FINAL REPORT

FISH HEALTH STUDY ASHTABULA RIVER NATURAL RESOURCE DAMAGE ASSESSMENT

Prepared for U.S. Fish and Wildlife Service by:

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INTRODUCTION

The Ashtabula River is located in northeast Ohio, flowing into Lake Erie at Ashtabula, Ohio. Tributaries include Fields Brook, Hubbard Run, Strong Brook, and Ashtabula Creek. The bottom sediments, bank soils and biota of Fields Brook have been severely contaminated by unregulated discharges of hazardous substances. Hazardous substances have migrated downstream from Fields Brook to the Ashtabula River and Harbor, contaminating bottom sediments, fish and wildlife. There are presently more than 1,000,000 cubic yards of contaminated sediment in the Ashtabula River and Harbor, much of which originated from Fields Brook. Contaminants include polychlorinated biphenyls (PCBs), chlorinated benzenes, chlorinated ethenes, hexachlorobutadiene, polyaromatic hydrocarbons (PAHs), other organic chemicals, heavy metals and low level radionuclides.

A Preassessment Screen, using existing data, was completed for the Ashtabula River and Harbor on May 18, 2001. Among the findings was that the fish community at Ashtabula contained approximately 45 percent fewer species and 52 percent fewer individuals than the Ohio EPA designated reference area, Conneaut Creek. The Ashtabula River and Conneaut Creek are similar in many respects, with the exception of the presence of contamination at Ashtabula. The difference in the fish communities between the two sites is believed to be at least partially a result of the hazardous substance contamination at Ashtabula. In order to investigate this matter further, the Trustees elected to conduct a study of the status and health of the aquatic biological communities of the Ashtabula River and Conneaut Creek in 2002-2004. The following document contains brief method descriptions (more detail available in attached Appendix A) and a summary of the data used to evaluate the health status of brown bullheads (*Ameiurus nebulosus*) and largemouth bass (*Micropterus salmoides*) collected from the above sites.

METHODS AND MATERIALS

Species and Sites: The health of brown bullhead, a fish species with a benthic diet (Keast 1985) that burrows into soft sediments (Loeb 1964) and largemouth bass, a fish species with a more pelagic life history, were evaluated. Fish were collected from sites in the Ashtabula River and the reference site, Conneaut Creek (Figure 1).

Figure 1. Collections Sites Within the Ashtabula River and Conneaut Creek Drainages. Collections of fish in the Ashtabula river were between RM 1.7 and RM 0.57. Collections in Conneaut creek were between RM 2 and RM 0.65, as well as the southwest section of Conneaut Harbor.

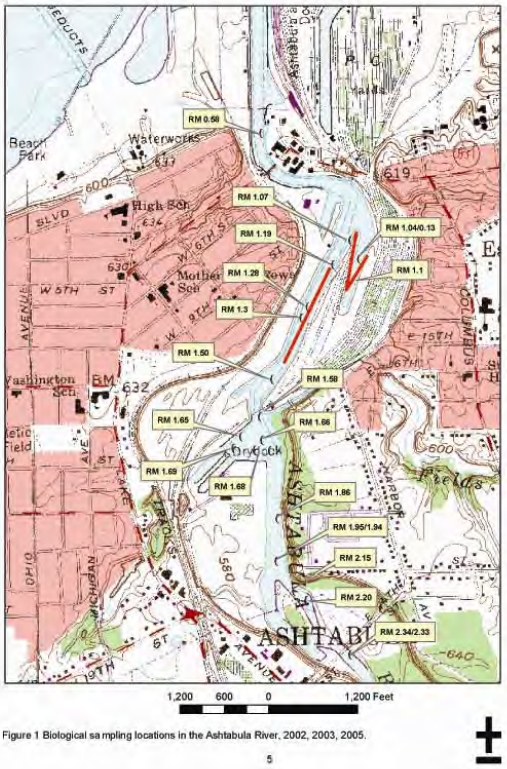

Figure 1 Biological sampling locations in the Ashtabula River, 2002, 2003, 2005.

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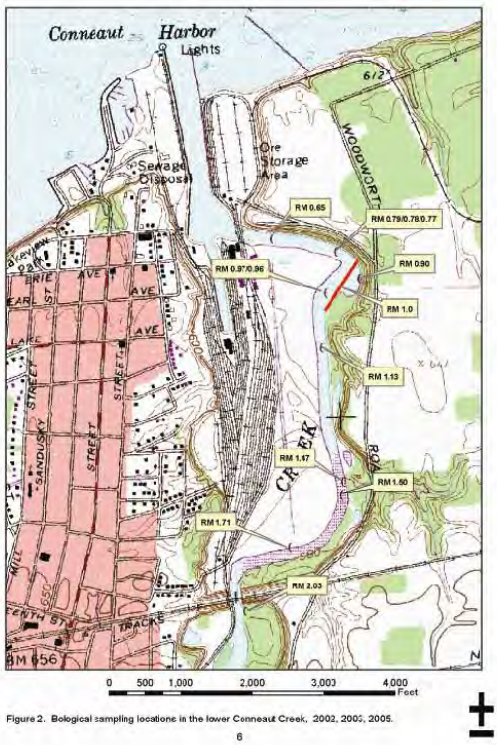

Figure 2. Biological sampling locations in the lower Conneaut Creek, 2002, 2003, 2005.

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Fish Health Assessments: A battery of variables was used to compare the fish health. These included: morphometric (length, weight, length at age, condition and hepatosomatic indices), necropsy-based observations (external and internal lesions/abnormalities), genotoxic (comet assay), immunological (bactericidal activity, respiratory burst, lymphocyte mitogenesis, cytotoxic cell activity), reproductive (gonadosomatic index, stage, percent atresia, reproductive hormones), thyroid hormones and histopathological (preneoplastic and neoplastic liver lesions, microscopic evaluation of gross skin lesions, macrophage aggregate measurements, parasites) analyses.

Fish Collection and Necrospy-based Assessment: Largemouth bass (LMB) and brown bullheads (BB) were sampled at each site over a one- or two- day sampling period using a pulsed DC electroshocking boat during October 2002, July 2003, October 2003 and April 2004. The target sample size was twelve 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 immediately prior to necropsy. Blood was drawn from the caudal vessels with a heparinized 5 cc syringe. Two drops of whole blood were used to produce blood smears, about 1mL of the blood was placed in cryovials and shipped in ice filled coolers (0 to 4 ºC) by overnight express mail to the U.S. Environmental Protection Agency, National Exposure Research Laboratory, Cincinnati, Ohio for the comet assay. The remaining blood was placed in a heparinized vacutainer on wet ice until centrifuged (within 24 hrs) for plasma collection.

A complete necropsy-based fish health assessment as described by Goede and Barton (1990) was completed. Briefly, fish were measured and weighed. External abnormalities including melanistic spots on body surfaces, raised lesions in the oral cavity and body surfaces, and missing, shortening and deformed nasal, maxillary, and chin barbels (for bullheads only) were recorded. Fish were aseptically necropsied and a portion of the anterior kidney was removed and placed into L-15 medium at 4^{o}C. Within an hour the tissue was homogenized with a sterile, hand-held tissue grinder, returned to the individual tube and samples were placed 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. Pieces of liver, kidney, spleen, gonad and any lesions were removed and fixed in Z-Fix®, an aqueous buffered zinc formalin (Anatech LTD, Battle Creek,

MI) for histological analyses. Pectoral spines were removed from bullheads and scales (right side above the pectoral fin and lateral line) from largemouth bass for aging. Carcasses were wrapped in foil and frozen for later chemical analyses.

The condition factor (K), hepatosomatic index (HSI) and gonadosomatic (GSI) were calculated by $K= 10^5 \times \text{weight} / \text{length}^3$, HSI = 100 × liver weight / body weight, GSI = 100 X gonad weight/(body weight – gonad weight), respectively. The number of bullhead with melanistic spots, raised lesions, and/or barbel deformities was documented. Prevalence was calculated by number of fish with each type of abnormalities ÷ total number of fish × 100.

Genotoxic Analysis - Comet assay: The comet assay was performed on erythrocytes of bullhead as described by Singh et al. (1988). Briefly, about 75 uL of 0.5% low melting point agarose (LMPA) (37°C) mixed with 5-10 uL of the blood sample was added to a slide which was already coated with a thin layer of 1.0% normal melting point agarose (NMA) according to Klaude et al. (1996). Agarose was chilled to 4°C to solidify, and a third layer of agarose (75 uL LMPA) was added. Slides were then slowly immersed into cold, freshly made lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% dimethylsulfoxide, pH 10) and refrigerated (4°C) for a minimum of 1 hour. Slides were gently removed from the lysing solution and immersed in freshly made pH>13 electrophoresis buffer (300 mM NaOH, 1 mM EDTA) for 20-60 minutes to allow unwinding of DNA and expression of alkali-labile damage.

Electrophoresis was then performed in the same buffer for 10-40 minutes at 25 V. After eletrophoresis, slides were neutralized with neutralization buffer (0.4 M Tris, pH 7.5) and stained with 100 uL of ethidium bromide of SYBR™ Green I stain (20 ug/mL ethidium bromide diluted by TE buffer (10 mM Tris-Hc1, 1 mM EDTA, pH 7.5) by 10,000 times). Slides were observed under a fluorescent microscope. DNA fragments following strand breaks migrated from the nuclear core and appeared as a comet like tail. Cells with increased DNA damage displayed increased migration and greater tail lengths and densities. A Komet analysis system developed by Kinetic Imaging Ltd (Liverpool, UK) linked to a CCD camera was used to quantify the length of DNA migration (tail length) and the percentage of migrated DNA (% tail DNA). Tail extent moment (= tail length × % tail DNA / 100) and Olive tail moment (= (tail mean – head mean) × % tail DNA / 100) introduced by Olive et al. (1990) were also calculated as measures of DNA damage. Two slides were prepared for each fish and 50 cells were scored on each slide.

Immunological Analyses

Bactericidal Activity*:* Macrophages and neutrophils are phagocytic cells of the innate immune system in fishes. These cells are responsible for killing and removing foreign organisms such as bacteria, other infectious agents and non-soluble organic substances from the body. They are also instrumental in removing cellular debris in tissues following necrosis. In the case of pathogen invasion, these cells serve as a first line of cellular defense. Both phagocytosis and destruction of the invading organisms with reactive oxygen or nitrogen intermediates are necessary to effectively prevent the progression of infection. Assays that exclusively determine the phagocytic activity of macrophages and neutrophils measure the cells ability to engulf invading organisms, while the bactericidal activity is a functional assay that measures the ability of adherent cells to destroy phagocytized bacteria. Both the bactericidal activity and respiratory burst assays measure the functional status of the adherent cell populations. Briefly, adherent cells were isolated from the anterior kidney and challenged with *Yersinia ruckeri* (an enteric salmonid pathogen), allowed to phagocytize and non-phagocytosed bacteria were washed off. The adherent cells are lysed after an incubation period and colony counts are performed to determine the number of viable, phagocytosed bacteria. Environmental chemicals including PCBs, PAHs, and heavy metals have been reported to reduce phagocytic activity in head kidney adherent cells (Rice and Schlenk 1995; Rozell and Anderson 1997; Bols et al. 2001; Karrow et al. 2001).

Respiratory Burst Assay: The respiratory burst activity of "professional" phagocytes, primarily macrophages and neutrophils, is a fundamental, innate pathway that facilitates microbial killing. Reactive oxygen species (ROS) produced by these cells include the superoxide anion (O^-), hydroxyl radicals (HO^-), hydrogen peroxide (H_2O_2) and the hypochlorite anion (ClO^-). Nitrogen intermediates, including nitric oxide, are also produced depending on the type of stimulus. These pathways have been shown to be modulated by numerous contaminants including PAHs and PCBs (Kelly-Reay and Weeks-Perkins 1994; Voie et al. 1998; Clemons et al. 1999; Regala et al. 2001). In fact, measuring the respiratory burst activity has been advocated as a useful bioindicator of fish health (Rice et al. 1996; Bols et al. 2001). The adapted peroxidase, luminol-enhanced chemiluminescent method allows for the detection of a variety of ROS production produced by these cells (Coteur et al. 2002). In short, the assay involves enriching for adherent

leukocytes in the same fashion as bactericidal activity assay above. Cells are then allowed to "rest" for 48 hours. Lipopolysaccharide is used to induce ROS production. The presence of luminol and horseradish peroxidase in the ROS stimulation solution allow for the detection of ROS via light emission that is read using a luminometer. ROS stimulation is calculated as the luminescence measured in stimulated cells/luminescence from unstimulated cells. An additional negative control containing superoxide dismutatse (SOD) is included in another set of replicate wells to determine the nature of the ROS.

Lymphocyte Mitogenesis: The lymphocyte mitogenesis assay quantifies the relative proliferative response of specific lymphocyte populations (T-cells, B-cells or a combination of the two) following stimulation with a polyclonal mitogen to unstimulated lymphocytes. Concanavalin A (Con A) and phytohemagglutinin (PHA) are T-cell mitogens, lipopolysaccharide (LPS) is a B-cell mitogen, and pokeweed mitogen (PWM) stimulates proliferation in both B- and T-cells. The presence of environmental contaminants, including PCBs, PAHs and metals, are known to alter the proliferative response of lymphocytes in fish (Arkoosh et al. 1996; Sakazaki et al. 2001; Reynaud et al. 2003; Iwanowicz et al. 2005). Thus, quantifying mitogenesis serves a sensitive tool to monitor chemically induced insult to the immune system. The method uses 5'-bromo-2'-deoxyuridine (BrdU), a pyrimidine nucleotide analogue, as a marker of DNA synthesis. Proliferating cells incorporate BrdU into their DNA. The incorporated BrdU is then detected with antibody Fab fragments specific for BrdU and relative incorporation determined using enzyme-linked immunoassay (ELISA) methodology (Gauthier et al. 2003).

Cytotoxic Cell Activity: Numerous teleost fishes possess a population of natural killer-like cells termed nonspecific cytotoxic cells (NCC). These cells are involved in protozoan parasite immunity (Jaso-Freidmann et al. 2000), mediate innate immune functions and may play a role during viral infections and clearance of tumor cells (Jaso-Freidmann et al. 1999).
The cytotoxic-cell assay is a functional assay that measures killing of adherent target cells by cytotoxic immune cells by measuring the release of a fluorescent viability dye. Calcein AM is a colorless, nonfluorescent uncharged molecule. Once inside a target cell non-specific esterases cleave the lipophilic blocking groups resulting in a highly fluorescent, charged molecule that is

more effectively retained within the cell than the parent compound. Thus release of the dye occurs following cell lysis or disruption in the cell membrane and reflects cytotoxic-cell activity. This method provides a useful way of comparing the relative cytotoxic-cell activity between groups of fishes (Iwanowicz et al. 2004). Very few studies have addressed the effect of contaminants on this cell population, however modulation of activity has been reported from fish collected in the contaminated Elizabeth River (Faisal et al. 1991).

