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Environmental Toxicology and Chemistry, Vol. 27, No. 8, pp. 1756–1767, 2008

Printed in the USA
0730-7268/08 $12.00 + .00

UTILIZATION OF PROTEIN EXPRESSION PROFILES AS INDICATORS OF ENVIRONMENTAL IMPAIRMENT OF SMALLMOUTH BASS (*MICROPTERUS DOLOMIEU*) FROM THE SHENANDOAH RIVER, VIRGINIA, USA

JENNIFER RIPLEY,*† LUKE IWANOWICZ,‡ VICKI BLAZER,‡ and CHRISTY FORAN†
†Department of Biology, West Virginia University, 53 Campus Drive, P.O. Box 6057, Morgantown, West Virginia 26506, USA
‡U.S. Geological Survey, Leetown Science Center, National Fish Health Research Laboratory, 11649 Leetown Road, Kearneysville, West Virginia 25430

(*Received* 9 *November* 2007; *Accepted* 13 *February* 2008)

Abstract—The Shenandoah River (VA, USA), the largest tributary of the Potomac River (MD, USA) and an important source of drinking water, has been the site of extensive fish kills since 2004. Previous investigations indicate environmental stressors may be adversely modulating the immune system of smallmouth bass (*Micropterus dolomieu*) and other species. Anterior kidney (AK) tissue, the major site of blood cell production in fish, was collected from smallmouth bass at three sites along the Shenandoah River. The tissue was divided for immune function and proteomics analyses. Bactericidal activity and respiratory burst were significantly different between North Fork and mainstem Shenandoah River smallmouth bass, whereas South Fork AK tissue did not significantly differ in either of these measures compared with the other sites. Cytotoxic cell activity was highest among South Fork and lowest among North Fork AK leukocytes. The composite two-dimension gels of the North Fork and mainstem smallmouth bass AK tissues contained 584 and 591 spots, respectively. South Fork smallmouth bass AK expressed only 335 proteins. Nineteen of 50 proteins analyzed by matrix-assisted laser desorption ionization–time of flight were successfully identified. Three of the four identified proteins with increased expression in South Fork AK tissue were involved in metabolism. Seven proteins exclusive to mainstem and North Fork smallmouth bass AK and expressed at comparable abundances serve immune and stress response functions. The proteomics data indicate these fish differ in metabolic capacity of AK tissue and in the ability to produce functional leukocytes. The variable responses of the immune function assays further indicate disruption to the immune system. Our results allow us to hypothesize underlying physiological changes that may relate to fish kills and suggest relevant contaminants known to produce similar physiological disruption.

Keywords—Two-dimension gel electrophoresis Anterior kidney Leukocytes Metabolism Fish kills

INTRODUCTION

The Shenandoah River (VA, USA), the largest tributary of the Potomac River (MD, USA) and an important source of drinking water, was ranked as America's fifth most endangered river in 2006 based on the magnitude of threat and the regional and national significance of the river (http://www.americanrivers.org). Extensive fish kills from April to July of 2005 were observed, with 80% mortality of the adult smallmouth bass (*Micropterus dolomieu*) and redbreast sunfish (*Lepomis auritus*) along 100 miles of the South Fork Shenandoah River. The previous spring, the North Fork Shenandoah River was the site of a nearly identical fish kill event. In 2006, fish kills were documented throughout the Shenandoah River in its North Fork, South Fork, and mainstem [1] (www.deq.virginia.gov/info/documents/2007.02.23ScienceTeamReport.pdf). More than 20 theories identifying the causes of these fish kills have been proposed, ranging from agricultural chemicals to unknown pathogens to changes in trophic cascades [1]. Whereas limited data have been unable to clearly support any one of these theories, several important observations have been documented. Investigation of the affected fish revealed bacterial and fungal lesions that penetrated to the dermal tissues of the skin. Opportunistic bacteria, including *Enterobacter* sp. and *Flavobacterium columnare*, which are common in freshwater game fish, and *Aeromonas salmonicida*, a notable primary pathogen of salmonids, were the dominant microbes [1]. The observation that adult fish rather than early life stages (i.e., larvae and juveniles) are the most gravely impacted implies a long-term exposure to sublethal stressors. Comprehensive fish health assessments during the fall of 2005 revealed a high frequency (80–100%) of male smallmouth bass with testicular oocytes, a condition often associated with exposure to estrogenic contaminants [2]. Overall, the prevalence and origin of these lesions and their occurrence in the adult population imply environmental stressors may be causing immune suppression.

Many classes of contaminants are immunotoxic, disrupting the critical role of the immune system in maintaining health. Xenobiotics can exert structural and functional changes to the immune system, including (but not limited to) organ damage, histopathologic effects in tissues and organs, abnormal cell proliferation and maturation, and altered cellular function [3]. These adverse immunotoxic effects from contaminant exposure include both suppression and enhancement of immune function. Ultimately, immunosuppression can result in decreased resistance to infection and cancer, whereas induction of immune responses can lead to allergies and autoimmune diseases [3]. The present study proposed a physiological investigation to examine the immunosuppression hypothesis indicated by initial observations of the Shenandoah River smallmouth bass.

Proteomics, or the study of the proteins expressed by a

* To whom correspondence may be addressed (jripley@mix.wvu.edu).
Published on the Web 3/3/2008.

Table 1. Intersex, age, size, and gross lesions and anomalies for smallmouth bass (*Micropterus dolomieu*) from the Shenandoah River (VA, USA)[a]

Site	Sample size (females /males)	Intersex males (%)	Age (years)	Total length (cm)	Weight (g)	Condition factor	Overall lesion prevalence (%)	Lesion prevalence (%)				
								External skin and fin	Gill	Liver	Kidney	Spleen
North Fork	10/10	50	2.4 ± 0.2	20.9–33.4	118–474	1.28 ± 0.02	100	55	30	95	50	45
South Fork	12/8	50	2.5 ± 0.3	18.9–35.4	87–523	1.26 ± 0.01	55	35	5	35	0	10
Mainstem	8/8	100	2.4 ± 0.4	22.0–40.3	137–816	1.33 ± 0.02	80	50	10	80	15	0

[a] Intersex and lesion data are presented as the percentage of collected bass with these traits, age and condition factor as the mean ± standard error, and total length and weight as the minimum–maximum.

genome, is gaining application as a measure of environmental stress, providing more meaningful data and a more accurate reflection of functional status compared with mRNA expression. The underlying principle is that a proteome differs from cell to cell and constantly changes via biochemical interactions with the genome and environment. Accordingly, environmental conditions drive the expression of a unique set of proteins in the exposed organism, tissue, or cell type [4]. Additionally, a proteomics approach utilizing matrix-assisted laser desorption ionization–time of flight mass spectrometry enables the investigation of differentially regulated proteins without a priori knowledge of protein sequences. In the present study, we first evaluated a proteomics approach as a tool to gauge the health of smallmouth bass in the Shenandoah River. Second, we compared anterior kidney (AK) protein profiles and immune function to assess the utilization of proteomics in identifying the severity of environmental stress. Proteins of this organ were targeted, because the AK is the primary site of hemopoiesis and leukopoiesis, serves as a secondary immune organ that supports mature resident leukocytes, and is comprised of cells integral to the stress response (i.e., chromaffin and interrenal cells). Physiological differences indicated by protein expression profiles are likely related to potential causes of altered immune function. The occurrence of fish kills and modulation of immune responses (data not shown) in the Shenandoah River varies both seasonally and annually [1], but the present study establishes a snapshot baseline for a future comparison to uncover trends that either predict or indicate an impacted site.

MATERIALS AND METHODS

Fish and tissue collection

Smallmouth bass were collected on July 25 to 28, 2006, from three sites along the Shenandoah River. The collection sites were located in the mainstem (near Winchester, VA, USA; 39°02′11.24″N, 78°00′15.07″W), North Fork (near Strasburg, VA, USA; 38°58′24.10″N, 78°21′11.79″W), and South Fork (near Elkton, VA, USA; 38°31′39.87″N, 78°35′41.75″W). Given the widespread nature of the fish kills in the Shenandoah and neighboring rivers, no regionally relevant rivers could be classified as legitimate reference sites. Smallmouth bass from fish farms were small fingerlings (length, 3–5 cm) and were not appropriate controls because of size and variable rearing conditions. Originally, the collection sites were selected based on fish kill data from the previous two years [1] such that the mainstem Shenandoah River would represent a control site; the South Fork a recent, severely impacted site; and the North Fork a recovering site. On concurrent collection of fish kill data and fish tissue, however, the classification of the sites was revised, and all sites were considered to be impacted.

