ASSESSING THE UTILITY AND FEASIBILITY OF IMMUNE FUNCTION ASSAYS TO UNDERSTAND SMALLMOUTH BASS *(MICROPTERUS DOLOMIEU)* HEALTH IN THE CHESAPEAKE BAY WATERSHED

by

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ABSTRACT

Skin lesions and mortality events of both adult and young smallmouth bass have occurred in the Chesapeake Bay, an area largely influenced by agriculture and heavily impacted by various toxic contaminants. Co-infections of bacterial, viral and parasite pathogens have been documented in both adult and young fish suggesting immunosuppression. To better understand the risk factors associated with these fish health issues, functional immune assays were developed. Adult bass were collected from two sites with different land use (forested versus agricultural). At both sites some level of immunosuppression was noted when compared to laboratory-reared bass. Histopathology of the wild bass indicated the presence of parasites and various inflammatory lesions. These results indicate immune function assays may add important information to fish health assessments, however, need to be used in conjunction with other lines of evidence to help inform managers on relationships among land use, contaminants and wild fish health.

DEDICATION

This thesis is dedicated to my Mom, Aunt Carol, and dog, Tidus, of course. He was absolutely no help in writing this but was always there to distract by whining and dropping tennis balls at my feet to play fetch.

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LIST OF ABBREVIATIONS

Smallmouth bass (SMB): Study organism

Young of the year (YOY): Fish born in the spring of the relative sampling season

Reactive oxygen species (ROS): Reactive molecules and free radicals derived from molecular oxygen; deleterious effects of ROS within the host cell are balanced by antioxidants

T cell receptor (TCR): Surface receptor on T lymphocytes responsible for recognizing fragments of antigen as peptides bound to major histocompatibility complex (MHC) molecules

Concanavalin A (Con A): Mitogen that stimulates T cells in adaptive immunity

Phytohaemagglutinin (PHAP): Mitogen that stimulates T cells in adaptive immunity

Lipopolysaccharide (LPS): Mitogen that stimulates B cells in adaptive immunity; also used as a stimulant to induce respiratory burst in cells; major component in the outer membrane of Gram-negative bacteria

Anterior kidney (AK): Main hematopoietic tissue in fish

Culture medium (CM): Liquid media containing nutrients to support survival and growth of cells in culture

Adherence medium (AM): Liquid media containing nutrients needed to support adherence of cells

Tryptic soy agar (TSA): Growth media for culturing a wide variety of microorganisms

Colony-forming unit (CFU): Individual colonies of bacteria; counts are used to measure the number of viable bacteria in a sample

Dulbecco's phosphate-buffered saline (DPBS): Balanced salt solution used for a variety of cell culture applications, such as washing, transporting or diluting cells and preparing reagents

Macrophage aggregate (MA): distinct groupings of macrophages within tissues that are analogous to germinal centers in mammals

Introduction

Fish health is routinely monitored because fish are widely used as an indicator species for environmental pollution of our rivers and streams (Schmitt et al. 1999). When fish health is adversely affected, it is an indicator that the aquatic ecosystem is not completely healthy. The aquatic environment is often a cocktail of chemical and biological contamination, where pollution becomes exacerbated by events like agricultural and storm water runoff which allow bacteria, nutrients, and synthetic chemicals from the land to enter aquatic communities (Mayes 2013). Wastewater treatment effluent or agricultural runoff include nutrients, hormones, pharmaceuticals, personal care products, and other compounds (Thorpe et al. 2005). Due to the elevated levels of pollution found in our freshwater ecosystems, biological monitoring for adverse effects has become an effective way of measuring the cumulative effects to ecological communities and field studies of fish health are an important aspect in this monitoring (Meador et al. 1993; Schmitt et al. 1999; Ekman et al. 2013).

Health problems in wild fishes in the Chesapeake Bay watershed and elsewhere, including fish kills and a high prevalence of visible lesions, concern management agencies as well as the public (Figures 1-3; Blazer et al. 2010; Blazer et al. 2014; Blazer et al. 2017). Fish kills can also have a negative impact on the economy. For example, following one of the major fish kill events in the Chesapeake Bay watershed, fishing was interrupted for more than 2,000 licensed anglers and caused the area to suffer about $700,000 in losses during 2005 (Papadakis 2006). Fish kill events and visible lesions affect many wild fish species as is shown in Figures 1-3, but this study will focus on smallmouth bass *Micropterus dolomieu.*

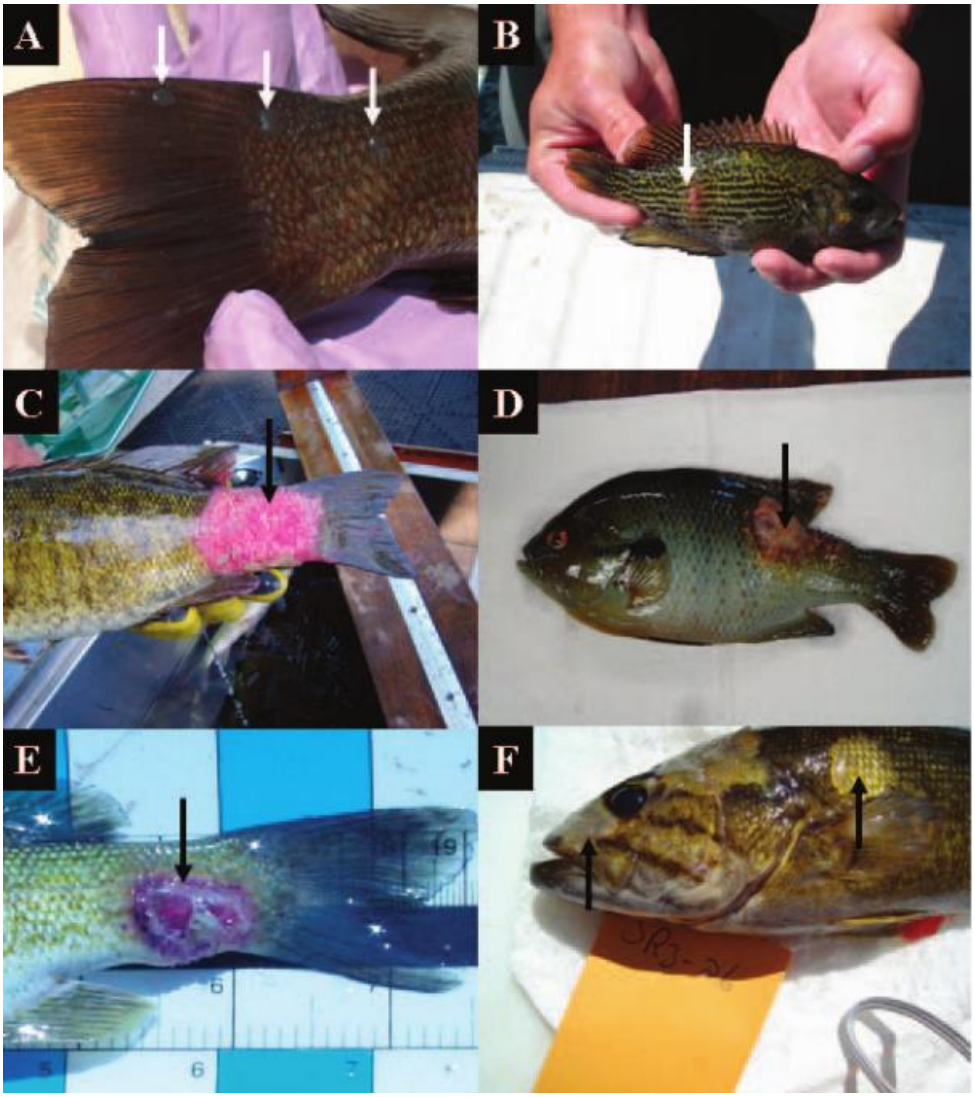

Figure 1. Examples from Blazer et al. 2010 depicting various gross lesions observed during mortalities in the Potomac River drainage: (A) raised, pale lesions (arrows) on the body surface and fins of a SMB, (B) small, raised, pale to reddened areas with epidermal erosion (arrow) on the lateral body surface of a redbreast sunfish, (C) large, reddened area on the body surface (arrow) of a SMB, (D) area of epidermal and dermal ulceration and necrosis with a reddened margin (arrow) on the lateral surface of a bluegill, (E) a deep eroded area extending into the underlying muscle (arrow) of a SMB, and (F) pale areas with patches of fungal hyphae (arrows) on the skin of a SMB (Blazer et al. 2010).

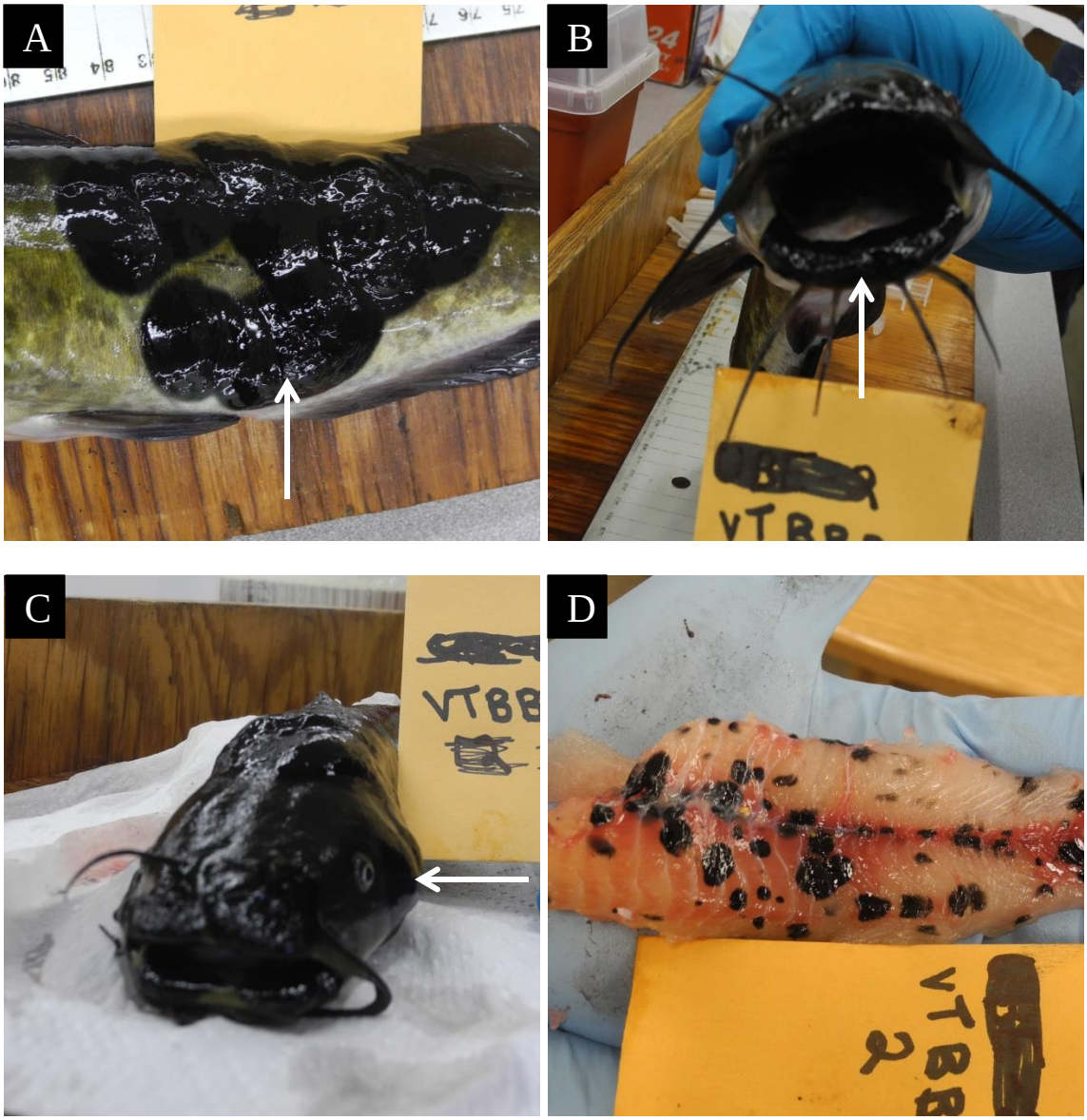

Figure 2. Melanomas observed in brown bullhead from Lake Memphremagog, Vermont during summer 2015 field sampling (V. Blazer unpublished data): (A) raised melanomas on side of body surface (B) raised melanoma covering bottom lip (C) raised melanoma behind eye (D) melanomas in muscle tissue.