Histopathology: Two to three transverse sections of large gonads or a longitudinal cut of small gonads were placed in one cassette, 3-4 pieces of liver in a second cassette, pieces of hind kidney, liver and spleen in a third cassette and any grossly observed abnormalities in subsequent cassettes. Tissues were routinely processed for histology, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). Two slides were prepared from the block containing spleen, kidney and liver and one slide was stained by the Perl's method for iron (Luna 1992) for macrophage aggregate analysis.

All tissues were examined for any microscopic changes and to attempt to determine causes for any grossly observable lesions. In the liver, foci of inflammation, bile duct proliferation, bile duct fibrosis, altered foci, neoplasia, bile duct parasites, and parasites in the hepatic parenchyma were noted. Gonads were sexed, stage of development noted, percent of atretic eggs calculated in females and pigmented cell accumulations in both sexes rated; intersex, Sertoli cell proliferation, parasites or any other abnormalities were noted, if present.

Splenic macrophage aggregate (MA) parameters were evaluated in both largemouth bass and bullheads. In addition, hepatic and kidney MA parameters were evaluated in bullhead. Aggregates greater than 50 μm^2 were counted and measured in 10 fields at 25X using a microscope fitted with a video camera. SigmaScan image analysis software (Jandel) was used. The number of aggregates per mm^2 and the mean size or area of those aggregates was measured. Using that data, the percent of tissue occupied by MA is calculated. Percent of area occupied by helminth parasites in largemouth bass liver was measured in the same way.

Hormone Analysis: Plasma samples from brown bullheads and largemouth bass were analyzed for the sex steroids 17β-estradiol and testosterone, and the thyroid hormones T_4 and T_3. Plasma samples were assayed in duplicate and values reported as pg/mL for the sex steroids and ng/mL for the thyroid hormones. Plasma samples were extracted twice in a ten fold excess of diethyl ether prior to radioimmune assay (RIA) analysis for the sex steroids. Extraction efficiencies were determined and all assays validated for brown bullheads and largemouth bass. Extraction was not necessary for the thyroid hormone assays. Hormone assays were run according using standard RIA methods (Sower and Schreck 1982). Antibodies used for the RIAs were: 17B-estradiol Ab (#244 anti-estradiol-6-BSA) purchased from the lab of Gordon Niswender, Testosterone antiserum (polyclonal R156/7) purchased from Coralie Munroe (UC Davis), T_4 and T_3 antibodies from Accurate Scientific.

Statistical Data Analysis: Statistical analysis of body weight and HSI data of fish were log transformed before the statistical analysis to increase normality and homogeneity of variance. Two sample t-test was used to compare the length, weight, K, and HSI between the Ashtabula and Conneaut Rivers. Fisher's (or Chi-square) test was performed to compare the prevalence of melanistic spots, raised lesions, and barbel deformities in fish between the two sites. Two way [site and season] ANOVA was used to detect the impact of sampling sites and seasons on the comet assay. The nonparametric Mann-Whitney-Wilcoxon Rank-Sum test was performed to compare the tail length, % tail DNA, tail extent moment and Olive tail moment of fish from the Ashtabula and Conneaut Rivers.

Data for immune function assays were tested for normality using the Shapiro-Wilks W test and homogeneity of variance via the Brown-Forsythe test of homogeneity of variances. One-way ANOVA or the Mann-Whitney U test was used to detect site differences at each sampling season. Differences were considered statistically significant when $P \leq 0.05$ and very significant when $P \leq 0.02$.

RESULTS AND DISCUSSION

Bullhead Necropsy-based Observations

No bullheads were caught in the Conneaut Creek during the 2002 sampling. Comparisons were made on a seasonal basis in 2003 and 2004 (Table 1). In July 2003, only five bullheads were collected from the Conneaut and there was no statistical difference in morphometric parameters between Ashtabula and Conneaut bullheads, although the Conneaut fish were approximately a year younger. No melanistic spots or raised lesions were found in the bullhead from Conneaut, while 16.7 and 8.3% respectively, were observed in bullhead from Ashtabula. In addition, two of the Ashtabula bullheads were emaciated. The raised lip lesions were diagnosed as papillomas by histology. Two additional fish had slightly raised lesions diagnosed as epidermal hyperplasia. Barbel deformities were observed at both sites.

In October 2003 a larger sample size of Conneaut bullhead were obtained and they were significantly longer, heavier and had a higher condition factors than those collected in the Ashtabula, although mean age for the two groups was similar. Melanistic spots and raised lesions were higher, although not statistically, at Conneaut, during this sampling period (Table 1). The two fish with raised lesions from Conneaut and one fish from Ashtabula were all diagnosed as papillomas microscopically. In addition, four of the bullhead from Ashtabula had deformed fins.

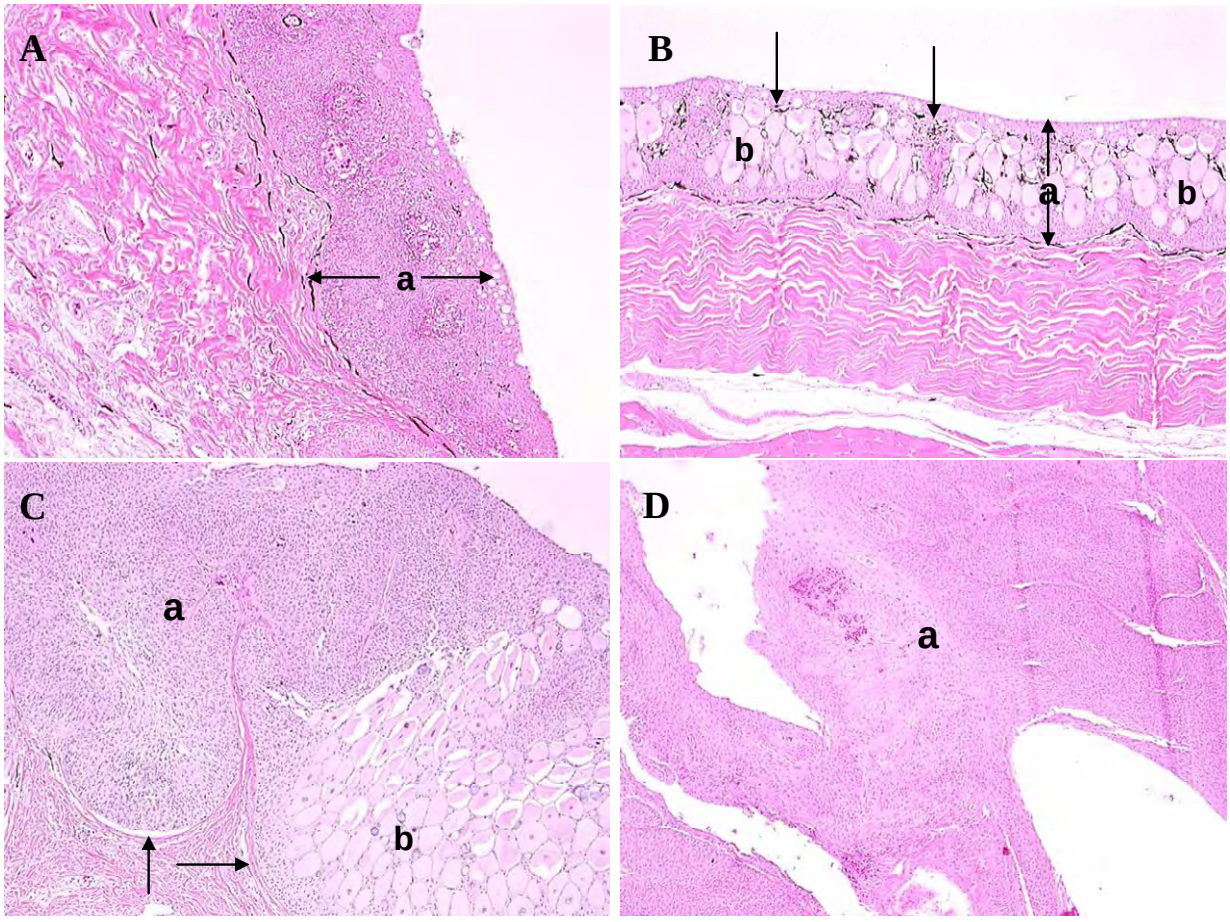
In April 2004, equal sample sizes were collected at both sites. The fish were similar in size and condition factor, although bullheads from Conneaut were approximately a year younger. The prevalence of melanistic spots and barbel deformities at Ashtabula was approximately double that observed at Conneaut (Table 1). Prevalence of raised lesions was significantly higher at Ashtabula. Histological evaluation of tissues collected from these lesions during April 2004 indicated that the two raised lesions from Conneaut bullhead were inflammation and epidermal hyperplasia (Figure 2A). Melanistic spots had slight epidermal hyperplasia with increased numbers of melanocytes within dermis and epidermis (Figure 2B). Of the five bullheads with raised lesions collected at Ashtabula, three had papillomas (Figure 2C) and two had squamous cell carcinomas (Figure 2D).

Table 1. SEASONAL COMPARISON OF MORPHOMETRIC PARAMETERS AND EXTERNAL ABNORMALITIES IN BROWN BULLHEAD SAMPLED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK, 2002-2004

Sampling season		**Fall (October) 2002**		**Summer (July) 2003**	
Site		**Ashtabula**	**Conneaut**	**Ashtabula**	**Conneaut**
Sample size (n)		27	0	24	5
Length (mm)		280.8 ± 8.0	NA	279.3 ± 7.5	268.8 ± 16.5
Weight (g)		286.3 ± 23.7	NA	314.7 ± 20.4	279.6 ± 52.5
Age		4.7 ± 0.3	NA	5.1 ± 0.3	3.8 ± 1.1
Condition factor		1.23 ± 0.04	NA	1.48 ± 0.11	1.37 ± 0.05
Hepatosomatic index		1.90 ± 0.06	NA	1.59 ± 0.10	1.72 ± 0.11
Prevalence (%)	Melanistic spots	18.5	NA	16.7	0
	Raised lesions	11.1	NA	8.3	0
	Barbel deformities	29.6	NA	79.2	60.0
Sampling season		**Fall (October) 2003**		**Spring (April) 2004**	
Sample size (n)		24	14	24	24
Length (mm)		273.1 ± 4.0	301.9 ± 9.5*	298.5 ± 5.5	300.5 ± 8.1
Weight (g)		259.6 ± 13.2	396.9 ± 42.4*	375.8 ± 20.0	374.1 ± 23.5
Age		4.0 ± 0.3	3.7 ± 0.6	5.4 ± 0.3	4.3 ± 0.3
Condition factor		1.25 ± 0.03	1.37 ± 0.03*	1.39 ± 0.03	1.36 ± 0.04
Hepatosomatic index		2.67 ± 0.10	2.81 ± 0.09	2.84 ± 0.16	2.49 ± 0.14
Prevalence (%)	Melanistic spots	0	7.1	8.3	4.2
	Raised lesions	4.2	14.3	20.8	8.3*
	Barbel deformities	37.5	21.4	45.8	20.8

Data presented as means ± standard errors; NA: not available; * indicates significantly different than Ashtabula at $p < 0.05$.

FIGURE 2. MICROSCOPIC APPEARANCE OF EXTERNAL LESIONS NOTED IN BROWN BULLHEAD



A. Epidermal hyperplasia as indicated by a thickening of the epidermis (a). B. Microscopic appearance of a melanistic area. Epidermis (a) is slightly thickened and has increased numbers of melanocytes (arrows) and alarm substance cells (b). C. Section of papilloma showing the transition between an area of proliferative Malpighian cells (a) and alarm substance cells (b). Arrows indicate the basement membrane which is intact in papillomas. D. Squamous cell carcinomas differ from papillomas in that there is rupture of the basement membrane and invasion of neoplastic cells into adjacent stroma (a). H&E stain.

Data from the four sample periods was also combined for statistical analysis. Overall, fish from Conneaut Creek weighed significantly more than fish from the Ashtabula River, were slightly longer in length, although somewhat younger. Condition factor and hepatosomatic index were also higher, although not significantly in bullheads from the Conneaut. Bullheads from the Ashtabula River had a significantly higher prevalence of barbel deformities than fish from Conneaut Creek (Table 2). Although there was not a statistical difference in the prevalence of

melanistic spots or raised lesions between sites, melanistic areas were more than double at Ashtabula and only bullheads from the Ashtabula River had large raised lesions (> 6mm in diameter). In addition, of the four bullheads with raised lesions from Conneaut Creek, only two were histologically diagnosed as papillomas and none as squamous cell carcinomas. The other two lesions were chronic inflammation and hyperplasia. Of the 11 fish with raised lesions collected in the Ashtabula river, six were diagnosed as papillomas, two as squamous cell carcinomas, two with epidermal hyperplasia and one with chronic inflammation.

Skin tumors, including papillomas and squamous cell carcinomas have been used as indicators of chemical exposure in bullhead and other species (Grizzle et al. 1981; Smith et al. 1989; Black and Baumann 1991; Pinkney et al. 2001). Although no cause-and-effect correlations have been made in wild populations, papillomas were induced in brown bullhead by repeatedly painting the skin with sediment extracts containing high levels of PAHs (Black et al.1985).

TABLE 2. COMPARISON OF MORPHOMETRIC PARAMETERS AND PREVALENCE OF RAISED LESIONS AND BARBEL ABNORMALITIES IN BROWN BULLHEAD SAMPLED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK DURING 2002-2004.

Site		**Ashtabula**	**Conneaut**
Sample size (n)		99	43
Length (mm)		282.3 ± 3.6	297.3 ± 5.9
Weight (g)		309.1 ± 10.7	370.5 ± 20.2*
Age		4.8 ± 0.1	4.0 ± 0.3
Condition factor		1.36 ± 0.04	1.53 ± 0.03
Hepatosomatic index		2.24 ± 0.09	2.50 ± 0.10
Prevalence (%)	Melanistic areas	11.1	4.7
	Raised lesions	10.1	9.3
	Large lesions	6.1	0
	Barbel abnormalities	47.5	25.6*

Data presented as means ± standard errors; * indicates significantly different than Ashtabula at $p < 0.05$.