Flowing northward, the mainstem Shenandoah River begins at Front Royal (VA, USA). Avtex Corporation, located in Front Royal, was at one time the world's largest producer of rayon. The plant was closed in 1989 and listed among the U.S. Environmental Protection Agency's Superfund sites, with polychlorinated biphenyls, arsenic, and carbon disulfide among the many contaminants released [5] (http://www.cbf.org/site/DocServer/Shenandoah_River_Basin_Fact_Sheet.pdf?docID=5873). Southern portions of both the North Fork and South Fork of the Shenandoah River, including the town of Elkton, transverse Rockingham County (VA, USA), the nation's top turkey-producing county (http://www.vafb.com/va_ag/facts_poultry.htm). Merck Pharmaceuticals, located on the South Fork Shenandoah River in Elkton, is a large source of nitrogen and phosphorous [5]. E.I. DuPont De Nemours and Company has released contaminants, particularly mercury, into the South Fork Shenandoah River [5]. Overall, 23 significant sewage treatment plants and industrial discharges release 55 million gallons of wastewater each day into the Shenandoah River watershed [6] (http://www.cbf.org/site/DocServer/Shenandoah_River_Basin_Fact_Sheet.pdf?docID=5873).

Smallmouth bass were collected by the Virginia Division of Game and Inland Fisheries using a pulsed DC electroshocking boat. The target sample size was 20 fish per site. Fish were weighed and measured, and any visible lesions or abnormalities were documented. A minimum total length of 20 cm was established in an attempt to collect only sexually mature individuals (Table 1). Condition factor (K) was calculated for each fish according to the formula $K = (W \cdot 100)/L^3$, where W is the fish weight (g) and L is the total length (cm). Fish were killed with a lethal dose of 3-aminobenzoic acid ethyl ester (MS-222; Sigma-Aldrich, St. Louis, MO, USA) immediately before aseptic necropsy. Blood was drawn from the caudal vessels with a heparinized, 3-ml syringe to deplete erythrocytes from the hematopoietic organs. A portion of the AK was removed and placed into a cryovial containing 50 μl of protease inhibitor cocktail in dimethyl sulfoxide (Sigma-Aldrich). The tissue sample was then snap-frozen on dry ice. The remaining tissue was placed into processing medium comprised of cold L-15 medium supplemented with 2% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA, USA), 100 U/ml of penicillin, 100 μl/ml of streptomycin, and 10 U/ml of sodium heparin. If a tissue sample was not large enough for both proteomics and immune analyses, it was not partitioned and, instead, was preferentially prepared for the functional immune assays. These samples were fragmented using a sterile Ten-Broek homogenizer (Fisher Scientific, Waltham,

MA, USA) and immediately stored on ice for transport. Pieces of liver, spleen, gill, posterior kidney, gonad, and any tissues with grossly apparent lesions were stored in Z-Fix™ (Anatech, Battle Creek, MI, USA), routinely processed for histology, embedded into paraffin, and sectioned (thickness, 6 μm) for microscopic examination. Otoliths were removed for aging by officials of the Virginia Division of Game and Inland Fisheries.

Leukocyte isolation

Anterior kidney leukocytes were isolated and processed as described by Iwanowicz et al. [7]. Briefly, fragmented AK tissue was suspended and allowed to settle for 20 min on ice. Supernatant was removed and transferred to a sterile polypropylene conical tube. Cells were pelleted by centrifugation at 500 *g* for 10 min at 4°C (Allegra 6R with rotor GH 3.8; Beckman Coulter, Fullerton, CA, USA). Cells were then washed by suspension in processing medium, centrifuged as described above, suspended in processing medium, and layered onto a Percoll cushion (32% Percoll in phenol red–free Hanks' balanced salt solution [HBSS]; Sigma-Aldrich). Samples were then centrifuged at 400 *g* for 40 min at 4°C, and the leukocyte fraction was removed from the Percoll/processing medium interface. Leukocytes were pelleted and washed as described above and resuspended in processing medium for counting. The number of viable leukocytes isolated from each fish was determined by trypan blue exclusion using 0.1% trypan blue in processing medium (leukocyte viability exceeded 95% for all fish). Cells were pelleted and suspended at 2×10^7 viable cells/ml in adherence medium comprised of L-15 medium supplemented with 0.1% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin before plating for innate immune function assays.

Bactericidal activity

Functional assessment of adherent AK leukocytes to kill the salmonid pathogen *Yersinia ruckeri* was determined using the method described by Harms et al. [8]. Briefly, 2×10^6 leukocytes suspended in adherence medium were added in quadruplicate to the wells of a 96-well plate for the bacterial challenge and in triplicate on the same plate for adherent cell enumeration. Smallmouth bass leukocytes from the three sites were included on all plates in this assay and in those to follow to account for interplate variability. Plates were incubated at 20°C for 2 h following cell plating. Media were then removed from all wells and replaced with culture media comprised of L-15 medium supplemented with 5% FBS, 100 U/ml penicillin, and 100 μg/ml of streptomycin. Cells were cultured at 20°C in a humidified chamber for 36 h to allow activated leukocytes to reach a resting state. Culture media were then removed, and the wells were washed with antibiotic-free, unsupplemented L-15 medium and filled with 100 μl of L-15 medium supplemented with 5% FBS but no antibiotics. Twenty-five microliters of a 48-h culture of *Y. ruckeri* (Hagerman strain; National Fish Health Research Laboratories no. 11.40) washed and suspended in HBSS (optical density at 600 nm, 1.5) were added to the treatment wells and a row of cell-free control wells. The plates were incubated for 4 h in the humidified chamber at 20°C. Media were 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 the percentage CFU reduction by the calculation $(1 - (CFU_{treated}/CFU_{control}) \cdot 100)$, where $CFU_{treated}$ is the mean CFU value for the replicate wells with adherent leukocytes and $CFU_{control}$ is the mean CFU value for the replicate wells with media only. A corrected percentage CFU reduction was defined as the percentage CFU reduction multiplied by the cell density correction, where cell density correction is the mean number of adherent cells from the same cell source used to determine bactericidal activity divided by 10^6.

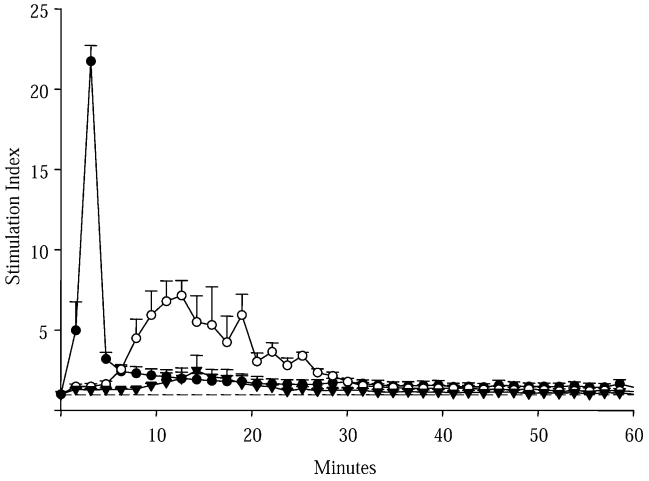

Fig. 1. Kinetics of the peroxidase luminol-enhances chemiluminescence assay using smallmouth bass (*Micropterus dolomieu*) anterior kidney leukocytes from an independent validation trial. Comparison of response kinetics to phorbol 12-myristate 13-acetate (PMA) and biologically relevant lipopolysaccharide (LPS) from different bacterial sources. Data points represent the mean stimulation index; bars represent the standard error ($n = 12$). Only positive errors bars are included for visual clarity. —●— = 100 ng/ml of PMA; —○— = 100 μg/ml of LPS (*Escherichia coli* 0111:B4); —▼— = 100 μg/ml of LPS (*Salmonella typhimurium*).

Respiratory burst activity

The production of extracellular reactive species was determined using the peroxidase luminol–enhanced chemiluminescence assay described by Coteur et al. [9] with modifications. In short, 10^6 leukocytes suspended in adherence medium were added to the wells of a white 96-well plate, incubated for adherence as described above, washed, and subsequently incubated in culture medium for 36 h. Culture medium was removed, and cells were washed with room-temperature HBSS. Cells were then treated in quadruplicate with 25 μl of either lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (100 μg/ml of LPS in HBSS), LPS superoxide dismutase (100 μg/ml of LPS and 352 U/ml of superoxide dismutase in HBSS), or HBSS alone and incubated for 1 min. Then, the peroxidase luminol–enhanced chemiluminescence reaction solution (500 μM luminol and 13.2 U/ml of horseradish peroxidase in HBSS) was added. Plates were immediately loaded into a SpectraFluorPlus microplate reader (gain, 180; integration, 500 ms; Tecan, San Jose, CA, USA), and luminescence was measured every 2 min for 20 min. Kinetic data from assay validation with smallmouth bass AK leukocytes identified that a modest, continuous production of free radicals occurs up to 30 min (Fig. 1). Stimulation index values for LPS-induced reactive species 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 stimulation index value for a given treatment was defined as the highest value determined during the 20-min read.