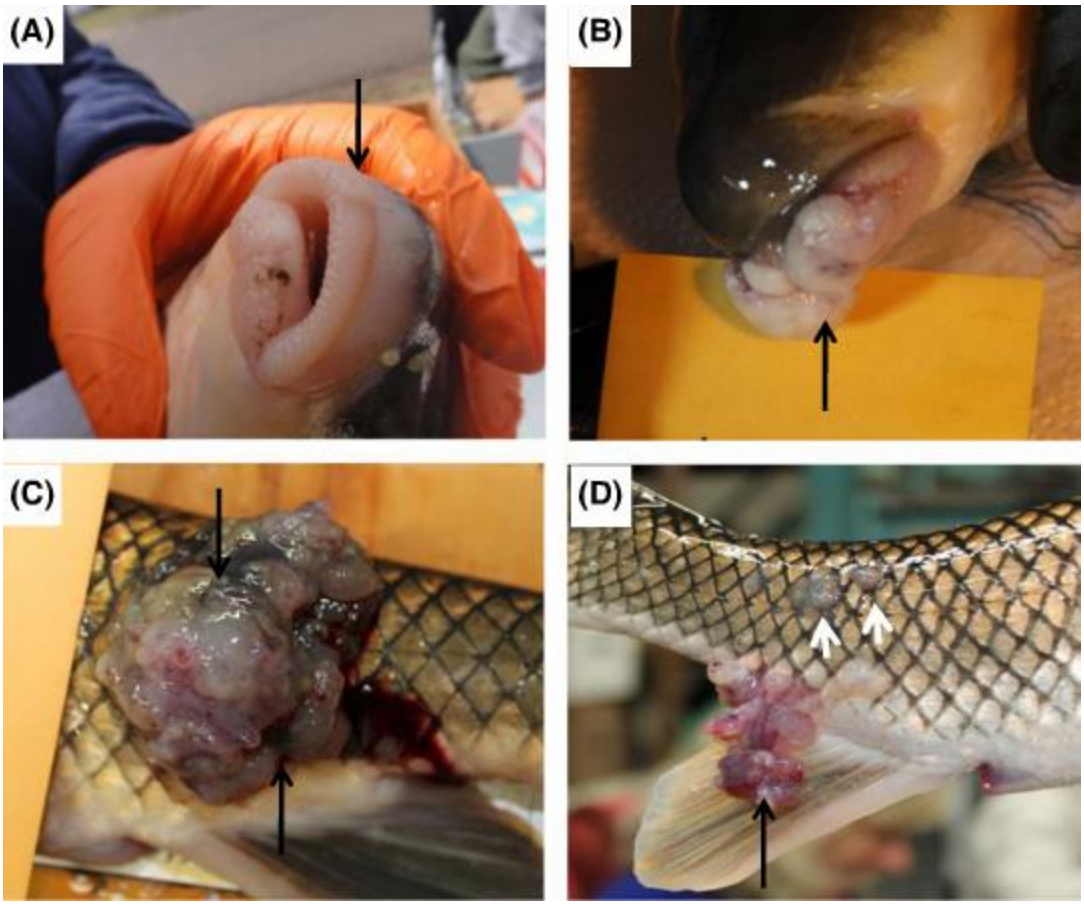

Figure 3. Examples from Blazer et al. 2017 depicting visible lesions/tumors observed on white sucker from Lake Michigan: (A) Slightly raised lip lesion (arrow), (B) Multiple, large, irregular, papillomatous lip lesions (arrow), (C) Large, greyish-red, multilobed body surface lesions (arrows), (D) Large, irregular, raised, pedunculated lesion (black arrow) and smaller pale, raised lesions (white arrowheads) on the body (Blazer et al. 2017).

Smallmouth bass in the Chesapeake Bay watershed

Smallmouth bass (SMB) are one of the more sensitive species to environmental conditions (Brown et al. 2009). They are a member of the sunfish family Centrarchidae and belong to the class Actinopterygii (ray-finned fishes), which are the most advanced of the bony fishes (Fofonoff et al. 2003; Brown et al. 2009). Within the class Actinopterygii, SMB belong to the Teleostei infraclass, the largest group of fishes, and

are referred to as a teleost species because they possess a movable jaw bone (Fofonoff et al. 2003; Brown et al. 2009). SMB are a freshwater species that live in the littoral zone of cool water streams and margins of clear water lakes (Scott and Crossman 1973; Page and Burr 1991; Brewer and Orth 2015), where the water temperature remains around 17-20 °C. Growth becomes restricted at 22 °C and temperatures greater than this have even shown displacement of smallmouth bass with largemouth bass *Micropterus salmoides* (Zweifel et al. 1999). SMB are a carnivorous, pelagic species native to the Mississippi River basin and Great Lakes (Scott and Crossman 1973; Page and Burr 1991; Stark and Echelle 1998).

Regarded as a desirable game fish, SMB have been extensively stocked within and outside their native range, including the Potomac (Smith 1907; Goldsborough and Clark 1908) and Susquehanna River basins (Jackson 2002). SMB were stocked in the Potomac River system from a native founding population in the Ohio River Basin, near Wheeling, WV (Smith 1907; Goldsborough and Clark 1908) and later introduced to the Susquehanna River system around 1870 using stock from the Potomac River in West Virginia (Milner 1874; Stillwell et al. 1895). Since being introduced in the 19th century, SMB have become one of the most abundant top predators in the Potomac and Susquehanna River basins (Jenkins and Burkhead 1994; Fofonoff et al. 2003; Borden and Stepien 2006).

The Potomac and Susquehanna Rivers are part of the Chesapeake Bay watershed along the Eastern Coast of the United States. The Chesapeake Bay is considered to have a warm temperate climate with highest precipitation in the summer (June-August) and lowest in the fall (September-November; Willard et al. 2015). Streamflow in this area is

highest between January and May (Willard et al. 2015). The health of this watershed is extremely important, as it is the largest estuary in North America and extends to over 64,000 square miles or 166,000 square kilometers (Beegle 2013; Phillips et al. 2015; Willard et al. 2015). The Chesapeake Bay watershed takes up almost 12,000 miles of shoreline, includes 150 major rivers and streams, provides spawning grounds for important species such as striped bass and blue crab, and provides homes to over 17 million people and over 3,600 species of wildlife and plants (Phillips et al. 2015). The economic value of this watershed is predicted to be $100 billion a year considering the annual seafood harvest, agricultural production, tourism and recreation it brings to the area (Phillips et al. 2015). The SMB fishery alone brings thousands of jobs and millions of dollars each year to all four states it encompasses: Pennsylvania, Maryland, Virginia, and West Virginia (Pelton et al. 2013).

Health of the Chesapeake Bay has been a concern for many years beginning with the first study in the early 1970s (Jaworski et al. 1971). Agricultural sources are a main contributor to pollution in the Bay, but despite efforts to improve on-farm nutrient management, nutrient loads have still been increasing in some areas (Beegle 2013; Paolisso et al. 2015). For these reasons, monitoring fish health in this watershed, especially for popular sport and recreational fish such as SMB, is an essential priority.

The Potomac and Susquehanna Rivers, both tributaries to the Chesapeake Bay, are sites where SMB have suffered major fish kills, disease, and sexual abnormalities, with reports beginning in spring 2002 for adults (Potomac; Blazer et al. 2010) and early summer 2005 for young of the year (Susquehanna; Chaplin et al. 2009; Smith et al. 2015). Previous fish health investigations have indicated SMB from both Potomac and

Susquehanna River basins to have a variety of bacterial infections, heavy parasite loads and sometimes viral and fungal infections (Chaplin et al. 2009; Blazer et al. 2010; Walsh et al. 2012; Blazer et al. 2014; Smith et al. 2015), but this study will focus on the Susquehanna River basin.

In the Susquehanna, young of the year (YOY) SMB have suffered disease related fish kill events in 2005, 2007, 2008 and also in 2006 and 2009 although to a lesser extent (Smith 2010). Figure 4 shows examples of visible lesions observed during investigations of the mortalities (Starliper et al. 2013). Investigations have found bacterial pathogens *Flavobacterium columnare* (Chaplin et al. 2009), *Pseudomonas aeruginosa* (Smith et al. 2015), and motile *Aeromonas* species (Starliper et al. 2013), parasites *Myxobolus inornatus* (Walsh et al. 2012) and trematode metacercariae (Smith 2010), as well as largemouth bass virus (Starliper et al. 2013). Reproductive endocrine disruption seen in the form of intersex and testicular oocytes in adult male fish has also been elevated in SMB and correlated to agricultural chemicals in the Susquehanna River basin (Blazer et al. 2014).

These observations lead to two main hypotheses: (1) SMB in the Susquehanna River basin are immunosuppressed, causing them to be susceptible to many opportunistic pathogens and (2) land use, particularly exposure to agricultural chemicals, affects immune function in SMB. Increasing evidence supports a link between the endocrine and immune system in fish (Weyts et al. 1999; Iwanowicz and Ottinger 2009; Casanova-Nakayama et al. 2011; Milla et al. 2011), so it is possible that the same chemicals or stressors contributing to intersex may also be contributing to immunosuppression.

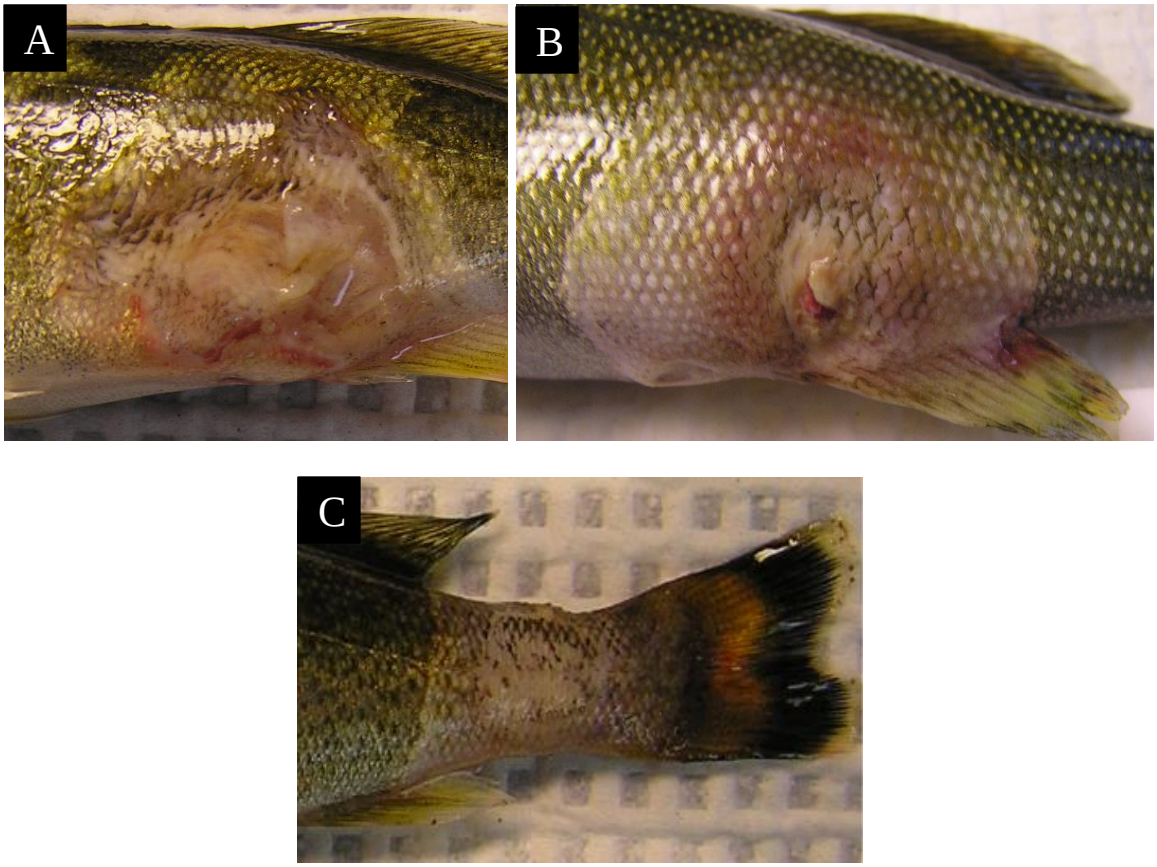

Figure 4. Examples from Starliper et al. 2013 depicting visible lesions observed on smallmouth bass sampled from various sites on the Susquehanna River: (A) Large area of extensive necrosis on side of body, (B) Severe necrotic lesion with a wide margin covering a large portion of the side, (C) Discoloration, scale loss and minor tissue necrosis on caudal peduncle (Starliper et al. 2013).

If these hypotheses are true, it is predicted that SMB collected at a highly disturbed agricultural site within the Susquehanna River basin will have a different immune response than at a less disturbed site. The main objective of my study was to develop and assess techniques to evaluate immune function in wild SMB. Validation of the techniques was achieved by comparing a) two sites with different land use and potential contaminant exposures and b) wild to laboratory-reared fish.

Fish immunology

Studying immune function in wild fish populations provides one method for determining health of the fish by providing information on how well a fish will respond to infectious disease. The primary line of defense in the immunity of teleost fishes is their mucosal body surface, where invaders can first be effectively blocked or neutralized (Hoar et al. 1997). Then, resistance to disease involves many aspects of both the innate and adaptive immune system – both cellular and humoral defenses (Hoar et al. 1997). The cells involved in the innate, or nonspecific, immune system of teleost species are very similar to higher vertebrates with the principal cells involved being neutrophils and macrophages (Secombes 1996; Ellis 1999). Other granulocytes (eosinophils, basophils) and nonspecific cytotoxic cells (NCCs; equivalent to mammalian natural killer cells) are also involved in the nonspecific immune response (Hoar et al. 1997). Innate humoral responses are led by protein or glycoprotein substances in the serum, mucus, and eggs of fish (Hoar et al. 1997). The importance of these innate responses is their lack of specificity; however, the innate cells lack memory that would allow them to respond quicker to pathogens upon subsequent exposure as in the adaptive immune response.

The primary cells involved in the adaptive immune response in teleosts are T and B lymphocytes (Hoar et al. 1997). Soluble factors called cytokines regulate or enhance adaptive immunity by mediating interactions between immune cells (Hoar et al. 1997). In humoral adaptive immunity, B-lymphocytes respond to antigens with specific antibodies and form memory cells to remember antigens they have already encountered (Hoar et al. 1997). The importance of the adaptive response is the memory capability. Due to the B-

lymphocytes' ability to recognize specific antigens, antibodies can be produced quicker in future infections leading to more efficiency in the immune response.