Largemouth Bass Necropsy-based Observations

Twenty-four largemouth bass were collected from each site at each sampling time with the exception of the Fall 2003 Conneaut sample when only 16 bass were collected. In Fall 2002 bass collected at Conneaut were approximately a year older than those collected at Ashtabula. They were also significantly larger. During the other sampling periods the mean age between sites was not significantly different and was approximately 3-3½ years. In summer 2003 there were no significant differences between sites. In Fall 2003 bass from the Conneaut were significantly heavier than those collected at Ashtabula. In Spring 2004 there were no significantly differences between the sites (Table 3).

TABLE 3. SEASONAL COMPARISON OF MORPHOMETRIC PARAMETERS IN LARGEMOUTH BASS SAMPLED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK, 2002-2004

Sampling season	**Fall (October) 2002**		**Summer (July) 2003**	
Site	**Ashtabula**	**Conneaut**	**Ashtabula**	**Conneaut**
Sample size (n)	24	24	24	24
Length (mm)	248.7 ± 3.5	322.9 ± 14.7*	294.7 ± 11.0	297.3 ± 10.6
Weight (g)	229.0 ± 16.8	559.6 ± 78.7*	355.1 ± 41.7	372.8 ± 39.6
Age	2.8 ± 0.1	3.8 ± 0.3	3.5 ± 0.5	3.3 ± 0.4
Condition factor	1.47 ± 0.09	1.42 ± 0.03	1.28 ± 0.04	1.36 ± 0.05
Hepatosomatic index	1.14 ± 0.07	1.17 ± 0.07	1.15 ± 0.09	1.10 ± 0.10
Sampling season	**Fall (October) 2003**		**Spring (April) 2004**	
Sample size (n)	24	16	24	24
Length (mm)	291.8 ± 8.3	318.6 ± 8.6	326.1 ± 10.9	342.3 ± 8.4
Weight (g)	412.3 ± 31.2	513.8 ± 53.9*	588.0 ± 72.3	610.3 ± 52.1
Age	3.3 ± 0.1	3.4 ± 0.2	3.5 ± 0.3	3.6 ± 0.2
Condition factor	1.73 ± 0.19	1.52 ± 0.03	1.60 ± 0.10	1.47 ± 0.05
Hepatosomatic index	1.36 ± 0.08	1.27 ± 0.05	2.29 ± 0.44	1.76 ± 0.10

Data presented as means ± standard error. * Indicates significantly different than Ashtabula at $p < 0.05$.

Data from all four sampling periods was combined for statistical analyses. The mean age of bass collected at the two sites was very similar, as were most other morphometric measurements. Bass collected at Conneaut were significantly heavier than those collected at Ashtabula and had higher condition factors and HSI (Table 4). Similar results were seen in a study of smallmouth bass *Micropterus salmoides* collected from PCB-contaminated and uncontaminated reaches of the Kalamazoo river. Bass collected from the uncontaminated area were slightly heavier and had a significantly higher condition factors and HSI (Anderson et al. 2003). Laboratory studies have shown that PCB exposure of fishes may result in developmental abnormalities, reduced growth rates and increased mortality (Niimi 1996; Monosson 1999).

TABLE 4. AN OVERALL COMPARISON OF MORPHOMETERIC PARAMETERS IN LARGEMOUTH BASS COLLECTED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK DURING 2002-2004.

Site	**Ashtabula**	**Conneaut**
Sample size (n)	96	88
Length (mm)	290.3 ± 5.2	320.4 ± 5.8
Weight (g)	396.1 ± 25.7	514.2 ± 29.6*
Age	3.3 ± 0.1	3.5 ± 0.1
Condition factor	1.52 ± 0.06	1.44 ± 0.02
Hepatosomatic index	2.37 ± 0.09	2.50 ± 0.10

Data presented as means ± standard errors; * indicates significantly different than Ashtabula at $p < 0.05$.

A variety of external lesions were noted in largemouth bass from both the Conneaut and Ashtabula sites. Most of these were inflammatory reactions in response to a variety of parasites including helminths, myxosporidians and protozoa. Raised growths on one fish from each site were diagnosed as papillomas. There did not appear to be differences between the sites in the number or types of external lesions in largemouth bass.

Bullhead Genotoxic Results

The comet assay, a method used to measure DNA strand breaks, showed variations between sampling seasons and sites in genetic damage (ANOVA, $p < 0.001$); thus the two rivers were compared by the season. There was no significant difference between fish sampled from the two sites in Fall 2003, however fish from the Ashtabula River had greater genetic damage than fish from the Conneaut River in Summer 2003 and Spring 2004 (Table 5).

TABLE 5. COMPARISON OF GENETIC DAMAGE BETWEEN BROWN BULLHEAD SAMPLED FROM THE ASHTABULA AND CONNEAUT RIVERS AT DIFFERENT SEASONS

Sampling season		**Fall (October) 2002**		**Summer (July) 2003**	
Site		Ashtabula	Conneaut	Ashtabula	Conneaut
Sample size (n)		27	0	24	5
Comet assay	Tail length (μm)	42.23 ± 1.14	NA	70.09 ± 1.71	55.94 ± 6.90*
	Tail DNA (%)	15.94 ± 0.63	NA	58.47 ± 2.97	36.68 ± 4.90*
	Tail extent Moment (μm)	8.32 ± 0.76	NA	44.08 ± 2.72	25.19 ± 4.95*
	Olive tail moment (μm)	3.00 ± 0.29	NA	17.38 ± 1.27	9.47 ± 1.56*
Sampling season		**Fall (October) 2003**		**Spring (April) 2004**	
Site		Ashtabula	Conneaut	Ashtabula	Conneaut
Sample size (n)		24	14	24	24
Comet assay	Tail length (μm)	50.60 ± 1.36	50.68 ± 1.04	39.92 ± 1.65	18.24 ± 1.30*
	Tail DNA (%)	42.07 ± 1.68	41.85 ± 1.90	23.25 ± 1.75	9.43 ± 0.62*
	Tail extent Moment (μm)	22.82 ± 1.41	22.48 ± 1.31	11.52 ± 1.08	2.59 ± 0.38*
	Olive tail moment (μm)	8.55 ± 0.57	8.22 ± 0.52	4.32 ± 0.41	1.23 ± 0.12*

Data presented as Means ± S.E.; NA: not available; * indicates Ashtabula was significantly higher than Conneaut at $p < 0.01$

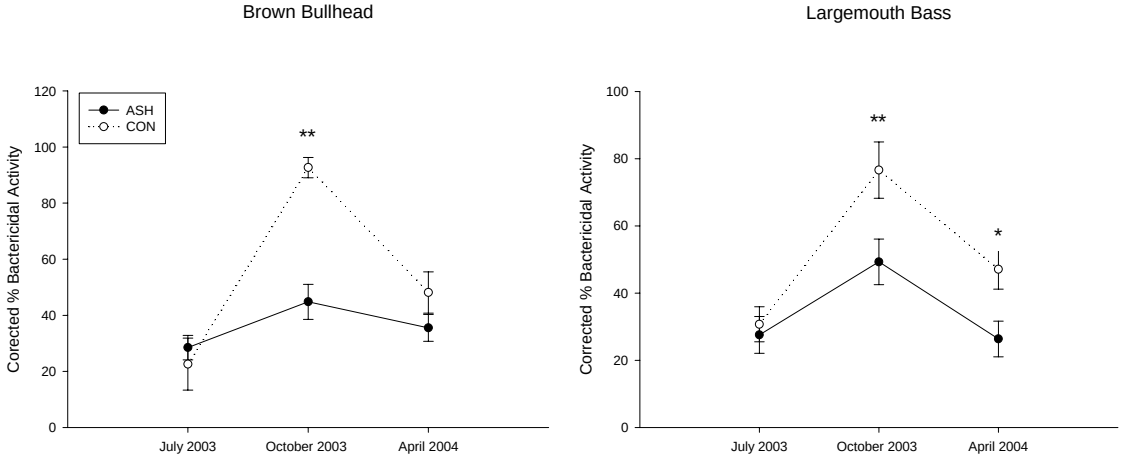
Previous studies in a number of species have indicated the genotoxicity (as measured by strand breaks) of PAHs (Nacci et al. 1996; Mitchelmore and Chipman 1998), however, the ability of PCBs to induce DNA strand breaks is less well-established and appears to vary significantly among species (Akcha et al. 2003). In dab *Limanda limanda* an increase in DNA fragmentation was demonstrated *in vivo* after exposure to PCBs 118 and 77 (Piechotta et al. 1999), while DNA strand breaks were positively correlated with certain PCB congeners along a pollution gradient in the North Sea (Everaarts 1995).

Immune Function Results

Adherent cell (macrophage) function:

The October 2003 and April 2004 samples were the most informative in the case of the bactericidal activity for both brown bullheads and largemouth bass. When differences were observed, bactericidal activity was lower in the Ashtabula River than the Conneaut River. This observation was consistent across species. Bactericidal activity was significantly lower ($P<0.001$, $P=0.020$) in bullheads and bass, respectively, from the Ashtabula in October 2003 (Figure 3). During April 2004 bactericidal activity significantly was lower ($P=0.040$)in Ashtabula River largemouth bass when compared to the Conneaut bass. No differences were observed during the July sampling for either species. These findings suggest that macrophages from fish in the Ashtabula would be less able to kill or inactivate engulfed infectious agents. Hence, they would be more prone to infectious diseases. Chinook salmon *Oncorhynchus tshawytscha* collected at PAH/PCB-contaminated sites had reduced host resistance when challenged with a bacterial pathogen (Arkoosh et al. 1998). In laboratory exposures salmon exposed to a PCB mixture had a significantly reduced primary and secondary immune response and an increased mortality after exposure to the bacterial pathogen *Vibrio anguillarum* (Arkoosh and Collier 2002).

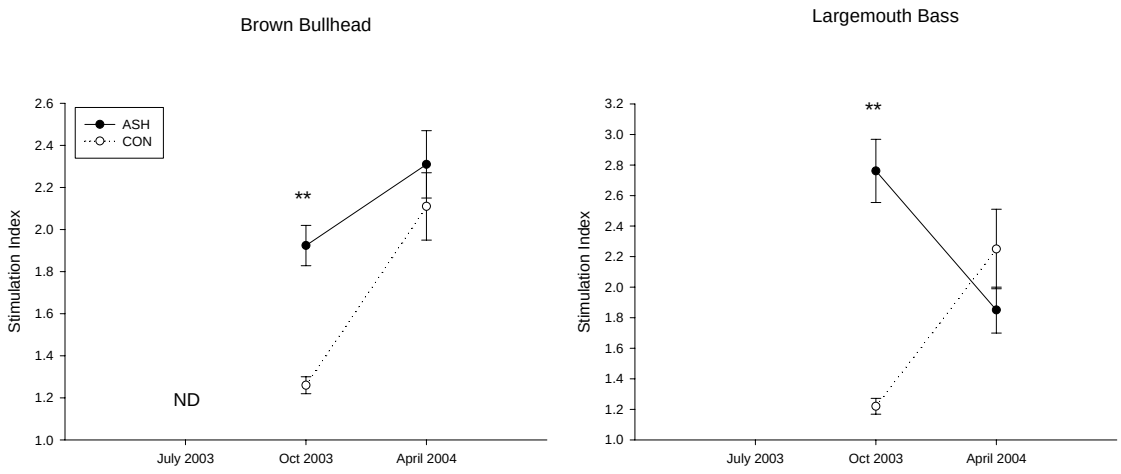
FIGURE 3. BACTERICIDAL ACTIVITY OF ADHERENT CELLS IN BROWN BULLHEADS AND LARGEMOUTH BASS COLLECTED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK 2003-2004



Data presented as means ± standard errors; * indicates a significant difference $P < 0.05$, ** indicates a significant difference $P \leq 0.02$.

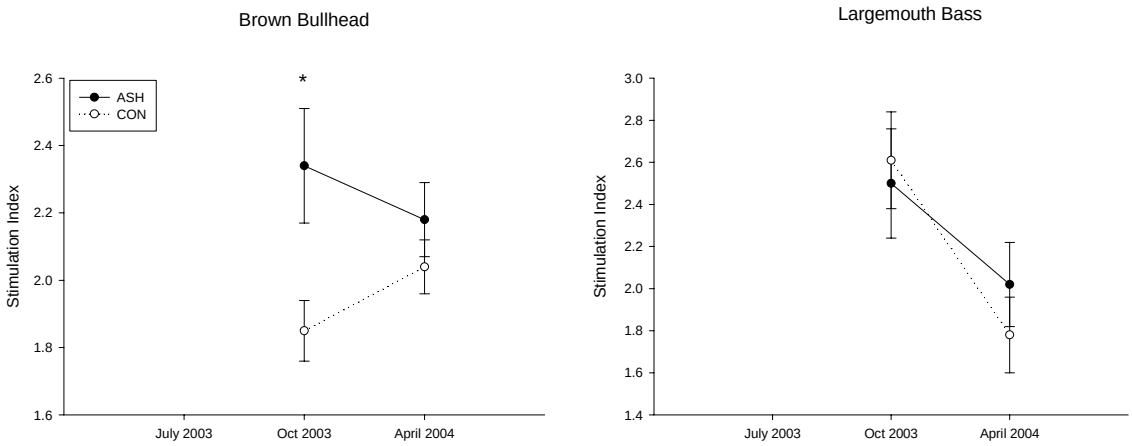
In contrast to bactericidal activity, respiratory burst activity in response to LPS stimulation tended to be higher in the Ashtabula compared to the Conneaut. Reactive oxygen species (ROS) induced by LPS were significantly higher in brown bullheads and largemouth bass collected from the Ashtabula River compared to those from the Conneaut ($P < 0.001$) in October 2003. Differences were not observed during April 2004 and the assay was not run during July 2003 (Figure 4). Similar differences were observed in the case of superoxide dismutase (SOD) insensitive ROS. A similar significant elevation was observed in Ashtabula bullheads ($P = 0.022$) during October 2003, but not in largemouth bass or during the Spring 2004 sampling (Figure 5).

FIGURE 4. RESPIRATORY BURST ACTIVITY, LPS INDUCIBLE REACTIVE OXYGEN SPECIES, IN BROWN BULLHEAD AND LARGEMOUTH BASS COLLECTED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK, 2003-2004



Data presented as means ± standard errors; * indicates a significant difference $P < 0.05$, ** indicates a significant difference $P \leq 0.02$.

FIGURE 5. RESPIRATORY BURST ACTIVITY, SOD INSENSITIVE REACTIVE OXYGEN SPECIES, IN BROWN BULLHEAD AND LARGEMOUTH BASS COLLECTED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK, 2003-2004



Data presented as means ± standard errors; * indicates a significant difference $P < 0.05$, ** indicates a significant difference $P \leq 0.02$.