Cytotoxic cell activity

The ability of smallmouth bass AK leukocytes to lyse epithelioma papulosum cyprini cells was determined using the calcein adherence medium release-based cytotoxic cell assay as described by Iwanowicz et al. [10]. Briefly, an 18-h culture of epithelioma papulosum cyprini cells plated at a density of 10^5 cells/well in 96-well plates were incubated with 5 μM calcein adherence medium in culture medium for 5 h. Cells were then washed four times with room-temperature Dulbecco's phosphate-buffered saline and cultured in culture medium for an additional 30 min. The medium was removed, and effector AK leukocytes suspended in culture medium were added in quadruplicate at target ratios of 10:1, 2:1, and 1:1. Lysis buffer (25 mM sodium borate and 0.1% Triton X-100 in culture medium, pH 9.0; Sigma-Aldrich) or culture medium alone was added in quadruplicate to another set of wells to determine total and spontaneous release, respectively. Plates were centrifuged at 50 *g* for 5 min and incubated at 20°C for 8 h in the dark. Fifty microliters of supernatant were then removed from all wells and loaded into the wells of a black 96-well plate preloaded with 2× lysis buffer. Supernatant from the total release wells were added to 1× lysis buffer. Fluorescence intensity was measured reading the plates from the bottom (excitation wavelength, 485 nm; emission wavelength, 535 nm; gain, 60). Cytotoxic cell activity was calculated as:

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

Two-dimension gel electrophoresis

In the laboratory, AK tissues were blotted dry, weighed, and homogenized for 120 s in protease inhibitor cocktail (1 μl per 10 mg; Sigma-Aldrich). The homogenate was sonicated and then centrifuged at 21,000 *g* for 30 min at 4°C (Allegra 25R with rotor TA-15-1.5; Beckman Coulter). Total protein concentration of the supernatant (1:199 dilution) was determined using a Bradford-based protein dye reagent (Bio-Rad, Hercules, CA, USA) and a standard curve of bovine serum albumin. Smallmouth bass AK proteins (400 μg) were mixed with lysis buffer (15 mM 1,2-diheptanoyl-*sn*-glycero-3-phosphatdiyl choline [Avanti Polar Lipids, Alabaster, AL, USA], 7 M urea, 2 M thiourea, 200 mM dithiothreitol, and 0.5% biolytes) to a final volume of 450 μl [11]. This detergent was shown to most effectively solubilize AK proteins for separation [11]. A trace of bromophenol blue was added to monitor the electrophoretic run. This solution was added to a 24-cm, pH 3 to 10, immobilized pH gradient strip (Amersham Biosciences, Piscataway, NJ, USA) and isoelectrically focused on an Ettan IPGphor system at 20°C according to the manufacturer's instructions (12-h rehydration, 1 h at 500 V, 1 h at 1,000 V, 8.3 h at 8,000 V, and hold at 500 V; Amersham Biosciences). For the second dimension, strips were equilibrated for 20 min by rocking in a solution of 10 mM dithiothreitol (50 mM Tris-HCl [pH 6.8], 6 M urea, 30% glycerol, and 2% sodium dodecyl sulfate), followed by 15 min in a 10 mM iodoacetamide equilibration solution. The immobilized pH gradient strips were laid on 12.5% sodium dodecyl sulfate–polyacrylamide gels and run at 4°C for 16 h at 2 W/gel (450 V, 300 mA) on an Ettan DALTsix electrophoresis unit (Amersham Biosciences). The cathode buffer consisted of 192 mM glycine, 25 mM Tris, and 0.1% sodium dodecyl sulfate at pH 8.3. The anode buffer was identical but twice as concentrated. The run was completed at 5 W/gel if necessary. Gels were fixed for 1 h in 50% ethanol and 3% phosphoric acid. Three 20-min washes with deionized water preceded 20- to 24-h staining with colloidal Coomassie blue G-250 (0.1% Coomassie Brilliant Blue G-250 [Sigma-Aldrich], 20% methanol, 10% phosphoric acid, and 10% ammonium sulfate). The detection limit with this staining procedure was 10 ng. Images were captured at high resolution (36.3 μm) with a GS-800 densitometer (Bio-Rad). All samples were randomized in two-dimensional electrophoresis and image analysis. Molecular masses of the proteins and isoelectric points (pI) were determined by coelectrophoresis with standard protein markers (6.5–205 kDa; Genomic Solutions, Ann Arbor, MI, USA) and pI calibration kits (broad range, pH 3–10; Amersham Biosciences), respectively.

Protein expression computer analysis

Gel images were analyzed by PDQuest (Ver 8.0.1; Bio-Rad). Only gels that showed good protein separation (i.e., no bands or protein fronts) were included in the analysis. Spot detection and background subtraction were performed on each gel, producing filtered, gaussian model images. Images were grouped by site. The gel image showing the highest number of spots and the best protein pattern was chosen as the reference template for each site. Spots that appeared on every image within a site were used as landmarks and matched to the reference template. Landmark spots were selected to create an approximate ''X'' shape across the sample area of each image to allow good alignment in both the horizontal and vertical directions. This landmarking aligned the images for corresponding proteins to be matched to each other. A minimum of 20 spots served as landmarks for each sample site. To confirm the software's alignment and matching, an additional 20 spots were manually matched throughout the gel. Composite images were created for each collection site. Proteins had to be expressed by a majority of individuals from a site to be included in the composite image (i.e., the maximum number of individuals that a protein could be absent from was two). Composite images were then landmarked and aligned by the process described above. The amount of protein in a spot was normalized in each gel by dividing the raw quantity of each spot by the total intensity value of all the pixels in the image. The normalized spot volume is referred to as abundance. Differentially induced and repressed proteins were determined by comparison of composite images. Differences were statistically compared by Wilcoxon or Kruskal–Wallis tests, depending on the number of collection sites expressing the particular protein.

Matrix-assisted laser desorption ionization–time of flight mass spectrometry

Spots were selected based on protein quantity, separation, and significance of their expression relationship between sites. Five spots were run in replicate by excising the matching spot from gels for two smallmouth bass collected from two sites. Each selected spot was cut into approximately 1-mm^2 pieces and destained with 30-min washes in a 1:1 mixture of 100 mM ammonium bicarbonate and methanol and then in 100 mM ammonium bicarbonate, followed by a 10-min wash in

Table 2. Immune function of anterior kidney leukocytes from smallmouth bass (*Micropterus dolomieu*) of the Shenandoah River (VA, USA)[a]

Site	Viable leukocytes (1×10^7)	Adherent cells (%)	Corrected bactericidal activity (%)	Stimulation index	Cytotoxic cell activity (%)
North Fork	12.8 ± 1.5 A	61.1 ± 3.4 A	19.2 ± 2.8 A	4.0 ± 0.6 A	2.1 ± 0.7 A
South Fork	13.8 ± 1.5 A	44.4 ± 4.0 B	11.8 ± 4.0 AB	5.3 ± 0.7 AB	8.7 ± 0.6 B
Mainstem	24.9 ± 3.5 B	44.7 ± 4.0 B	8.4 ± 2.7 B	8.2 ± 1.6 B	5.4 ± 1.4 C

[a] Values are presented as the mean ± standard error. Those with the same uppercase letter are not significantly different ($p < 0.05$).

acetonitrile. The gel pieces were dried under vacuum for 5 min at room temperature and covered with 50 μl of 2 μg/ml of sequencing-grade trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate buffer and left at 37°C overnight. The samples were sonicated for 15 min in ammonium bicarbonate and then desalted and concentrated using ZipTip C18 (Millipore, Bedford, MA, USA). Peptides were then eluted in 4 μl of 2% acetic acid and 50% acetonitrile. One microliter of this sample was mixed with an equivalent volume of matrix solution (50 mM α-cyano-4-hydroxy-cinnamic acid in 49.5% ethanol, 49.5% acetonitrile, and 1% of 0.1% trifluoroacetic acid). One microliter of the resulting mixture was loaded onto the matrix-assisted laser desorption ionization sample plate and allowed to dry. Measurements were performed using a Micromass matrix-assisted laser desorption ionization reflectron instrument (Waters, Milford, MA, USA) operated in positive-ion mode with a pulse voltage of 3,500 V.

Molecular masses of the tryptic peptide profiles were searched against the Swiss-Prot database (Swiss Institute of Bioinformatics, Geneva, Switzerland) using ProteinLynx (Waters) and the National Center for Biotechnology Information nucleotide database using Mascot (Matrix Science, Boston, MA, USA). Only peptide fragments with a relative abundance greater than 100 were submitted for searching. Database queries were carried out for monoisotopic peptide masses using the following conditions: Peptide mass tolerance of ±50 ppm, one as the maximum number of missed tryptic cleavages, molecular mass range depending on the apparent relative molecular mass of the protein spot in the selected gel, and all pI ranges. Carbamidomethyl modification of cysteine residues was selected as an allowed modification. Because of the limited number of fish fully covered in the sequence databases, positive identification of the protein was assigned if the sequence coverage was at least 20% and the identified protein molecular mass and pI values approximately corresopnded to the experimental values. ProteinLynx (significance score, >7.5) and Mascot (significance score, >78) scores were considered even though a significant match was improbable because of the lack of protein sequences for smallmouth bass. Protein function was determined from the Swiss-Prot database.