The majority of immune function studies conducted with fish utilize laboratory reared fish held under defined conditions. Unfortunately, that does not mimic the real-world environmental conditions to which fish are exposed. A number of studies have successfully used methods for detecting and comparing immunomodulation in wild fishes (Harms et al. 2000; Gauthier et al. 2003; Iwanowicz et al. 2012), including a few studies that have utilized SMB (Anderson et al. 1997; Ripley et al. 2008). The assays chosen include bactericidal killing ability, respiratory burst activity and mitogen-induced leukocyte proliferating ability.

The bactericidal assay detects how well leukocytes are able to kill or inactivate pathogens by exposing the cells to bacteria and measuring the percentage of bacteria killed. Both bactericidal and respiratory burst activity address adherent cell (macrophage) function (Iwanowicz et al. 2012). The respiratory burst assay measures production of reactive oxygen species (ROS) in adherent cells. Producing ROS is a mechanism within phagocytic cells (primarily macrophages and neutrophils) for killing bacteria and other organisms that have been engulfed (Ellis 1999). ROS are produced when phagocytes containing many hydrolytic enzymes are activated by phagocytosis (Ellis 1999). ROS can also be released from phagocytic cells and aid in destruction of tumor cells, certain parasites, natural killer activity, and selected targets in antibody-dependent cellular cytotoxicity; and have been found to induce cytogenetic changes in cultured mammalian cells (reviewed by Zoschke and Messner 1984). Measuring ROS production is most often used as an indicator of the innate immune system, as phagocytosis has been highly

correlated with elevated oxygen consumption in mammals (Baldridge and Gerard 1932; Biller-Takahashi et al. 2013), as well as increased cytokine release and inflammatory response in fish (Neumann et al. 2000; Rieger et al. 2010; Biller-Takahashi et al. 2013). However, in addition to their role as antimicrobial defenders of the innate immune system, there is also evidence that ROS play a role in the adaptive immune response.

Multiple studies have shown that T lymphocytes can produce ROS with activating stimuli to the T cell receptor (TCR; reviewed by Williams and Kwon 2004). ROS production in activated T lymphocytes has been found to help regulate protein kinase activation, gene expression, and/or cell proliferation/transformation (Williams and Kwon 2004). Although there is conflicting evidence on the exact roles of ROS in T lymphocyte function, it is clear that a balance of ROS is needed for proper activation of lymphocytes (Williams and Kwon 2004). In both innate and adaptive immunity, antioxidants scavenge excessively produced ROS and any imbalances between these (antioxidants and ROS) can be damaging to cells (Poljsak et al. 2013). An antioxidant is any substance (including antioxidant enzymes and non-enzymatic compounds) that delays, prevents, or removes oxidative damage (Halliwell and Gutteridge 2007).

A complication in evaluating the respiratory burst and ROS production as a killing mechanism is that free radicals and other ROS are produced in a wide range of physiological processes. They are also produced by UV radiation and from the metabolism of xenobiotics (Winterbourn 2008). Hence, ROS can act as secondary messengers in intracellular signaling (beneficial role) but can also cause damage. Oxidative stress and potentially pathology occurs when the antioxidant status is

overwhelmed. Exposure to many environmental stressors can induce oxidative stress in aquatic organisms (Valavanidis et al. 2006; Lushchak 2011).

A measure of background ROS in anterior kidney cells from wild fish provides information on the oxidation status of the cells in regards to environmental factors versus *in vitro* stimulation, meaning it provides a measurement of what the cells were experiencing in the field at the time of collection. Lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate (PMA) are stimulants commonly used to induce respiratory burst in cells *in vitro* (Zoschke and Messner 1984; Ripley et al. 2008). Stimulating cells *in vitro* gives a measure of how inducible the cells are relative to their oxidation status from the environment. LPS was selected as the stimulant for this study because it more closely resembles a bacterial encounter (Iwanowicz et al. 2012).

The mitogen-induced proliferation assay measures cell proliferation in response to specific mitogens, which can provide a functional assessment of leukocytes in regards to various pathogens (Ahmed et al. 1994). Mitogen stimulation evaluates the ability of lymphocytes in the adaptive immune response (B and T cells) to respond to pathogens. Different mitogens are utilized to stimulate different subsets of lymphocytes. Concanavalin A (Con A) and phytohaemagglutinin (PHAP) are used to stimulate T cells, while lipopolysaccharide (LPS) is used to stimulate B cells (Hoar et al. 1997). Response of fish leukocytes to mitogenic stimulation has been found to be influenced by oxidative stress caused by a number of environmental factors including heavy metals (Zelikoff 1993; O'Halloran et al. 1998), pesticides (Dunier and Siwicki 1993), and other immunotoxins such as the hormones cortisol (Ellsaesser and Clem 1987; Tripp et al.

1987; Wang and Belosevic 1994; Espelid et al. 1996) and estradiol (Wang and Belosevic 1994).

Mitogenesis methods development

One aspect of my research was developing a method for measuring mitogenesis of smallmouth bass leukocytes with image-based flow cytometry. For the measurement of mitogenesis, one of the most important assay design considerations is the detection strategy, including the compound used to detect cell proliferation and the diagnostic technology used to analyze the results. Several techniques have been adapted for quantification of cell proliferation in mammals involving both colorimetric and radioactive detection strategies as well as indirect versus direct measurement (Mosmann 1983; Wagner et al. 1999; Gauthier et al. 2003). Adaptations to some of these methods have been developed for fish leukocytes (Daly et al. 1995; Gauthier et al. 2003).

The simplest method for detecting mitogenesis in cells involves the indirect incorporation of the tetrazolium salts MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) or XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt), where both salts are reduced by mitochondrial dehydrogenases in live cells into a pigment that can be measured with a microplate absorbance reader (Mosmann 1983; Wagner et al. 1999; Gauthier et al. 2003). The MTT method was introduced in 1983 for mammalian cell lines (Mosmann 1983) and was later adapted to brook trout (Daly et al. 1995). Tetrazolium salt incorporation provides a rapid and large scale assay, but it is of limited accuracy because it does not directly measure DNA synthesis. Rather it measures metabolic activity as the salts are reduced by NAD(P)H-dependent cellular oxidoreductase enzymes (Mosmann 1983).

A more accurate method of proliferation analysis involves direct measurement of DNA synthesis. This was originally performed through incorporation of the radioactive nucleoside methyl-^{3}H-thymidine (tritiated thymidine) into new strands of chromosomal DNA during mitotic cell division followed by cell lysate filtration and quantification of radioactivity in DNA recovered from the cells through autoradiography or scintillation techniques (Wagner et al. 1999; Gauthier et al. 2003; Haugland 2005; Salic and Mitchison 2008; Cavanagh et al. 2011). Although this method is reliable and sensitive, detection of incorporated tritiated thymidine in DNA of replicating cells requires specialized equipment and produces radioactive waste that not all laboratories are equipped to manage (Wagner et al. 1999; Gauthier et al. 2003). Detection by autoradiography has also proven to be labor intensive and slow to detect (several months), thus not suitable for high-throughput studies (Salic and Mitchison 2008).

More recently developed methods for detecting mitogenesis have eliminated the need for radioisotopes, through incorporation of a thymidine analog, either BrdU (5'-bromo-2'-deoxyuridine) or EdU (5-ethynyl-2'-deoxyuridine), directly into newly synthesized DNA (Wagner et al. 1999; Haugland 2005). BrdU incorporation methods are based on anti-BrdU antibody detection (Gratzner 1982). Exposing the incorporated BrdU epitope requires denaturation of DNA and permeabilization of cell membranes, resulting in tissue damage and degradation of most protein epitopes (Wagner et al. 1999; Salic and Mitchison 2008; Cavanagh et al. 2011). BrdU incorporation is more efficient than tritiated thymidine, but detection still lasts several hours and compromises structural integrity of the cell (Salic and Mitchison 2008).

Incorporation of EdU has been introduced as an alternative to the BrdU assay because the technique is simple, rapid and sensitive but does not require destruction of cell structure (Salic and Mitchison 2008). Detection remains highly sensitive but is much faster than BrdU (30 minutes vs. several hours) and does not require DNA denaturation for the detection reagent to gain access to the DNA (Salic and Mitchison 2008). Standard aldehyde-based fixation and detergent permeabilization are sufficient for detection of EdU incorporation (Salic and Mitchison 2008; Cavanagh et al. 2011). Detection is based on click chemistry or Huisgen's 1,3-dipolar cycloaddition, a copper catalyzed covalent reaction between an azide (coupled to fluorescent dye) and an alkyne (ethynyl moiety of EdU; Salic and Mitchison 2008; Cavanagh et al. 2011). One advantage to this method is that incorporated EdU has been found to be very stable, allowing fixed cells to be stained months later without a loss of signal (Salic and Mitchison 2008). Also because cell structure is not compromised, the EdU method (unlike BrdU) allows for multiparameter downstream analysis in image-based flow cytometry (Cappella et al. 2008).

In this study, I utilized the EdU methodology to detect mitogenesis (S phase cells) in smallmouth bass leukocyte populations and analyze those populations in image-based flow cytometry for identification and characterization of subpopulations based on cell size and morphology. Compared to standard flow cytometry, image-based flow cytometry has introduced identification of objects based on a collection of real multicolor images for individual cells (vs. side/front scatter) with high spatial resolution and high fluorescence sensitivity, helping to avoid artifacts and false positives during analysis (Basiji et al. 2007; Zuba-Surma and Ratajczak 2011). Although standard flow cytometry provides important quantitative information about cells based on their fluorescence, it

does not provide details on the location of fluorescent signals or cellular morphology (Zuba-Surma and Ratajczak 2011).

Image-based flow cytometry fully integrates the advanced capabilities of both standard flow cytometry (*i.e.* statistical power, fluorescence sensitivity, speed, and phenotyping abilities) and fluorescence microscopy (*i.e.* detailed imagery, spatial resolution, quantitative morphology, and functional insight) with extended digital imaging analysis software, called IDEAS® (Basiji et al. 2007; Zuba-Surma and Ratajczak 2011). This software can analyze over 40 quantitative features per image, including fluorescence intensity, cell size, shape, texture, shape changes, location of probes within, on or between cells, and co-localization of multiple probes for individual cells or selected subpopulations (Basiji et al. 2007; Zuba-Surma and Ratajczak 2011). While multiple applications of image-based flow cytometry have been established by several studies and the list of publications is still growing (reviewed by Zuba-Surma and Ratajczak 2011), the availability of methods developed for investigating piscine leukocytes with image-based cytometry are limited although methods have been developed for standard flow cytometry (Sizemore et al. 1984; Lin et al. 1999; Scharsack et al. 2001).

Scientists have been performing health surveys of smallmouth bass in the Chesapeake Bay region for many years; however, health of the immune system on the cellular level has not been a routine part of their investigations. My research was designed to assess the validity of using immune function assays to evaluate immunosuppressive mechanisms that could be contributing to the mortalities and disease experienced by SMB in the Susquehanna River. The objectives of this thesis were to:

1. Provide biological endpoints that can be used to assess immune function and more comprehensively understand fish health in the study area.
2. Determine the feasibility and utility of immune function assays on wild smallmouth bass using anterior kidney (AK) tissue collected in the field to perform the three immune function assays mentioned above (bactericidal, respiratory burst, and mitogenesis). This was done at two sites (one within the Susquehanna basin and one out-of-basin) with varying land use and compared to smallmouth bass raised in the laboratory.
3. Develop and evaluate an image-based flow cytometry mitogenesis method that could be used for smallmouth bass leukocytes.

This study was initiated as part of a larger USGS collaborative study to understand endocrine disruptors, other chemicals of emerging concern and their effects on fish in the Chesapeake Bay watershed. The comparison of responses in bass collected from a site in the Susquehanna to an out-of-basin site plus laboratory fish provides an assessment of the utility of immune function assays.

Based on previous research, there is a need for integration of immune function research with previous bacteriological, virological, parasitological, and histopathological reports made on SMB collected during and prior to the mortality events in both the Susquehanna and Potomac River basins. Integrating studies of immune function with pathogen identification, pathological responses, water quality analyses, tissue contaminant concentrations, and land use assessments will allow for a better understanding of the causes and risk factors contributing to the skin lesions and disease (adults/YOY) and mortality events (YOY) observed in SMB from the Susquehanna. This

integration will play a significant part in reporting on the overall health of the Chesapeake Bay watershed.

Methods

Site description

Three sampling sites were chosen for this study: Upper Juniata River (40.56082, -78.0694), Tionesta Lake (41.4753121, -79.438331), and USGS Fish Health Research Laboratory in Kearneysville, WV. The two wild fish sites were chosen based on land use (Table 1), previous fish health issues, and river basin location (Figure 5). The laboratory fish were obtained from a commercial source as young-of-year and maintained in spring water at the USGS Fish Health Research Laboratory, Kearneysville, WV.

The Upper Juniata (JUP) site is located within the Susquehanna River basin where YOY SMB have suffered high prevalences of fish kills, skin lesions, and disease over the last two decades (Chaplin et al. 2009; Blazer et al. 2010; Walsh et al. 2012; Blazer et al. 2014; Smith et al. 2015). The Juniata River is the second largest tributary to the Susquehanna River. It forms at the confluence of Frankstown Branch and Little Juniata River in central Pennsylvania and empties into the mainstem Susquehanna in southeastern Pennsylvania. Land cover upstream from JUP is a mix of forested, agricultural, and developed (Table 1a).

Tionesta Lake (TL) is the out-of-basin site for this study located in the Ohio River basin. Tionesta Lake in northwest Pennsylvania is a US Army Corps of Engineers owned impoundment built as flood control for the Allegheny and upper Ohio Rivers. The lake empties into the Allegheny River by the Tionesta Dam. Tionesta Lake is located on the southwestern edge of the Alleghany National Forest. Land cover upstream from the Tionesta Lake sampling site is largely forested (Table 1a). Additional land use descriptions upstream of both sites (JUP and TL) are displayed in Table 1b.