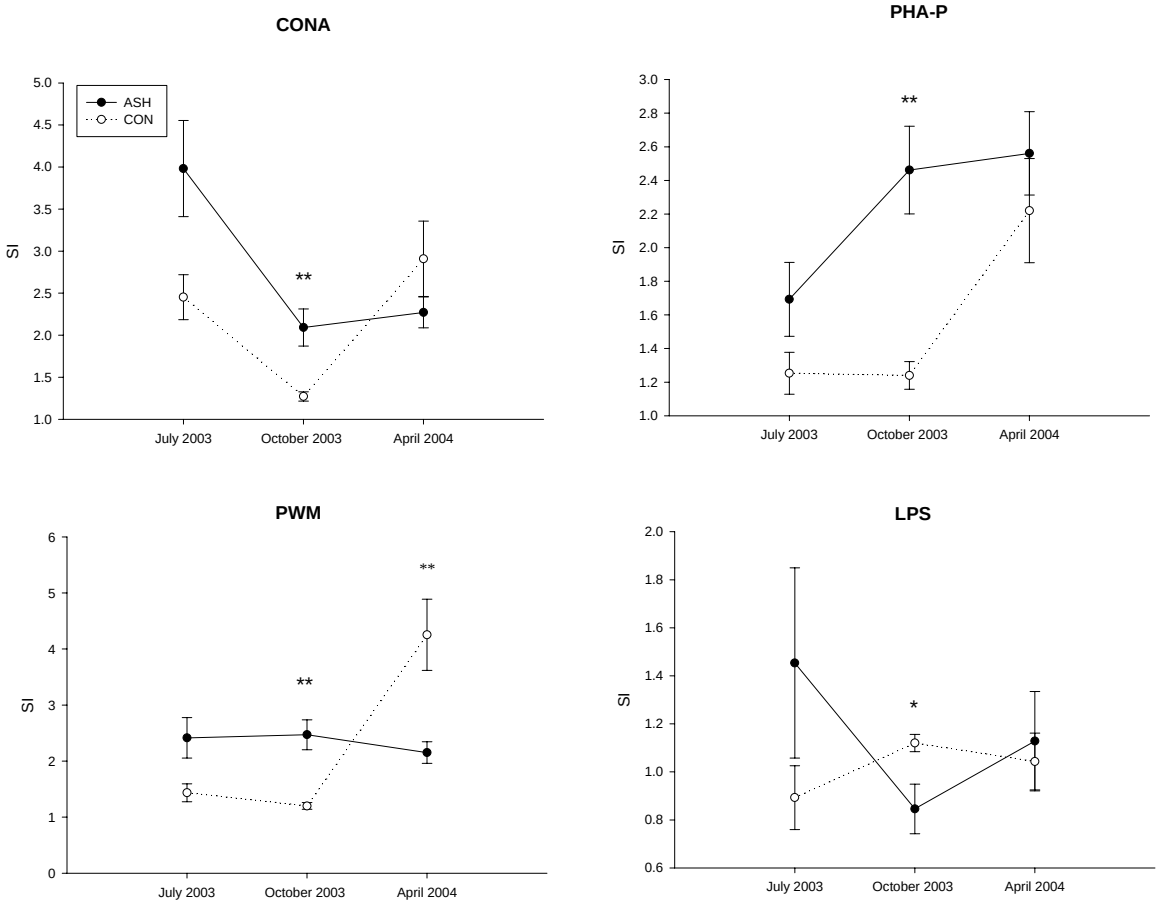
Similar observations have been made in mummichogs *Fundulus heteroclitis* collected from PAH contaminated sites and medaka in laboratory experiments (Kelly-Reay and Weeks-Perkins 1994; Carlson et al. 2002). PAH contamination is documented to lessen the ability of macrophages to kill bacteria (bactericidal 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 1989, 1991). No differences were noted in these cell populations at the species level during the July 2003, suggesting an overriding seasonal effect. Such seasonal effects are natural and not unexpected based on similar environmental monitoring research (Chesapeake Bay tributary health project) with other species (white perch). There tends to be an overall suppression in adherent cell population activity as water temperatures increases during the summer months (unpublished data). The above data is consistent with the effects caused by PAH/PCB and heavy metal exposure.

Lymphocyte mitogenesis:

Due to poor cell adherence of largemouth bass leukocytes during this assay, data from these fish are not reported here. Similar to the bactericidal and respiratory burst assays, October 2003 samples appear to be most informative for the mitogenesis assay. Only five (5) brown bullheads were collected form the Conneaut River during July 2003. In general the stimulation index for all T-cell mitogens was higher in bullheads from the Ashtabula when compared to Conneaut. During October 2003, the stimulation indices for all mitogens differed significantly, and were higher in the Ashtabula River brown bullheads (n = 24); Con A ($P = 0.007$), PHA-P ($P < 0.001$) and PWM ($P < 0.001$) when compared to those from the Conneaut (n = 14). Conversely stimulation indices were lower in the Ashtabula during this sample period for LPS stimulated lymphocytes ($P = 0.044$). Significant differences ($P = 0.014$) in mitogenesis during April 2004 were observed in PWM sensitive lymphocytes (Figure 6).

Increased proliferative responses in 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 (Smailowicz et al. 1989; Arkoosh et al. 1996; Segre et al. 2002; Iwanowicz et al. 2005; Keller et al. 2006). In general the PHA-P response of bullhead from the Ashtabula was higher than that of fish from the Conneaut, and this response is significantly correlated with a number of contaminants measured in this study, including PCBs (see Table 11 – Contaminant body burden section). 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.

Figure 6. **LYMPHOCYTE PROLIFERATIVE RESPONSES TO THE MITOGENS CONA A, PHA-P, PWM AND LPS IN BROWN BULLHEADS COLLECTED FROM THE ASHTABULA AND CONNEAUT CREEK IN 2003-2004**

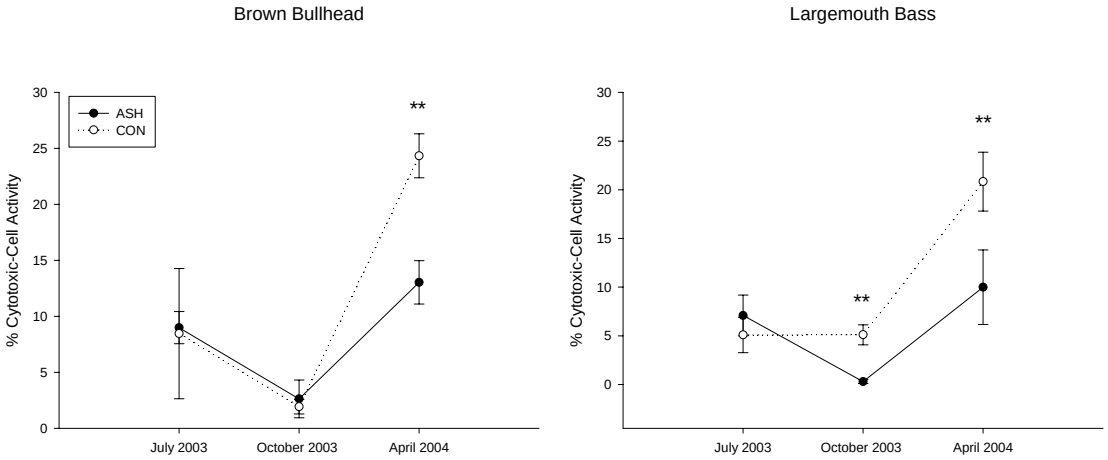


Data presented as means ± standard errors; * indicates a significant difference $P < 0.05$, ** indicates a significant difference $P \leq 0.02$.

Cytotoxic-cell activity:

Cytotoxic-cell activity evaluated at a 10:1 (effector to target cell) ratio revealed significant a depression in Ashtabula River brown bullhead and largemouth bass during April 2004 (P<0.001). Lower activity in this cell population was noted in Ashtabula largemouth bass during the October 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 (Figure 7).

FIGURE 7. CYTOTOXIC CELL ACTIVITY IN BROWN BULLHEAD AND LARGEMOUTH BASS FROM CONNEAUT CREEK AND ASHTABULA RIVER, 2003-2004, AT EFFECTOR TO TARGET CELL RATIOS OF 10:1



Data presented as means ± standard errors; * indicates a significant difference $P < 0.05$, ** indicates a significant difference $P \leq 0.02$.

Cytotoxic cell activity for bullhead and largemouth bass was generally lower in the Ashtabula River than the Conneaut. This cell population is most known for activity against parasites and virally infected cells (Jaso-Friedmann et al. 2000; Somamoto et al. 2002). However, they are also tumoricidal in fish (Cuesta et al. 2003) as well as other vertebrates (Herberman and Ortaldo 1981). 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). It is interesting to note that a helminth parasite was observed within the liver and splenic tissue of largemouth bass at both sites (Figure 8). Based on subjective observations it appeared there were more parasites, replacing a greater percent of tissue, particularly in the spleen. Hence, using image analysis the amount of tissue occupied by parasites was measured. At all collections times except July 2003, significantly greater numbers (greater tissue occupied) of parasites were observed in bass collected at Ashtabula when compared to those collected at Conneaut Creek (Figure 9).

Figure 8. Helminth Parasites Within the Liver and Splenic Tissue of Largemouth Bass Collected in the Ashtabula River and Conneaut Creek.

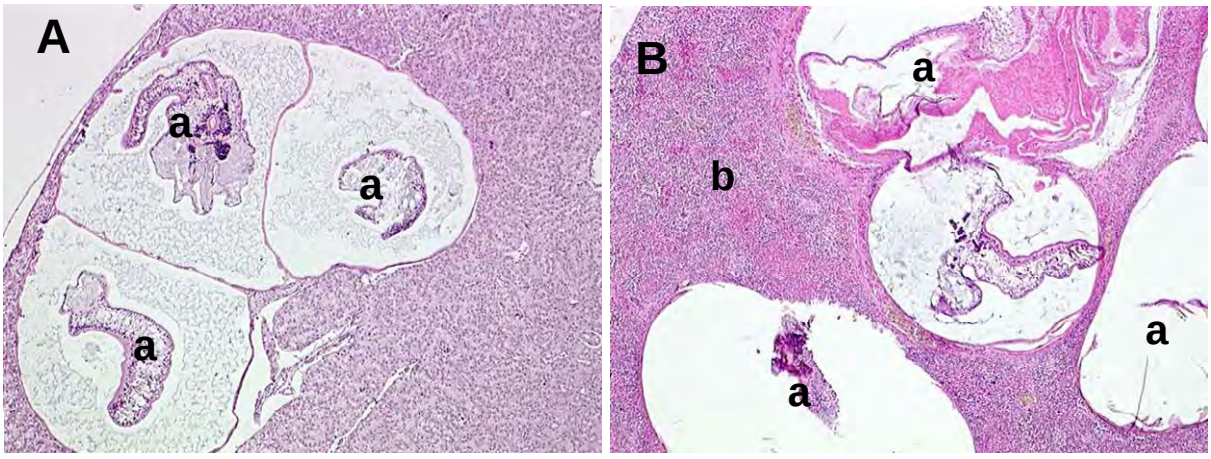
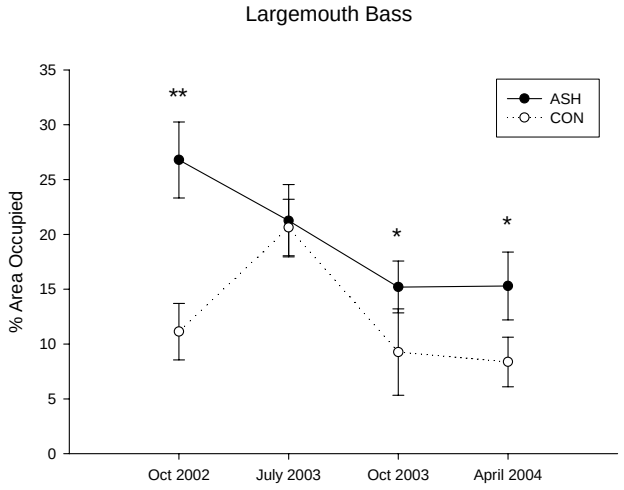


Figure 8. A. Section of liver from a largemouth bass collected in the Ashtabula river, illustrating three helminth parasite cyst containing live parasites (a). B. Splenic tissue in bass from the Ashtabula river with numerous helminth parasites (a) replacing normal tissue (b).

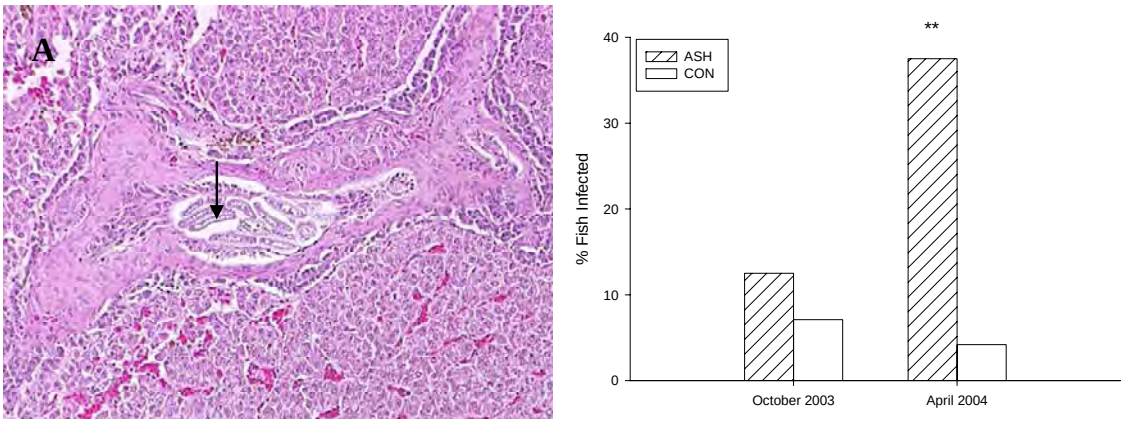
FIGURE 9. SEASONAL COMPARSION OF PERCENT SPLENIC AREA OCCUPIED BY HELMINTH PARASITES IN LARGEMOUTH BASS COLLECTED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK 2002-2004



Data presented as means ± standard errors; * indicates a significant difference $P < 0.05$, ** indicates a significant difference $P \leq 0.02$.

A similar observation was made with a myxosporidian parasite (Figure 10A) observed within the bile ducts of brown bullhead. In this case we compared the percentage of fish infected based on histological evaluation in October 2003 and April 2004. Significantly more bullhead collected in the Ashtabula were infected when compared to those collected in Conneaut Creek (Figure 10B).

FIGURE 10. MYXOSPORIDIAN PARASITE OBSERVED WITHIN THE BILE DUCTS OF BROWN BULLHEAD



A. Sporoplasm of a myxosporidian parasite (arrow) within the bile ducts of brown bullhead. B. Seasonal comparison of the percentage of bullhead infected at each site. ** indicates significantly different at $P < 0.005$.