Statistical analyses

All statistical analyses were performed with SAS® (Cary, NC, USA) JMP 5.1 at $\alpha = 0.05$. Normality and homogeneity of data sets were assessed before the use of parametric statistics. One-way analysis of variance (ANOVA) was used to examine distinctions between sites in the size and condition of fish. Differences in immune function were investigated by Kruskal–Wallis tests.

RESULTS

Numerous male smallmouth bass collected from the South Fork, North Fork, and mainstem Shenandoah River had intersex characteristics (Table 1). Bass collected from the three sites were not significantly different in age, length, weight, or condition factor (one-way ANOVAs, all $p > 0.05$) (Table 1). Thus, no potential age or size class differences warranted investigation. Lesions and anomalies included parasites (trematode metacercariae, leaches, gill monogenetic trematodes, myxozoans, and larval cestodes in the liver, spleen, and kidney); raised, pale, or reddened areas on the body surface; and skin erosions. These gross lesions and anomalies were most prevalent in North Fork smallmouth bass. Mainstem Shenandoah River smallmouth bass were intermediate in severity, and South Fork smallmouth bass were the least impacted (Table 1). Condition factor did not appear to correlate with lesion prevalence, because South Fork and mainstem smallmouth bass had the lowest and highest values, respectively.

Anterior kidney leukocyte immune function

The number of viable leukocytes was significantly higher for mainstem Shenandoah River smallmouth bass AK tissue compared with South Fork or North Fork smallmouth bass AK tissues, which were not different from each other (one-way ANOVA, $F = 9.610$, $p = 0.0003$; post-hoc Tukey-Kramer, $q = 2.41$, $\alpha = 0.05$) (Table 2). The percentage of adherent cells was significantly greater for North Fork AK tissue than for either South Fork or mainstem AK tissue, which were not different from each other ($F = 6.53$, $p = 0.003$) (Table 2). Bactericidal activity (Kruskal–Wallis, $k = 8.51$, $p = 0.01$; post-hoc Tukey–Kramer, $q = 2.41$, $\alpha = 0.05$) and respiratory burst ($k = 10.03$, $p = 0.007$) (Table 2) were significantly different between North Fork and mainstem smallmouth bass AK leukocytes. Anterior kidney leukocytes from North Fork smallmouth bass had the highest bactericidal activity and the lowest stimulation index. South Fork smallmouth bass AK leukocytes did not significantly differ in either of these immune measures compared with the other sites (Table 2). Cytotoxic cell activity significantly differed among sites, with the highest activity exhibited by South Fork smallmouth bass AK leukocytes and the lowest by North Fork smallmouth bass AK leukocytes ($k = 21.58$, $p < 0.0001$) (Table 2). Basal reactive species production was not different between collection sites ($k = 3.24$, $p = 0.20$). Statistical analyses restricted to the samples utilized in the proteomics characterization followed the same trends but were not significant because of small sample sizes.

Protein expression profiles of smallmouth bass AK tissue from the Shenandoah River differed between collection sites. South Fork smallmouth bass AK tissues expressed the fewest number of proteins, with only 335 spots on the composite gel ($n = 5$ fish; range, 221–355) (Figs. 2A and 3). The composite

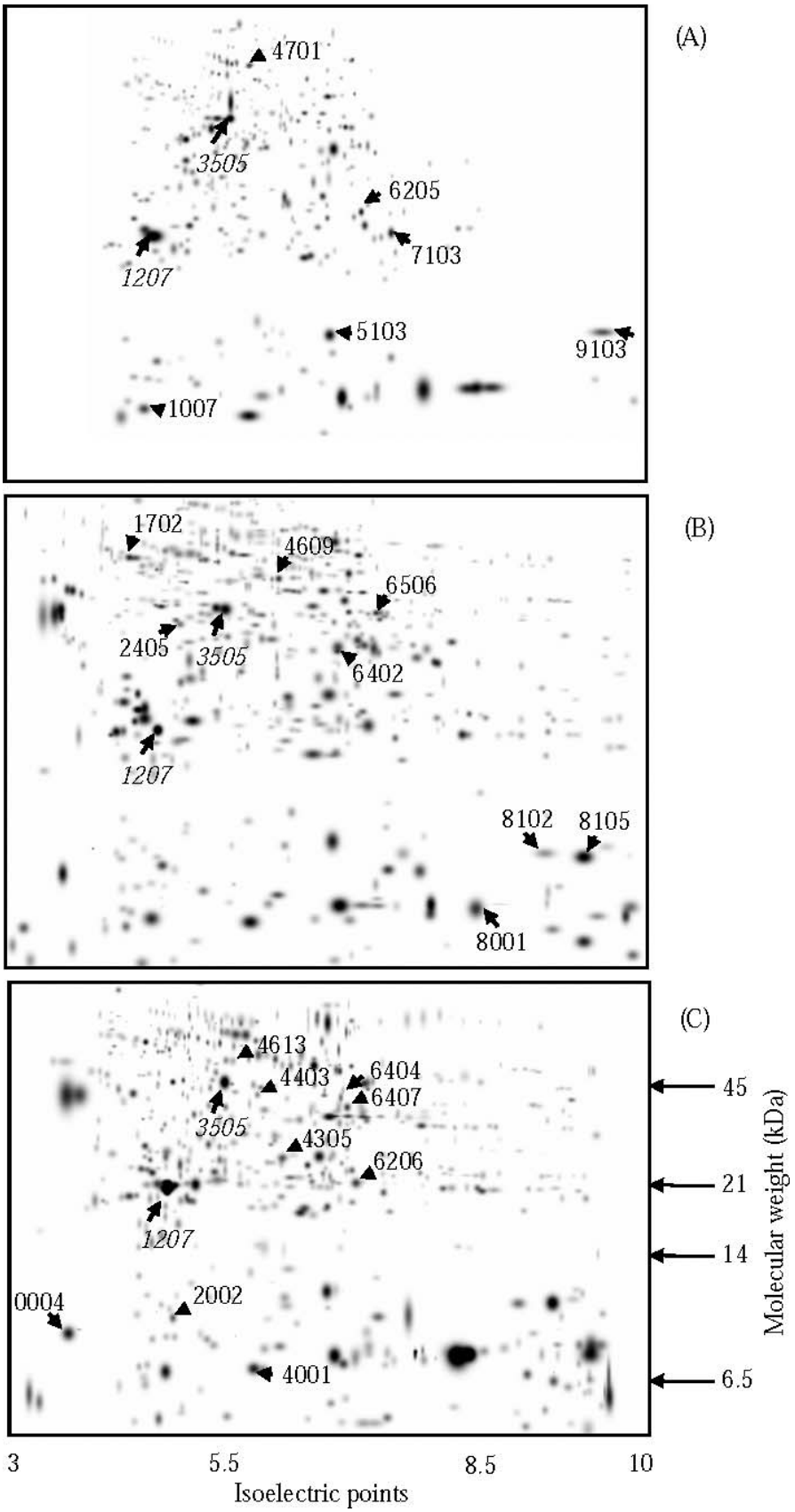

Fig. 2. Composite two-dimensional images created for smallmouth bass (*Micropterus dolomieu*) anterior kidney tissue collected during July 2006 from the (**A**) South Fork, (**B**) North Fork, and (**C**) mainstem of the Shenandoah River (VA, USA). Spots 3505 and 1207 (italics) are example landmarks used to align the gels both within and between sites. **A** and **B**. Protein spots successfully identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry are indicated by the arrows and listed in Table 4. **C**. Protein spots potentially indicative of bacterial infection are identified by the arrows and listed in Table 5.

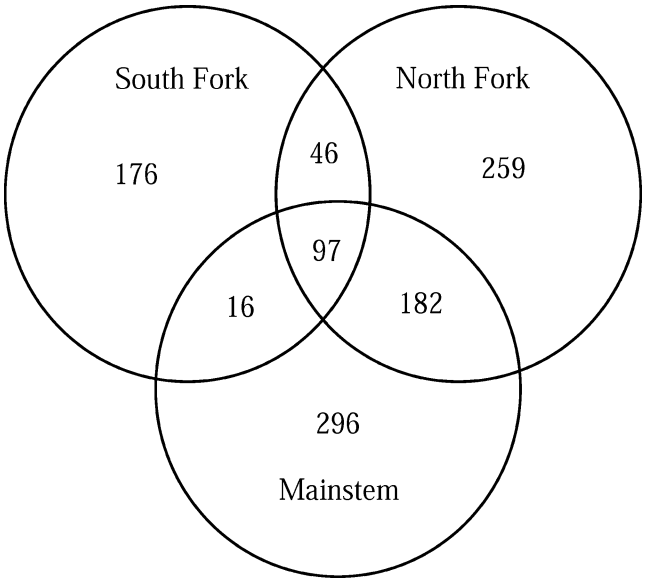

Fig. 3. Number of proteins expressed by Shenandoah River (VA, USA) smallmouth bass (*Micropterus dolomieu*) anterior kidney tissue. Proteins shared by two or more sites are indicated in the overlapping areas.

gels of the North Fork and mainstem smallmouth bass AK tissue contained 584 and 591 spots, respectively (North Fork: $n = 6$ fish; range, 512–592; mainstem: $n = 4$; range, 503–612) (Figs. 2B and C and 3). Depressed protein expression by smallmouth bass AK collected from the South Fork is indicated by the low number of proteins shared with the other two sites and the small number of exclusive proteins (Fig. 3).