Table 1a-b. Land use in upstream drainage area of each sampling site. GIS land cover data (Stephanie Gordon, USGS, personal communication) was collected from National Land Cover Database 2011.

(a) Percent land cover in upstream drainage area. Upstream drainage area = total upstream cumulative drainage area at the downstream end of the National Hydrography Dataset (NHD) Flowline feature in ArcGIS (Esri, Redlands, CA).

		Percent Land Cover in Upstream Drainage Area						
Site	**Upstream drainage area (km^2)**	**Cultivated**	**Developed**	**Forested**	**Herbaceous**	**Shrub**	**Water**	**Wetland**
Upper Juniata	1024.189	23.6	12.0	63.7	0.11	0.02	0.37	0.002
Tionesta Lake	1235.941	2.4	2.0	85.3	0.85	6.78	0.33	2.33

(b) Additional land use descriptions. Population density is from 2010 census block data for the National Hydrography Dataset (NHD, Plus Version 2) Flowline catchment in ArcGIS (Esri, Redlands, CA). All counts are upstream totals per catchment. Pesticides applied were >99% herbicides at both sites. NA = data not available.

Site	**Population density (upstream total per catchment)**	**Impervious coverage in upstream drainage area (%)**	**Total concentrated animal feeding operations (CAFOs)**	**Landfills**	**Total pesticide applied (kg) 2001-2015**	**Active septic facilities**
Upper Juniata	2606	3.07	8959	3	353548	58
Tionesta Lake	748	0.21	NA	NA	9345	NA

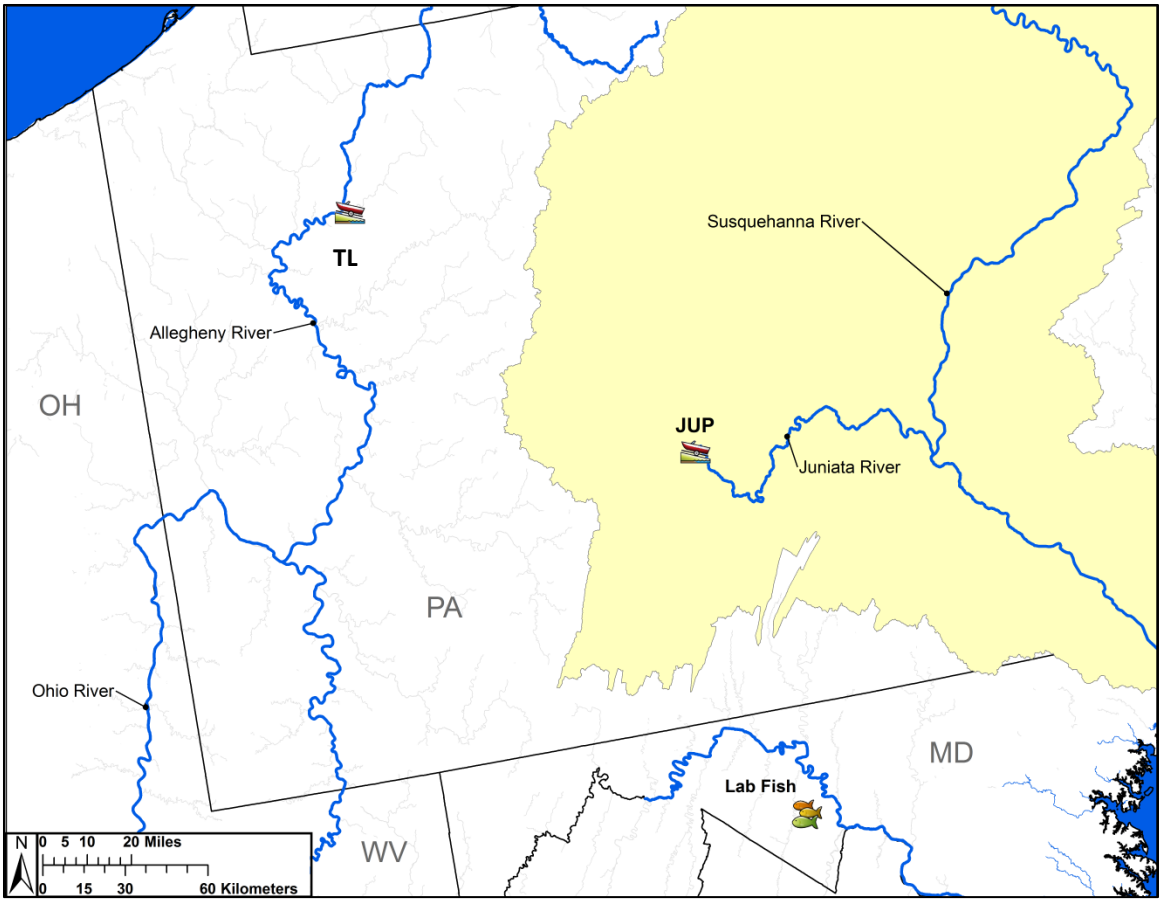

Figure 5. Map displaying sampling locations. TL = Tionesta Lake, JUP = Upper Juniata River, Lab = USGS Fish Health Research Laboratory in Kearneysville, WV. Yellow = Susquehanna River basin.

Field sampling

All sampling methods were modified from Schmitt et al. (1999). At each site, attempts were made to collect 20 adult smallmouth bass by boat electrofishing in May 2016. All captured fish were placed immediately in a live well to be necropsied on site. Fish were euthanized with an overdose of tricaine methanesulfonate (MS-222). The fish were then weighed and bled from the caudal vessels. Length, weight, and any visible abnormalities, both external and internal, were recorded for all individuals. Anterior kidney was excised using aseptic technique and placed in 10 ml of cold L-15 media

supplemented with 290 μg ml^{-1} L-Glutamine, 10 units ml^{-1} sodium heparin, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 2% fetal bovine serum (FBS, Hyclone (GE Life Sciences), Characterized, U.S. origin; supplement media designated L-15/2%). 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. Spleen tissue was taken for histology. Samples were transported to the lab for next day processing and kept at 4 °C overnight prior to leukocyte isolation (12-16 h holding time). All procedures in the lab were performed using sterile technique in a biological safety cabinet.

Lab fish care

Age-0 smallmouth bass were obtained from Schultz's Fish Hatchery (Lake Ariel, PA) in November 2016. Fish were housed indoors at the USGS Fish Health Research Laboratory (Kearneysville, WV) in 1287 L circular tanks (n = 100 per tank initial density). Tanks were supplied with flow-through spring water heated to an in-tank temperature of 20^{o}C using a heat exchanger located outside the holding facility. Total tank-volume replacement occurred about four times daily (4 L/min). Tank illumination was provided by a combination of natural and fluorescent light with photoperiod matching natural light supply. Fish were initially fed small (~ 1 g) fathead minnows (*Pimephales promelas*) supplied by Schultz's Fish Hatchery and obtained at the same time as the smallmouth bass. Subsequent supplies of small fathead minnows were obtained from Anderson Minnows (Lonoke, AR). The bass were transitioned to age-0 rainbow trout (*Onchorhynchus mykiss*; surplus stock, USDA National Center for Cool and Coldwater Aquaculture, Kearneysville, WV) as they became willing to accept the

larger prey. Target feed rates were approximately 4% body weight per day. Actual feed rates varied as a result of size differences in individual bass as well as differences in individual feeding performance. Feed rates for individual smallmouth bass were not determined. Fish were acclimated to holding conditions and feeding regime for a minimum of 6 months prior to sampling. Lab sampling occurred in May 2017.

Leukocyte isolation

Anterior kidney leukocytes were isolated following a procedure modified from Sharp et al. (1991). Cell suspensions were pelleted by centrifugation at 500 RCF and 4 °C for 10 min, then washed 3x by suspension in 10 ml L-15/2%, centrifuging as above between each wash. Following wash steps, cells were resupended in 6 ml of L-15/2%, passed through a 70 μm cell strainer to remove clumps or clots, and then layered onto 6 ml of 32% Percoll concentration in Hanks balanced salt solution (HBSS) without phenol red in 15 ml centrifuge tubes. Percoll tubes were centrifuged at 4 °C for the time and force appropriate for the taxa (Centrarchidae – 45 min at 500 RCF). Leukocytes were removed from the Percoll interface and pelleted by centrifugation at 500 RCF and 4 °C for 10 min. Isolated leukocytes were washed 1x as above and resuspended in 10 ml of L-15/2% for counting. Total numbers of viable leukocytes were determined by trypan blue exclusion using the Countess™ II automated cell counter (ThermoFisher, Pittsburgh, PA). Countess™ II viable-cell counts were optimized for smallmouth bass leukocytes prior to use in this study. Final instrument settings were validated using parallel cell counts from a propidium iodide exclusion assay with data acquisition performed using the Amnis FlowSight® (MilliporeSigma, Billerica, MA) image-based flow cytometer (data not shown).

Following isolation, leukocytes were resuspended at 2 x 10^7 cells ml^{-1} in culture medium (CM; L-15 media containing 290 μg ml^{-1} L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 5% FBS; L-15/5% P/S) for plating mitogenesis assay or adherence medium (AM; L-15 media containing 290 μg ml^{-1} L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 0.1% FBS; L-15/0.1% P/S) for plating bactericidal and respiratory burst assays (Figure 6). Bactericidal and respiratory burst assays were plated simultaneously followed by plating of mitogenesis assay. Unless otherwise noted, all incubations for immune function assays were performed in an open zip-lock plastic bag lined with paper towel saturated with deionized water (humidified container) at 17 °C. In May 2017, cell yield from lab fish was significantly less than from the field, so assays for lab fish were ran in 384-well tissue culture plates rather than 96-well plates. Media amounts were divided by 4, but all concentrations remained the same.

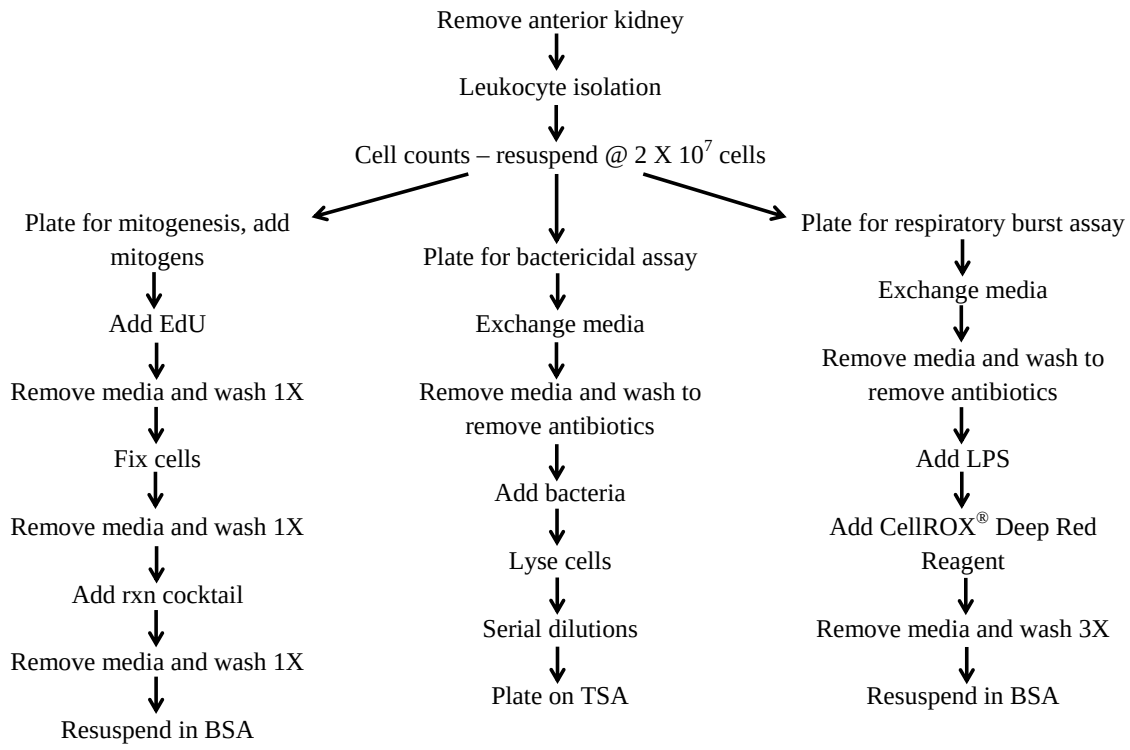

Figure 6. Methods flow chart. Following isolation and resuspension of leukocytes, cells were plated for three immune cell functional assays. Bactericidal and respiratory burst were plated simultaneously with cells suspended in adherence medium (L-15/0.1% P/S). Cells were then centrifuged; adherence medium was removed and cells were resuspended in culture medium (L-15/5% P/S) before plating mitogenesis assay.

Adherent cell (macrophage) bactericidal assay

Following leukocyte isolation, functional assessment of adherent AK leukocytes to kill the motile *Aeromonas* species *A. sobria* was determined using a procedure modified from Harms et al. (2000). In short, 2 x 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 counting adherent cell enumeration (see

plate layout, Figure 7). After 2 hours at 17 °C incubation, media was removed from all wells to remove non-adherent cells 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 before adding bacteria cultures. Following 36 h incubation, wells were washed to remove antibiotics prior to the addition of bacteria. A 48 h culture of *A. sobria* was added at 25 µl well^{-1} to treatment wells and a row of cell-free control wells, leaving some wells bacteria-free and available for counting adherent cell enumeration (see plate layout, Figure 7). After the addition of bacteria, the plate was returned to the humidified container in the 17 °C incubator for 4 h incubation. After 4 hours, media was removed from the treatment wells and replaced with 50 µl well^{-1} of lysis buffer. Serial dilutions were performed for each row A-D and H, plated onto tryptic soy agar (TSA) plates, and colony-forming units (CFUs) were determined after 24 h incubation. Cell counts were performed for rows E-G using the Countess™ II automated cell counter (ThermoFisher).