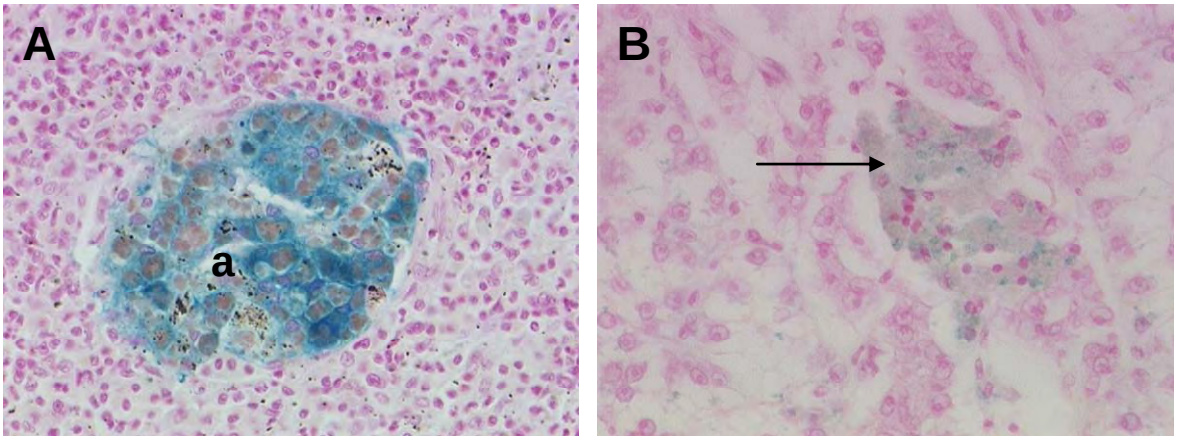
Histopathology Results

Macrophage Aggregate Parameters

Seasonal Comparisons – Bullhead and Bass

Macrophage aggregates (MA) are structures observed within the spleen, kidney and liver of fishes (Figure 11). Their relative distribution and pigment content differs among species and also within species in relation to age, environmental stressors, infectious disease and nutrition (Wolke 1992; Agius and Roberts 2003). Splenic MA, and to a lesser degree liver and kidney MAs, have been used extensively as indicators of exposure to degraded environments (Blazer et al. 1997; Fournie et al. 2001) and recently have been used to assess the effects of remediation on fish health in Burlington Harbor, Lake Champlain (Facey et al. 2005).

Figure 11. Microscopic Appearance of Macrophage Aggregates Measured in the Spleen and Liver of Largemouth Bass and Brown Bullhead



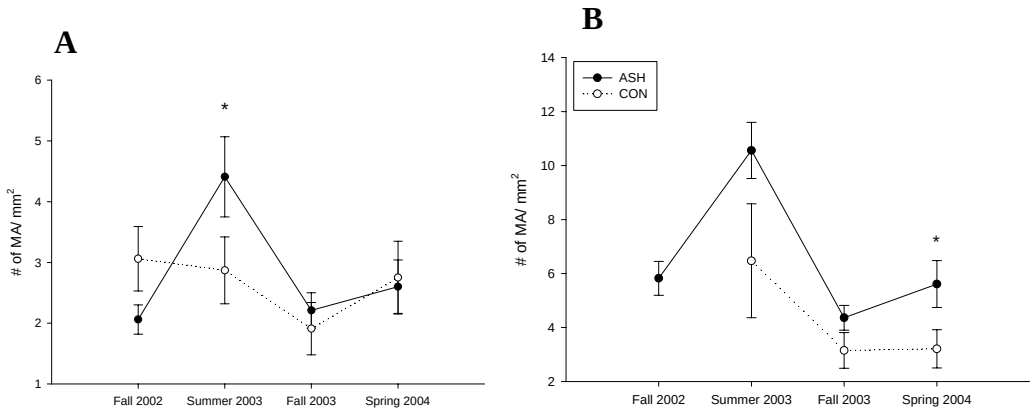
A. Splenic macrophage aggregate (a) of largemouth bass stained by the Perl's method which allows for visualization of the three pigments commonly observed. Hemosiderin, an iron-containing stains blue, melanin appears black and ceroid/lipofuscin, a lipopigment resulting from oxidative damage appears yellowish-brown. B. Hepatic macrophage aggregates of brown bullhead (arrow) often contain more ceroid/lipofuscin than hemosiderin.

The number of aggregates per mm^2, the mean size (area) of aggregates and the percent of tissue occupied by aggregates were evaluated in spleen and liver of bullhead and spleen of largemouth bass. Statistical analysis was not run if $n < 12$. The parameter of shape factor was never significantly different so graphs for this measurement are not included below. In all cases, when significant differences were found measurement parameters were higher in the Ashtabula. More MA aggregates and a higher percent of tissue were occupied by splenic MA in bullheads collected at Ashtabula versus Conneaut during all sampling periods (Figure 12). However, significant differences were noted in number of macrophage aggregates per mm^2 (P=0.014) and percent area occupied (P=0.009) only during the Spring of 2004. Statistical comparisons were not made for Fall 2002 and Summer 2003 due to low sample size from the Conneaut.

A significantly greater number of macrophage aggregates per mm^2 were observed in largemouth bass collected from the Ashtabula during the summer of 2003 (P=0.019) compared to bass from the Conneaut. In Fall 2002, the number of aggregates and the percent tissue occupied were higher, although not significantly, in bass collected from Conneaut (Figure 12). However, the mean age of bass during this collection time was approximately a year older at

Conneaut, when compared to Ashtabula (Table 3). It was previously shown that age has a significant influence on MA parameters in largemouth bass collected at reference sites (Blazer et al. 1987).

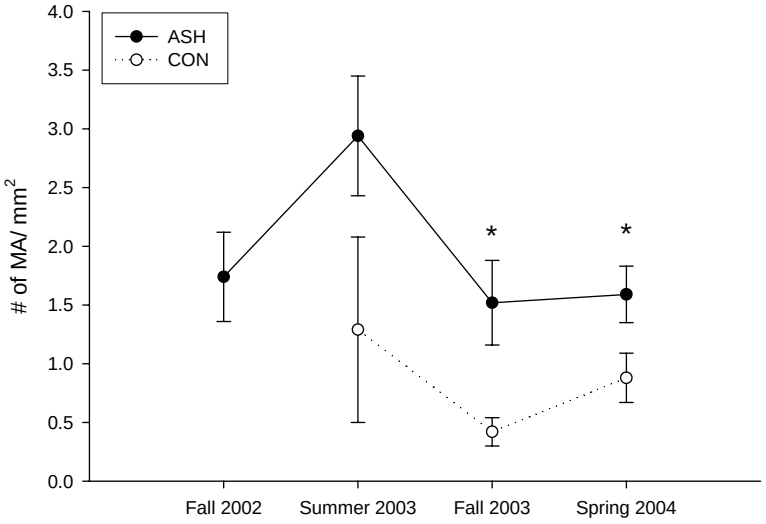
FIGURE 12. SEASONAL COMPARISON OF SPLENIC MACROPHAGE AGGREGATES IN BROWN BULLHEAD AND LARGEMOUTH BASS SAMPLED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK, 2002-2004



Mean number of MA/ mm^2 in (A) bullhead and (B) bass spleens; means ± standard errors;
* indicates a significant difference P < 0.05, ** indicates a significant difference P < 0.02

There were greater differences between the sites in hepatic (versus splenic) MAs of bullheads (Figure 13). Significant differences were noted in the number of macrophage aggregates per mm^2 during the Fall of 2003 (P=0.002) and Spring of 2004 (P=0.002). In both cases more macrophage aggregates were noted in bullheads collected from the Ashtabula. The percent area occupied by macrophage aggregates was significantly higher (P<0.001) in bullheads collected from the Ashtabula during the Spring of 2004 compared to those from the Conneaut.

FIGURE 13. SEASONAL COMPARSION OF HEPATIC MACROPHAGE AGGREGATES IN BROWN BULLHEAD SAMPLED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK, 2002-2004



Mean number of MA/ mm^2 in bullhead livers; means ± standard errors; * indicates a significant difference $P < 0.05$, ** indicates a significant difference $P < 0.02$

Comparisons of Macrophage Aggregate Parameters

When all data was combined the mean age of Ashtabula bass was similar to that of Conneaut bass, as were MA parameters (Table 6). Ashtabula bullheads were slightly older (4.8 versus 4.0 years), however splenic MA number, percent of spleen occupied by MA and number of hepatic MA were all approximately double that observed in Conneaut bullheads.

Increase in the number or size of macrophage aggregates have 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). In addition, numerous field and laboratory studies have used them as indicators of environmental stress (reviewed by Wolke 1992; Aguis and Roberts 2003). Exposure to a variety of 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) and PCBs (Pierce et al. 1980; Anderson et al. 2003) have all been shown to increase MAs. 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 at the contaminated site (Blazer et al. 1994). Bowser et al. (1990) also found increased hepatic macrophage aggregates in bullhead collected at a site contaminated with PCBs and metals when compared to a reference site.

TABLE 6. COMPARISON OF MACROPHAGE AGGREGATE PARAMETERS IN LARGEMOUTH BASS AND BROWN BULLHEAD COLLECTED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK, 2002-2004

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

*Indicates a significant difference between sites at $p < 0.05$.

Brown Bullhead – Liver Pathology

The brown bullhead has been designated a key indicator species for Great Lakes Areas of Concern (AOC) because they are bottom-dwelling fish with a small home range that are known to take up contaminants from food and sediments (Maccubbin et al. 1985; Baumann 1989; Smith et al. 1994) and because liver (Harshbarger and Clark 1990; Baumann and Harshbarger 1995; Baumann et al. 1991) neoplasms have been associated with PAH and other contaminant exposure in this species. There is also good evidence for the influence of PAH and PCB exposure on the development of liver 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), winter flounder *Pseudopleuronectes americanus*

(Gardner et al. 1989), marine flatfish *Platichthys flesus* (Grinwis et al. 2001; Kohler et al. 2002) and walleye *Stizostedium vitreum vitreum* (Barron et al. 2000).

One of the 14 beneficial use impairments at AOC is listed as "fish tumors and other deformities", defined as occurring when "the incidence rate of fish tumors and other deformities exceeds rates at unimpacted or control sites or when survey data confirm the presence of neoplastic or preneoplastic liver tumors in bullhead or suckers" (IJC, 1989; www.epa.gov/lakeerie/buia/reports). Hence, over the years numerous surveys have been undertaken to assess the incidence of liver tumors, as well as other contaminant-associated microscopic lesions, at AOCs. However, a major problem has been a lack of consistent criteria for evaluating histological changes in bullhead livers. For instance, in some studies there is no distinction between preneoplastic, altered foci and neoplastic lesions, or no good descriptions of individual lesions (Black 1983; Pyron et al. 2001). Baumann et al. (2000) presents a compilation of liver tumor (neoplasm) prevalence at AOCs by location and year. However, a comparison of the papers/reports from which these data were obtained demonstrates the lack of consistent criteria. For instance, a prevalence of 7% was noted in the Ashtabula River in 1991. In that study neoplasia included only hepatocellular carcinoma or cholangiocarcinoma, not hepatocellular adenoma, cholangioma or altered foci (Mueller and Mac 1994). Conversely, the prevalences reported for the Black river in 1982 (60%) and the Detroit river in 1985-87 (9%) included carcinomas, adenomas and in some cases, altered foci (Baumann et al. 1990; Maccubbin and Ersing 1991). For this reason, at the request of PA Sea Grant, we have developed a paper describing diagnostic criteria for proliferative liver lesions in bullheads (Blazer et al. Submitted).

Bullhead livers, as in most other teleosts, are composed of hepatic tubules. Hepatocyte appearance of teleosts can vary greatly due to sex, maturity, diet, season, contaminant exposure and other factors (Hinton and Couch 1998; Rocha and Monteiro 1999). Pancreatic tissue is commonly observed around the portal veins and bile ducts may be observed within this tissue. In liver sections from bullheads collected at reference sites bile ducts are often sparse and not prominent in sections examined by light microscopy (Figure 14A), although bile preductules and ductules are numerous and are located within the hepatic parenchyma (Hinton and Couch 1998).

Proliferative lesions of the liver are separated into nonneoplastic and neoplastic lesions of either hepatocellular or biliary origin. Nonneoplastic lesions include altered foci and bile duct

proliferation and fibrosis, while neoplastic lesions include hepatocellular adenoma, hepatocellular carcinoma, cholangioma and cholangiocarcinoma.