Anterior kidney protein expression

Thirty-seven proteins were differentially expressed between at least two collection sites (Table 3). The greatest difference was observed for spot 8009, which exhibited a 129-fold higher abundance in South Fork versus North Fork Shenandoah River smallmouth bass AK tissue (Fig. 4). Twenty-five of these 37 proteins showed an increased expression in South Fork smallmouth bass AK tissue.

Nineteen of 50 protein spots excised for matrix-assisted laser desorption ionization–time of flight mass spectrometry were identified as probable smallmouth bass AK proteins (Table 4). The other 31 spots either did not fulfill the criteria described above or were not matched to a comparable species. It is possible that protein function may be different between species; however, considering most matches involved fairly ubiquitous proteins, similar function could be assumed. Three of the four identified proteins with increased expression in South Fork smallmouth bass AK tissue were involved in metabolism. A protein playing a role in circulation and respiration (spot 9103) also displayed elevated abundance. Seven proteins exclusive to mainstem and North Fork smallmouth bass AK tissues were expressed at comparable abundances. These proteins served immune or stress functions (Table 4). The remaining identified proteins, involved in respiration, structure, and motility, were expressed by all the smallmouth bass AK tissues. Nine of the 50 analyzed spots were considered to be indicative of bacterial infection because of homology with a bacterial species and the fact that the AK is not known to harbor bacteria (Table 5). Four were classified as hypothetical proteins with no identified function. Two spots were related to bacterial alteration of DNA replication (spot 4403) and transcriptional activity (spot 2002) (Table 4), and the remaining three proteins were involved in metabolism. Protein expression was not correlated with bactericidal activity (Spearman ρ, all $p > 0.5$) or the cytotoxicity activity of the cells (Spearman ρ, all $p > 0.5$) for these five spots.

DISCUSSION

The proteomics data indicate that smallmouth bass collected from three sites in the Shenandoah River differ in the metabolic capacity and immunological physiology of AK tissue. The variable responses in the functional immune assays further corroborate the suggestion of site-associated modulation of the immune system. Moreover, the presence of bacteria-related proteins implies the prevalence of active or covert bacterial infection or processed proteins trafficked by macrophages from another location in the body at all three sites. Comparison of immune function and protein expression of AK tissue presents a complementary picture of the physiological disruption experienced by these smallmouth bass.

Anterior kidney protein expression

Smallmouth bass AK protein expression provides a basis for hypothesizing site differences in AK function. Two identified proteins significantly elevated in South Fork samples are indicative of increased erythrocyte production. β-Globin is a structural element of hemoglobin, a major component of erythrocytes, and critical for oxygen transport. Triosephosphate isomerase B is a glycolytic enzyme known to be active in trout red blood cells [12]. The other two elevated proteins have complementary roles, with adenosine triphosphate synthase producing adenosine triphosphate and adenosine triphosphatase releasing energy from adenosine triphosphate. Together, increased expression of these proteins in South Fork smallmouth bass AK samples allows us to hypothesize that these fish had enhanced capacity for oxygen delivery to tissues and elevated metabolic rates in erythrocytes and hematopoietic tissue. Moreover, site comparisons revealed South Fork smallmouth bass AK expressed fewer proteins (or expressed proteins below our detection limits) and that those proteins that were expressed were at significantly elevated levels. These changes provide further evidence for modulation of AK function.

Seven identified proteins were not expressed in South Fork smallmouth bass AK tissues at measurable levels but were expressed at comparable levels in smallmouth bass AK tissues collected at the other two sites. The absence of these proteins potentially impinges on the responsiveness of the immune system. The C-type lysozyme is a muramidase that damages bacterial cell walls by catalyzing hydrolysis of 1,4-β-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues in a peptidoglycan and between *N*-acetyl-D-glucosamine residues in chitodextrins. It is expressed weakly in myeloblasts, moderately in immature macrophages, and strongly in both mature macrophages and macrophage-rich tissues. Additionally, lysozyme is present in cytoplasmic granules of polymorphonuclear neutrophils. Immunoglobulin proteins, or antibodies, are expressed as either integral membrane proteins of B cells or are secreted from these cells into circulation as soluble antibody. They are best known as the proteins of adaptive immunity specific for components of invading microbes and eukaryotic parasites that have been identified by the immune system during previous infection. Mitochondrial isocit-

Table 3. Differential expression of smallmouth bass (*Micropterus dolomieu*) anterior kidney proteins common to the North Fork, South Fork, and mainstem collection sites in the Shenandoah River (VA, USA)[a]

Trend	Spot no.	Abundance ($\times 10^3$) South Fork	North Fork	Mainstem	*p*
South Fork > mainstem ≈ North Fork	*9103*	20.37 ± 1.21	1.88 ± 0.10	0.73 ± 0.05	0.0211
	7103	10.64 ± 0.57	2.20 ± 0.12	2.44 ± 0.15	0.0101
	4202	1.35 ± 0.09	0.61 ± 0.05	0.57 ± 0.06	0.0487
	4201	2.87 ± 0.19	0.24 ± 0.06	0.49 ± 0.08	0.0181
	4101*	3.89 ± 0.11	0.75 ± 0.08	1.06 ± 0.08	0.0231
	3303	3.04 ± 0.26	0.75 ± 0.04	0.96 ± 0.05	0.0073
	3101	3.27 ± 0.14	0.59 ± 0.04	0.48 ± 0.07	0.0052
	1106	2.32 ± 0.22	1.08 ± 0.11	0.72 ± 0.04	0.0181
South Fork > mainstem > North Fork	8009*	35.40 ± 1.08	0.27 ± 0.19	3.29 ± 0.18	0.0093
	6411*	20.57 ± 1.31	6.15 ± 0.30	9.13 ± 0.21	0.0181
	6009*	5.80 ± 0.12	1.26 ± 0.09	3.76 ± 0.23	0.0048
	2609	1.04 ± 0.08	0.19 ± 0.03	0.63 ± 0.07	0.0116
South Fork ≈ mainstem > North Fork	*4701*	1.96 ± 0.11	0.20 ± 0.08	1.62 ± 0.19	0.0390
	3507	1.31 ± 0.18	0.18 ± 0.08	0.91 ± 0.12	0.0116
	2007	1.57 ± 0.11	0.20 ± 0.06	1.14 ± 0.21	0.0064
	1207	56.97 ± 1.28	13.24 ± 2.33	62.07 ± 1.10	0.0051
South Fork > North Fork > mainstem	7003*	59.50 ± 1.20	15.20 ± 1.24	1.39 ± 0.10	0.0079
	5206	3.53 ± 0.11	2.80 ± 0.15	1.26 ± 0.17	0.0116
	4613	1.18 ± 0.08	0.32 ± 0.05	1.24 ± 0.06	0.0475
	1002	14.34 ± 0.52	3.83 ± 0.20	0.69 ± 0.03	0.0048
South Fork ≈ North Fork > mainstem	7306	1.71 ± 0.36	1.48 ± 0.08	0.64 ± 0.04	0.0453
	6602	1.64 ± 0.17	1.25 ± 0.12	0.37 ± 0.07	0.0302
	6008	3.39 ± 0.11	3.95 ± 0.13	0.83 ± 0.04	0.0198
	3505	9.77 ± 0.23	12.90 ± 0.48	0.81 ± 0.06	0.0056
	2002	6.61 ± 0.32	5.87 ± 0.10	3.18 ± 0.12	0.0222
North Fork > mainstem > South Fork	6801*	1.02 ± 0.07	6.95 ± 0.26	10.76 ± 0.91	0.0273
	6604*	0.84 ± 0.09	4.08 ± 0.18	1.90 ± 0.11	0.0181
	4810	0.24 ± 0.07	1.09 ± 0.06	0.59 ± 0.07	0.0116
	3812	0.54 ± 0.08	2.25 ± 0.13	1.17 ± 0.06	0.0079
	3502	0.25 ± 0.04	1.64 ± 0.05	0.84 ± 0.03	0.0181
North Fork > mainstem ≈ South Fork	4402	0.80 ± 0.08	1.61 ± 0.09	0.77 ± 0.08	0.0233
North Fork ≈ mainstem > South Fork	5204*	1.08 ± 0.16	2.72 ± 0.12	2.83 ± 0.15	0.0406
	4403	0.52 ± 0.04	1.57 ± 0.06	1.39 ± 0.09	0.0124
North Fork > South Fork > mainstem	5102	5.17 ± 0.18	11.23 ± 0.61	1.75 ± 0.07	0.0079
Mainstem > South Fork ≈ North Fork	*8001*	15.91 ± 2.09	29.72 ± 3.34	94.35 ± 1.17	0.0108
	3602	0.27 ± 0.08	0.59 ± 0.05	1.69 ± 0.09	0.0273
Mainstem > North Fork > South Fork	*6407*	1.38 ± 0.09	5.48 ± 0.17	3.15 ± 0.13	0.0273