Bactericidal activity was expressed as the percentage CFU reduction and as corrected CFU reduction. The percentage CFU reduction was achieved using equation 1, where $\%CFU_r$ is the total reduction, $CFU_{treated}$ is the mean CFU value for replicate wells with adherent leukocytes, and $CFU_{control}$ is the mean CFU value for replicate wells with media only.

$$\%CFU_r = \left(1 - \frac{CFU_{treated}}{CFU_{control}}\right) * 100$$

Equation 1. Percentage CFU reduction.

A corrected CFU reduction percentage (%CFU_{rc}) was determined using equation 2 by multiplying percentage CFU reduction by the cell density correction factor, which accounts for the actual number of adherent cells from each cell source.

$$\%CFU_{rc} = (\%\ CFU\ reduction)(Cell\ density\ correction)$$

Equation 2. Corrected CFU reduction.

The cell density correction factor is defined in equation 3, where $AC_{\bar{x}}$ represents the mean number of adherent cells from replicates in rows E-G.

$$Cell\ density\ correction = \frac{1}{\left(AC_{\bar{x}}/_{1 * 10^6}\right)}$$

Equation 3. Cell density correction.

Macrophage Bactericidal Assay - Plate 1												
	1	2	3	4	5	6	7	8	9	10	11	12
A A.SOBRIA	FISH 1	FISH 1	FISH 1	FISH 1	FISH 2	FISH 2	FISH 2	FISH 2	FISH 3	FISH 3	FISH 3	FISH 3
B A.SOBRIA	FISH 4	FISH 4	FISH 4	FISH 4	FISH 5	FISH 5	FISH 5	FISH 5	FISH 6	FISH 6	FISH 6	FISH 6
C A.SOBRIA	FISH 7	FISH 7	FISH 7	FISH 7	FISH 8	FISH 8	FISH 8	FISH 8	FISH 9	FISH 9	FISH 9	FISH 9
D A.SOBRIA	FISH 10	FISH 10	FISH 10	FISH 10	FISH 11	FISH 11	FISH 11	FISH 11	FISH 12	FISH 12	FISH 12	FISH 12
E CELL COUNTS	FISH 1	FISH 1	FISH 1	FISH 2	FISH 2	FISH 2	FISH 3	FISH 3	FISH 3	FISH 4	FISH 4	FISH 4
F CELL COUNTS	FISH 5	FISH 5	FISH 5	FISH 6	FISH 6	FISH 6	FISH 7	FISH 7	FISH 7	FISH 8	FISH 8	FISH 8
G CELL COUNTS	FISH 9	FISH 9	FISH 9	FISH 10	FISH 10	FISH 10	FISH 11	FISH 11	FISH 11	FISH 12	FISH 12	FISH 12
H MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA

Macrophage Bactericidal Assay - Plate 2												
	1	2	3	4	5	6	7	8	9	10	11	12
A A.SOBRIA	FISH 13	FISH 13	FISH 13	FISH 13	FISH 14	FISH 14	FISH 14	FISH 14	FISH 15	FISH 15	FISH 15	FISH 15
B A.SOBRIA	FISH 16	FISH 16	FISH 16	FISH 16	FISH 17	FISH 17	FISH 17	FISH 17	FISH 18	FISH 18	FISH 18	FISH 18
C A.SOBRIA	FISH 19	FISH 19	FISH 19	FISH 19	FISH 20	FISH 20	FISH 20	FISH 20				
D A.SOBRIA												
E CELL COUNTS	FISH 13	FISH 13	FISH 13	FISH 14	FISH 14	FISH 14	FISH 15	FISH 15	FISH 15	FISH 16	FISH 16	FISH 16
F CELL COUNTS	FISH 17	FISH 17	FISH 17	FISH 18	FISH 18	FISH 18	FISH 19	FISH 19	FISH 19	FISH 20	FISH 20	FISH 20
G CELL COUNTS												
H MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA	MEDIA

Figure 7. Plate layout for macrophage bactericidal activity. Rows A-D are treated with *A. sobria*, rows E-G are for counting adherent cell enumeration, and row H is the bacteria-free control.

Respiratory burst assay

Respiratory burst was determined via the procedures described by Coteur et al. (2002) and Iwanowicz et al. (2012) with modifications. Respiratory burst was plated at the same time as the bactericidal assay so incubation times could run simultaneously. Lipopolysaccharide (LPS) was used as the stimulant in this assay. Isolated leukocytes at 2 x 10^7 cells ml^{-1} in AM (L-15/0.1% P/S) were added in duplicate to a white 96-well tissue

culture plate for the LPS challenge and in duplicate on the same plate for negative controls containing only media (see plate layout, Figure 8). Alongside the bactericidal assay, respiratory burst plates were incubated for two hours at 17 °C before removing media from all wells and replacing 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 before performing respiratory burst assay. Following 36 h incubation, wells were washed to remove antibiotics prior to the addition of LPS (100 μg ml^{-1}) or media (L-15/5% NO P/S). Treatments (LPS or media) were added at 25 μl $well^{-1}$. After the addition of LPS or media, the plate was returned to the humidified container in the 17 °C incubator for 4 h incubation. After exposing the cells to LPS, extracellular reactive oxygen species (ROS) in live cells were determined using a CellROX® fluorogenic probe, which exhibits a strong fluorescent signal in the presence of oxidation.

After 4 hours, CellROX® Deep Red Reagent was added at a final concentration of 5 μM to the cells and incubated for 30 minutes at room temperature protected from light. After 30 min incubation, well contents were removed and cells were washed three times with Dulbecco's phosphate-buffered saline (DPBS). Cells were then preserved by fixation with 100 μl of 3.7% formaldehyde in DPBS for 15 minutes. The fixative was removed and 200 μl of 1% BSA in DPBS $well^{-1}$ was added. The fluorescent signal was analyzed within 2 hours on both the SpectraMax® M4 microplate reader (Molecular Devices) and the Amnis FlowSight® image-based flow cytometer (MilliporeSigma, Billerica, MA).

Respiratory Burst Assay - Plate Layout												
	1	2	3	4	5	6	7	8	9	10	11	12
A LPS	FISH 1	FISH 1	FISH 2	FISH 2	FISH 3	FISH 3	FISH 4	FISH 4	FISH 5	FISH 5	FISH 6	FISH 6
B LPS	FISH 7	FISH 7	FISH 8	FISH 8	FISH 9	FISH 9	FISH 10	FISH 10	FISH 11	FISH 11	FISH 12	FISH 12
C LPS	FISH 13	FISH 13	FISH 14	FISH 14	FISH 15	FISH 15	FISH 16	FISH 16	FISH 17	FISH 17	FISH 18	FISH 18
D LPS	FISH 19	FISH 19	FISH 20	FISH 20								
E MEDIA	FISH 1	FISH 1	FISH 2	FISH 2	FISH 3	FISH 3	FISH 4	FISH 4	FISH 5	FISH 5	FISH 6	FISH 6
F MEDIA	FISH 7	FISH 7	FISH 8	FISH 8	FISH 9	FISH 9	FISH 10	FISH 10	FISH 11	FISH 11	FISH 12	FISH 12
G MEDIA	FISH 13	FISH 13	FISH 14	FISH 14	FISH 15	FISH 15	FISH 16	FISH 16	FISH 17	FISH 17	FISH 18	FISH 18
H MEDIA	FISH 19	FISH 19	FISH 20	FISH 20								

Figure 8. Plate layout for respiratory burst assay. Rows A-D are treated with LPS and rows E-H are the negative controls.

Stimulation index (SI) values were used to compare the variation between LPS-treated and unstimulated (negative control) leukocytes. For data collected using the microplate reader SI values were calculated based on equation 4, where OD_t is the mean optical density (fluorescence) of replicate wells that were treated with LPS and OD_c is the mean optical density (fluorescence) of the unstimulated negative control leukocytes in replicate wells.

$$SI = \frac{OD_t}{OD_c}$$

Equation 4. Stimulation indices for LPS-induced respiratory burst for data collected with the SpectraMax® M4 microplate reader.

For data collected using the image-based flow cytometer SI values were calculated based on equations 5a-c. Using data from the pooled replicate of leukocytes from the same fish, the ratio of positive to negative cells in the stimulated replicate (F_t) was divided by the number of positive to negative cells in unstimulated (negative control) replicate (F_c).

$$\mathbf{5a}. \qquad SI = \frac{F_t}{F_c}$$

$$\mathbf{5b}. \qquad F_t = \frac{positively\ fluoresced\ cells}{negative\ cells}$$

$$\mathbf{5c}. \qquad F_c = \frac{positively\ fluoresced\ cells}{negative\ cells}$$

Equation 5a-c. Stimulation indices for LPS-induced respiratory burst for data collected with the Amnis FlowSight® image-based flow cytometer.

Mitogenesis assay

The mitogen-induced proliferative response was evaluated following the bromo-deoxyuridine (BrdU) based ELISA procedure from Gauthier et al. (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) was used as an alternative to the second half of the BrdU assay.

Isolated leukocytes at 2 x 10^7 viable cells ml^{-1} in CM (L-15/5% P/S) were plated at 50 µl $well^{-1}$ to be treated at 50 µl $well^{-1}$ with either a mitogen or mitogen-free media (negative control wells; see plate layout, Figure 9). Working concentrations of mitogens were prepared as described for the bromo-deoxyuridine (BrdU) based ELISA procedure from Gauthier (Gauthier et al. 2003).

Mitogenesis CON A & PHA-P: Plate 1												
	1	2	3	4	5	6	7	8	9	10	11	12
A CON A	FISH 1	FISH 1	FISH 2	FISH 2	FISH 3	FISH 3	FISH 4	FISH 4	FISH 5	FISH 5	FISH 6	FISH 6
B CON A	FISH 7	FISH 7	FISH 8	FISH 8	FISH 9	FISH 9	FISH 10	FISH 10	FISH 11	FISH 11	FISH 12	FISH 12
C PHA-P	FISH 1	FISH 1	FISH 2	FISH 2	FISH 3	FISH 3	FISH 4	FISH 4	FISH 5	FISH 5	FISH 6	FISH 6
D PHA-P	FISH 7	FISH 7	FISH 8	FISH 8	FISH 9	FISH 9	FISH 10	FISH 10	FISH 11	FISH 11	FISH 12	FISH 12
E MEDIA	FISH 1	FISH 1	FISH 2	FISH 2	FISH 3	FISH 3	FISH 4	FISH 4	FISH 5	FISH 5	FISH 6	FISH 6
F MEDIA	FISH 7	FISH 7	FISH 8	FISH 8	FISH 9	FISH 9	FISH 10	FISH 10	FISH 11	FISH 11	FISH 12	FISH 12
G												
H												

Mitogenesis CON A & PHA-P: Plate 2												
	1	2	3	4	5	6	7	8	9	10	11	12
A CON A	FISH 13	FISH 13	FISH 14	FISH 14	FISH 15	FISH 15	FISH 16	FISH 16	FISH 17	FISH 17	FISH 18	FISH 18
B CON A	FISH 19	FISH 19	FISH 20	FISH 20								
C PHA-P	FISH 13	FISH 13	FISH 14	FISH 14	FISH 15	FISH 15	FISH 16	FISH 16	FISH 17	FISH 17	FISH 18	FISH 18
D PHA-P	FISH 19	FISH 19	FISH 20	FISH 20								
E MEDIA	FISH 13	FISH 13	FISH 14	FISH 14	FISH 15	FISH 15	FISH 16	FISH 16	FISH 17	FISH 17	FISH 18	FISH 18
F MEDIA	FISH 19	FISH 19	FISH 20	FISH 20								
G												
H												

Mitogenesis LPS: Plate 1												
	1	2	3	4	5	6	7	8	9	10	11	12
A LPS	FISH 1	FISH 1	FISH 2	FISH 2	FISH 3	FISH 3	FISH 4	FISH 4	FISH 5	FISH 5	FISH 6	FISH 6
B LPS	FISH 7	FISH 7	FISH 8	FISH 8	FISH 9	FISH 9	FISH 10	FISH 10	FISH 11	FISH 11	FISH 12	FISH 12
C LPS	FISH 13	FISH 13	FISH 14	FISH 14	FISH 15	FISH 15	FISH 16	FISH 16	FISH 17	FISH 17	FISH 18	FISH 18
D LPS	FISH 19	FISH 19	FISH 20	FISH 20								
E MEDIA	FISH 1	FISH 1	FISH 2	FISH 2	FISH 3	FISH 3	FISH 4	FISH 4	FISH 5	FISH 5	FISH 6	FISH 6
F MEDIA	FISH 7	FISH 7	FISH 8	FISH 8	FISH 9	FISH 9	FISH 10	FISH 10	FISH 11	FISH 11	FISH 12	FISH 12
G MEDIA	FISH 13	FISH 13	FISH 14	FISH 14	FISH 15	FISH 15	FISH 16	FISH 16	FISH 17	FISH 17	FISH 18	FISH 18
H MEDIA	FISH 19	FISH 19	FISH 20	FISH 20								

Figure 9. Plate layouts for mitogenesis assay. Rows A-D are the treated with mitogens (light blue-Con A, dark blue-PHAP, brown-LPS) and rows E-H are the negative control leukocytes.