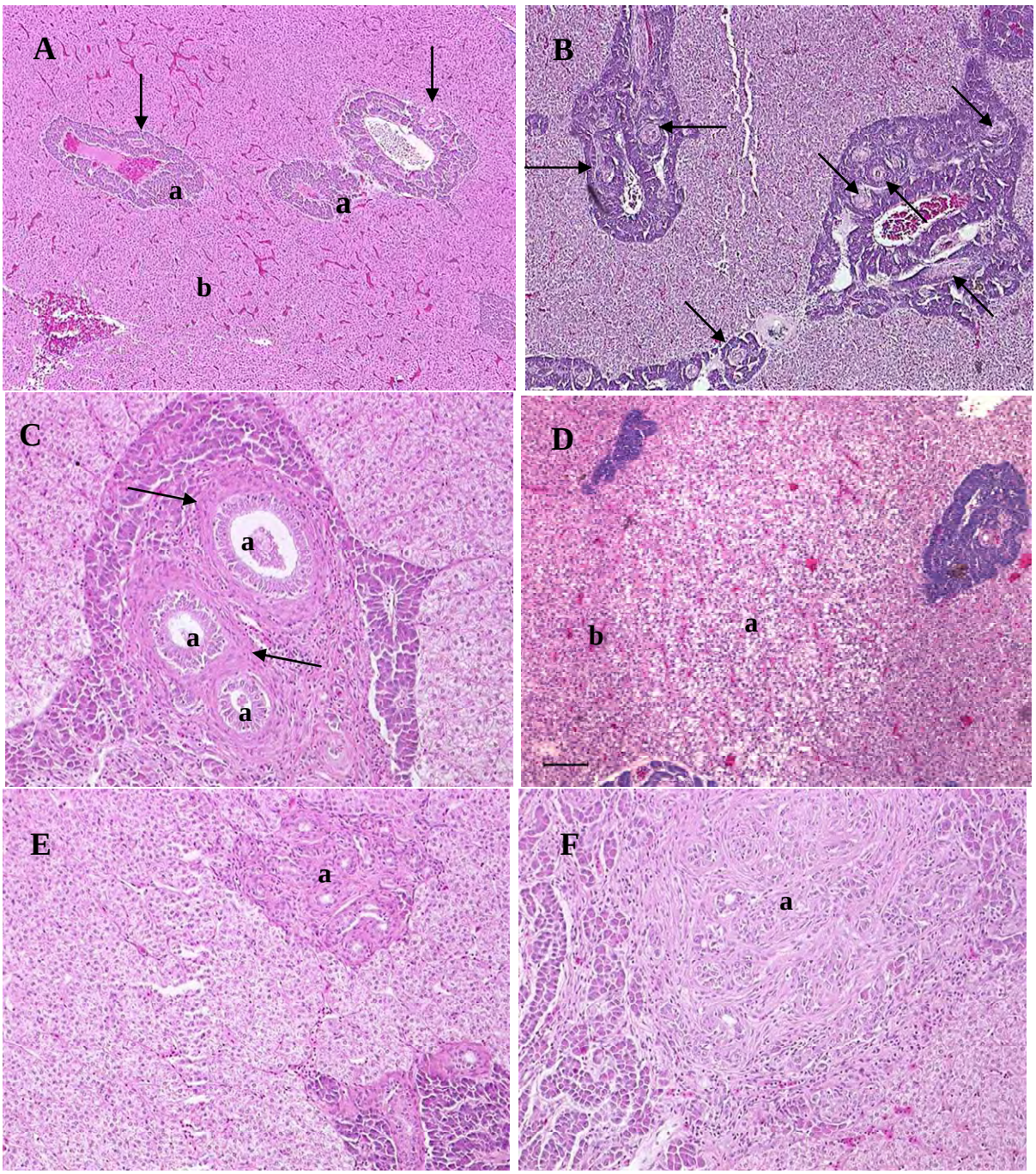
A number of microscopic lesions were noted in the liver of brown bullheads in this study. Bile duct proliferation (Figure 14B), bile duct fibrosis (Figure 14C), altered foci (Figure 14D) and cholangioma (Figure 14E) were observed in fish collected at both sites, while cholangiocarcinoma (Figure 14F) was only observed in bullhead from Ashtabula. Hepatic cell neoplasia was not observed at either site.

A number of the proliferative lesions (bile duct proliferation, altered foci and bile duct neoplasia) were observed at higher prevalences in bullhead collected in the Ashtabula river (Table 7). Only one fish (4 years of age) was found to have neoplastic biliary changes (cholangioma) in the Conneaut samples. In the Ashtabula samples, seven fish (ages 6,6,6,6,7,7,9) were observed with cholangiomas and six (ages 2,3,3,4,6,8) had cholangiocarcinomas. Hence, at Ashtabula a more aggressive, malignant neoplasia was observed. Grinwis et al. (2001) found a higher number of proliferating hepatocytes in flounder exposed to PCB-126, suggesting it may play a role in tumor development.

TABLE 7. COMPARISON OF THE PREVALENCE OF MICROSCOPIC LESIONS OBSERVED IN BROWN BULLHEAD LIVERS COLLECTED AT CONNEAUT CREEK AND ASHTABULA RIVER 2002-2004

Parameter	Conneaut Creek (n=43)	Ashtabula River (n=99)
Foci of necrosis	**9.3**	**15.2**
Bile duct proliferation	**25.6**	**48.5**
Bile duct fibrosis	**51.2**	**58.6**
Altered foci	**16.3**	**21.2**
Cholangioma	**2.3**	**7.1**
Cholangiocarcinoma	**0**	**6.1**
Total bile duct neoplasia	**2.3**	**13.1**

FIGURE 14. MICROSCOPIC APPEARANCE OF HEPATIC LESIONS IN BROWN BULLHEAD



A. Normal bullhead liver illustrating hepatopancreatic tissue (a) which contains sparse bile ducts (arrows) and is found within the hepatic parenchyma (b). B. Bile duct proliferation (arrows) occurs when many more, but normal-appearing bile ducts are observed. C. In some cases fibrosis (arrows) was observed around otherwise normal bile ducts. D. Altered cell foci (a) blend imperceptibly into normal hepatic parenchyma (b). E. Cholangioma (a), a less invasive, neoplasm and F. Cholangiocarcinoma, a malignant neoplasm of bile ducts (a) were observed.

Reproductive Health – Brown Bullheads

Gonadosomatic index:

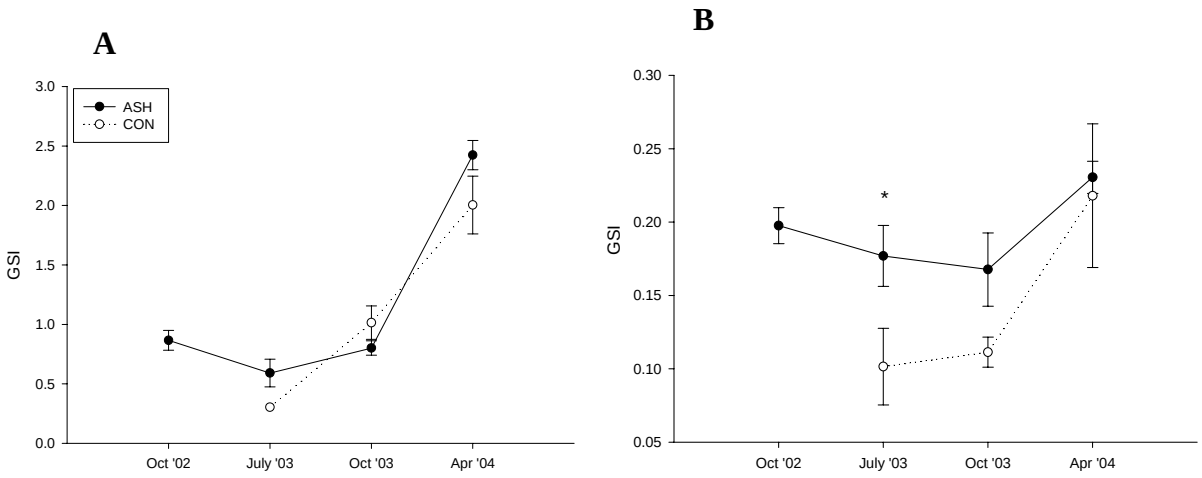
Gonad weights were obtained in the field and the gonadosomatic index (GSI) calculated using the following formula: (Gonad weight X 100) / (total body weight – gonad weight). GSI is widely used as an index of gonadal activity as pronounced variations in gonad size throughout the reproductive cycle. Although, it has been shown, at least in some species, this is not an accurate indicator of gonadal activity (DeVlaming et al. 1982). However, used in conjunction with other indicators for comparing individuals collected at the same time seasonally it has been used as an indicator of impaired gonadal development due to exposures to mercury, leachate from refuse dumps and municipal sewage effluent (Friedmann et al. 1996; Noaksson et al. 2001; Diniz et al. 2005).

Previous reports of the reproductive natural history of brown bullheads in the Northeastern U.S. indicate that the size/age fish we collected were sexually mature and that reproductive activity in this area generally begins in May and spawning may occur in late May-early June, but may differ annually due to climatic factors (Burke and Leatherland 1984; Burke et al. 1984; Blumer 1985; Rosenblum et al. 1987).

The GSI for both male and female brown bullheads followed a seasonal pattern with the highest GSIs occurring during the April sampling. No statistical differences between sites were observed during any season in female bullheads. In general males bullheads from the Ashtabula had a higher GSI than those from the Conneaut; however, statistical differences were only observed during July 2003 (P=0.039). Average mean age of male brown bullheads from the Conneaut was about one year less in during all seasons.

Microscopic evaluation of the gonads of both male and female bullhead did not reveal any significant differences between those collected in the Ashtabula river and those collected in Conneaut creek.

FIGURE 15. SEASONAL COMPARISON OF GONADOSOMATIC INDEX IN BROWN BULLHEADS COLLECTED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK, 2002-2004



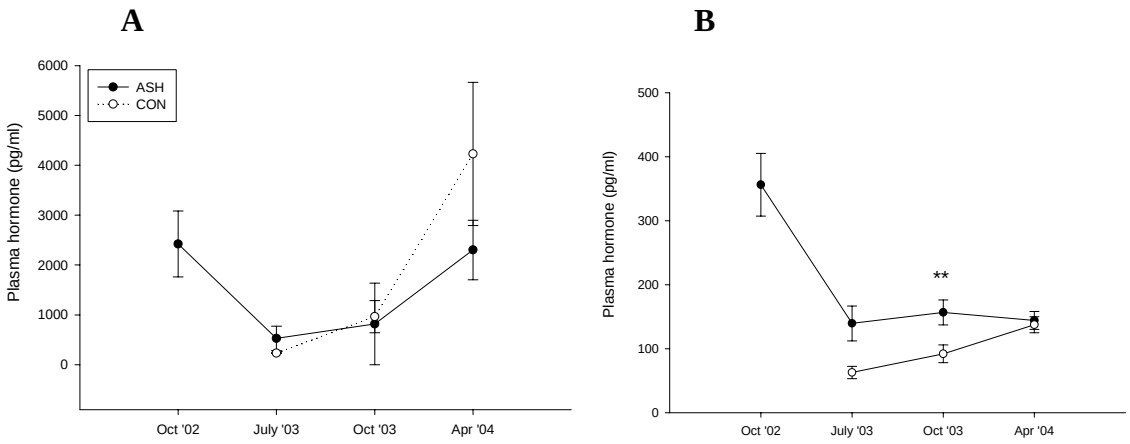
Mean GSI of (A) females and (B) males; means ± standard errors; * indicates a significant difference $P < 0.05$, ** indicates a significant difference $P < 0.02$.

Reproductive Hormones:

Extraction efficiencies of bullhead plasma were 91±2.4% and 85±3.5% for E_2 and T, respectively. Statistically significant differences were observed in sex hormone concentrations of brown bullheads. In general male bullhead from the Ashtabula had higher concentrations of E_2 than males from the Conneaut (Figure 16). Statistical differences were observed during October 2003 (P=0.016). Similarly, female bullhead from the Ashtabula had higher plasma T than females from the Conneaut (Figure 17). Statistically significant differences were observed during October 2003 and April 2004 (P=0.041 and 0.022, respectively). Male bullhead from the Ashtabula also had significantly higher T during October 2003 ($P \leq 0.001$).

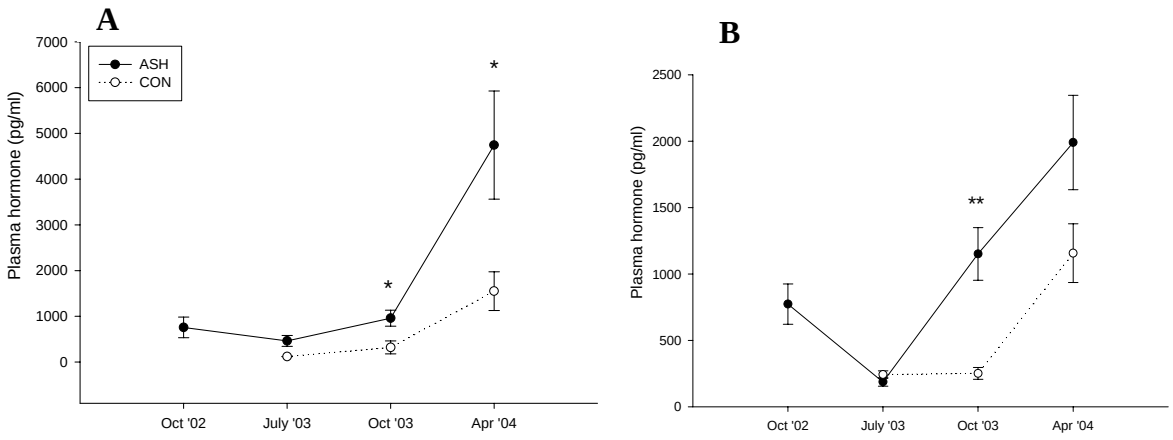
Previous studies with brown bullheads have shown altered steroid hormone levels in fish from contaminated sites. For instance, male brown bullheads collected from Lake Apopka, contaminated with organochlorine pesticides, and other domestic, agricultural and industrial pollution, have significantly higher plasma E_2 than males from uncontaminated sites. Likewise, females have higher testosterone levels than those from similar reference fish (Gallagher et al. 2001). These observations are consistent with those made in bullheads from the Ashtabula.

FIGURE 16. SEASONAL COMPARION OF PLASMA 17B- ESTRADIOL IN BROWN BULLHEADS COLLECTED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK



Mean plasma hormone concentrations of (A) females and (B) males; means ± standard errors; * indicates a significant difference $P < 0.05$, ** indicates a significant difference $P < 0.02$.

FIGURE 17. SEASONAL COMPARISON OF PLASMA TESTOSTERONE IN BROWN BULLHEADS COLLECTED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK



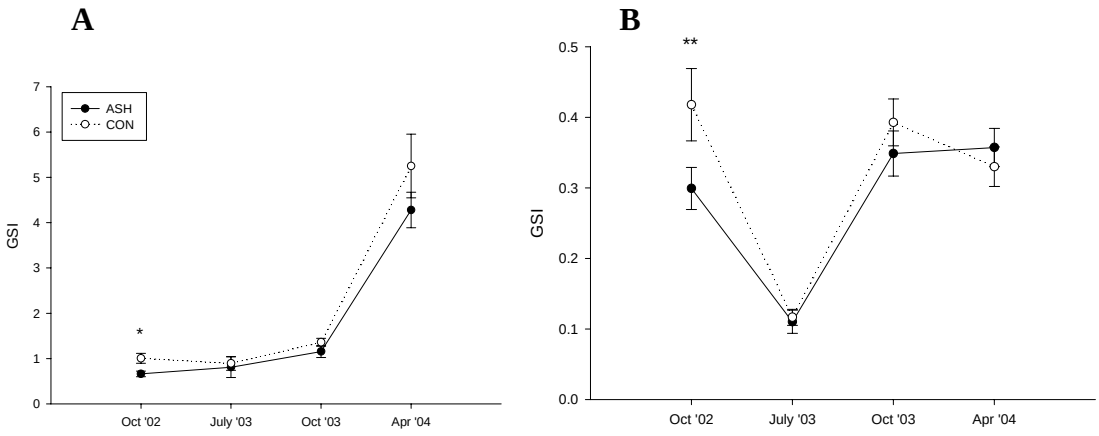
Mean plasma hormone concentrations of (A) females and (B) males; means ± standard errors; * indicates a significant difference $P < 0.05$, ** indicates a significant difference $P < 0.02$.

