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Prepared in cooperation with Pennsylvania Department of Environmental Protection

Development of a Suite of Functional Immune Assays and Initial Assessment of Their Utility in Wild Smallmouth Bass Health Assessments

Open-File Report 2020–1077

U.S. Department of the Interior
U.S. Geological Survey

Cover. Photographs showing the collection and laboratory processing of smallmouth bass tissue samples. Photographs by Vicki Blazer, U.S. Geological Survey (USGS), Kay Briggs, USGS, Cheyenne Smith, West Virginia University, and Heather Walsh, USGS.

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Conversion Factors

International System of Units to U.S. customary units

Multiply	By	To obtain
	Length	
centimeter (cm)	0.3937	inch (in.)
millimeter (mm)	0.03937	inch (in.)
micrometer (µm)	0.00003937	inch (in.)
meter (m)	3.281	foot (ft)
kilometer (km)	0.6214	mile (mi)
	Volume	
milliliter (mL)	0.03382	ounce, fluid (fl. oz)
liter (L)	33.81402	ounce, fluid (fl. oz)
liter (L)	2.113	pint (pt)
liter (L)	1.057	quart (qt)
liter (L)	0.2642	gallon (gal)
liter (L)	61.02	cubic inch (in^3)
	Mass	
gram (g)	0.03527	ounce, avoirdupois (oz)
kilogram (kg)	2.205	pound avoirdupois (lb)

Temperature in degrees Celsius (°C) may be converted to degrees Fahrenheit (°F) as follows:

$$°F = (1.8 \times °C) + 32.$$

Abbreviations

AF647	Alexa Fluor 647
BrdU	Bromo-deoxyuridine
BSA	Bovine serum albumin
bv.	Biovar
CFU	Colony forming units
Con A	Concanavalin A
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
EdU	5-ethynyl-2′-deoxyuridine
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
L-15	Leibovitz L-15 medium
LPS	Lipopolysaccharide
PHA	Phytohemagglutinin
PHA-P	Phytohemagglutinin from *Phaseolus vulgaris*
PMA	Phorbol 12-myristate 13-acetate
P/S	Penicillin-streptomycin
RCF	Relative centrifugal field
ROS	Reactive oxygen species
SI	Stimulation index
TSA	Tryptic soy agar
TSB	Tryptic soy broth

Development of a Suite of Functional Immune Assays and Initial Assessment of Their Utility in Wild Smallmouth Bass Health Assessments

By Cheyenne R. Smith,[1,2] Christopher A. Ottinger,[1] Heather L. Walsh,[1] and Vicki S. Blazer[1]

Abstract

Methods were developed for measuring immune function in *Micropterus dolomieu* (smallmouth bass). The ultimate objective is to monitor and evaluate changes over time in immune status and disease resistance in conjunction with other characteristics of fish health and environmental stressors. To test these methods for utility in ecotoxicological studies, 192 smallmouth bass, age 2 years and older, were collected from three sites within the Susquehanna River Basin and one site in the Ohio River Basin during spring and fall 2016 and 2017. The anterior kidney was aseptically removed and homogenized for leukocyte isolation. Leukocytes were tested for bactericidal activity against two species of bacteria; respiratory burst activity when stimulated with phorbol 12-myristate 13-acetate; and mitogenesis activity when stimulated with concanavalin A, phytohemagglutinin, and lipopolysaccharide. Tissues were preserved for histopathological analyses.

Two of the sites were part of a monitoring program at which surface-water samples were collected monthly (bimonthly in spring) for chemical contaminants. Significant seasonal and (or) site differences in all three immune function tests were observed. Interpretations of seasonal trends in immune function of wild fish or correlations with environmental variables and other factors are difficult to make owing to the complex nature of the immune response and the environment. Differences in immune function could potentially be related to a variety of confounding factors; therefore, additional endpoints and repeated sampling over an extended period are essential to draw conclusions on the immune status of wild fish.

Introduction

Micropterus dolomieu (smallmouth bass) are an economically important sportfish throughout the Chesapeake Bay watershed. Large-scale episodic mortality and disease events have occurred since 2002 in the Potomac River and 2005 in the Susquehanna River, along with many of their tributaries (Blazer and others, 2007; Blazer and others, 2010; Smith and others, 2015; Walsh and others, 2018). Disease related mortality has mainly affected adults in the Potomac River (Blazer and others, 2010) and young-of-year in the Susquehanna River (Smith and others, 2015; Walsh and others, 2018). Over time, young-of-year smallmouth bass abundance in the Susquehanna River has declined, resulting in less recruitment, reduced adult populations, and interruptions to recreational fishing (Arway and Smith, 2013). Consequently, Federal and State wildlife management agencies in all four affected states—Pennsylvania, Maryland, West Virginia, and Virginia—have reported large economic losses relating to the decline in smallmouth bass (Pelton and others, 2013).

No consistent cause or causes for disease or mortalities have been identified. Bacterial infections, heavy parasite loads, sometimes viral and fungal infections, and often co-infections of multiple parasites and pathogens have been observed. Pathogens isolated from smallmouth bass collected in the Potomac River have included *Flavobacterium columnare*, *Enterobacter* sp., *Aeromonas hydrophila*, and *Aeromonas veronii* biovar (bv.) sobria (Blazer and others, 2010; Starliper and others, 2013). In the Susquehanna River, researchers have observed or isolated bacteria (mainly *Flavobacterium columnare* and motile *Aeromonas* spp.), largemouth bass virus, and parasites, including trematode metacercariae, larval cestodes, and the myxozoan *Myxobolus inornatus* (Chaplin and others, 2009; Starliper and others, 2013; Smith and others, 2015; Walsh and others, 2018). The mixed infections and lack of any one consistent cause indicate that these fish are immunocompromised and hence susceptible to a variety of opportunistic pathogens.

Other effects on smallmouth bass health have been observed in the affected areas, including signs of estrogenic endocrine disruption, as evidenced by testicular oocytes

[1]U.S. Geological Survey.

[2]West Virginia University Division of Forestry and Natural Resources.

(intersex) and vitellogenin in male fish (Blazer and others, 2007; Iwanowicz and others, 2009; Blazer and others, 2014), and exposure to chemical contaminants, as evidenced by the increased presence of macrophage aggregates and oxidative damage in liver, spleen, and kidney tissues, and gill and skin lesions (Blazer and others, 2010). Numerous chemical contaminants linked to intersex, immunomodulation, and reduced disease resistance have been measured in affected locations, including up to 135 different compounds in the water at smallmouth bass nesting sites (Alvarez and others, 2009; Blazer and others, 2010; Reif and others, 2012; Kolpin and others, 2013; Walsh and others, 2018). Complex interactions between water quality, contaminants, pathogens, parasites, and other environmental stressors appear to be affecting smallmouth bass immunity, making it difficult to pinpoint any specific cause(s) for disease or death.

The immune system can be divided into two interacting components—innate and acquired immunity. Innate immunity is the first line of defense against infectious agents or other foreign material and is especially important in cold-blooded organisms such as fishes (Magnadóttir, 2006). It has also been the most studied aspect of fish immunity in terms of environmental stressors (Bols and others, 2001). Bactericidal activity and the respiratory burst of phagocytic cells, primary aspects of innate immunity, are closely related. Bactericidal activity assesses the ability of anterior kidney adherent phagocytes (macrophages, neutrophils) to kill bacteria; respiratory burst measures the phagocytic cells' ability to produce reactive oxygen species (ROS) with and without stimulation.

Reactive oxygen species are essential for the activity of professional phagocytes, which in fishes includes macrophages, neutrophils, dendritic cells, monocytes, and potentially B cells (Rehberger and others, 2017; Biller and Takahashi, 2018). Upon ingestion of bacteria or other microscopic pathogens, phagocytic cells undergo respiratory burst (an increase in oxygen consumption of up to 20 times resting levels) to form potent bactericidal compounds (Slaninova and others, 2009; Biller and Takahashi, 2018). In a healthy organism, antioxidant enzymes produced in the host cell counteract damage caused by ROS (Biller and Takahashi, 2018). An overproduction of ROS, and thus an imbalance between ROS and antioxidant formation, is toxic to cells and leads to oxidative stress (Slaninova and others, 2009; Lushchak, 2016; Biller and Takahashi, 2018). High levels of oxidative stress over time can potentially have a profound effect on disease susceptibility (Tort, 2011). Phagocytosis may ultimately promote activation of the acquired immune response (Biller and Takahashi, 2018), which is measured by the mitogenesis assay.

Acquired immunity refers to learned cell- and humoral-mediated responses capable of memory and building tolerance to specific antigens where the main cells involved are lymphocytes (Zelikoff, 1998). However, lymphocytes in fish can also behave as nonspecific immune cells resembling innate-like lymphocytes in humans (that is, γδT cells, mucosal associated invariant T cells, natural killer T cells, and B1-B cells), potentially performing activities such as phagocytosis, major histocompatibility complex independent direct killing by T-cells, and spontaneous antibody production in B-cells (Scapigliati and others, 2018). Mitogenesis tests the ability of lymphocytes (T- and B-cells) to proliferate in response to specific mitogens, proteins that induce cell division. Mitogens were chosen on the basis of evidence from previous studies that each is specific to either B- or T-cells. In fishes, this has been shown in *Oncorhynchus mykiss* (rainbow trout), where concanavalin A (Con A) and phytohemagglutinin (PHA) were specific to T-cells and lipopolysaccharide (LPS) was specific to B-cells (Boardman and others, 2012). Proliferation of anterior kidney lymphocytes occurring in the absence of mitogen-based induction in the experiment represents the level of background proliferation present at the time the fish were collected and will be referred to here as the untreated background response.

These immune characteristics can be affected by such factors as disease status (Magnadóttir, 2010), pesticides (Dunier and Siwicki, 1993; Slaninova and others, 2009; Lushchak, 2016), polycyclic aromatic hydrocarbons (Faisal and others, 1991; Lushchak, 2016), perfluoroalkyl acids (Lee and others, 2019), reproductive hormones or compounds mimicking their action (Wang and Belosevic, 1994; Harris and Bird, 2000; Szwejser and others, 2016), heavy metals (Zelikoff, 1993; O'Halloran and others, 1998; Lushchak, 2016), and other external factors such as temperature and water quality (Makrinos and Bowden, 2016). To better understand the adverse effects of these multiple environmental stressors on the immune system of smallmouth bass, a suite of functional immune assays for bactericidal activity, respiratory burst activity, and mitogenesis were developed. This suite includes methods that have been previously used in other fish species, most often in laboratory studies. Each assay was optimized using 20 laboratory-held smallmouth bass (N = 60) fed with live minnows to best approximate natural conditions and immune response of wild fish (Ottinger and others, 2019), and then tested with 192 wild smallmouth bass from stream sites with varying land use in Pennsylvania. The optimization samples were used for method development and are not included in this report. Ultimately, understanding the effects of these complex mixtures of environmental stressors, as well as individual risk factors, on fish health will require a comprehensive assessment of immune function, along with identification of infectious agents, parasites, pathology, and physiological and molecular endpoints.

Materials and Methods

Immune Functional Assays

Immune functional assays were completed in the laboratory using isolated anterior kidney leukocytes from wild smallmouth bass. Following isolation and resuspension of leukocytes, cells were plated for three immune cell

functional assays (fig. 1). Bactericidal and respiratory burst were plated simultaneously with cells suspended in adherence medium (L-15 media containing 290 micrograms (µg) per mL^{-1} L-glutamine, 100 units (U) per mL^{-1} penicillin, 100 µg per mL^{-1} streptomycin, and 0.1% fetal bovine serum (FBS); L-15/0.1% penicillin-streptomycin (P/S)). Remaining cells were resuspended in culture medium (L-15 media containing 290 µg per mL^{-1} L-glutamine, 100 units (U) per mL^{-1} penicillin, 100 µg per mL^{-1} streptomycin, and 5-percent FBS; L-15/5% P/S) and plated for mitogenesis assay.