[a] Abundance values are presented as the mean ± standard error. Spot numbers in italics correspond to proteins in the annotated composite gel images of Figure 2 and Tables 4 and 5. Asterisks indicate proteins that were subjected to matrix-assisted laser desorption ionization–time of flight analysis but not successfully identified.

rate dehydrogenase catalyzes an irreversible reaction in the citric acid cycle. The activity of this enzyme is induced in lymphocytes following mitogen stimulation [13]. Neutrophil cytosolic factor 4 ($p40^{phox}$) is a cytosolic regulatory component of the superoxide-producing phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase preferentially expressed in leukocytes of myeloid lineage. It is an important regulatory component of the respiratory burst system in phagocytes. Heat shock proteins are induced in fish in response to several stressors, including heat, hypoxia, metal exposure, and altered oxidation–reduction reaction status of cells [14]. In particular, heat shock protein 70 has been shown to facilitate antigen presentation and has been recognized as an endogenous immunostimulant [15]. Vimentin is an intermediate filament protein of the cytoskeleton expressed during cell differentiation in cell types of mesodermal origin. It is expressed in

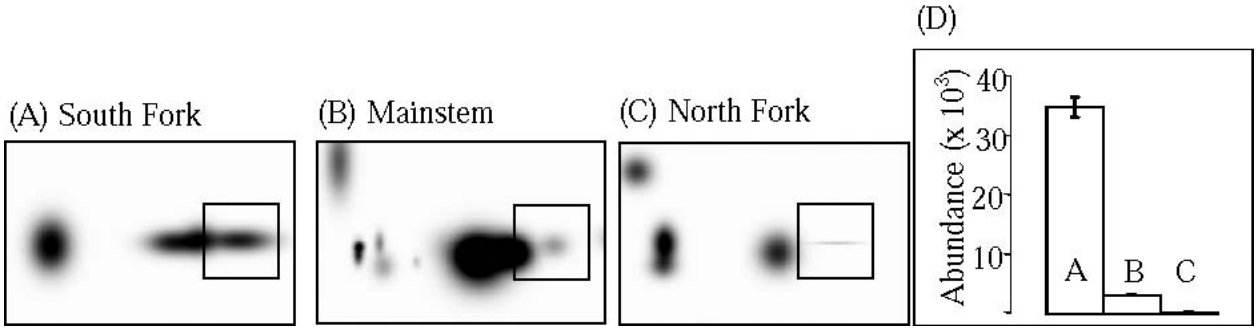

Fig. 4. (**A–C**) Illustration of the protein expression relationship in the Shenandoah River (VA, USA): South Fork > mainstem > North Fork (spot 8009). (**D**) Graph of protein abundance displayed in **A** through **C**. Bars represent the mean ± standard error.

Table 4. Identified anterior kidney proteins and trends in protein expression between South Fork (S), mainstem (M), and North Fork (N) Shenandoah River (VA, USA) smallmouth bass (*Micropterus dolomieu*)[a]

Spot no.	Gel[b]	Exp. pI	Exp. MW	Homology	Species	Theor. pI	Theor. MW	Score	Coverage (%)	Matched peptides	Trend	Protein function[c]
9103	S_M4, S_M5	9.5	10.6	β-Globin	*Oncorhynchus mykiss*	9.2	13.3	7.4/7.5	70	7	S > M ≈ N	R
8105	M_M1, N_F2	9.3	10	Lysozyme	*Gallus gallus* (Chicken)	9.3	14.8	59/62	71	8	M ≈ N	I
8102	M_F4	8.9	10.2	Immunoglobulin heavy-chain variable region	*Homo sapiens*	8.9	8.4	46	84	5	M ≈ N	I
8001	M_F1, M_M3	8.2	7.4	Hemoglobin subunit β-1	*Arctogadus glacialis* (Arctic cod)	8.3	14.9	52/50	33	4	M > S ≈ N	R
7103	S_F1	7.4	20	Triosephosphate isomerase B	*Xiphophorus maculatus* (southern platyfish)	7.6	26.8	72	57	11	S > M ≈ N	M
6506	M_F2	7.2	40.8	Mitochondrial isocitrate dehydrogenase 2-like	*Oreochromis mossambicus* (Mozambique tilapia)	7.6	50.9	80	43	19	M ≈ N	O, S
6402	N_M3	6.8	32.8	Glyceraldehyde 3 phosphate dehydrogenase	*O. mykiss*	6.8	36.3	6.9	26	6	N only	M
6205	S_M4	7	23.3	Heat shock 70-kDa protein 8	*Danio rerio*	7.8	20.6	88	61	11	M ≈ N	S
5103	M_F1	6.7	11	Na,K-ATPase α subunit	*Homo sapiens*	6.3	19.4	54	39	4	S ≈ M ≈ N	M
4701	S_M5	5.9	55	V-type ATPase β subunit	*O. mykiss*	5.6	55.7	6.7	25	8	S ≈ M > N	O, M
4609	M_F4	6.1	49.6	Neutrophil cytosolic factor 4	*O. mykiss*	6.6	40.5	5.9	33	10	M ≈ N	I, B, U, M
3505	N_M6	5.6	41.6	β-Actin	*O. mykiss*	5.5	41.7	6.5	42	16	S ≈ M ≈ N	D
2405	N_F5	5	38.3	Heat shock 70-kDa protein 8, isoform b	*O. mykiss*	5.1	58.3	6.4	21	12	N ≈ M	I, S, M, B
1702	M_F2	4.4	55.9	Vimentin	*O. mykiss*	5.2	53	7.6	30	12	M ≈ N	I, D
1207	S_F2	4.7	21	ATP synthase β subunit	*O. mykiss*	4.9	18.7	6.1	57	6	S ≈ M > N	M
1007	N_M3	4.6	7	ATPase inhibitor precursor	*Siniperca chuatsi* (Chinese perch)	9.5	6	53	56	3	S ≈ M ≈ N	M

[a] When the trend omits (S), the protein was not expressed by smallmouth bass from the South Fork. Successful identification was determined in part by significant ProteinLynx (scores < 10) or Mascot (scores > 10) scores. ATP = adenosine triphosphate; ATPase = adenosine triphosphatase; Exp. MW = experimental molecular weight; Exp. pI = experimental isoelectric point; Theor. MW = theoretical molecular weight; Theor. pI = theoretical isoelectric point.

[b] Identification of the gel from which the protein was excised for matrix-assisted laser desorption ionization–time of flight. Notation indicates mainstem (M), North Fork (N), or South Fork (S), followed (after the underscore) by the sex (male [M] or female [F]) and the identification number. Identification of two gels indicates the same spot was excised from two different gels.

[c] Protein function was assigned according to reports from Swiss-Prot database (Swiss Institute of Bioinformatics, Geneva, Switzerland) and the current literature. Functions are indicated as follows: B = protein binding and folding; D = structure and motility; I = immune; M = metabolism; O = osmoregulation; R = circulation and respiration; S = stress; U = cell communication.

leukocytes and plays an important role in lymphocyte adhesion and extravasation, and it has been identified as a ''kill me'' signal to natural killer cells presented on the surface of microbe-infected monocytes [16,17]. Overall, the expression pattern of these proteins indicates that North Fork and mainstem Shenandoah River smallmouth bass likely have more functional leukocytes and a greater capacity to respond to an immune challenge. The nine identified bacterial proteins, however, do not indicate any consistent differences in bacterial load in analyzed AK tissues.