Reproductive Health – Largemouth Bass

Gonadosomatic Index:

The reproductive cycle of largemouth bass has been described, with much of the research being with the Florida strain *M. salmoides floridanus* in culture situations (Rosenblum et al. 1994; Gross et al. 2002). In a study in Texas, GSI peaked February to March for males and March-April for females, declined to the lowest value in September and October and then began to rapidly rise (Rosenblum et al. 1994). In one study, no significant differences were reported for GSI in largemouth bass downstream of pulp and paper mills in Florida (Sepúlveda et al. 2004). However in another study, female bass captured at sites in the St. John's river with either close proximity to a paper mill (high PAHs, PCBs and chlorinated benzenes compared to the reference site) or influenced by marine shipping (high PCBs compared to the reference site) had significantly lower GSI during the spawning season than those from the reference site. Only males collected at the site influenced by the paper mill had lower GSI compared to those from the reference site (Sepúlveda et al. 2002). In our study we saw no significant difference in GSI in either sex between sites (Figure 18), nor were there any histological differences evident.

FIGURE 18. SEASONAL COMPARIONS OF GSI IN LARGEMOUTH BASS COLLECTED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK, 2002-2004

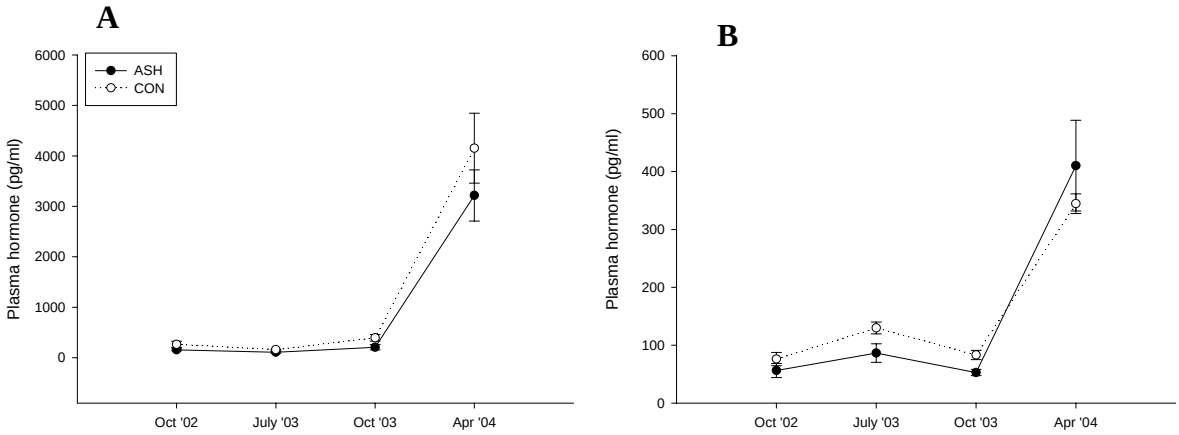


Mean GSI of (A) females and (B) males; means ± standard errors; * indicates a significant difference $P < 0.05$, ** indicates a significant difference $P < 0.02$.

Reproductive Hormones, Largemouth Bass:

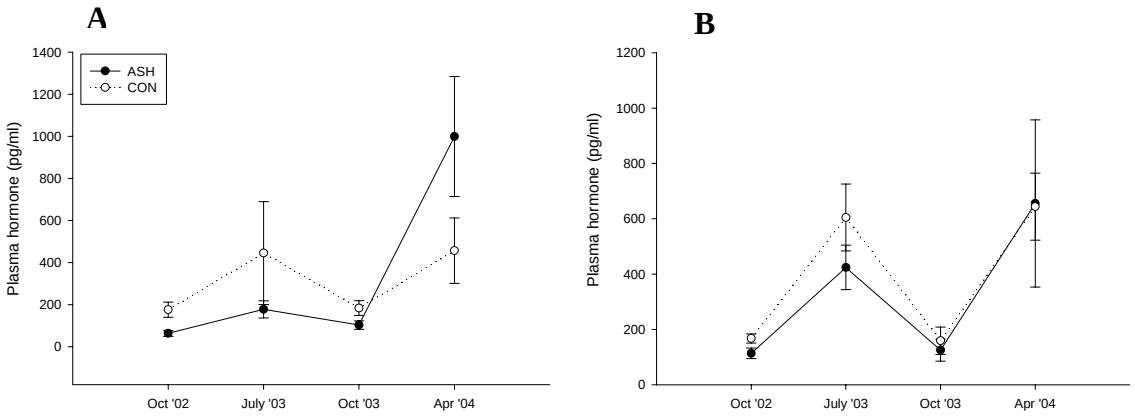
Extraction efficiencies were 86±3.1% and 81±2.55% for largemouth bass plasma E_2 and T, respectively. Statistical differences were not observed in plasma sex hormone concentration in male or female largemouth bass at any season (Figures 19 and 20).

FIGURE 19. SEASONAL COMPARISON OF PLASMA 17B-ESTRADIOL IN LARGEMOUTH BASS COLLECTED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK, 2002-2004



Mean plasma hormone concentrations of (A) females and (B) males; means ± standard errors; * indicates a significant difference P < 0.05, ** indicates a significant difference P < 0.02.

FIGURE 20. SEASONAL COMPARISON OF PLASMA TESTOSTERONE LEVELS IN LARGEMOUTH BASS COLLECTED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK, 2002-2004



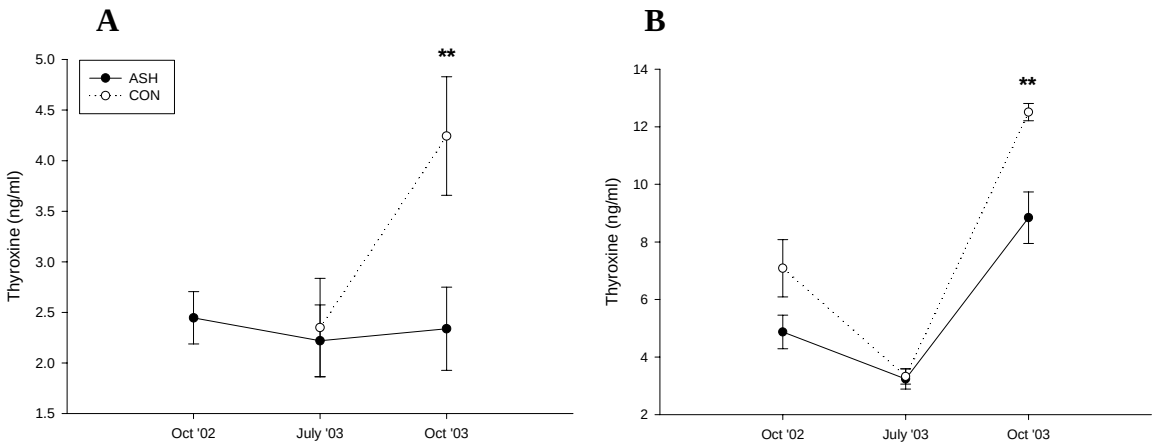
Mean plasma hormone concentrations of (A) females and (B) males; means ± standard errors; * indicates a significant difference P < 0.05, ** indicates a significant difference P < 0.02.

Endocrine modulation was not evident in largemouth bass from the Ashtabula or Conneaut as measured by GSI, hormones parameters, or microscopic observations.

Thyroid Hormone:

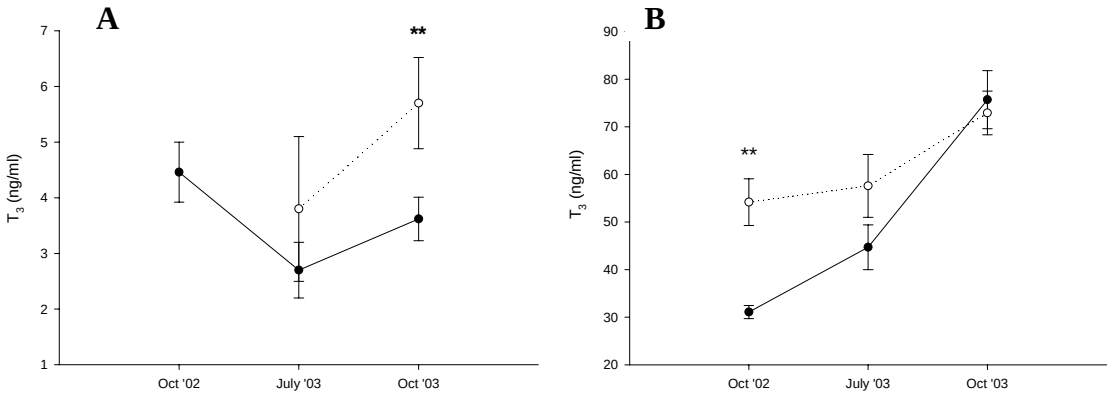
The thyroid hormone, T_4, was significantly lower in both brown bullhead and largemouth bass collected from the Ashtabula than in those collected from the Conneaut during October 2003 (P=0.013 and 0.014, respectively; Figure 21). Plasma concentrations of T_3 were also lower in Ashtabula bullheads during October 2003 (P=0.010) and Ashtabula largemouth bass during October 2003 ($P \leq 0.001$; Figure 22).

FIGURE 21. SEASONAL COMPARISON OF PLASMA THYROXINE (T_4) IN BROWN BULLHEAD AND LARGEMOUTH BASS COLLECTED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK



Mean plasma hormone concentrations of (A) bullhead and (B) largemouth bass; means ± standard errors; * indicates a significant difference $P < 0.05$, ** indicates a significant difference $P < 0.02$.

FIGURE 22. SEASONAL COMPARISON OF PLASMA TRIIODOTHYRONINE (T_3) IN LARGEMOUTH BASS COLLECTED FROM THE ASHTABULA RIVER AND CONNEAUT CREEK



Mean plasma hormone concentrations of (A) bullhead and (B) largemouth bass; means ± standard errors; * indicates a significant difference $P < 0.05$, ** indicates a significant difference $P < 0.02$.

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, exposure to PCBs is associated with decrease levels of circulating T_4 and sometimes T_3 (Persky et al. 2001; Brown et al. 2004). The observations of decreased thyroid hormone concentrations in both bullheads and largemouth bass from the Ashtabula are consistent with those associated with PCB hormone disruption.

CONTAMINANT BODY BURDENS

We were provided with the contaminant body burden data by the Fish and Wildlife Service. Total PCB body burdens in both largemouth bass and bullhead were significantly higher in fish collected in the Ashtabula when compared to those collected in Conneaut Creek (Figure 23). Numerous other contaminants, including chlordane, dieldrin, DDT, DDE and DDD were found at significantly higher levels in fish of both species collected in the Ashtabula (Figure 24).

Figure 23. **PCB TOTAL BODY BURDEN IN BROWN BULLHEADS AND LARGEMOUTH BASS COLLECTED FROM THE ASHTABULA AND CONNEAUT CREEK IN OCTOBER 2003 AND APRIL 2004**

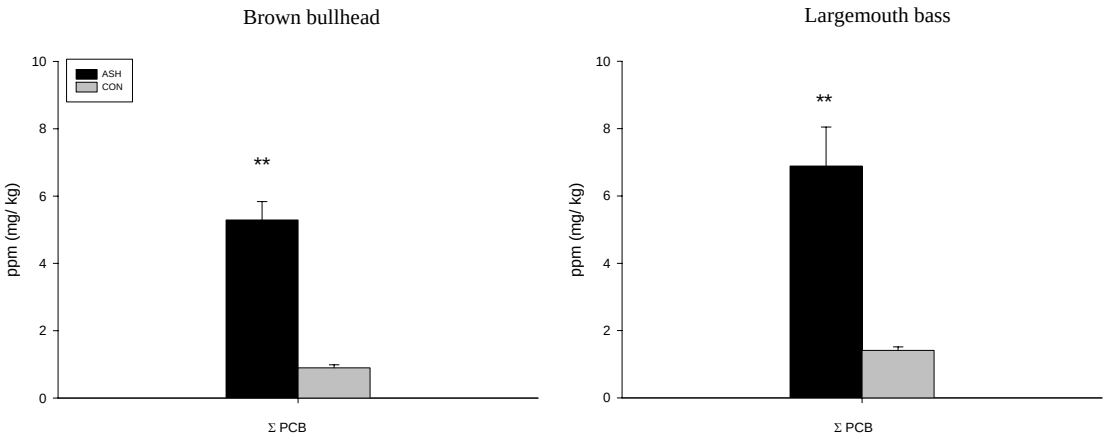
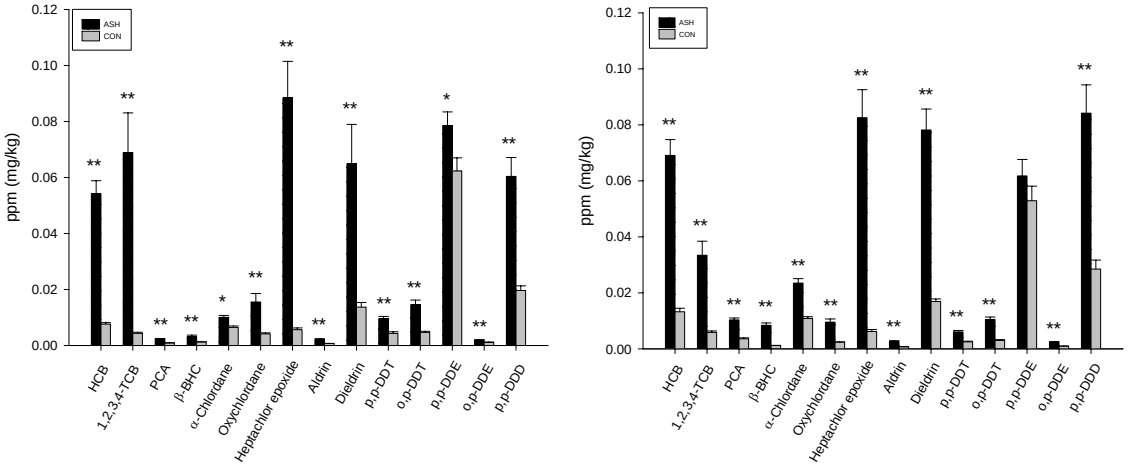


Figure 24. **CONTAMINANT TOTAL BODY BURDEN IN BROWN BULLHEADS AND LARGEMOUTH BASS COLLECTED FROM THE ASHTABULA AND CONNEAUT CREEK IN OCTOBER 2003 AND APRIL 2004**



Data presented as means ± standard errors; * indicates a significant difference $P < 0.05$, ** indicates a significant difference $P < 0.02$.

A number of contaminants were correlated with immune function. In general contaminant body burdens significantly negatively correlated with bactericidal activity, cytotoxic-cell activity and the LPS proliferative response. Significant positive correlations were observed with PHA-P mitogenesis and LPS stimulated respiratory burst (Table 11).