Leukocyte Isolations and Plating

Anterior kidney leukocytes were isolated following a procedure modified from Sharp and others (1991). Cell suspensions were pelleted by centrifugation at 500 relative centrifugal field (RCF) and 4 degrees Celsius (°C) for 10 minutes (min), then washed 3 times by suspension in 10 milliliters (mL) Leibovitz L-15 medium with 2-percent fetal bovine serum (L-15/2%), centrifuging as above between each wash. Following wash steps, cells were resuspended in 6 mL of L-15/2%, passed through a 70-micron (µm) cell strainer to remove clumps or clots, and layered onto 6 mL of 32-percent percoll concentration in Hanks' Balanced Salt solution (Sigma-Aldrich, St. Louis, Mo.) without phenol red in 15 mL centrifuge tubes. Cells on percoll were centrifuged at 500 RCF at 4 °C for 45 min. Leukocytes were removed from the media/percoll interface and pelleted by centrifugation at 500 RCF at 4 °C for 10 min. Isolated leukocytes were resuspended in L-15/2% for counting. Total numbers of viable leukocytes were determined by trypan blue exclusion using the Countess™ II automated cell counter (ThermoFisher, Waltham, Mass.). Countess™ II viable-cell counts were optimized for smallmouth bass leukocytes prior to use in this study using laboratory fish. Final instrument settings were validated using parallel cell counts from a propidium iodide exclusion assay, with data acquisition performed using the Amnis FlowSight® imaging flow cytometer (Luminex Corporation, Austin, Tex.).

Following leukocyte counts, cells were pelleted by centrifugation, resuspended at 2×10^7 cells mL^{-1} in adherence medium and plated at 100 microliter (µL) well^{-1} for the bactericidal and respiratory burst assays or culture medium and plated at 50 µL well^{-1} for the mitogenesis assay. The bactericidal and respiratory burst assays were plated simultaneously. Remaining cells were concentrated by centrifugation (500 RCF at 4 °C for 10 min), and the adherence medium was replaced with culture medium before plating the mitogenesis assay. Isolated leukocytes were plated in 96-well Corning™ Costar™ flat-bottom cell culture plates (Corning Cat. No. 3596; ThermoFisher). Leukocyte incubations for all three assays occurred within a humidified container at 17 °C to provide the closest fit to the water temperatures from which the source fish were obtained. A minimum of 2.5 mL of isolated leukocytes resuspended at 2×10^7 cells per mL^{-1} were required to complete all immune function assays (1.1 mL for bactericidal assay against two different bacteria; 0.6 mL for respiratory burst assay; and 0.8 mL for mitogenesis assay). In some cases when leukocyte counts were low, not all assays were completed.

Bactericidal Activity of Adherent Cells (Phagocytes)

When adequate cell numbers were available to complete all immune function assays, the bactericidal activity against two different bacteria—*Aeromonas veronii* bv. sobria and *Yersinia ruckeri*—were tested. When adequate cell numbers were not available, only bactericidal activity against *A.veronii* bv. sobria was tested. *Aeromonas veronii* bv. sobria has been isolated from affected adult and young-of-year smallmouth bass. *Yersinia ruckeri* is a cold-water pathogen (Kumar and others, 2015) to which smallmouth bass are less likely to be exposed; however, it is a widely distributed gram-negative bacterium that has been previously used in bactericidal assays.

Functional assessment of adherent anterior kidney leukocytes to kill bacteria was determined following a procedure from Harms and others (2000) with modifications (table 1).

Briefly, 2×10^7 viable cells mL^{-1} in AM (L-15/0.1% P/S) were added in quadruplicate to a 96-well tissue culture plate for the bacterial challenge and in triplicate on the same plate for adherent cell enumeration. One row on the same plate contained media-only control wells to measure bacterial growth in the absence of cells. After plating, leukocytes were incubated for 2 hours at 17 °C. After 2 hours, the media were removed from all wells to remove non-adherent cells and replaced with CM (L-15/5% P/S). Cells were cultured for 36 hours in a humidified container to allow activated leukocytes to reach a resting state before adding bacterial cultures.

Following 36-hour incubation, wells were washed to remove antibiotics prior to the addition of bacteria. Forty-eight hour cultures of bacteria grown in tryptic soy broth (TSB) were added to the treatment and cell-free control wells, leaving some wells bacteria free and available for adherent cell enumeration. After bacteria were added, the plate was incubated in a humidified container at 17 °C for 4 hours. After 4 hours, the media were removed from treatment and cell-free control wells (not bacteria-free rows) and replaced with a lysis buffer. Serial dilutions were performed for all treatments and controls, plated onto tryptic soy agar (TSA) plates, and colony-forming units (CFUs) were counted after 24 hours. Cell counts were performed for bacteria-free rows using the Countess™ II automated cell counter (ThermoFisher, Waltham, Mass.). Bactericidal activity was expressed as the percentage CFU reduction corrected for mean adherent cell counts:

$$\%CFU_r = \left[\left(1 - \frac{CFU_{treated}}{CFU_{control}}\right) * 100\right]\left[\frac{1}{\overline{AC}_{\neq 1} * 10^6}\right], \qquad (1)$$

where

$\%CFU_r$ is the percentage reduction in colony forming

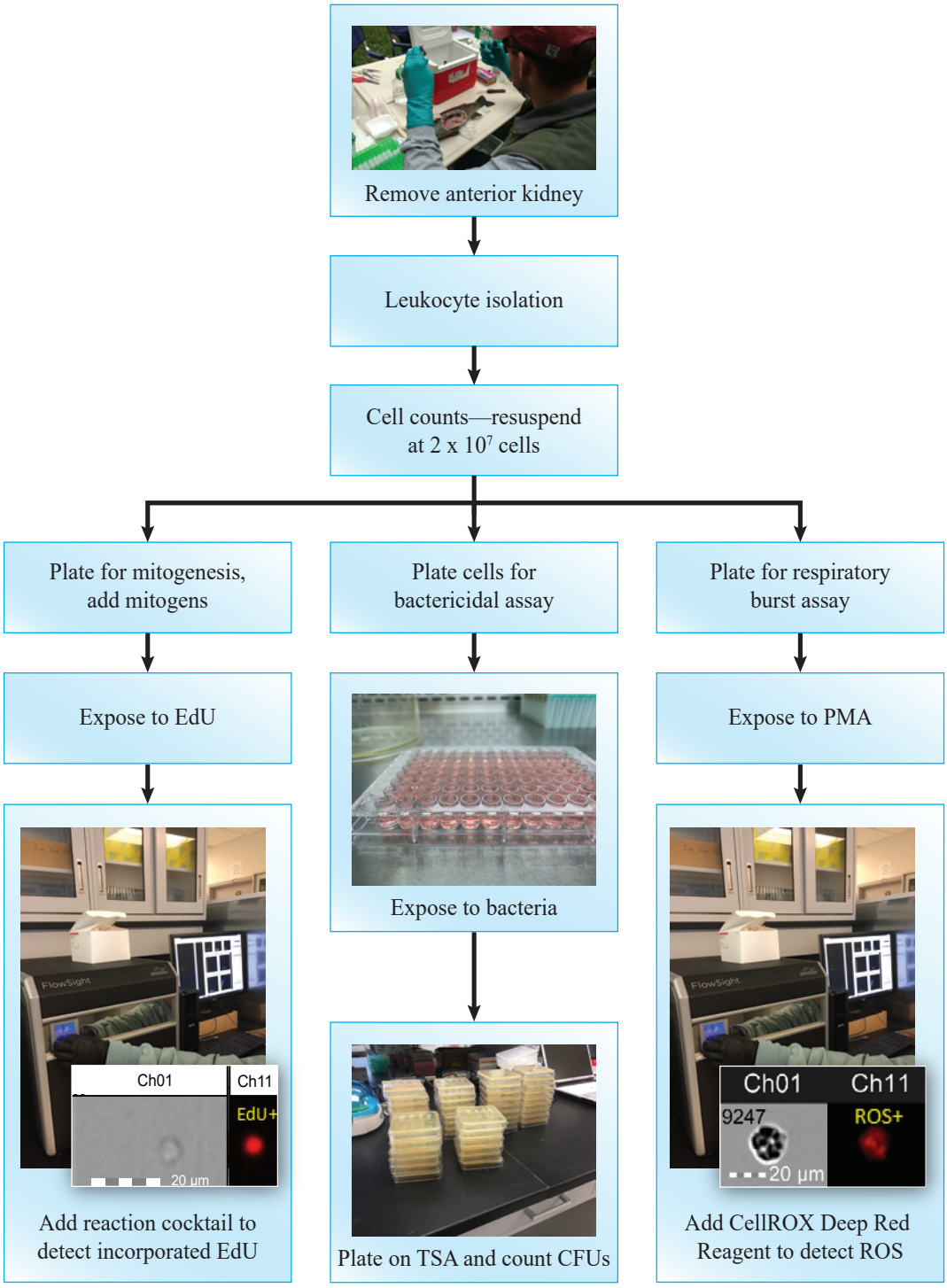

Figure 1. Workflow for immune cell functional assays. Following isolation and resuspension of leukocytes, cells were plated for three immune cell functional assays. Bactericidal and respiratory burst were plated simultaneously with cells suspended in adherence medium (L-15/0.1% P/S). Remaining cells were resuspended in culture medium (L-15/5% P/S) and plated for mitogenesis assay. (EdU, 5-ethynyl-2'-deoxyuridine; TSA, tryptic soy agar; CFUs, colony forming units; PMA, phorbol 12-myristate 13-acetate: ROS, reactive oxygen species; L-15/0.1% P/S, L-15 media containing 290 micrograms (µg) per mL^{-1} L-glutamine, 100 units (U) per mL^{-1} penicillin, 100 µg per mL^{-1} streptomycin, and 0.1% fetal bovine serum (FBS); L-15/5% P/S, L-15 media containing 290 µg per mL^{-1} L-glutamine, 100 units (U) per mL^{-1} penicillin, 100 µg per mL^{-1} streptomycin, and 5-percent FBS)

Table 1. Bactericidal activity protocol.