Corroboration of AK protein expression with functional immune assays

Comparisons with AK immune function generally support the hypotheses generated by the proteomics results. For instance, AK-derived adherent leukocytes in South Fork and mainstem Shenandoah River smallmouth bass exhibited a more exaggerated LPS-induced production of extracellular reactive species than those from the North Fork Shenandoah River. The inducible respiratory burst response in phagocytes is mediated primarily by the multicomponent enzyme, leukocyte NADPH oxidase. This enzyme is comprised of flavocytochrome b558 and a number of cytosolic phox proteins, including $p40^{phox}$. Interestingly, of these proteins, the $p40^{phox}$ down-regulates NADPH oxidase activity, which is in functional contrast to other phox protein regulatory roles [18]. The absence of $p40^{phox}$ in the South Fork smallmouth bass AK protein profile may mechanistically explain the observed higher reactive species. We are not aware of contaminants that specifically modulate $p40^{phox}$, but elevated reactive species is a common observation in fish from impacted areas. Confounding this observation is that the respiratory burst response was greatest in smallmouth bass from the mainstem Shenandoah River, which expressed detectable amounts of $p40^{phox}$. The production of reactive spe-

Table 5. Proteins indicative of bacterial infection identified from the South Fork (S), mainstem (M), and North Fork (N) Shenandoah River (VA, USA) smallmouth bass (*Micropterus dolomieu*) anterior kidneys[a]

Spot no.	Gel[b]	Exp. pI	Exp. MW	Homology	Species	Theor. pI	Theor. MW	Mascot score	Coverage (%)	Matched peptides	Trend	Protein function[c]
6407	M_F3	7	34.9	Hypothetical protein MXAN_7286	*Myxococcus xanthus*	7.2	40.7	58	46	14	M ≈ N > S	X
6404	S_M4	6.9	38.1	UDP-glucose 6-dehydrogenase	*Shewanella baltica*	6.3	44.2	74	48	14	S ≈ M ≈ N	M
6206	S_M5	7.1	21.5	Hypothetical protein Strop-DRAFT_4220	*Salinispora tropica*	7.6	13.7	49	36	2	S ≈ M ≈ N	X
4613	M_F2	5.8	48.1	Citrate synthase (fragment)	*Bartonella grahamii*	5.9	36.5	54	26	7	S ≈ M > N	M
4403	N_M6	5.9	38.1	DNA gyrase subunit B	*Vibrio* sp.	6.4	31.4	55	41	8	M ≈ N > S	T
4305	M_M3	6.3	25.3	Hypothetical protein Sama-DRAFT_2968	*Shewanella amazonensis*	6.7	14.7	48	45	3	S ≈ M ≈ N	X
4001	S_F3	5.9	6.9	Phosphoglycerate mutase	*Bacillus* sp.	6.3	11.9	48	53	3	S ≈ M ≈ N	M
2002	N_F5	4.8	9.5	ENSANGP-00000029042	*Anopheles gambiae*	4.3	16.5	42	36	3	S ≈ N > M	L
0004	M_F2	3.8	9.1	Hypothetical protein WD0192	*Wolbachia* endosymbiont of *Drosophila melanogaster*	4.6	6.7	55	85	5	M ≈ N	X

[a] Exp. MW = experimental molecular weight; Exp. pI = experimental isoelectric point; Theor. MW = theoretical molecular weight; Theor. pI = theoretical isoelectric point; UDP = uridine diphosphate.

[b] Identification of the gel from which the protein was excised for matrix-assisted laser desorption ionization–time of flight. Notation indicates mainstem (M), North Fork (N), or South Fork (S), followed (after the underscore) by the sex (male [M] or female [F]) and the identification number.

[c] Protein function was assigned according to reports from Swiss-Prot database (Swiss Institute of Bioinformatics, Geneva, Switzerland) and the current literature. Functions are indicated as follows: L = translation regulation; M = metabolism; T = topological transitions of DNA; X = unknown.

cies is directly related to the metabolic activity of the cell and the buffering capacity of antioxidant defenses that are commonly affected by contaminants.

The higher natural killer-like cytotoxicity in AK tissue from smallmouth bass of the South Fork Shenandoah River may reflect potentially greater numbers of such cells or cells with elevated activity. The AK also is the richest tissue source of active natural cytotoxic cells [19,20]. Cytotoxicity mediated by nonspecific natural cytotoxic cells of tilapia is positively correlated with circulating stress-activating serum factors induced by temperature stress [21]. Lower cytotoxic cell activity from AK tissues in North Fork and mainstem Shenandoah River smallmouth bass may be the result of lower metabolic activity of these cells or of trafficking of this cell population to peripheral tissues, such as the liver, spleen, posterior kidney, and skin in response to the observed lesions.

The present proteomics results indicate that North Fork and mainstem Shenandoah River smallmouth bass AK tissues likely have increased numbers of, and more vigorous, leukocytes. This may be an indication of more healthy immune systems, but it also may be the result of the higher lesion prevalence and, consequently, a differential increase in activated leukocytes. The percentage of adherent AK leukocytes in fish from the North Fork was considerably higher than that generally observed in this species, whereas that of the South Fork and mainstem fish was typical. This observation could be the result of a site-specific recruitment of such cells because of parasitic infestation (trematodes are common in AK tissue) or an increased population of resident macrophages or macrophage aggregates. Whereas bactericidal activity was highest in AK tissues of fish from this site, the increased capacity is not simply the result of more adherent cells, because the bactericidal activity reported here was corrected for cell number. Bactericidal activity of smallmouth bass leukocytes follows a definite seasonal trend in which this immune parameter is depressed during the summer months. Of particular note, the killing capacity of adherent cells from AK tissues of South Fork and mainstem smallmouth bass were the lowest that we have observed for centrarchids sampled during the summer. Generally, immune function results are consistent with an interpretation of metabolic activation and immunosuppression in South Fork smallmouth bass AK tissues relative to those in the North Fork.

Hypothesized sources of observed physiological disruption

The present hypothesis of metabolic activation and immunosuppression in smallmouth bass from areas of the South Fork, North Fork, and mainstem Shenandoah River does not implicate one specific factor as the source of physiological disruption. Environmental conditions in the Shenandoah River include general stressors, such as eutrophication, increasing temperature, and chemical contamination [1]. Each factor has the potential to activate the stress response, resulting in altered circulating concentrations of cortisol. Cortisol will inhibit the release of thyroid hormone (triiodothryonine) and result in a reduced uptake of glucose into some tissues [22,23], thereby leading to a general diminution in metabolism. Immunosuppression is a well-characterized effect of increased cortisol concentrations, with reductions in circulating immune cell numbers and bactericidal activity coinciding with the inflam-

matory response [24,25]. Unfortunately, given the method of fish capture used in the present study, precapture levels of plasma cortisol could not be assessed. Thyroid hormones are the classic mediators of cellular metabolism, causing increased oxygen consumption. Elevated thyroid hormone, as an effect of increasing temperatures, stimulates more leukocytes into circulation [26,27]. Because leukocytes play a role in the regulation of circulating thyroid hormone [28], suppressed immune cell numbers and elevated thyroid hormone levels are an unlikely physiological combination. If the proteomics hypothesis is the result of an endogenous response, both cortisol and thyroid hormone activity should be elevated.

The Shenandoah River contains measurable levels of arsenic from the poultry industry (Barron, cited in Garman and Orth [1]). Arsenic exposure causes decreased leukocyte activity and adherence [29]. Reactive species production increases with arsenic exposure [30]. Therefore, the variability in immune response between collection sites suggests differential arsenic exposure. Arsenic exposure, however, decreases cellular metabolism [31] and the activational effects of the glucocorticoid receptor [32]. The expression of heat shock proteins [33] and the estrogen receptor is elevated following exposure [34]; these effects are consistent with our proteomics results and abnormally high incidences of intersex smallmouth bass from these sites [2]. The Shenandoah River drainage basin includes important agricultural areas for corn, soy, and apples. A 1994 U.S. Geological Survey study of herbicides in streams of the Potomac River Basin found detectable concentrations in more than half of the collected samples [35]. The most common herbicide was atrazine (maximum concentration, 0.73 μg/L). Some halogenated herbicides, including atrazine, have been linked to immunosuppression in amphibians [36]. In goldfish, exposure to a mixture of herbicides (atrazine, simazine, diuron, and isoproturon) increased reactive species production and circulating lysozyme activity but reduced pathogen resistance and antibody titer following infection [37]. A similar mechanism of immunosuppression also has been shown in amphibians exposed to a pesticide mixture containing atrazine [38]. Therefore, decreased lysozyme expression revealed by the protein profile does not coincide with the expected pattern of herbicide exposure. Herbicides such as atrazine, alachlor, and acetochlor, however, have been described as having thyroid hormone activity in amphibians [36]. In an agricultural setting where poultry litter commonly is used as a fertilizer, varying mixtures of arsenic and atrazine would be expected. We hypothesize that these contaminants, aggravated by stressful environmental conditions and increasingly resistant pathogens [39], could explain the indications via protein expression of variable metabolism and immune function.

The analysis of protein expression profiles allowed us to develop a number of testable hypotheses regarding the type and source of fish health impacts on Shenandoah River smallmouth bass. Despite the efforts of numerous researchers and agencies, no single pattern or cause has emerged that accounts for the loss of smallmouth bass [1]. The value of the proteomics analysis is that we can infer changes in tissue function from expression profiles [4]. Based on relative protein content and immune function assays, we hypothesize that smallmouth bass from three sites in the Shenandoah River differ in basal or AK metabolic activity and immune function. We can further hypothesize that these physiological changes are potentially related to circulating cortisol concentrations as well as exposure to arsenic and herbicides. This hypothesis predicts that the mixture of these factors should be directly related to the frequency and severity of Shenandoah River smallmouth bass fish kills.

