vitreum vitreum* (Barron et al. 2000).

Skin tumors, including papillomas and squamous cell carcinomas have been used as indicators of chemical exposure in brown bullhead and other species (Grizzle et al. 1981; Smith et al. 1989; Black and Baumann 1991; Pinkney et al. 2001; Blazer et al. 2009a). Although no cause- and-effect correlations have been made in wild populations, papillomas have been experimentally induced in brown bullhead by repeated dosing of the skin with sediment extracts with high levels of PAHs (Black et al. 1985). In the present study neoplastic lesions were observed in brown bullhead from both rivers. These lesions, however, were generally more prevalent in brown bullhead from the Ashtabula River. Perhaps of greater significance, the neoplastic lesions observed in brown bullhead from the Ashtabula River were more aggressive, malignant tumors compared to the benign tumors noted in fish from Conneaut Creek.

Factors, including contaminants that affect thyroid status in wildlife have been reviewed by Rolland (2000). In general it is accepted that PCBs disrupt the thyroid axis. While the specific mechanisms of thyroid disruption by PCBs have not been clarified, laboratory exposure of fish to PCBs is associated with decrease levels of circulating T_4 and sometimes T_3 (Persky et al. 2001; Brown et al. 2004; Iwanowicz et al. 2009). Different PCB congeners, especially coplanar versus noncoplanar, have different effective doses and mechanisms of action. These include impacts on responsiveness of the thyroid to TSH, clearance of thyroid hormones from the blood and interaction with thyroid binding proteins (Brouwer et al. 1998; Chauhan et al. 1999). Circulating thyroid hormones in fish may also be impacted by other contaminants, including estrogenic compounds (McCormick et al. 2005), and we cannot be conclusive regarding which of the many contaminants may be affecting plasma thyroid hormones. However, the observations of decreased thyroid hormone concentrations in both brown bullhead and largemouth bass from the Ashtabula River, and the strong negative correlation of plasma T_4 and T_3 with PCB body burdens are consistent with PCB-induced thyroid hormone disruption.

We observed seasonal changes in plasma thyroid hormones in both species, with low levels occurring in summer and increases in spring. Although seasonal changes in thyroid hormones are common in fish (Leatherland 1982), there is limited published information on plasma thyroid hormones in these two species. Hazen et al. (1978) observed higher levels of plasma T_4 in summer compared to winter in a reservoir population of largemouth bass. Spring increases in both plasma T_4 and T_3 were observed in feral populations of brown bullhead (Burke and Leatherland 1983). We also observed remarkably high levels of circulating T_3 in largemouth bass, especially in spring. These values are not an artifact of our assay, as they were run side-by-side with brown bullhead, and extra validation steps were carried out to ensure their validity (data not shown). To our knowledge these are the first published values for plasma T_3 in this species.

Differences in plasma thyroid hormones between rivers were seasonally dependent. Thyroid hormones were not different in summer when levels were low, whereas spring

levels which were lower in the Ashtabula River. This suggests that thyroid hormone disruption may only be evident when the thyroid is stimulated. One possible implication of this finding is that PCBs (or other contaminants) in the Ashtabula have interfered with the capacity of the thyroid to respond to thyroid stimulating hormone, as has been demonstrated in rats (Byrne et al. 1987). However, the mechanism of PCB impacts on the thyroid in fish are largely unexplored, and more research will be necessary to establish this connections.

Degradation of aquatic ecosystems and the biological health of organisms inhabiting these habitats is an unfortunate consequence of global industrialization. Assessing the health of biological communities is often a necessary requirement before a particular area can be officially classified as impacted, or delisted from a pre-existing impaired status. The multifactoral scope of biological impact imparted by factors such as contaminants presents a challenge in regards to conclusively identifying biological perturbation. Single, simple biomarkers are generally not sufficient for such investigations. Biological indicators that gauge disruption on multiple scales of biological (ecological) relevance are therefore required to assess the adequacy of the environment, monitor trends over time, provide early warning of environmental degradation, or diagnose the cause of an existing problem (Cairns et al. 1993). Fish are presently the preferred sentinel vertebrate of aquatic ecosystems as they are stellar integrators of natural and anthropogenic stressors (Hodson et al. 1996; van der Oost et al. 2003; Johnson et al. 2007). Here two species, brown bullhead and largemouth bass, were utilized as sentinels and numerous biomarkers evaluated to qualify differences between biological health in the Ashtabula River *versus* a reference site (Conneaut Creek). Consistent differences in biomarkers from both species particularly in regards to immune function and related histopathology were identified. These biomarker responses were supported by the body burden chemical analysis and documented biological effects of these chemicals.

In short, this study in conjunction with others provides evidence that brown bullhead and largemouth bass of the Ashtabula River are biologically different from those from Conneaut Creek, and this difference is best explained by the presence of contaminants. Findings from this study and other associated investigations led to remediation recommendations in 2006 and settlement of natural resource damage claims under CERCLA. These actions primarily included dredging of the Ashtabula River harbor. Future research at these sites would be highly beneficial to evaluate the effects of such remediation.

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