[HBBS, Hanks' balanced salt solution; P/S, penicillin-streptomycin; %, percent; L-15, Leibovitz L-15 media; OD, optical density; h, hour; min, minute; nm, nanometer; RCF, relative centrifugal field; mL, milliliter; NO P/S, no penicillin-streptomycin]

Task	Step[1]
	Day 1
Plating isolated leukocytes	1. Incubate bacterial cultures in tryptic soy broth (TSB) on shaker for 48 hours at room temperature. 2. Plate 2×10^7 cells ml^{-1} in adherence medium (L-15/0.1% P/S) and one row of media-only control wells. 3. Incubate for 2 hours. 4. Replace adherence medium in all wells with culture medium (L-15/5% P/S). 5. Incubate for 36 hours.
	Day 3
Preparation of bacteria	1. After 48 h, prepare bacteria by centrifuging for 10 min at 2,000 RCF and wash 3 times[2]. 2. Determine the density of bacteria by absorbance at 600 nm on a microplate reader and adjust the stock solution to a working density of 0.15 OD_{600} by diluting the stock solution in HBSS to a final volume of 10 mL.
Final preparation of macrophages and addition of bacteria	3. After 36 h and prior to adding bacteria, wash all wells[3] in 36 h macrophage plates 1x to remove antibiotics, and add L-15/5% NO P/S. 4. Add bacteria to all wells except wells being used for adherent cell counts 5. Incubate for 4 hours.
Macrophage lyses, bacteria dilution and plating on agar	6. Approximately 1 h before the end of 4-h incubation, prepare lysis buffer (0.2% Tween 20), load 180 µL of HBSS into rows A-F of serocluster plates (1 serocluster plate for each row of treated cells or bacteria-only controls), and label one tryptic soy agar (TSA) plate for each fish plus three for bacteria-only controls. 7. At the end of the 4 h incubation, remove media from treated rows and bacteria-only control rows and replace with 50 µL of lysis buffer. 8. Mix well and transfer 20 µL from each row into row A of a corresponding serocluster plate for serial dilutions. 9. Perform serial dilutions by mixing and transferring 20 µL from row A to corresponding wells in row B. 10. Repeat the mixing and transferring process from row B to C, C to D, D to E, and E to F exchanging pipet tips in between. 11. After completing serial dilutions, plate 10 µL drops by column on TSA plates, mixing well contents prior to removal and changing tips between each column. 12. Plate 4 columns of dilutions per TSA plate to correspond to the 4 replicates for each fish and control. 13. Allow drops to air dry with plates partially uncovered, and then store covered plates inverted for 24 hours.
Adherent cell counts	14. Following the plating of bacterial dilutions, count the number of adherent cells in the wells replicated for each fish using the Countess™ automated cell counter (combine 10 µL from wells with 10 µL of trypan blue, mix well, and add 10 µL to chamber slide for analysis).
	Day 4
Count CFUs	1. Count colony forming units (CFUs) and record for each fish and bacteria-only control.

[1]Incubations should occur at species-appropriate temperature in a humidified container, and cells should be washed by pipetting up and down without resuspending the cells to minimize cell loss unless otherwise noted.

[2]Wash buffer: HBSS.

[3]Wash buffer: Unsupplemented L-15.

units by treated cells compared to controls (bacteria only),

$CFU_{treated}$ is the number of colony forming units on agar plates with treated cells,

$CFU_{control}$ is the number of colony forming units on agar plates with bacteria only, and

$AC\bar{x}$ is the mean adherent cell count.

Respiratory Burst Potential of Phagocytes

Respiratory burst was determined via procedures described by Coteur and others (2002) and Iwanowicz and others (2012) with modifications (table 2).

Leukocytes for respiratory burst were plated at the same time as the bactericidal assay so incubation times could run simultaneously. Briefly, isolated leukocytes at 2×10^7 viable cells per mL^{-1} in AM (L-15/0.1% P/S) were added in duplicate to a 96-well tissue culture plate for each treatment, including negative controls. Plates were incubated for 2 hours at 17 °C before media were removed from all wells and replaced with CM (L-15/5% P/S). Cells were then cultured for 36 hours in a humidified container to allow activated leukocytes to reach a resting state. Following 36-hour incubation, wells were washed to remove antibiotics, and plated leukocytes were treated with either phorbol 12-myristate 13-acetate (1 µg/mL PMA in dimethyl sulfoxide; DMSO), DMSO carrier control, or media only (negative control; L-15/5% NO P/S) for 5 minutes at room temperature. After 5 minutes, CellROX® Deep Red Reagent was added at a final concentration of 5 micromolars (µM) to the cells and incubated for 30 minutes at 17 °C, protected from light. After 30 minutes, media were removed, cells were washed three times with Dulbecco's phosphate-buffered saline (DPBS), and then resuspended in 1% bovine serum albumin (BSA) in DPBS before being analyzed for the fluorescent signal. The signal was analyzed on the Amnis FlowSight® imaging flow cytometer (Luminex Corporation, Austin, Tex.) within 2 hours, acquiring at least 10,000 events for each sample. Instrument settings were Illumination, 642 laser at 100.0 millivolts (mV), 785 laser at 5.00 mV; Fluidics, minimum flow speed.

Stimulation index (SI) values were calculated to compare the respiratory burst potential of treated leukocytes to control leukocytes treated with DMSO as a carrier control:

$$SI = \frac{F_t}{F_c} \text{ and} \quad (2)$$

$$F = \left(\frac{count\ of\ positively\ fluoresced\ cells}{count\ of\ total\ gated\ cells\ (single\ and\ focused)}\right)*100 \quad (3)$$

where

SI is the stimulation index,

F_t is the percentage of positive cells in the treated replicate,

F_c is the percentage of positive cells in the DMSO-stimulated carrier control replicate, and

F is the percentage of positive cells in the total population.

Table 2. Respiratory burst protocol.

[mL, milliliter; L-15/0.1%; P/S, penicillin-streptomycin; h, hour; µg, microgram; DMSO; µM, micromolar; min, minute; °C, degrees Celsius; BSA, bovine serum albumin; DPBS, Dulbecco's phosphate buffered saline; PMA, phorbol 12-myristate 13-acetate]

Task	Step[1]
	Day 1
Plating isolated leukocytes	1. Plate 2×10^7 cells mL^{-1} in adherence medium (L-15/0.1% P/S) in duplicate for each treatment.
	2. Incubate for 2 hours.
	3. Replace adherence medium in all wells with culture medium (L-15/5% P/S).
	4. Incubate for 36 hours.
	Day 3
CellROX® reagent staining and detection	1. After 36 h and prior to adding stimulant, wash all wells[2] in 36 h plates 1× to remove antibiotics, and add L-15/5% NO P/S.
	2. Add PMA (1 µg mL^{-1}), DMSO carrier control or media only (L-15/5% NO P/S) to corresponding wells.
	3. Incubate for 5 minutes.
	4. Add the CellROX® Reagent (5 µM) and incubate for 30 min at 17 °C in the dark.
	5. Remove medium, wash 3 times[3], and resuspend in 1% BSA in DPBS.
	6. Analyze on a FlowSight using 40× magnification within 2 hours.

[1]Incubations should occur at species-appropriate temperature in a humidified container, samples should be protected from light throughout protocol following addition of CellROX® Reagent, and cells should be washed by pipetting up and down without resuspending the cells to minimize cell loss unless otherwise noted.

[2]Wash buffer: Unsupplemented L-15.

[3]Wash buffer: DPBS.

The percentage of positive leukocytes in the total population from negative control samples provided a measure of background reactive oxygen species. SI values were calculated using data from the pooled replicate of leukocytes (two replicates per treatment for each fish) from the same fish.

Mitogenesis Assay

Mitogenesis was evaluated using mitogens obtained from Millipore Sigma (Burlington, Mass.) and following the bromo-deoxyuridine (BrdU) based enzyme-linked immunosorbent assay (ELISA) procedure from Gauthier and others (2003) with modifications. Ethynyl-deoxyuridine (EdU) was used instead of BrdU, and the Click-it® EdU (5-ethynyl-2'-deoxyuridine) Flow Cytometry Assay Kit (Molecular Probes, Eugene, Ore.) was used as an alternative for labeling and detection (table 3).

Plated leukocytes were treated at 50 µL well^{-1} with either a mitogen or mitogen-free media (negative control wells). Mitogens used were concanavalin A (Con A) at 10 µg per mL^{-1} (0.25 µg well^{-1} final concentration), phytohemagglutinin from *Phaseolus vulgaris* (PHA-P) at 20 µg per mL^{-1} (0.5 µg well^{-1} final concentration), and lipopolysaccharide from *Escherichia coli* (*E. coli*) O111:B4 (LPS) at 100 µg per mL^{-1} (2.5 µg well^{-1} final concentration). Working solutions of mitogens were prepared in L-15/5%. Mitogen treated and control wells were duplicated in the same plate. Plates were incubated for 24 hours before adding EdU. Following incubation with the mitogens, 25 µL well^{-1} of EdU in unsupplemented L-15 was added (EdU final concentration = 2.6 µM well^{-1}) to all wells. Following an additional 18-hour incubation, incorporation of EdU/cell replication was detected by Alexa Fluor 647 (AF647) using the Click-iT® EdU (5-ethynyl-2'-deoxyuridine) Flow Cytometry Assay Kit (Molecular Probes, Eugene, Ore.), following the manufacturers protocol (MP 10419) for fixation, permeabilization, and labeling with volume adjustments; 100 µL well^{-1} of wash permeabilization reagent, 25 µL well^{-1} of fixative, and 100 µL well^{-1} of reaction cocktail were used.

After labeling leukocytes with Alexa Fluor 647 (AF647), the cells were washed and resuspended in 200 µL of Click-iT® saponin-based permeabilization and wash reagent in

Table 3. Mitogenesis protocol.

[EdU, 5-ethynyl-2'-deoxyuridine; µg, microgram; mL, milliliter; Con A, concanavalin A; PHA-P, phytohemagglutinin from *Phaseolus vulgaris*; L-15, Leibovitz L-15 media; %, percent; FBS, fetal bovine serum; h, hour; µM, micromolar; DNA, deoxyribonucleic acid; ºC, degrees Celsius; DPBS, Dulbecco's phosphate buffered saline]

Task	Step[1]
	Day 1
Assay setup	1. Prepare working concentrations of mitogens (100 µg mL^{-1} LPS, 10 µg mL^{-1} Con A, 20 µg mL^{-1} PHA-P) in L-15 media with 5% FBS 2. Plate 2×10^{7} cells ml^{-1} in L-15 media with 5% FBS and add working concentrations of mitogens or L-15 media with 5% FBS (no mitogen, non-treated cells) 3. Incubate overnight (24 h) to allow mitogens time to stimulate the cells
	Day 2
Add EdU	1. After 24 h, add diluted EdU in unsupplemented L-15 to plates at 13 µM final concentration and incubate for an additional 18 h to allow incorporation of EdU into newly synthesized DNA
	Day 3
Click-iT® reaction	1. Wash cells[2] and remove supernatant without dislodging adherent cells/pelleting the cells
Fix and permeabilize cells	2. Fix and permeabilize the cells based on the manufacturer's instructions for the Click-iT® EdU Flow Cytometry Assay Kit (Molecular Probes) for 15 minutes at room temperature 3. Wash cells[2], remove supernatant (being careful not to dislodge pellet) and add 1X Click-iT® saponin-based permeabilization/wash reagent (resuspend cells and mix well only if storing plate for later) 4. At this point, fixed cells can be stored at 4 °C to finish the assay later (up to 1 week was investigated without signal loss) or proceed directly to click labeling
Detection of incorporated EdU	5. Perform click labeling following manufacturer's instructions for 30 minutes at room temperature 6. Wash cells[3] and remove supernatant (do not dislodge pellet) then resuspend (dislodge pellet and mix well) in 1X Click-iT® saponin-based permeabilization/wash reagent
Analyze on FlowSight	7. Analyze on a FlowSight using 40x magnification immediately or store at 4 °C for up to 1 week

[1]Incubations should occur at species-appropriate temperature in a humidified container, samples should be protected from light throughout protocol following addition of EdU, and cells should be washed by pipetting up and down without resuspending the cells to minimize cell loss unless otherwise noted.

[2]Wash buffer: 1% BSA in DPBS.

[3]Wash buffer: 1X Click-iT® saponin-based permeabilization/wash reagent.

preparation for analysis using the Amnis FlowSight® imaging flow cytometer (Luminex Corporation, Austin, Tex.) or the SpectraMax M4 microplate reader (Molecular Devices, San Jose, Calif.) for method comparison. Cells from replicate wells (two wells per treatment for each fish) were pooled prior to data acquisition using the Amnis FlowSight®, and at least 10,000 events were acquired for each sample. Instrument settings were Illumination, 642 laser at 100.0 mV, 785 laser at 5.00 mV; Fluidics, minimum flow speed. Cell analysis was performed using Image Data Exploration and Analysis Software (IDEAS® 6.2; Amnis/Luminex Corporation, Austin, Tex.).