Mitogens used were concanavalin A (Con A) at 10 μg ml^{-1} (0.25 μg $well^{-1}$ final concentration), phytohemagglutinin (PHA-P) at 20 μg ml^{-1} (0.5 μg $well^{-1}$ final concentration), and lipopolysaccharide from *E. coli* O111:B4 (LPS) at 100 μg ml^{-1} (2.5 μg $well^{-1}$ final concentration). Mitogen treated and control wells were replicated in duplicate in the same plate. After addition of mitogens, plates were incubated in a humidified container at 17 °C for 24 hours before the addition of EdU. Following 24 h incubation, twenty-five μl $well^{-1}$ of EdU in unsupplemented L-15 was added (EdU final concentration = 13 μM $well^{-1}$) to mitogen treated and control wells. Plates were returned to humidified container protected from light and incubated an additional 18 h. Total incubation time with mitogens was 42 h. After 18 h incubation, cell replication was detected using the Click-iT® EdU (5-ethynyl-2'-deoxyuridine) Flow Cytometry Assay Kit (Molecular Probes, Eugene, OR) following the manufacturer's protocol (MP 10419).

Briefly, culture supernatants were removed from all wells and leukocytes were washed with 100 μl $well^{-1}$ of solution consisting of 1% bovine serum albumin (BSA) in Dulbecco's phosphated buffered saline (1% BSA in DPBS). The 1% BSA in DPBS was removed and replaced with 25 μl $well^{-1}$ of Click-iT® fixative (Component D) for leukocytes to be fixed at room temperature for 15 min. Fixative was removed and cells were washed as described above before adding 100 μl $well^{-1}$ of 1X Click-iT® saponin-based permeabilization and wash reagent (Component E). The DBPS- based Click-iT reaction cocktail containing Alexa Fluor® 647 azide dye was prepared according to the manufacturers protocol (MP 10419) and 100 μl $well^{-1}$ was added for labeling incorporated EdU. Labeling occurred over 30 min at room temperature protected from light. After labeling, reaction cocktail was removed and cells were washed 1x with 100 μl

well^{-1} of Click-iT saponin-based permeabilization and wash reagent and then 200 µl well^{-1} of the same reagent was added in preparation for analysis using the Amnis FlowSight® image-based flow cytometer (MilliporeSigma, Billerica, MA).

For analysis on the flow cytometer, cells from replicate wells were pooled prior to data acquisition. Instrument settings were as follows: Illumination – 642 laser at 100.0 mV, 785 laser at 5.00 mV; Fluidics – minimum flow speed. Cell analysis was performed using IDEAS® version 6.2 (Amnis/MilliporeSigma). Primary gating was performed on initial cell-image data to isolate individual round cells that were in good focus. This subset was then divided further to fluorescent positive and negative cells by cell width. Cell widths considered ranged from 4 to 20 µm. Following initial analysis, a template was created containing the primary and secondary gating. This template was used to analyze all data sets to provide consistent analytics across samples.

Stimulation index (SI) values were used to compare the variation between mitogen-treated and unstimulated (negative control) leukocytes by dividing fluorescence of stimulated cells by fluorescence from unstimulated cells from the same fish. Calculations were made for data collected using the image based flow cytometer using equations 5a-c.

In an attempt to validate the cell sizes in my study, I looked at a subpopulation of cells isolated from a small subset of laboratory-reared fish microscopically during one of the mitogenesis assay runs. Smears of cell suspensions taken directly from the mitogenesis plate were made on glass slides and stained with Hema 3™ (FisherScientific, Hampton, NH) or Giemsa stain (Sigma-Aldrich, St. Louis, MO).

Statistical analysis

All data were tested for normality using Shapiro Wilkes W test and homogeneity of variances using Levene's test. Differences were considered statistically significant when $p \leq 0.05$. Statistical analyses were performed using R (version 3.2.2). Nonparametric statistical tests were used because sample sizes were small (<30) and data were not normally distributed. Chi-square (2x2 table) was used to compare histology (presence, absence data) between the two field sites. Fisher's exact test was used when expected counts were below 5. Kruskal-Wallis was used to compare bactericidal activity and mitogenesis results across multiple categories (*i.e.* field sites or cell sizes). Significant results were followed up with post hoc testing using pairwise Wilcoxon rank sum testing. The Mann-Whitney U test was used to compare respiratory burst results because there were only two groups to compare (field sites). This test is the nonparametric equivalent to an independent two sample t-test. All graphs were created using SPSS version 24 (IBM Analytics, Armonk, NY).

Results

A total of 72 smallmouth bass were collected from the Upper Juniata River (n=20), Tionesta Lake (n=12) and the USGS Fish Health Research Laboratory in Kearneysville, WV (n=40). Wild SMB were collected in May 2016 and the lab SMB were collected in May 2017. Lab fish were sampled twice because they were smaller and cell yields were only enough for one immune assay at a time (20 fish for bactericidal assay and 20 fish for mitogenesis assay). Condition factors were determined for individual fish. There were no significant differences in condition factors between the three sites (Figure 10).

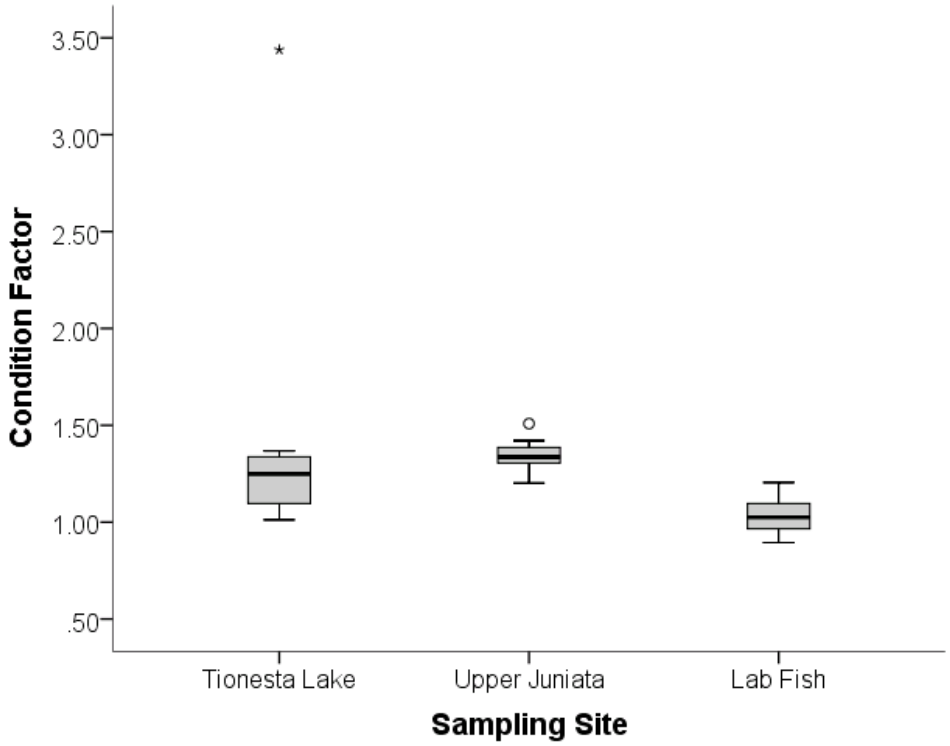

Figure 10. Condition factors for smallmouth bass. Determined using Fulton's condition factor equation, $K = W / L^3$, (Nash et al. 2006), where K is Fulton's condition factor, W is weight of the fish and L represents length of the fish. Horizontal bar = median. Box = interquartile (IQ) range which contains the middle 50% of the data. Error bars extend to highest and lowest values which are no greater than 1.5 times the IQ range. Small circle and asterisk indicate outliers not used in the analysis. Small circle = outlier more than 1.5 times the IQ range. Asterisk = extreme outlier more than 3 times the IQ range.

Development of mitogenesis assay

Adaptive immune responses were determined by measuring mitogenesis in isolated leukocytes from smallmouth bass anterior kidney. Responses to mitogens were considered positive if the stimulation index was 1.00 or greater. Different concentrations of EdU were not tested here. Data collected using image-based flow cytometry was compared to a more classical method (microplate reader) to validate the newer method.

No differences were seen in stimulation indices between collection methods by comparing the total population of cells.

Cell population analysis

Analysis of mitogenesis using image-based flow cytometry was separated into five subgroups of cell sizes based on relative proportions within the total gated population (2739-9319 cells gated, 27-47% of total population): Total gated (4-20 μm), 4-7 μm, 7-12 μm, 12-15 μm, and 15-20 μm (Figure 11). This allowed a more detailed analysis. Cells between 7 and 12 μm were the most abundant out of the total gated population from all sites.

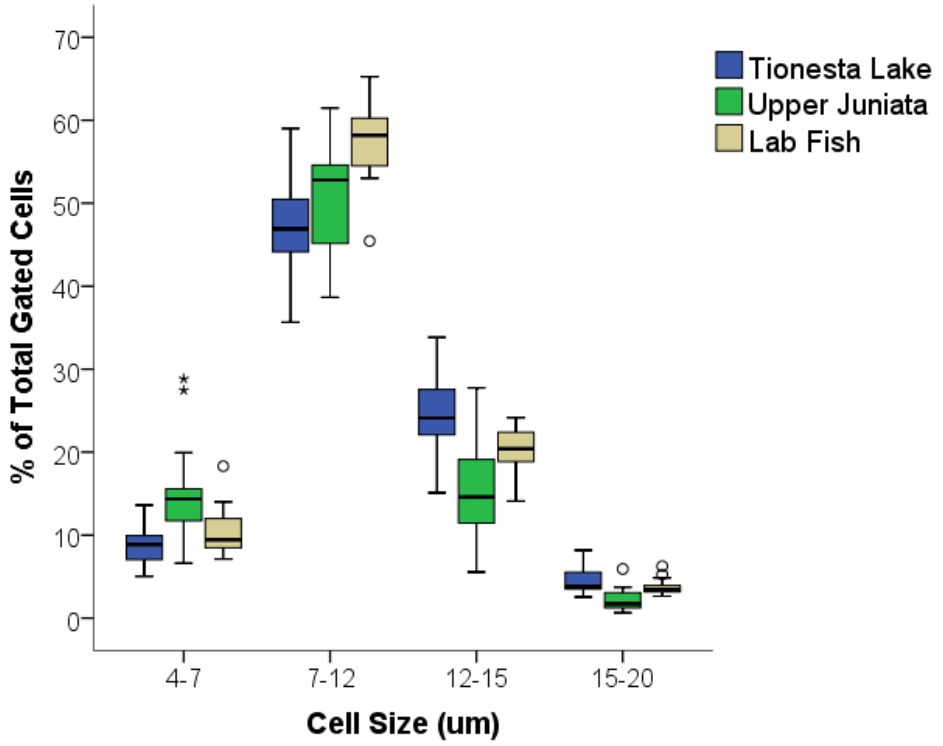

Figure 11. Cell size distributions as percentage of the total gated cell population. Total events captured on the Amnis FlowSight® image-based flow cytometer ranged from 10,000 to 20,000. Total events in focus ranged from 2739 to 9319 cells. Horizontal bar = median. Box = interquartile (IQ) range which contains the middle 50% of the data. Error bars extend to highest and lowest values which are no greater than 1.5 times the IQ range. Small circle and asterisk indicate outliers not used in the analysis. Small circle = outlier more than 1.5 times the IQ range. Asterisk = extreme outlier more than 3 times the IQ range.

Lymphocyte proliferation in laboratory-reared fish

Total gated leukocytes from laboratory-reared fish were most sensitive to Con A and PHAP when compared to LPS ($p = 0.007$ and $p < 0.001$, respectively; Figure 12). The difference in stimulation indices between Con A and PHAP for total gated cells was not significant (Figure 12).

Differences in stimulation indices were dependent upon cell size. In laboratory-reared fish, more positive responses to Con A were seen in the smaller cells and more positive responses to PHAP were seen in the larger subpopulations of cells. LPS positively stimulated both the smallest and largest subpopulations. No significant differences were found between subpopulations in response to Con A, but the smaller cells (4-12 μm) did have more positive responses than the larger cells (12-20 μm) as indicated by the red line in Figure 12. Larger cells (15-20 μm) responded significantly more to PHAP than the smaller cells (4-12 μm; $p = 0.01$). Larger cells (15-20 μm) also responded significantly more to LPS than smaller cells (7-15 μm; $p < 0.001$) and although not significant, the smallest cell population (4-7 μm) did have more positive responses than 7-15 μm cells as indicated by the red line in Figure 12.