Table 8. **CORRELATION BETWEEN CONTAMINANTS AND IMMUNE PARAMETERS IN BROWN BULLHEADS FROM THE ASHTABULA AND CONNEAUT CREEK IN OCTOBER 2003 AND APRIL 2004**

	COR BACT	Cytotox 10:1	CON A	PHA-P	PWM	LPS	SOD - ROS	LPS ROS
Σ PCB	**-0.38 ****	**-0.28 ***	0.18	**0.32 ***	0.09	**-0.24 ***	0.13	**0.34 ****
HCB	**-0.42 ****	**-0.27 ***	0.13	**0.25 ***	0.16	-0.15	0.19	**0.26 ***
PCA	**-0.23 ***	**-0.55 ****	0.04	**0.22 ***	-0.02	-0.04	0.08	0.04
1,2,3,4 TCB	-0.19	-0.18	-0.02	0.10	0.09	-0.12	< -0.01	0.14
β-BHC	**-0.35 ****	**-0.33 ***	0.07	**0.25 ***	0.04	-0.20	0.03	0.20
α-chlordane	**-0.29 ***	**-0.30 ***	0.09	**0.28 ***	0.02	-0.08	0.10	**0.28 ***
Oxychlordane	**-0.34 ****	**-0.31 ***	0.19	**0.31 ***	0.14	**-0.24 ***	0.15	**0.33 ***
Heptachlor epoxide	**-0.36 ****	**-0.22 ***	0.19	**0.29 ***	0.16	**-0.30 ***	**0.25 ***	**0.35 ****
Aldrin	**-0.33 ****	**-0.38 ****	0.17	**0.31 ***	0.09	-0.10	0.13	**0.27 ***
Dieldrin	**-0.41 ****	**-0.32 ***	0.16	**0.30 ***	0.06	-0.17	0.12	**0.31 ***
p,p-DDT	-0.20	**-0.39 ****	0.02	0.18	-0.08	-0.16	< 0.01	0.13
o,p-DDT	**-0.32 ***	**-0.27 ***	0.15	**0.29 ***	0.04	-0.21	0.12	**0.33 ***
p,p-DDE	-0.09	0.14	0.15	0.13	0.14	0.06	0.03	**0.27 ***
o,p'-DDE	**-0.40 ****	**-0.38 ****	0.06	0.18	0.01	-0.11	0.18	**0.29 ***
p,p-DDD	**-0.27 ***	-0.13	**0.22 ***	**0.25 ***	0.14	- 0.15	0.07	**0.33 ****

Spearman Rank Order (n=86) correlation analysis. Significant correlations highlighted in bold *P*=0.05 denoted by *, significance at p=0.002 denoted by **. Data pooled to include fish from both rivers from October and April

SUMMARY

Both species of fish accumulated similar levels of PCBs (Figure 23) and other contaminants (Figure 24), however, the bullhead appeared to be more impacted than the bass. Both species captured at Conneaut were significantly heavier than those at Ashtabula, while Conneaut bullhead also had a higher condition factor and hepatosomatic index. Conneaut bullhead had a lower incidence of skin lesions including melanistic spots, barbel abnormalities and malignant skin tumors. In summer (2003) and spring (2004) less genetic damage was observed in bullhead from the Conneaut.

Both species exhibited significant immunomodulation with fish collected in the Ashtabula showing a lower bactericidal activity of phagocytic cells and a lower activity of cytotoxic cells, particularly in the Fall and Spring. Immunosuppression was further substantiated by an increased parasite load of both species collected in the Ashtabula River. A number of immune function activities were negatively correlated to total body burdens of various contaminants, including PCBs (Table 8).

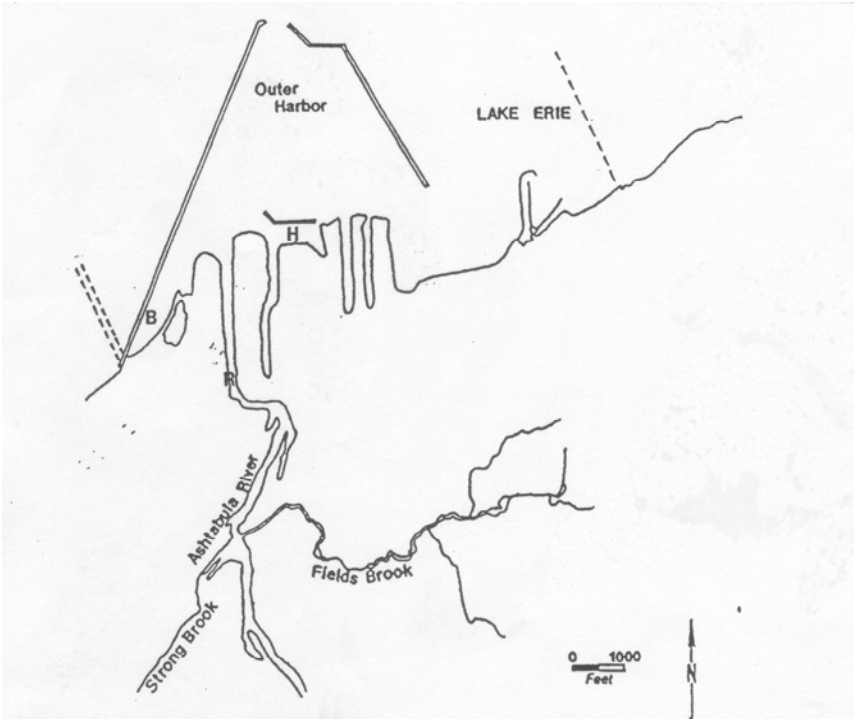
Bullhead collected in the Ashtabula had a number of microscopic liver lesions which have been used a biomarkers of contaminant exposure. These included more livers with necrosis (cell death), a greater number of macrophage aggregates and a higher number of individuals with nonneoplastic and neoplastic proliferative lesions such as bile duct proliferation, cholangioma and cholangiocarcinoma (Table 7). These liver lesions are consistent with contaminant exposures, particularly PAHs and PCBs.

Overall, a comparison of numerous parameters of both largemouth bass and brown bullhead collected in Conneaut Creek and the Ashtabula River indicated a significantly impaired health and condition of fishes living in the Ashtabula River

COMPARISON OF CURRENT FINDINGS WITH PREVIOUS STUDIES

A number of collections of brown bullheads have been made over the years for which we had morphometric and histologic data for comparison. In July 1990, brown bullhead were collected with boat electroshocking, by personnel from the Fish and Wildlife Service Great Lakes National Fisheries Research Center (data and tissue blocks provided to us by M.E. Mueller) from three sites in the Ashtabula harbor-river system (Figure 25), 44 from the breakwater (B), 39 from the harbor (H) and 15 from the river (R). In the current study bullheads were only collected from the river area.

FIGURE 25. MAP OF THE ASHTABULA RIVER SAMPLING AREAS IN JULY 1990.

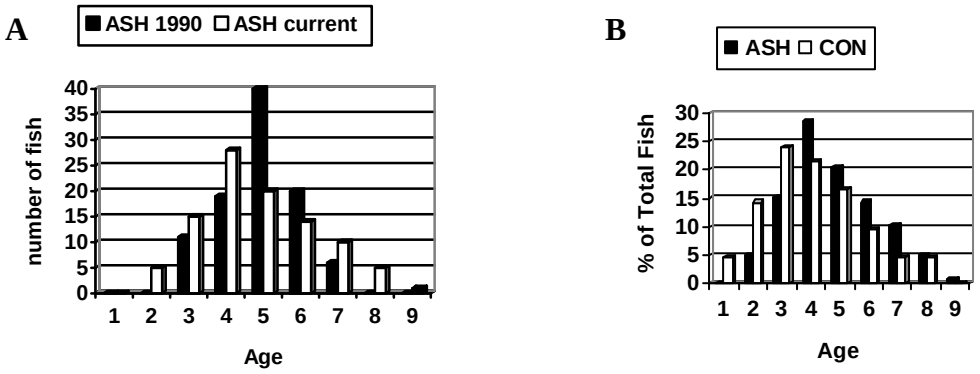


As in the recent study, bullheads larger than 240 mm in length were sacrificed using MS-222, weighed, measured and necropsied. Gross abnormalities were recorded, the liver weighed for hepatosomatic indices, tissues taken for histological evaluation and a pectoral spine taken for aging. The following results were obtained.

Physical Characteristics and Age Structure

A total of 40 males, 57 females and one unknown sex were collected. The average age for both sexes was 5 years, ranging from 3-7. The age structure is illustrated in Figure 26 and compared to that of bullheads collected during 2002-2004. In 1990, 98 bullheads were collected and in the current study 99 were collected. Figure 26A compares the two studies based on total fish numbers during each collection, while Figure 26B presents the percent of each age group collected in the current study.

FIGURE 26. AGE STRUCTURE OF BROWN BULLHEAD SAMPLED AT ASHTABULA 1990 AND 2002-2004.



In 1990, the average length for all fish was 311 mm, ranging from 260-362 mm and was similar at all three sites (Table 9). The average weight for fish from all sites was 428 g, ranging from 235-709. Fish collected at the river site were slightly less in weight than from the other two sites.

TABLE 9. MEAN ± STANDARD DEVIATION LENGTH AND WEIGHT OF BROWN BULLHEAD COLLECTED IN THE ASHTABULA RIVER, JULY 1990.

Parameter	Harbor	Breakwater	River	All
Length (mm)	319 ± 25.2	311 ± 26.5	312 ± 32.9	311 ± 41.3
Weight (g)	441 ± 104	438 ± 127	369 ± 120	428 ± 118

External Abnormalities

In 1990, external abnormalities noted included skin discolorations, stubbed barbels and lip papillomas. Skin discolorations and stubbed barbels were common at all sites with a total occurrence of 41% and 35%, respectively. The river and harbor areas had the highest frequency (Table 10). Papillomas were observed in 16% of the fish sampled with the lowest occurrence in fish collected from the river (Table 10). Histological analyses determined the areas of skin discoloration were pigmented areas with marked proliferation of epidermal cells and the presence of rete epithelial pegs projecting into the dermis. In addition there was proliferation and migration of melanophores or pigment cells into the superficial areas of the epidermis. Grossly, these areas presented themselves as either dark spots on the skin or raised darkened areas. Chi-square analysis showed a higher probability of skin discoloration in fish taken from the harbor. Stubbed barbels appeared histologically as areas of severe proliferation of the outer epithelium resulting in a blunted or stubbed appearance rather than the normal barbel which tapers to a slender tip. Lip papillomas were similar histologically to the skin lesions, showing a thickened epithelium.

TABLE 10. LESIONS PRESENT IN BROWN BULLHEAD FROM THE ASHTABULA RIVER IN 1990*

Lesion	River	Breakwater	Harbor	Total
Sample size	*15*	*44*	*39*	*98*
Hepatic microscopic lesions				
Altered foci	8 (53.3)	5	5	18 (18.4)
Hepatocellular adenoma	0	0	0	0
Hepatocellular carcinoma	2	1	0	3 (3.1)
Cholangioma	2 (13.3)	1	0	3 (3.1)
Cholangiocarcinoma	1 (6.7)	0	0	1 (1.0)
External abnormalities				
Raised lesions	1 (6.7)	8	7	16 (16.3)
Barbel deformities	8 (53.3)	15	12	35 (37.7)
Skin discoloration	7 (46.7)	13	21	41 (41.8)

*Presented as total number of fish with individual lesions, values in parentheses are percent of fish affected at that particular site.

Lake Erie Ecological Investigations

During 1998-2000 USGS conducted an ecological study at selected AOC around Lake Erie. The Ashtabula River was one of those and fish were collected at this site in 2000. This study included sediment contaminant evaluation, benthic community studies, fish community studies and bullhead health. Table 11 compares the prevalence of raised lesions over time.

TABLE 11. HISTORICAL COMPARISON OF PREVALENCE OF RAISED LESIONS AND BARBEL DEFORMITIES IN BROWN BULLHEAD SAMPLED FROM THE ASHTABULA RIVER.

Sampling year	Sample size	Raised lesions (%)	Barbel deformities (%)
1990	98 (15)*	16.3 (6.7)	35.7 (53.3)
2000	45	6.7	22.2
2002-2004	99	10.1	47.5
Statistical analysis	No significant difference in prevalence of raised lesions ($p > 0.05$), but fish from 2002-2004 and 1990 (when only river fish were included) had higher prevalence of barbel deformities than fish from 2000 ($p < 0.05$).		

* Indicates results when only fish collected in the river were included.

Table 12 compares the sediment contaminant concentrations at the various sites in the 1998-2000 study. The Ashtabula sample contained lower levels of PAHs than most of the other sites, while having the second highest level of PCBs.

TABLE 12. CONCENTRATIONS OF SELECTED CHEMICALS (µG/G DRY WEIGHT) IN SEDIMENTS AT SELECTED AOCS, 1998-2000.

Site [a]	DET*	OTT	OWC	BLA	CUY	ASH	BUF	NIA
Sampling year	2000	1999	2000	1998	1999	2000	1998	1998
PAHs [b]	17.42	9.41	5.25	5.42	19.07	3.91	7.59	1.02
PCBs	0.51	2.93	0.08	0.15	0.46	1.03	0.25	0.06
DDTs [c]	0.082	0.081	0.033	0.030	0.023	0.013	0.028	0.005
Heavy metals [d]	1.09× 10^3	0.88× 10^3	0.33× 10^3	0.69× 10^3	1.17× 10^3	0.65× 10^3	1.00× 10^3	0.35× 10^3

*Detroit River (DET), Ottawa River (OTT), Old Woman Creek (OWC), Black River (BLA), Cuyahoga River - harbor (CUY), Ashtabula River (ASH), Buffalo River (BUF), and Niagara River (NIA)

[a] Sediment data were taken from Smith et al. 2003; Passino-Reader et al. 2005.

[b] Sum of concentrations of anthracene, benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, chrysene, dibenz(a,h)anthracene, fluoranthene, fluorene, naphthalene, C1-, C2-, C3-, and C4- naphthalene, perylene, phenanthrene, and pyrene.

[c] Sum of concentrations of o,p'- and p,p'- DDD (dichlorodiphenyldichloroethane), o,p'- and p,p'- DDE (dichlorodiphenyldichloroethylene), o,p'- and p,p'- DDT (dichlorodiphenyltrichloroethane). For the concentration below the detection limit, half of the detection limit was used.

[d] Sum of concentrations of Ba, Cd, Cr, Cu, Hg, Mn, Ni, Pb, Sr, V. For the concentration below the detection limit, half of the detection limit was used.

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