IDEAS® Gating Strategy for Data Analysis

Primary gating of imaging flow cytometry data collected from 10,000 events isolated individual round cells that were in good focus by brightfield (fig. 2). Gating was optimized using data from laboratory and wild smallmouth bass.

The population of round cells in focus is labeled R1 (fig. 2A). Doublet discrimination resulted in a population (R2) of single cells in focus (fig. 2B). Masking of brightfield images was performed using the adaptive erode function with 89 erosion coefficient to generate custom masks to increase the accuracy of size specific analysis (fig. 2C). Secondary gating of masked images divided the R2 population into three cell populations (R3, R4, and R5) on the basis of cell diameter and side scatter, as a measure of cell density (fig. 2D), and positive staining for AF647/EdU was based on fluorescence intensity. The threshold for AF647 detection was set at an intensity of 100 using visual inspection of the histograms and cell images (fig. 2E). Following initial analysis, a template was created containing the primary and secondary gating. This template was used to analyze all datasets to provide consistent analytics across samples.

Field Methods

Wild fish sampling was done in conjunction with ongoing fish health assessment and monitoring studies at four sites in Pennsylvania: Upper Juniata River (latitude 40.56082, longitude 78.0694), Pine Creek (41.282964, 77.321489), and West Branch Mahantango Creek (40.6478, 76.94296) within the Susquehanna River Basin and Tionesta Lake (41.4753121, 79.438331) in the Ohio River Basin (fig. 3). Attempts were made to collect 20 adult smallmouth bass from each site by boat electrofishing in spring and fall 2016 and 2017. Captured fish were placed immediately in a live well and transported to shore to be necropsied on site following the protocol by Blazer and others (2018).

Fish were euthanized, according to the Leetown Science Center's Institutional Animal Care and Use Committee guidelines, with a lethal dose (250 mg/mL) of tricaine methanesulfonate (MS-222; Western Chemical Laboratories, Ferndale, W.V.). The fish were measured (nearest millimeter), weighed (nearest gram) and bled from the caudal vessels. Fulton's condition factors were determined for individual fish using the equation $K = W/L^3$, where K = condition factor, W = weight of the fish, and L = length of the fish (Nash and others, 2006). Any visible abnormalities, external and internal, were recorded. The anterior kidney was aseptically excised and placed in 10 mL of cold L-15 media supplemented with 290 µg per mL-1 L-Glutamine, 10 units mL^{-1} sodium heparin, 100 units mL^{-1} penicillin, 100 µg per mL^{-1} streptomycin, and 2 percent FBS (characterized, U.S. origin; Hyclone, GE Life Sciences). Supplemental media were designated subsequently as L-15/2%. Anterior kidney tissues in L-15/2% were homogenized into a single cell suspension using a sterile, manual Tenbroeck tissue grinder. The cell suspension for each fish was returned to the original sample tube and kept on ice. Samples collected in the field were transported to the lab for next day processing and kept at 4 °C overnight prior to leukocyte isolation (12–16 hours holding time). Unless otherwise stated, reagents were obtained from Sigma Aldrich (St. Louis, Mo.).

Following aseptic removal of the anterior kidney, a necropsy was performed; tissues were removed and fixed in Z-fix™ (Anatech LTD, Battle Creek, MI) for histopathological analyses and RNALater™ (ThermoFisher, Waltham, Mass.) for gene expression analyses. Otoliths were removed for aging. Tissues for histopathology were routinely processed, embedded into paraffin, sectioned at 5 µm and stained with hematoxylin and eosin (Luna, 1992). Overall parasite intensity was rated on a scale of 0 to 4 for the sections of spleen and liver, and when possible the type of parasite was identified. The number of parasites associated with each rating was 0 = 0 parasites; 1 = 1–4 parasites, 2 = 5–9 parasites, 3 = 10–14 parasites, and 4 = 15+ parasites for spleen tissue and 0 = 0 parasites, 1 = 1–19 parasites, 2 = 20–39 parasites, 3 = 40–59 parasites, and 4 = 60+ parasites for liver tissue.

Statistical Analyses

Statistics were calculated using R 3.5.3 (R Core Team, 2019), *ggplot2* (Wickham and others, 2016), *ggpubr* (Kassambara, 2018), *cowplot* (Wilke, 2019), *ggpmisc* (Aphalo, 2016), and *plyr* (Wickham, 2011) packages. Individual fish comparisons among seasons at each site were made using unpaired Wilcoxon rank-sum tests. The Kruskal-Wallis test was used for multiple comparisons. If significant, post hoc analysis was performed using the Dunn test (Dunn, 1964) for groups with unequal numbers of observations, adjusted with the Benjamini-Hochberg method. An α-level of 0.05 was used to indicate significant results.

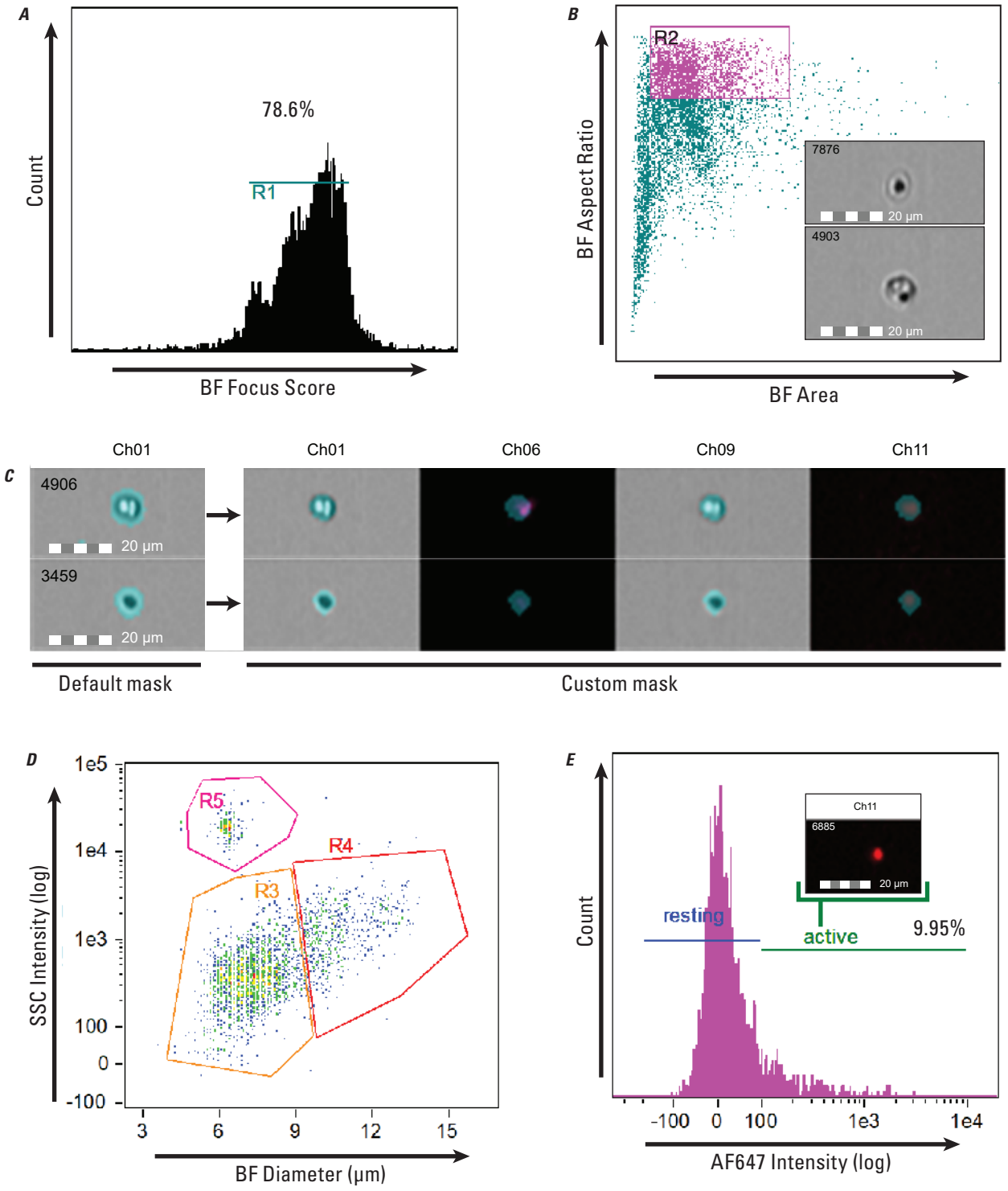

Figure 2. Image-based flow cytometry gating strategy for IDEAS software. A, Primary gating of cells in focus (R1). B, Doublet discrimination identifies single cells (R2). C, Representative images of smallmouth bass leukocytes with masks (cyan) overlaid; default cell mask (Ch01) generated by IDEAS software versus custom mask (Ch01, Ch06, Ch09, Ch11) generated to increase the accuracy of cell diameter calculations. D, Secondary gating of cell populations (R3, R4, R5) present based on a density scatterplot of intensity versus cell diameter. E, Percentage of replicating immune cells (for example, positive for AF647/EdU staining) of the total gated population (R2). (µm, micron; BF, brightfield; SSC, side scatter; AF647/EdU, Alexa Fluor 647 or 5-ethynyl-2'-deoxyuridine; %, percent)