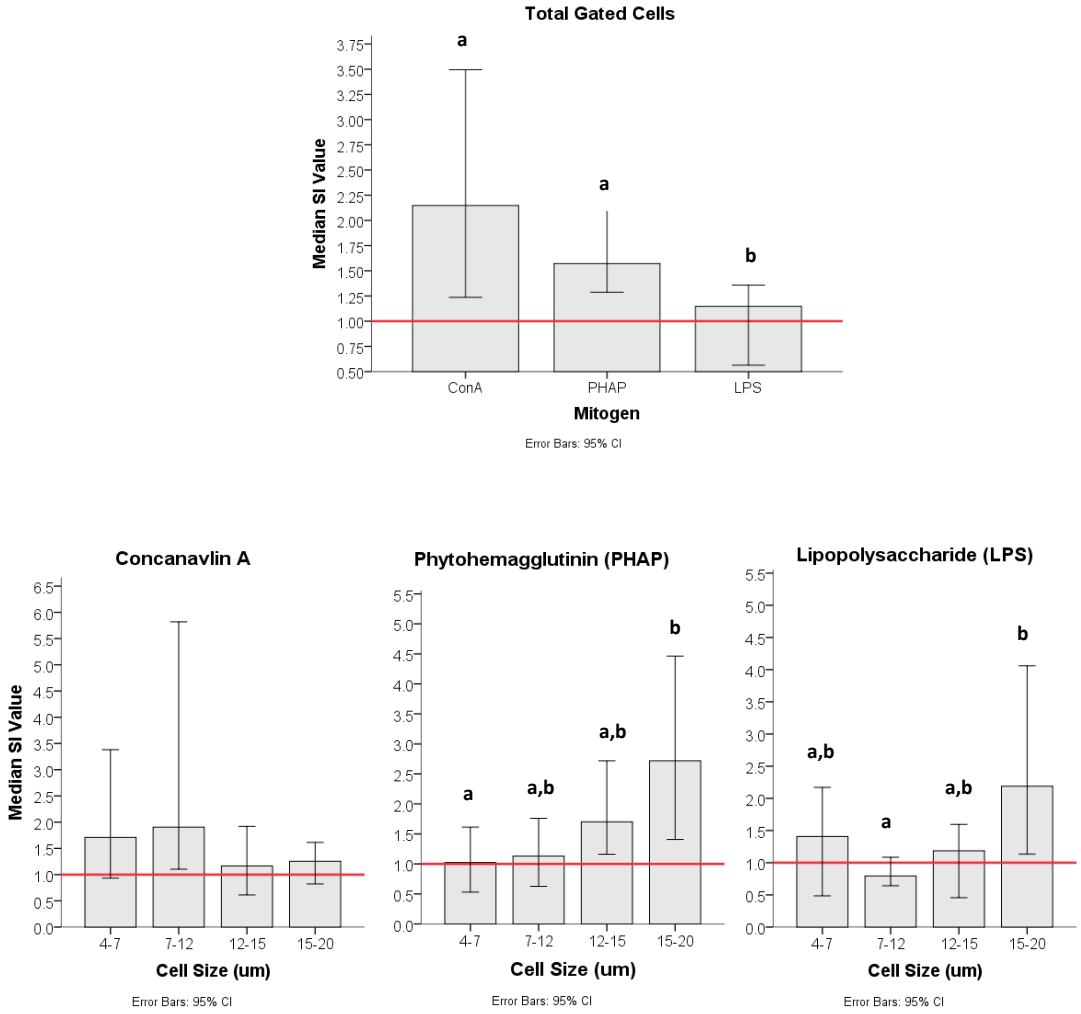

Figure 12. Laboratory-reared smallmouth bass leukocyte responses to EdU mitogenesis assay. Displayed are responses for total gated cells (top) and for each subpopulation (bottom). Fish were sampled in May 2017 from USGS National Fish Health Research Laboratory in Kearneysville, WV and data were collected on the Amnis FlowSight® image-based flow cytometer (MilliporeSigma). Mitogenesis was measured by fluorescence of EdU incorporated into DNA of S phase cells. Red line indicates a positive stimulation index (SI > 1). Error bars indicate 95% confidence intervals (CI). Groupings with different letters are significantly different (Kruskal-Wallis and Wilcoxon rank sum post-hoc test, $p < 0.05$). No letter indicates no significant differences.

Total gated cell analysis

In general, the stimulation indices for all mitogens were highest in the lab fish when compared to wild fish (Figure 13). During May of 2016, the stimulation indices for all mitogens did not differ significantly among field sampling sites (TL and JUP) when looking at the total gated cell population (Figure 13). Similar to the lab fish, leukocytes isolated from SMB collected in the field also showed the most sensitivity to Con A (Figure 13).

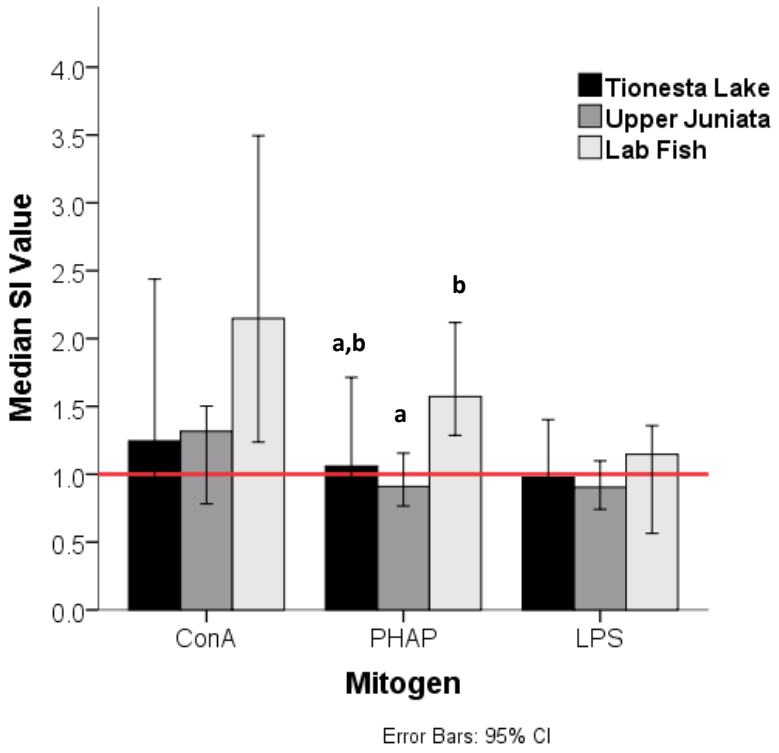

Figure 13. Smallmouth bass leukocyte mitogenesis responses for the total gated cell population. Mitogenesis was measured by fluorescence of EdU incorporated into DNA of S phase cells using the Amnis FlowSight® image-based flow cytometer (MilliporeSigma). The red line indicates a positive stimulation index (SI >1). Error bars = 95% CI. Sites with different letters are significantly different (Kruskal-Wallis and Wilcoxon rank sum post-hoc test, $p < 0.05$). No letter indicates no significant differences.

Analysis of subpopulations

Comparable to the lab fish, differences in stimulation indices from wild fish were also dependent upon cell size (Figures 14-16). Leukocytes isolated from SMB collected at Upper Juniata showed no positive responses to PHAP or LPS when looking at total gated population (Figure 13), but they do show positive responses to PHAP in the 12-15 μm subpopulation (Figure 14). Leukocytes isolated from SMB collected at Tionesta Lake showed no positive responses to LPS when looking at total gated population (Figure 13), but they do show positive responses to LPS in the 4-7 μm subpopulation (Figure 16).

The highest stimulation indices for T cell mitogens (PHAP and Con A) overall including lab fish were seen in a large range of cell sizes (7-20 μm; Figures 14 and 15, respectively), while the highest stimulation indices for the B cell mitogen (LPS) overall were only seen in the smallest (4-7 μm) and the largest (15-20 μm) cell sizes (Figure 16).

The trends for wild SMB leukocyte responses to PHAP are similar to those of the lab fish, with larger cells responding more than the smallest cells (Figure 14). Unlike the lab fish, however, the wild fish collected from Upper Juniata exhibited greatest sensitivity to PHAP in the 12-15 μm subpopulation rather than the 15-20 μm subpopulation (Figure 14). Unlike the lab fish, leukocyte responses to Con A in fish collected from Upper Juniata exhibited an opposite trend with the larger cells showing more sensitivity to Con A than the smaller cells (Figure 15).

The trends for wild SMB leukocytes in response to LPS are similar to the lab fish except for the largest subpopulation (15-20 μm; Figure 16). Leukocytes from SMB collected at the Upper Juniata site showed no response to LPS in any subpopulation

(Figure 16), which is consistent to what is seen in the total gated cell population shown in Figure 13.

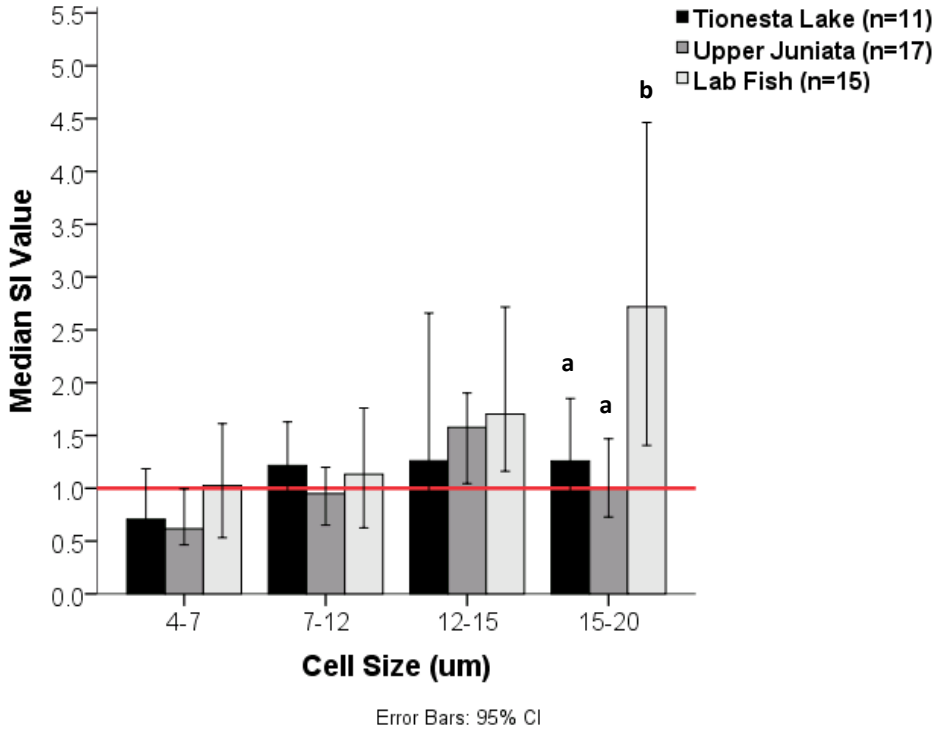

Figure 14. Smallmouth bass leukocyte mitogenesis responses to PHAP grouped by cell size. Mitogenesis was measured by fluorescence of EdU incorporated into DNA of S phase cells using the Amnis FlowSight® image-based flow cytometer (MilliporeSigma). The red line indicates a positive stimulation index (SI >1). Error bars = 95% CI. Sites with different letters are significantly different (Kruskal-Wallis and Wilcoxon rank sum post-hoc test, $p < 0.05$). No letter indicates no significant differences.

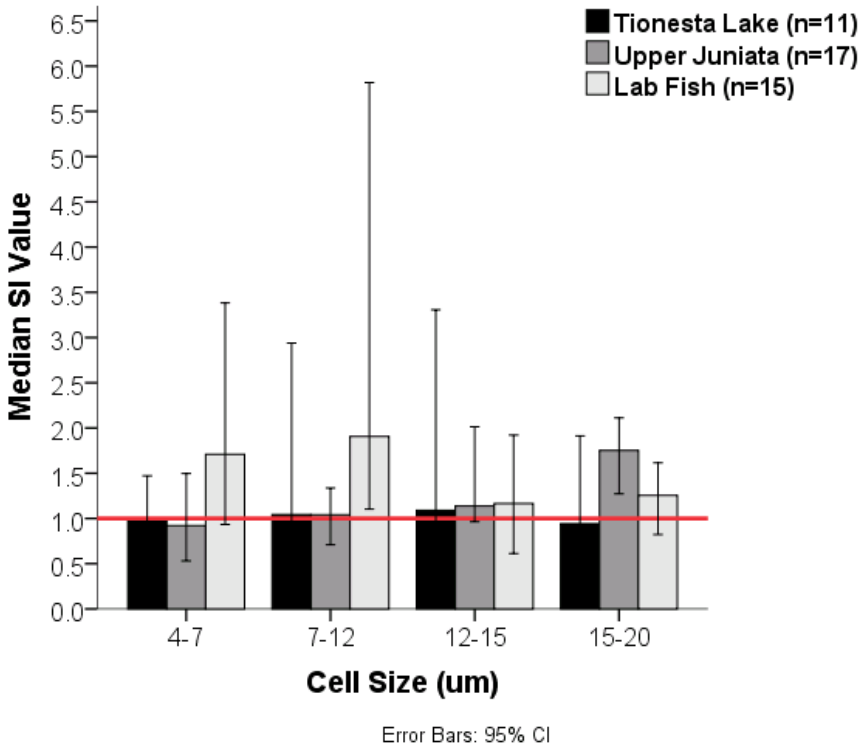

Figure 15. Smallmouth bass leukocyte mitogenesis responses to Con A grouped by cell size. Mitogenesis was measured by fluorescence of EdU incorporated into DNA of S phase cells using the Amnis FlowSight® image-based flow cytometer (MilliporeSigma). The red line indicates a positive stimulation index (SI >1). Error bars = 95% CI. No differences among sites.