Acknowledgement—Matrix-assisted laser desorption ionization–time of flight mass spectrometry was performed by S. Wolffe at the West Virginia University Proteomics Core facility. The present study was funded by the Hardy County Rural Development Authority, Virginia Department of Environmental Quality, Virginia Department of Game and Inland Fisheries, and U.S. Geological Society Priority Ecosystem Chesapeake Bay Program. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

REFERENCES

1. Garman G, Orth D. 2007. Fish kills in the Shenandoah River basin: Preliminary report of the Shenandoah Basin science team. Virginia Department of Environmental Quality, Richmond, VA, USA.
2. Blazer V, Iwanowicz L, Iwanowicz D, Smith D, Young J, Hedrick J, Foster S, Reeser S. 2007. Intersex (testicular oocytes) in smallmouth bass (*Micropterus dolomieu*) from the Potomac River and selected nearby drainages. *J Aquat Anim Health* 19:242–253.
3. Krzystyniak K, Tryphonas H, Fournier M. 1995. Approaches to the evaluation of chemical-induced immunotoxicity. *Environ Health Perspect* 103:17–22.
4. Nesatyy VJ, Suter MJF. 2007. Proteomics for the analysis of environmental stress responses in organisms. *Environ Sci Technol* 41:6891–6900.
5. Alliance for the Chesapeake Bay. 1994. Shenandoah River fact sheet. Richmond, VA, USA.
6. Chesapeake Bay Foundation. 2005. Shenandoah River fact sheet. Annapolis, MD, USA.
7. Iwanowicz L, Lerner D, Blazer V, McCormick S. 2005. Aqueous exposure to Aroclor 1254 modulated the mitogenic response of Atlantic salmon anterior kidney T cell: Indications of short- and long-term immunomodulation. *Aquat Toxicol* 72:305–314.
8. Harms C, Ottinger C, Blazer V, Densmore C, Pieper L, Kennedy-Stoskopf S. 2000. Quantitative polymerase chain reaction for transforming growth factor–β applied to a field study of fish health in Chesapeake Bay tributaries. *Environ Health Perspect* 108:447–452.
9. Coteur G, Warnau M, Jangoux M, Dubois P. 2002. Reactive oxygen species (ROS) production by amoebocytes of *Asterias rubens* (Echinodermata). *Fish Shellfish Immunol* 12:187–200.
10. Iwanowicz L, Densmore C, Ottinger C. 2004. Calcein AM release-based cytotoxic cell assay for fish leukocytes. *Fish Shellfish Immunol* 16:127–137.
11. Babu G, Wheeler D, Alzate O, Periasamy M. 2004. Solubilization of membrane proteins for two-dimensional gel electrophoresis: Identification of sarcoplasmic reticulum membrane proteins. *Anal Biochem* 325:121–125.
12. Ferguson R, Storey K. 1991. Glycolic and associated enzymes of rainbow trout (*Oncorhynchus mykiss*) red cells: In vitro and in vivo studies. *J Exp Biol* 155:469–485.
13. Yang L, Luo H, Vinay P, Wu J. 1996. Molecular cloning of the cDNA of mouse mitochondrial NADP-dependent isocitrate dehydrogenase and the expression of the gene during lymphocyte activation. *J Cell Biochem* 60:400–410.
14. Iwama G, Thomas P, Forsyth R, Vijayan M. 1998. Heat shock protein expression in fish. *Rev Fish Biol Fish* 8:35–56.
15. Milani V, Noessner E, Ghose S, Kuppner M, Ahrens B, Scharner A, Gastpar R, Issels R. 2002. Heat shock protein 70: Role in antigen presentation and immune stimulation. *Int J Hyperthermia* 18:563–575.
16. Garg A, Barnes PF, Porgador A, Roy S, Wu S, Nanda JS, Griffith DE, Girard WM, Rawal N, Shetty S, Vankayalapati R. 2006. Vimentin expressed on *Mycobacterium tuberculosis*–infected human monocytes is involved in binding to the NKp46 receptor. *J Immunol* 177:6192–6198.
17. Nieminen M, Henttinen T, Merinen T, Marttila-Ichihara F, Eriksson JE, Jalkanen S. 2006. Vimentin function in lymphocyte adhesion and transcellular migration. *Nat Cell Biol* 8:156–162.
18. Sathyamoorthy M, de Mendez I, Adams AG, Leto TL. 1997. $p40^{phox}$ down-regulates NADPH oxidase activity through interactions with its SH3 domain. *J Biol Chem* 272:9141–9146.

19. Faisal M, Ahmed II, Peters G, Cooper EL. 1989. Natural cytotoxicity of tilapia leukocytes. *Dis Aquat Org* 7:17–22.
20. Evans DL, Cooper EL. 1990. Natural killer cells in ectothermic vertebrates. *BioScience* 40:745–749.
21. Ruiz J, Leary JH, Jaso-Friedmann L. 2001. Phosphorylation-induced activation of tilapia nonspecific cytotoxic cells by serum cytokines. *Dis Aquat Organ* 46:129–137.
22. Vijayan MM, Flett PA, Leatherland JF. 1988. Effect of cortisol on the in vitro hepatic conversion of thyroxine to triiodothyronine in brook charr (*Salvelinus fontinalis* Mitchill). *Gen Comp Endocrinol* 70:312–318.
23. Vijayan MM, Ballantyne JS, Leatherland JF. 1991. Cortisol-induced changes in some aspects of the intermediary metabolism of *Salvelinus fontinalis*. *Gen Comp Endocrinol* 82:476–486.
24. Maule AG, Schreck CB. 1990. Changes in number of leukocytes in immune organs of juvenile coho salmon (*Oncorhynchus kisutch*) after acute stress or cortisol treatment. *J Aquat Anim Health* 2:298–304.
25. Wang WB, Li AH, Cai TZ, Wang JG. 2005. Effects of intraperitoneal injection of cortisol on nonspecific immune functions of *Ctenopharyngodon idella*. *J Fish Biol* 67:779–793.
26. Fabris N, Mocchegiani E, Provinciali M. 1995. Pituitary–thyroid axis and immune system: A reciprocal neuroendocrine–immune interaction. *Horm Res* 43:29–38.
27. Parker SJ, Specker JL. 2006. Salinity and temperature effects on whole-animal thyroid hormone levels in larval and juvenile striped bass, *Morone saxatilis*. *Fish Phys Biochem* 8:507–514.
28. Klein JR. 2006. The immune system as a regulator of thyroid hormone activity. *Exp Biol Med* 231:229–236.
29. Lage CR, Nayak A, Kim CH. 2006. Arsenic ecotoxicology and innate immunity. *Integr Comp Biol* 46:1040–1046.
30. Lantz RC, Hays AM. 2006. Role of oxidative stress in arsenic-induced toxicity. *Drug Metab Rev* 38:791–804.
31. Hermann AC, Kim CH. 2005. Effects of arsenic on zebrafish innate immune system. *Mar Biotechnol* 7:494–505.
32. Kaltreider RC, Davis AM, Lariviere JP, Hamilton JW. 2001. Arsenic alters the function of the glucocorticoid receptor as a transcription factor. *Environ Health Perspect* 109:245–251.
33. Del Razo LM, Quintanilla-Vega B, Brambila-Colombres E, Calerson-Aranda ES, Manno M, Albores A. 2001. Stress proteins induced by arsenic. *Toxicol Appl Pharmacol* 177:132–148.
34. Davey JC, Bodwell JE, Gosse JA, Hamilton JW. 2007. Arsenic as an endocrine disruptor: Effects of arsenic on estrogen receptor–mediated gene expression in vivo and in cell culture. *Toxicol Sci* 98:75–86.
35. Fisher GT. 1995. Selected herbicides in major streams in the Potomac River basin upstream from Washington, DC. U.S. Geological Survey NAWQA Fact Sheet 107-95. U.S. Geological Survey, Towson, MD.
36. Forson DD, Storfer A. 2006. Atrazine increases ranavirus susceptibility in the tiger salamander, *Ambystoma tigrinum*. *Ecol Appl* 16:2325–2332.
37. Fatima M, Mandiki SN, Douxfils J, Silvestre F, Coppe P, Kestemont P. 2007. Combined effects of herbicides on biomarkers reflecting immune–endocrine interactions in goldfish. Immune and antioxidant effects. *Aquat Toxicol* 81:159–167.
38. Hayes TB, Case P, Chui S, Chung D, Haeffele C, Haston K, Lee M, Mai VP, Marjuoa Y, Parker J, Tsui M. 2006. Pesticide mixtures, endocrine disruption, and amphibian declines: Are we underestimating the impact? *Environ Health Perspect* 114(Suppl 1):40–50.
39. Pruden A, Pei R, Storteboom H, Carlson KH. 2006. Antibiotic resistance genes as emerging contaminants: Studies in Northern Colorado. *Environ Sci Technol* 40:7445–7450.