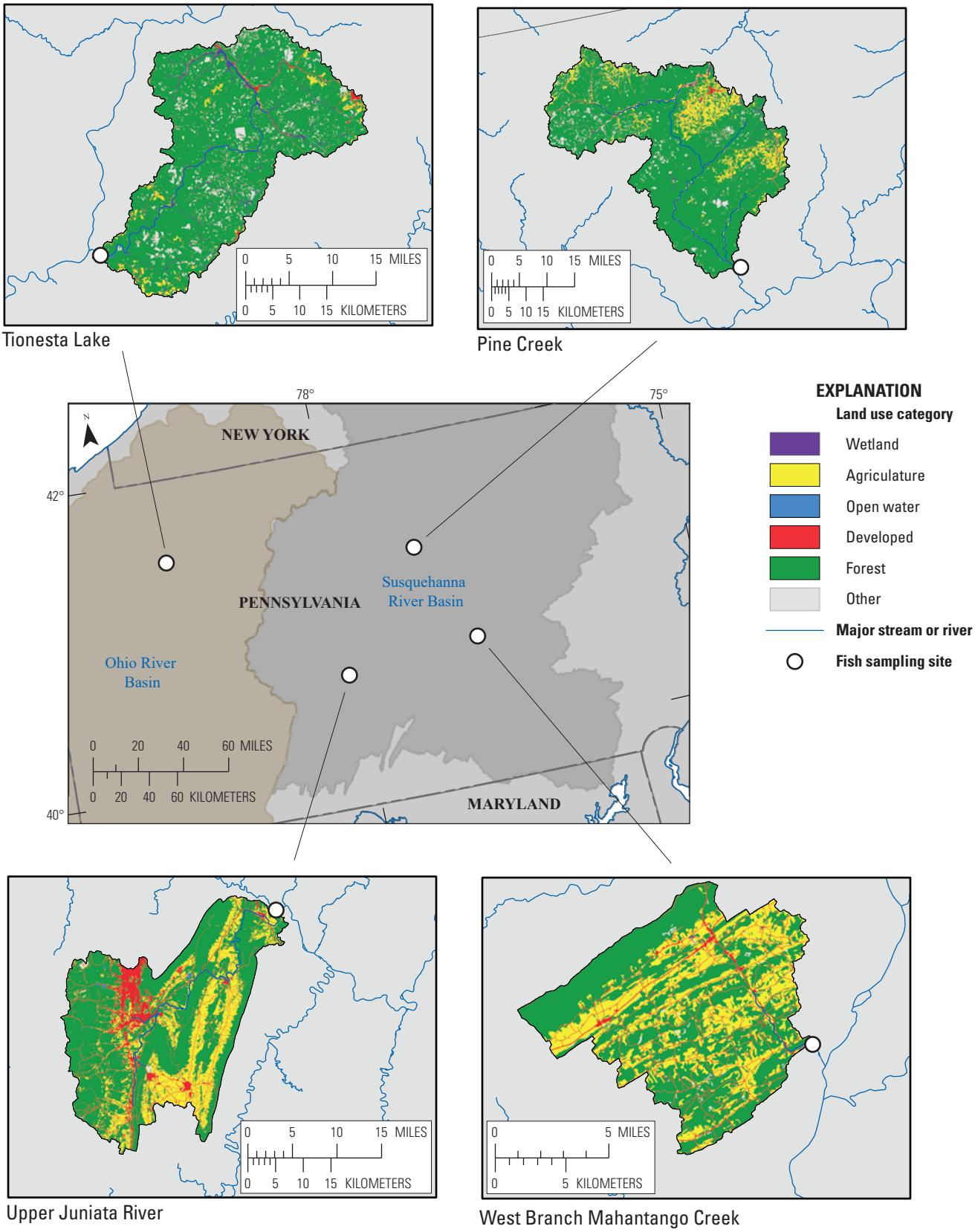

Figure 3. Fish collection sites at Tionesta Lake, Pine Creek, Upper Juniata River, and West Branch Mahantango Creek, Pennsylvania, with major land uses.

Biometric Data and Immune Function Results

Biometric Data

In spring and fall 2016, a total of 112 smallmouth bass were collected, age 2 and older (range = 2–13) from four sites—Tionesta Lake (n = 12, 2 females and 10 males), Pine Creek (n = 40, 17 females and 23 males), Upper Juniata River (n = 20, 5 females and 15 males) and West Branch Mahantango Creek (n = 40, 19 females and 21 males). In the spring and fall 2017, a total of 80 smallmouth bass were collected, age 2 and older (range = 2–12), from two sites—Pine Creek (n = 40, 23 females and 17 males) and West Branch Mahantango Creek (n = 40, 18 females and 22 males). The mean age was 4.6 ± 2.1 years in 2016 and 3.9 ± 2.0 years in 2017 and was not significantly different among sites (fig. 4).

Immune Function

Comparisons among all sampling sites could be made only for spring 2016 when all four sites were sampled and only for the mitogenesis assay. Tionesta Lake and Upper Juniata River were sampled only in spring 2016; no data are available on bactericidal or respiratory burst activity for these two sites owing to optimization of the assays.

Seasonal Differences in Bactericidal Activity

In 2016, median bactericidal activity against *A.veronii* bv. sobria was significantly elevated at West Branch Mahantango during fall when compared to spring (fig. 5A; $p < 0.001$). In fact, the median bactericidal activity against *A.veronii* bv. sobria was 0 percent during spring at West Branch Mahantango and during both seasons at Pine Creek in 2016. In 2017, seasonal differences were not significant at either

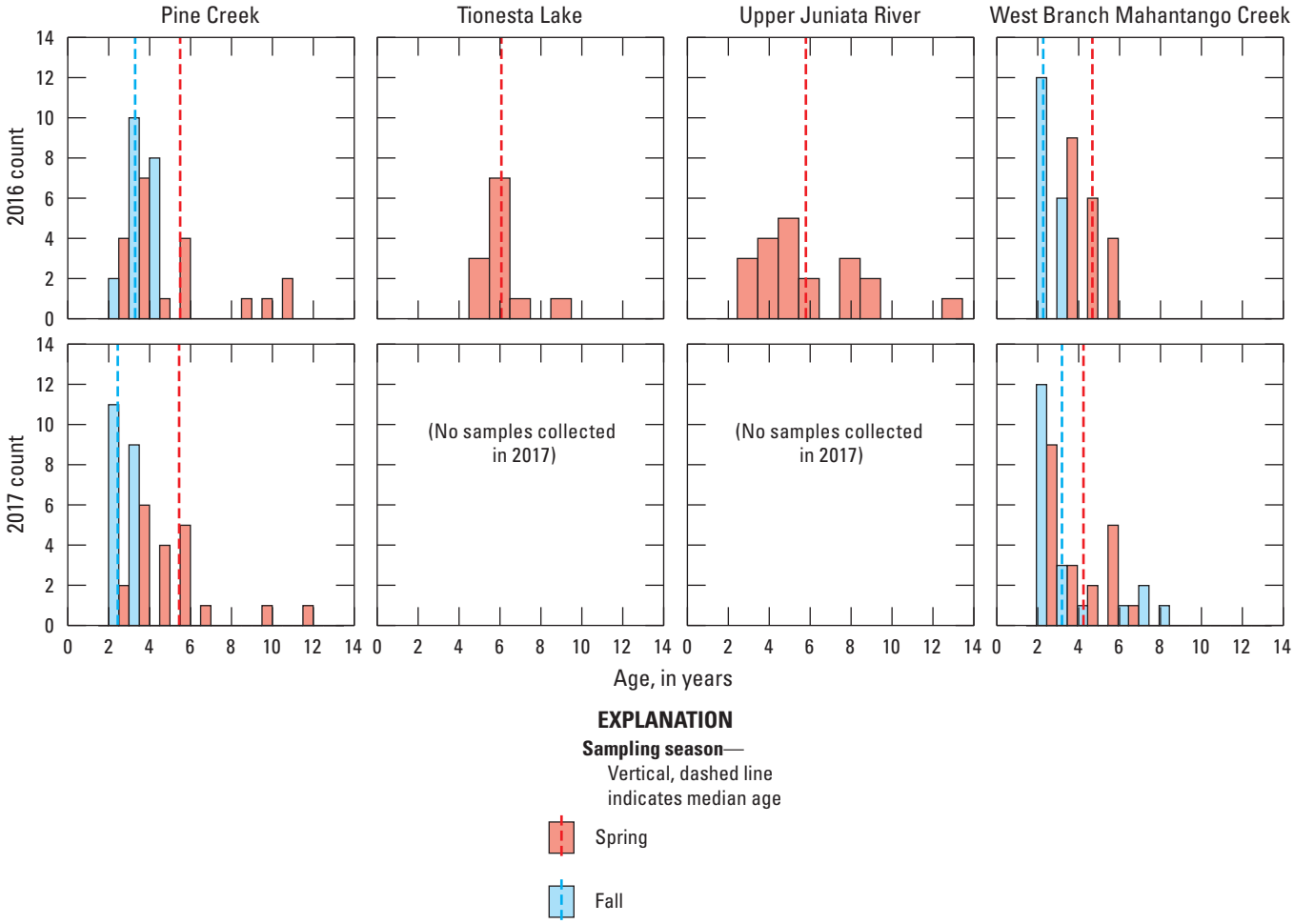

Figure 4. Age distribution of smallmouth bass collected from Pine Creek, Tionesta Lake, Upper Juniata River, and West Branch Mahantango Creek, Pennsylvania, 2016–17. Top row, age of the 112 smallmouth bass collected in spring and fall 2016; bottom row, age of the 80 smallmouth bass collected in spring and fall 2017. Vertical dashed lines indicate mean age for each season.

site; however, median bactericidal activity against *A.veronii* bv. sobria was elevated in the fall at Pine Creek, the mostly forested site, but during spring at West Branch Mahantango Creek, the more agricultural site (fig. 5A).

No seasonal comparisons were made for bactericidal activity against *Y. ruckeri* in 2016 because the bacterium was not added to the assay until fall 2016. The only seasonal comparison within the same sampling year that could be made for bactericidal activity against *Y. ruckeri* was for fish collected at West Branch Mahantango Creek in 2017 where, although not significant, bactericidal activity was greater in the spring (fig. 5B). Bactericidal activity at Pine Creek against *Y. ruckeri* was not tested during fall 2017 owing to low cell yields. Bactericidal activity against *A. veronii* bv. sobria, a known pathogen of smallmouth bass, was prioritized in cases of low cell yields.

Seasonal Differences in Reactive Oxygen Species and Respiratory Burst Potential

No seasonal comparisons were made for 2016 because the respiratory burst assay was completed only in fall of that year owing to optimization of the assay. No data on respiratory burst for Tionesta Lake or Upper Juniata River were available because those sites were sampled only in spring 2016 when the respiratory burst assay was being optimized. In 2017, background levels of reactive oxygen species (ROS) were higher in fall compared to spring (fig. 6A) at Pine Creek ($p < 0.001$) and West Branch Mahantango Creek ($p < 0.05$). No, or exceptionally low, respiratory burst activity occurred in leukocytes from fish collected during fall after being stimulated with PMA (fig. 6B). Respiratory burst was greater during spring at Pine Creek and West Branch Mahantango Creek in 2017, although only significantly greater at Pine Creek (fig. 6B; $p < 0.001$).

Mitogenesis

Untreated background proliferation of anterior kidney lymphocytes differed seasonally with 2016 and 2017 data pooled. Background mitogenesis was significantly elevated at Pine Creek in spring when compared to fall (fig. 7; $p < 0.001$). The opposite was true for West Branch Mahantango Creek, although it was not significant (fig. 7). Seasonal comparisons could not be made for Tionesta Lake or Upper Juniata River.

Leukocytes were considered stimulated by mitogens if the stimulation index (SI) value was greater than or equal to 1.25, meaning the treated leukocytes proliferated at least 1.25 times more than untreated leukocytes. A cutoff of 1.25 was used because it allows room for errors with the data collection

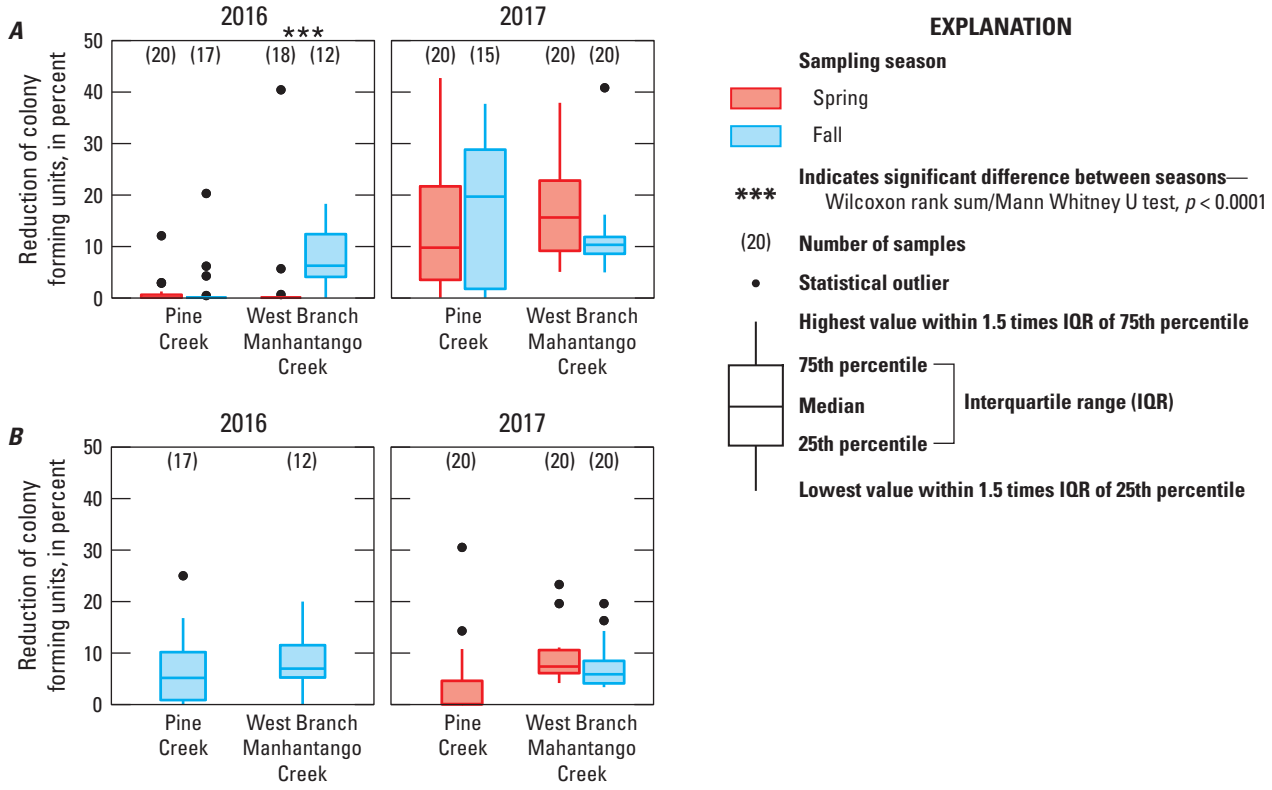

Figure 5. Bactericidal activity against A, *Aeromonas veronii* biovar (bv.) sobria and B, *Yersinia ruckeri* in spring and fall 2016 and 2017. Median (line in box), interquartile range (box), and statistical outliers (black dots) are provided. (*** indicates significant differences between seasons [Wilcoxon rank sum/Mann Whitney U test, $p < 0.001$])

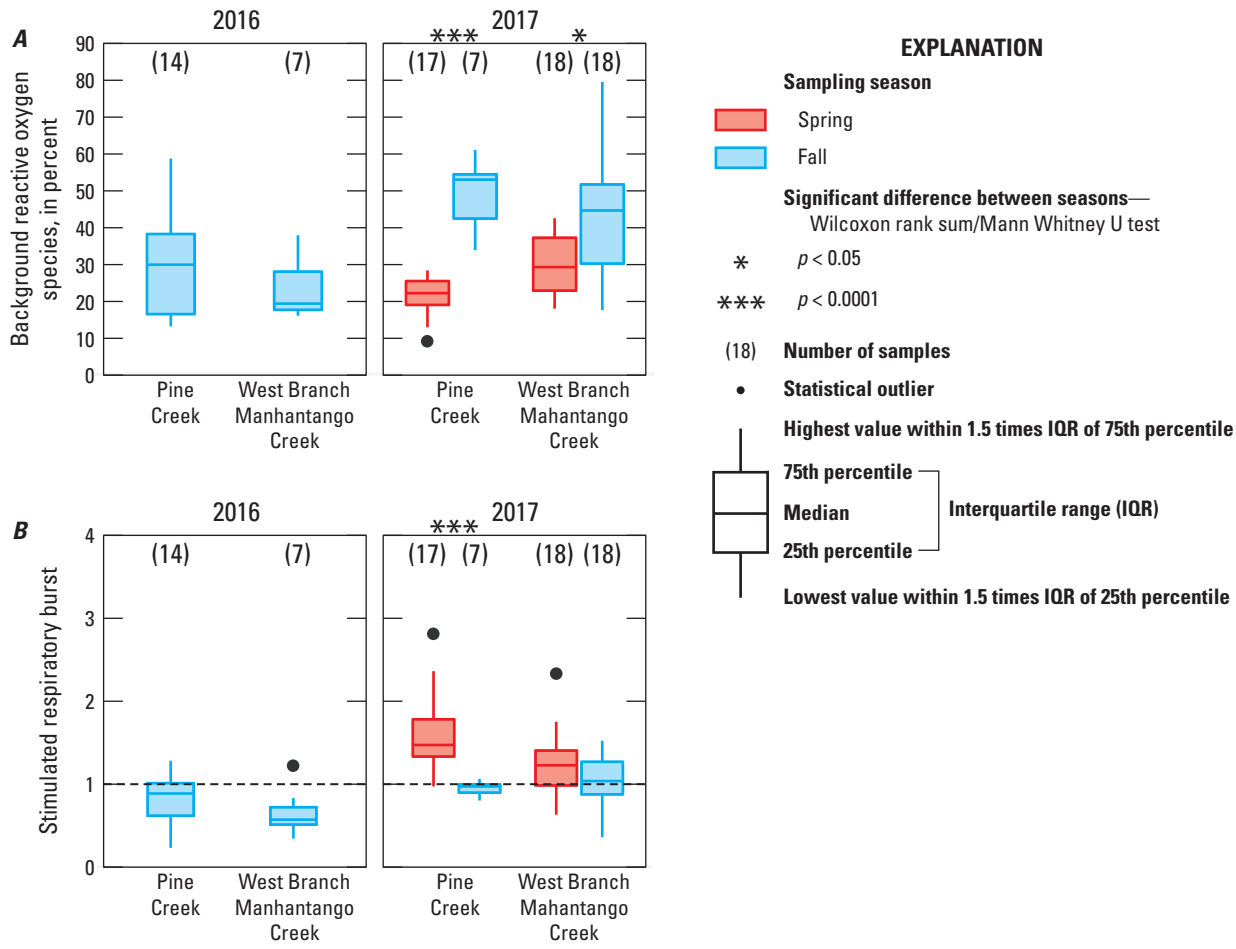

Figure 6. Production of reactive oxygen species. A, untreated leukocytes and B, leukocytes treated with phorbol 12-myristate 13-acetate (PMA). The dashed line at 1 represents the point where stimulated ROS production becomes greater than unstimulated/background ROS production. Median (line in box), interquartile range (box), and statistical outliers (black dots) are provided. (ROS, reactive oxygen species; %, percent; WB, West Branch. Asterisks indicate significant differences between seasons [Wilcoxon rank sum/Mann Whitney U test, * = p < 0.05; ** = p < 0.01; *** = p < 0.001])

and makes the analysis more robust. Individual fish that were stimulated by mitogens (SI ≥ 1.25) had lower levels of background proliferation than individuals that were not stimulated (SI < 1.25). At all sites, stimulated mitogenesis dropped below 1.25 as untreated background proliferation increased (fig. 8A-C).

The number of fish that were stimulated by mitogens (SI ≥ 1.25) was dependent upon site, season, and year. In spring 2016, more fish were not stimulated (SI < 1.25) than stimulated (SI ≥ 1.25) at all sites and for all mitogens (fig. 9A-C). In fall 2016, Con A was the only mitogen with more fish stimulated than not stimulated; this was true at two sites, Pine Creek and West Branch Mahantango Creek (fig. 9A). In spring 2017, no fish at Pine Creek were stimulated by PHA-P or LPS, and only five fish were stimulated by Con A. For West Branch Mahantango Creek in spring 2017, more fish were not stimulated than stimulated by all mitogens. In fall 2017 at Pine Creek, more fish were stimulated than not stimulated for mitogens when data from both seasons were collected (PHA-P and LPS), and at West Branch Mahantango Creek, the number of fish stimulated by Con A or LPS was equal to the number of fish which were not stimulated by either of those mitogens and more fish were not stimulated by PHA-P than stimulated.

Mean age varied among individual fish that were stimulated by mitogens and individuals that were not (table 4). During fall, stimulated individuals were younger than individuals not stimulated, except for PHA-P stimulated mitogenesis at West Branch Mahantango Creek where the average ages were the same for both groups. During spring, the relation between age and mitogenesis was not consistent. At some sites, stimulated individuals were younger, and at other sites they were older, than individuals that were not stimulated. Age also varied by mitogen. Correlations between age and mitogenesis were beyond the scope of this analysis, but the relation is worth investigating in a more robust statistical analysis.

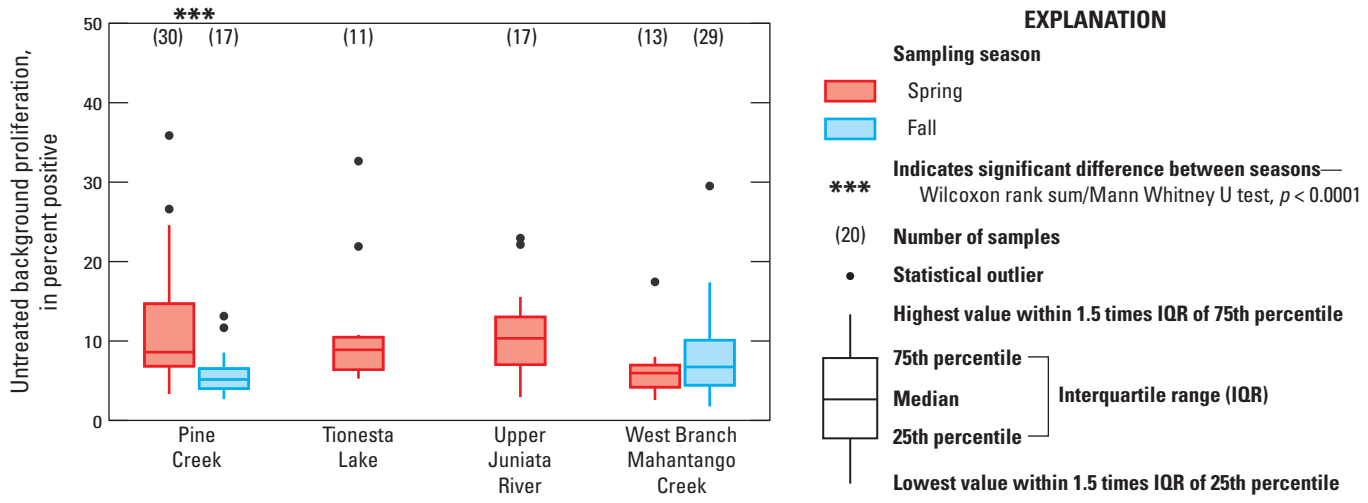

Figure 7. Untreated background lymphocyte proliferation grouped by season for all sites, 2016–17. Median (line in box), interquartile range (box), and statistical outliers (black dots) are provided. (Asterisks indicate significant differences between seasons [Wilcoxon rank sum/Mann Whitney U test, *** = p < 0.001])

Factors Potentially Affecting Immune Response

Microscopic Observations

Wild fish were infected by parasites and contained pathological lesions in spleen, liver, kidney, and other tissues. Microscopic lesions varied among individuals at a site and among sites and seasons. Some individuals had very few encysted areas containing killed and degenerating parasites (fig. 10A), whereas others had more intense infections with live and dead encysted parasites (fig. 10B). At some sites only trematode parasites were observed, whereas at other sites migrating cestodes (fig. 10C), encysted nematodes (fig. 10D), and myxozoan parasites were observed. At the Upper Juniata River site, smallmouth bass had focal granulomas (chronic inflammation) in the spleen and sometimes in the liver consistent with Mycobacterial infections (fig. 10E).

Parasite intensity varied among sites and between seasons. Pine Creek, the more forested site with low chemical contaminant concentrations had consistently higher parasite ratings than West Branch Mahantango Creek, except in fall 2017 when ratings were similar (table 5). The opposite trend was observed for Tionesta Lake and Upper Juniata River; Tionesta Lake, the more forested site, had consistently lower parasite ratings than Upper Juniata River for spring 2016 when both sites were sampled (table 5). Unfortunately, chemical contaminant concentrations were not determined for these two sites.

Chemicals in Surface Water

Wild fish were potentially exposed to mixtures of organic compounds measured in surface water. Pine Creek and West Branch Mahantango Creek were part of a monitoring program, and surface-water grab samples were analyzed for a variety of organic compounds, including pesticides, phytoestrogens, hormones, pharmaceuticals, wastewater indicators, and mycotoxins bimonthly in late spring/early summer (April–June) or monthly the rest of the year (July–March) from 2012 to 2017 (Williams and others, 2019). On average, water samples were collected 0 to 6 days before fish sampling occurred except in fall 2016, when water samples at Pine Creek and West Branch Mahantango Creek were collected 34 and 35 days before fish sampling, respectively.

More organic compounds and higher concentrations of mixtures were measured at West Branch Mahantango Creek than at Pine Creek (fig. 11). Herbicides (atrazine, metolachlor) and a fungicide (boscalid) were the only compounds detected at Pine Creek and only during spring 2016, whereas West Branch Mahantango Creek also had endocrine disrupting phytoestrogens (daidzein, equol, formononetin, genistein) and phenolic compounds (p-cresol). In 2016, higher concentrations of many chemicals were detected in spring than fall.

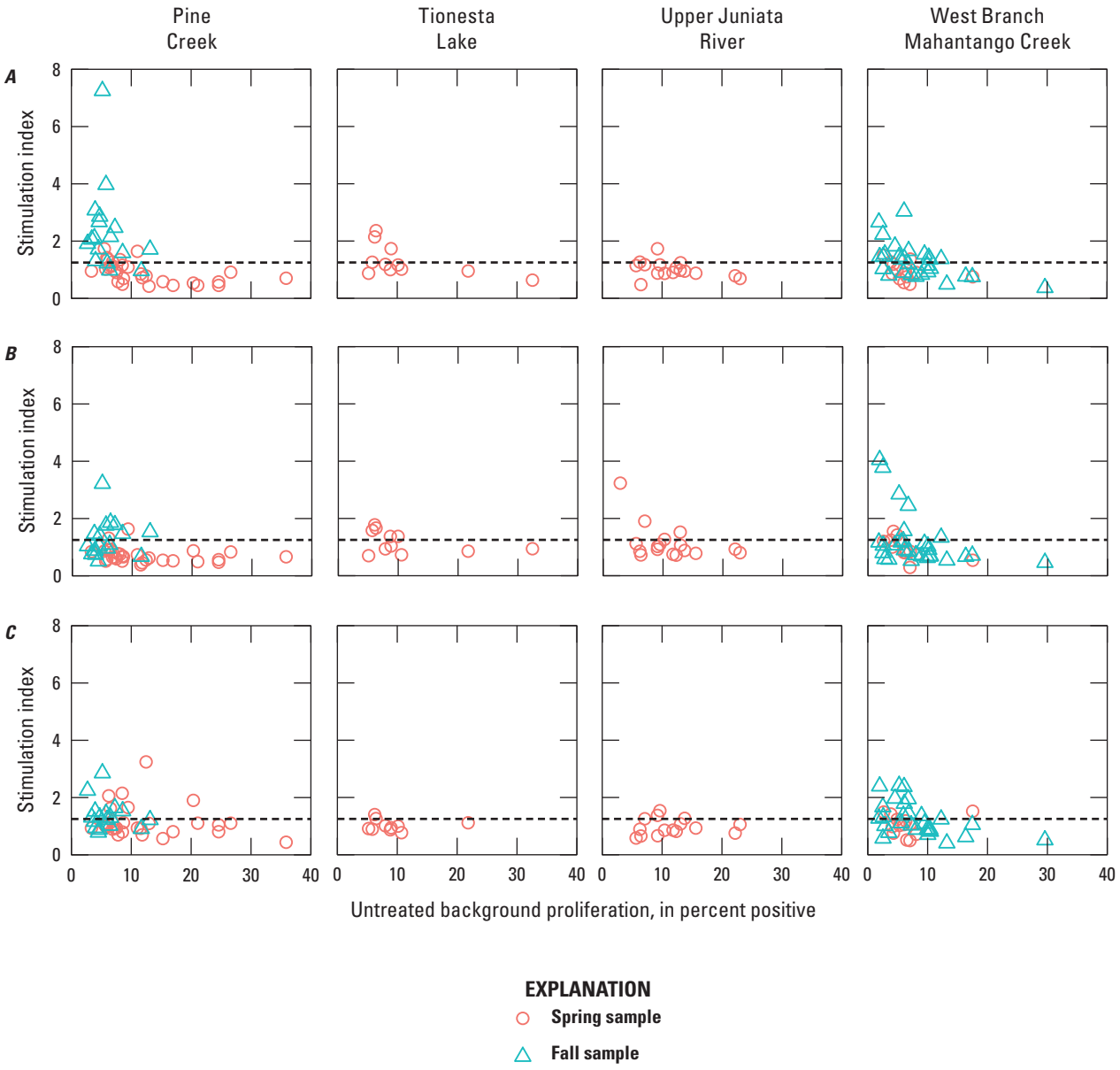

Figure 8. Lymphocyte stimulation by mitogens. A, Concanavalin A, B, Phytohemagglutinin from *Phaseolus vulgaris*, and C, Lipopolysaccharide versus untreated background proliferation of lymphocytes for all sampling sites. The dashed line at 1.25 represents the point where stimulated mitogenesis becomes greater than untreated background proliferation. Seasons are differentiated by shape and color.

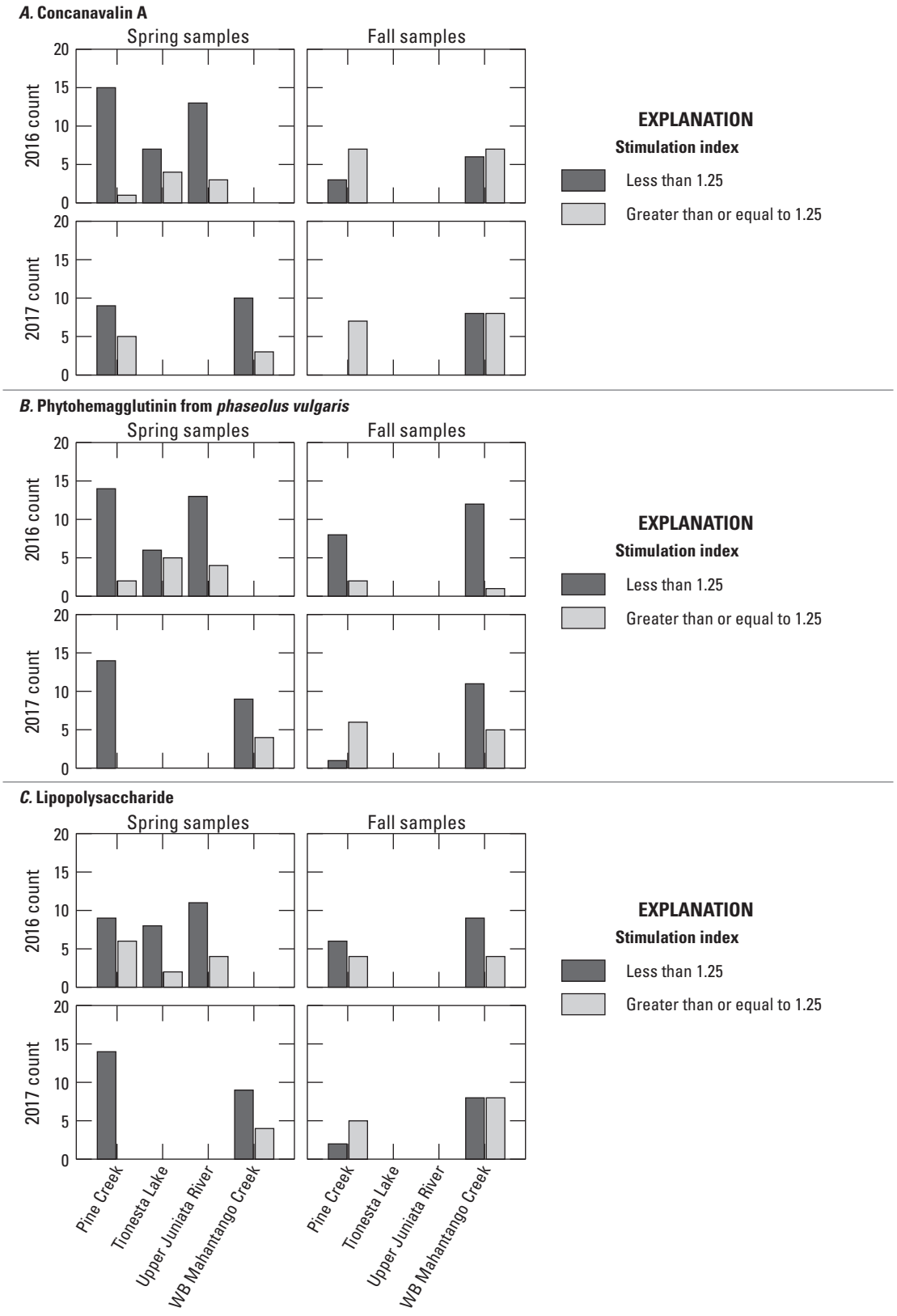

Figure 9. Number of fish from each site that were stimulated (simulation index greater than or equal to 1.25) versus not stimulated (simulation index less than 1.25). A, Concanavalin A, B, Phytohemagglutinin-P, and C, Lipopolysaccharide.

Table 4. Mean (± standard deviation) age of mitogen-stimulated and non-stimulated individual fish. The number of fish with a stimulation index greater than 1.25 of the total sample size and the average age of each group (stimulated or not) are listed by site, season, and mitogen.

[SI, simulation index; ≥, greater than or equal to; WB, West Branch; Con A, concanavalin A; PHA-P, phytohemagglutinin from *Phaseolus vulgaris*; LPS, lipopolysaccharide]

Mitogen	Season	Site	Sample size	SI≥1.25	Mean age stimulated	Mean age not stimulated
Con A	Spring	Pine Creek	30	6	4.2 ± 0.8	6.0 ± 2.5
		Tionesta Lake	11	4	6.5 ± 1.7	5.9 ± 0.7
		Upper Juniata River	16	3	7.7 ± 1.5	4.6 ± 1.6
		WB Mahantango Creek	13	3	3.3 ± 0.6	4.7 ± 1.4
	Fall	Pine Creek	17	14	2.9 ± 0.7	3.7 ± 0.6
		WB Mahantango Creek	29	15	2.7 ± 1.1	3.4 ± 2.2
PHA-P	Spring	Pine Creek	30	2	7.0 ± 5.7	5.5 ± 2.1
		Tionesta Lake	11	5	6.4 ± 1.5	5.8 ± 0.8
		Upper Juniata River	17	4	4.8 ± 2.4	5.5 ± 2.0
		WB Mahantango Creek	13	4	5.5 ± 1.0	3.9 ± 1.3
	Fall	Pine Creek	17	8	2.9 ± 0.8	3.2 ± 0.7
		WB Mahantango Creek	29	6	3.0 ± 1.7	3.0 ± 1.8
LPS	Spring	Pine Creek	29	6	4.7 ± 3.1	5.9 ± 2.1
		Tionesta Lake	10	2	5.5 ± 0.7	6.3 ± 1.3
		Upper Juniata River	15	4	6.5 ± 3.0	4.9 ± 1.4
		WB Mahantango Creek	13	4	4.3 ± 1.3	4.4 ± 1.5
	Fall	Pine Creek	17	9	2.9 ± 0.8	3.3 ± 0.7
		WB Mahantango Creek	29	12	2.4 ± 0.5	3.5 ± 2.1

Summary of Findings

The immune function findings for wild smallmouth bass at four Pennsylvania sites with differing land use provide evidence for seasonal changes as well as site differences. During spring, background levels of reactive oxygen species were low, and respiratory burst occurred when stimulated in vitro for smallmouth bass at all sites. However, during fall background levels of reactive oxygen species were high at all sites and respiratory burst after stimulation in vitro did not occur (fig. 6). Bactericidal activity and mitogenesis responses varied by sampling site and season. During spring, bactericidal activity was high at West Branch Mahantango Creek but low at Pine Creek (fig. 5). Untreated background mitogenesis was high at Pine Creek during spring (fig. 7) with little additional proliferation occurring after stimulation by mitogens (fig. 9). Spring was the only season when chemicals, specifically herbicides atrazine, boscalid, and metolachlor, were measured at Pine Creek (fig. 11).

For mitogenesis at each site, when untreated background proliferation was high, lymphocytes were no longer stimulated in vitro by mitogens (fig. 8). The level of untreated background proliferation for this to occur varied seasonally and geographically (by site). Untreated background proliferation tended to be higher in older smallmouth bass and these individuals were less likely to mount a proliferative response to mitogens in the laboratory. Some contaminants are known to accumulate with age (Brousseau and Fournier, 2013; Su and others, 2017). Such accumulations may be responsible for the increases in background lymphocyte proliferation observed in older individuals, but such a response has yet to be specifically demonstrated.

Differences observed in the immune response could potentially be related to a variety of factors, including land use and chemical inputs, but differences could be related to parasite loads and other infectious agents. Seasonal changes in immune function have been reported in many fish species (Bowden et al 2007; Magnadóttir, 2010; Szwejser and others,

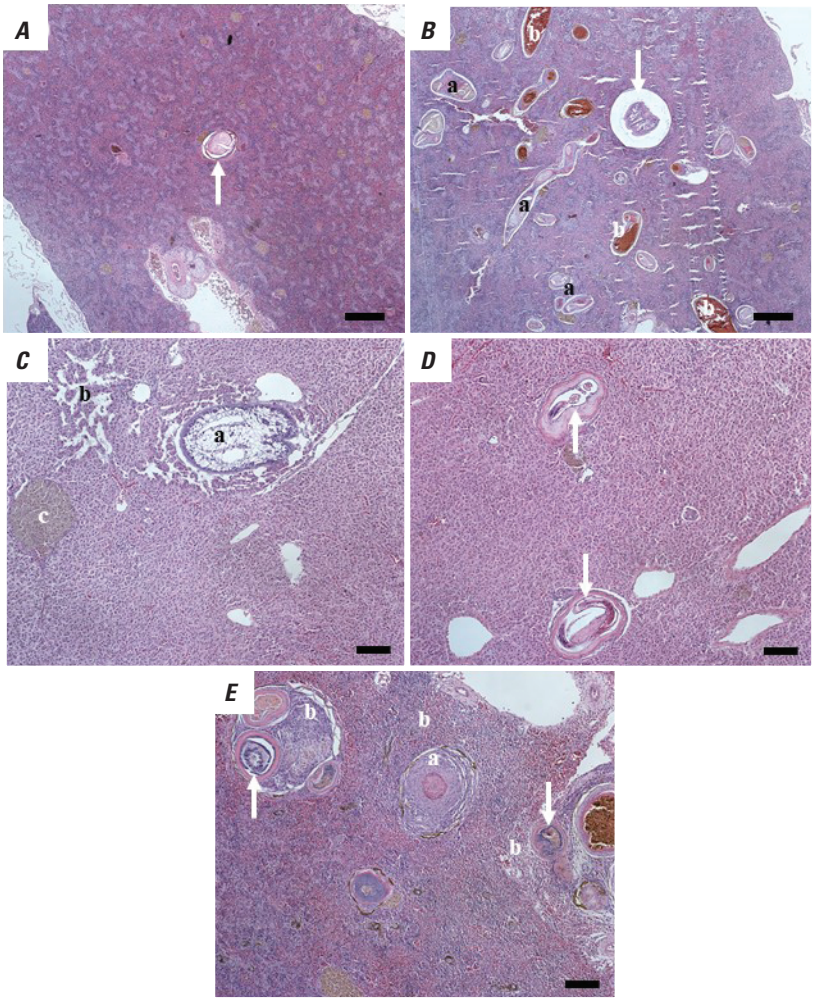

Figure 10. Microscopic pathology in smallmouth bass spleen and liver tissue. A, Section of spleen from a Tionesta Lake smallmouth bass illustrating cyst (arrow) with remnants of a helminth parasite, probably trematode. Scale bar equals 50 microns. B, Section of spleen from a Tionesta Lake smallmouth bass with an encysted live trematode (arrow). Numerous encysted areas containing degenerating parasites (a) and yellowish brown ceroid or lipofuscin (b). Scale bar equals 50 microns. C, Section of liver from an Upper Juniata River smallmouth bass with a migrating cestode (a) and associated tissue disruption and necrosis (b); a macrophage aggregate (c) is also present. Scale bar equals 30 microns. D, Section of liver from an Upper Juniata River smallmouth bass with encysted nematodes (arrows). Scale bar equals 30 microns. E, Focal granuloma (a) typical of Mycobacterial infections and focal encysted areas (arrow) with inflammation (b). Scale bar equals 30 microns. Hematoxylin and eosin stain were used.

Table 5. Frequency of parasites by tissue, year, season, and site. The number of fish classified under each parasite rating of the total sample size for that site is listed.

[WB, West Branch; SD, standard deviation]

Tissue	Year	Season	Sampling site	Sample size	Parasite rating					
					0	1	2	3	4	Mean ± SD
Liver	2016	Fall	Pine Creek	20	0	5	7	5	3	2.30 ± 1.03
			WB Mahantango Creek	20	4	9	4	2	1	1.35 ± 1.09
		Spring	Pine Creek	20	1	17	1	1	0	1.10 ± 0.55
			Tionesta Lake	12	7	5	0	0	0	0.58 ± 0.51
			Upper Juniata River	20	1	15	4	0	0	1.15 ± 0.49
			WB Mahantango Creek	20	6	14	0	0	0	0.70 ± 0.47
	2017	Fall	Pine Creek	20	0	7	10	0	3	1.95 ± 1.00
			WB Mahantango Creek	20	0	12	5	2	1	1.60 ± 0.88
		Spring	Pine Creek	19	0	14	3	0	2	1.47 ± 0.96
			WB Mahantango Creek	20	10	10	0	0	0	0.50 ± 0.51
Spleen	2016	Fall	Pine Creek	20	2	7	7	2	2	1.75 ± 1.12
			WB Mahantango Creek	20	6	11	2	0	1	0.95 ± 0.94
		Spring	Pine Creek	19	10	9	0	0	0	0.47 ± 0.51
			Tionesta Lake	12	10	2	0	0	0	0.17 ± 0.39
			Upper Juniata River	19	6	11	1	0	1	0.89 ± 0.94
			WB Mahantango Creek	20	15	5	0	0	0	0.25 ± 0.44
	2017	Fall	Pine Creek	20	5	7	5	3	0	1.30 ± 1.03
			WB Mahantango Creek	20	6	6	6	0	2	1.30 ± 1.22
		Spring	Pine Creek	20	5	10	3	1	1	1.15 ± 1.04
			WB Mahantango Creek	19	18	1	0	0	0	0.05 ± 0.23

2016) relating to multiple factors, including fluctuations in water temperatures and dissolved oxygen; changes in photoperiod; changes in hormones, such as estrogens; and exposure to parasites and pathogens which also varies seasonally. In summary, it has been shown that confounding factors such as age, sex, reproductive status, exposure to contaminants, health status of the organism, and any other physical stressors, such as hypoxic conditions, could affect immune function in fish and must be considered in the overall analysis (Farkas and others, 2003).

Caution is needed when interpreting immune function in wild fish as either normal or suppressed owing to environmental stressors without ancillary data such as disease status or the presence of parasites that may have stimulated various aspects of the immune response. Many different characteristics will be required to establish baseline immune function for these wild smallmouth bass populations. The incorporation of additional endpoints such as plasma antibodies, hormone concentrations, and gene expression will be important in future studies as well as repeated sampling over a long period to establish seasonal trends and document patterns or changes in the data relating to environmental variables or other factors.

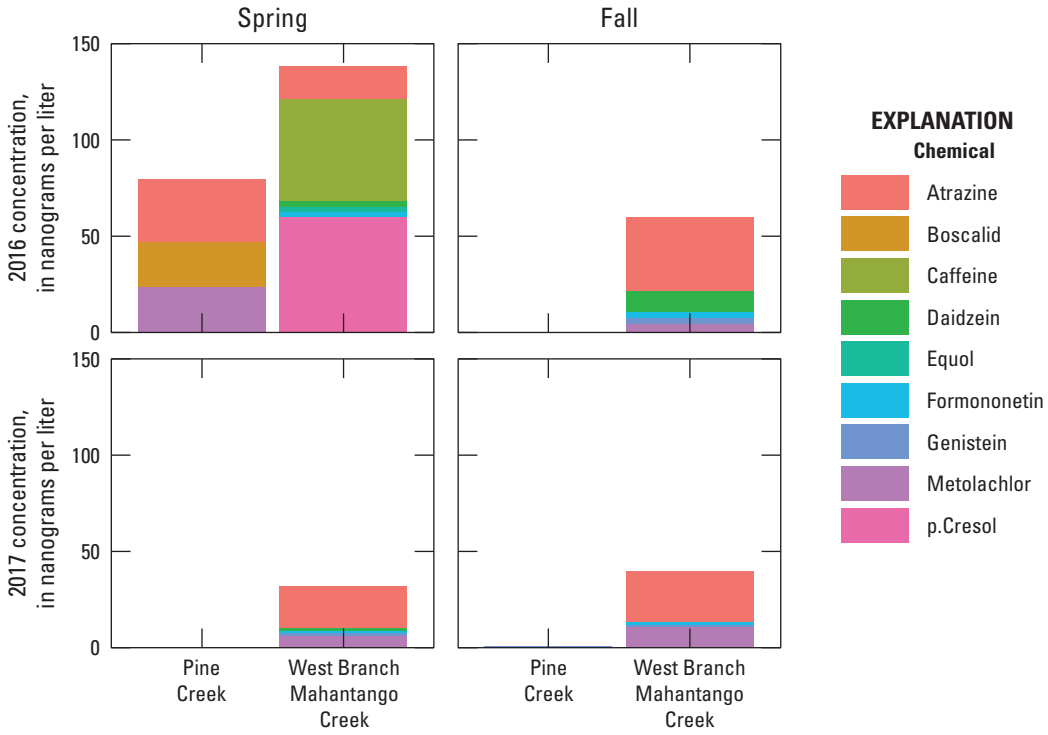

Figure 11. Chemical concentrations at selected sites before fish sampling occurred for immune function. On average, water samples were collected 0 to 6 days before fish sampling occurred except in fall 2016, when water samples at Pine Creek and West Branch Mahantango Creek were collected 34 and 35 days before fish sampling, respectively.

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For additional information, contact:
Director, Leetown Science Center
U.S. Geological Survey
11649 Leetown Road
Kearneysville, WV 25430

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