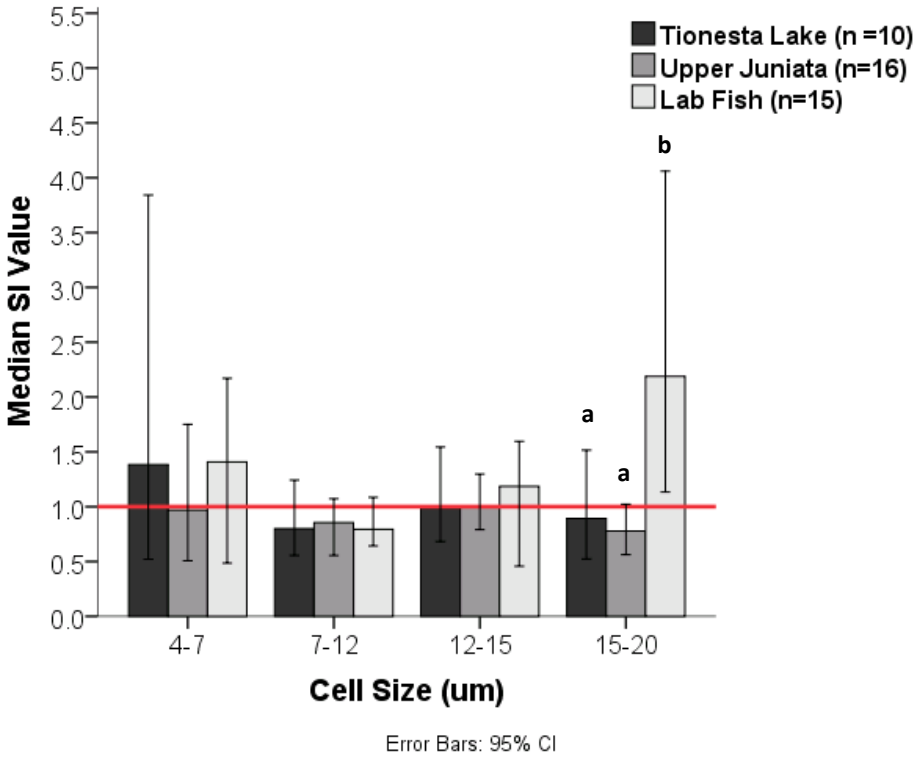

Figure 16. Median mitogenesis responses to LPS grouped by cell size. Mitogenesis was measured by fluorescence of EdU incorporated into DNA of S phase cells using the Amnis FlowSight® image-based flow cytometer (MilliporeSigma). The red line indicates a positive stimulation index (SI >1). Error bars = 95% CI. Sites with different letters are significantly different (Kruskal-Wallis and Wilcoxon rank sum post-hoc test, $p < 0.05$). No letter indicates no significant differences.

Adherent cell (macrophage) function

Nonspecific immune responses were determined by measuring bactericidal and respiratory burst activity in adherent cells isolated from smallmouth bass anterior kidney. Bactericidal activity was significantly lower in smallmouth at Tionesta Lake (Mdn = 0%) during May 2016 compared to smallmouth from the Upper Juniata (Mdn = 4.12%, $p = 0.0119$) or the lab (Mdn = 26.63%, $p = 0.0033$). No significant differences were observed between SMB from the Upper Juniata and the lab fish. Although not significant for the

Upper Juniata, bactericidal activity was still highest in smallmouth from the lab when compared to both field sites (Figure 17). Figure 18 microscopically shows macrophages isolated from laboratory fish engulfing *A. sobria*.

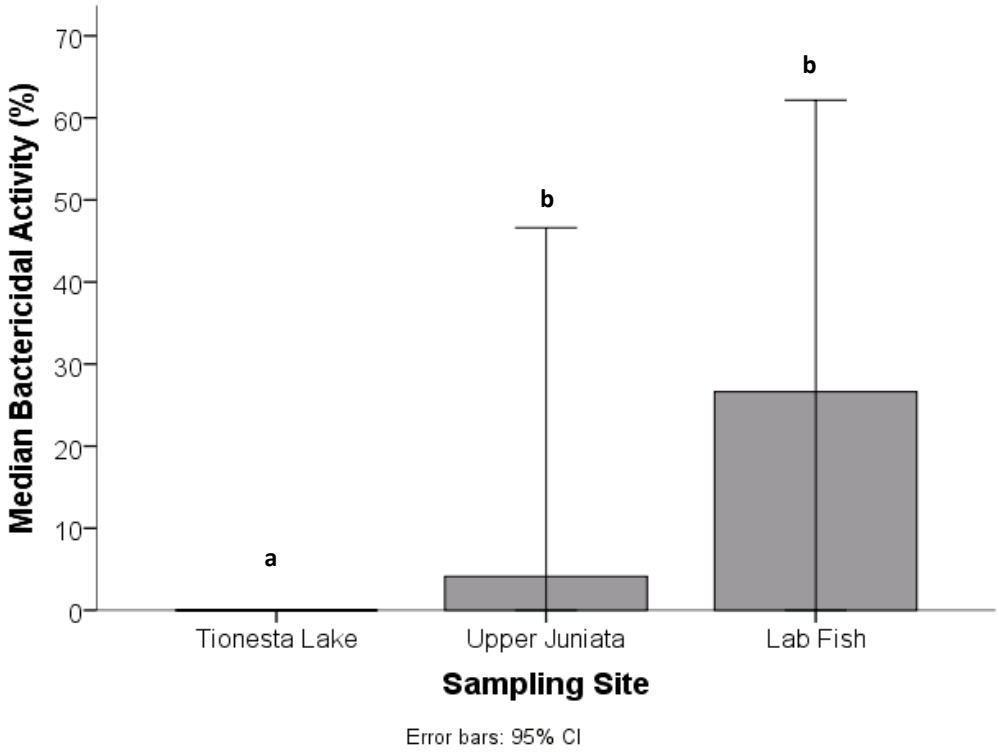

Figure 17. Bactericidal activity towards *A. sobria*. Activity was expressed as the percentage CFU reduction achieved when SMB leukocytes were exposed to *A. sobria* and subsequently plated on tryptic soy agar. Error bars = 95% CI. Sites with different letters are significantly different (Kruskal-Wallis and Wilcoxon rank sum post-hoc test, $p < 0.05$).

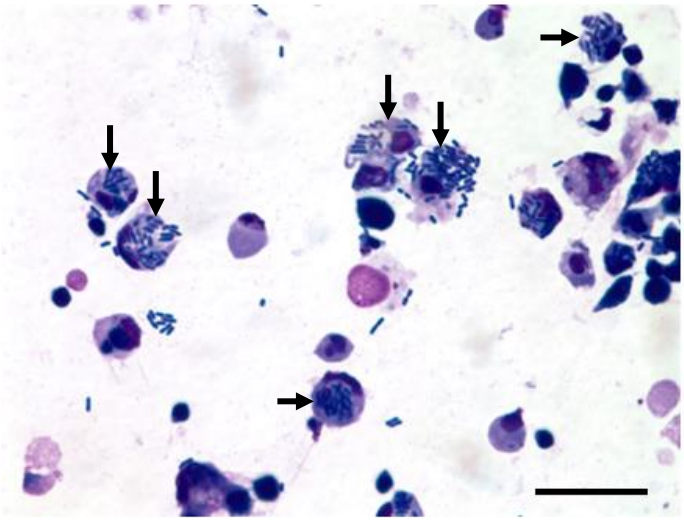

Figure 18. Macrophages engulfing *A. sobria* (arrows). Blood smear collected from laboratory fish in Kearneysville, WV. Cell suspensions were taken directly from the bactericidal plate during assay and smeared on glass slides. Hema 3™ stain, bar equals 20 μm.

Similar results were observed in smallmouth in regards to background ROS activity. Background ROS was statistically lower at Tionesta Lake (Mdn = 31.44) during May 2016 compared to Upper Juniata (Mdn = 35.12, p = 0.0036; Figure 19). Bactericidal activity and background ROS activity in smallmouth collected from Upper Juniata were negatively associated based on trendline (Figure 20). A Spearman's rank-order correlation was run to better determine the relationship in Figure 20 and the results were significant (r_s = -0.491, p = 0.05). No comparison can be made for Tionesta Lake due to the absence of bactericidal activity at this site.

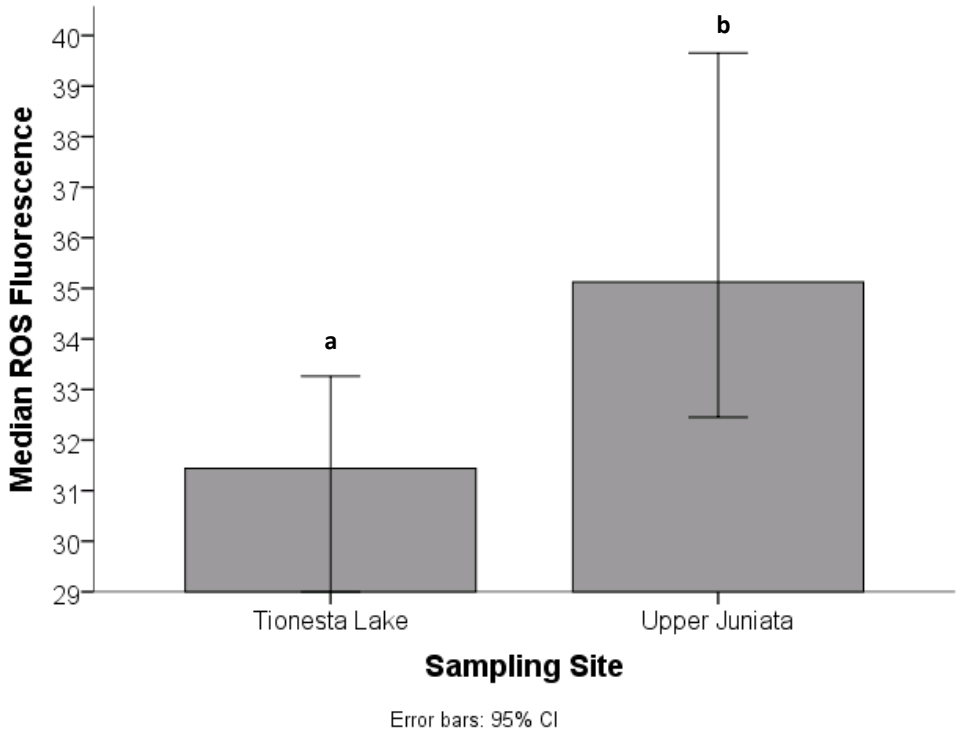

Figure 19. Background (unstimulated by LPS) respiratory burst in SMB leukocytes. Respiratory burst was measured by fluorescence of CellROX® Deep Red Reagent using a SpectraMax® M4 microplate reader. This measurement represents the total fluorescence of all cells in each well of a 96-well plate. The fluorescence measured by the microplate reader is proportional to the amount of ROS produced by the cells. Error bars = 95% CI. Sites with different letters are significantly different (Mann-Whitney U test, $p < 0.05$).

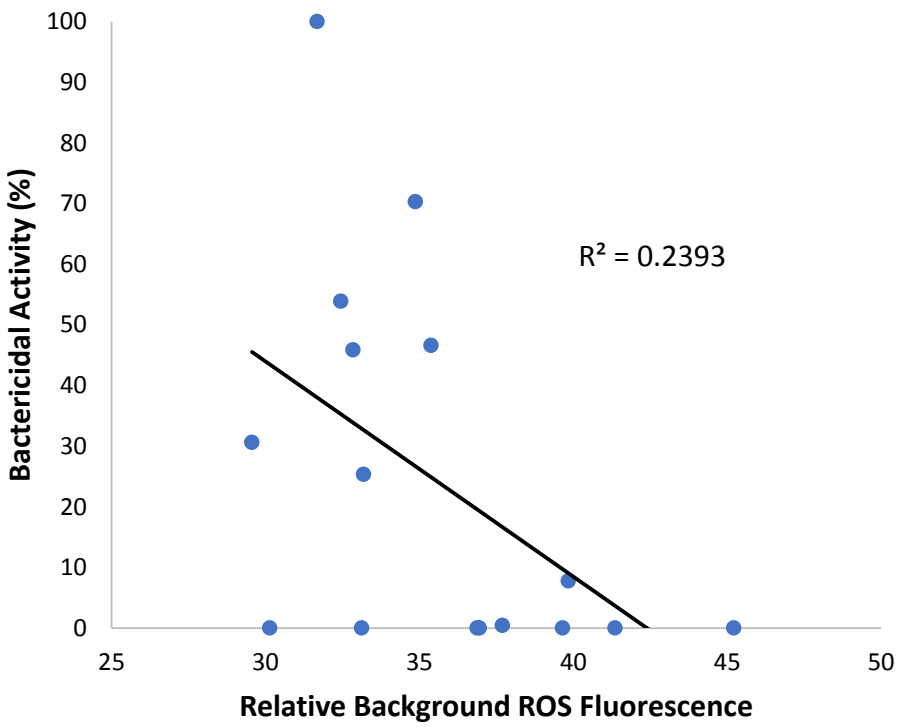

Figure 20. Bactericidal activity relative to background ROS fluorescence in SMB collected from the Upper Juniata River. Bactericidal activity was determined against the pathogen *Aeromonas sobria*. Background ROS fluorescence was measured using the SpectraMax® M4 microplate reader. Spearman's rank order correlation, r_s = -0.491, p = 0.05.

In regards to LPS-induced respiratory burst activity, no differences were observed between Tionesta Lake and the Upper Juniata River. LPS did not produce strong ROS responses in smallmouth bass leukocytes. The median stimulation index for both wild sites was 1.0, meaning the cells stimulated with LPS did not respond any more than the cells not stimulated with LPS. No comparisons are made to the lab fish because the respiratory burst assay was run using a different method for the lab fish.

Histopathology

The entire anterior kidney was used for the functional assays and so the microscopic pathology of the spleen was used to identify factors that may modulate the immune response. No parasites or signs of infectious disease were present in the laboratory fish with the exception of a few fish which had a nematode free in the peritoneal cavity. Wild fish from the two sites had very different pathological lesions. Bass from Tionesta Lake had very few encysted trematodes that appeared to be alive but did have many granulomas that appeared to contain killed and degenerating parasites (Figure 21A). Macrophage aggregates were also present (Figure 21A). In some cases, these granulomas contained no identifiable material (Figure 21B). These granulomas were often surrounded by epitheloid-appearing macrophages (Figure 21B). Spleen tissue from bass collected at the Upper Juniata site had granulomas that did not appear to be parasite-related. Many were small, encysted areas that contained lipofusin or ceroid and others that contained basophilic material (Figure 21C). There were clusters of these granulomas often replacing much of the normal spleen tissue and often surrounded by chronic inflammation (Figure 21C). In addition there were granulomas similar to those seen in Mycobacterial infections in fish as well as encysted parasites (Figure 21D). The prevalence of granulomas in the spleen was significantly higher (χ^2=8.3, p=0.004) in SMB from the Upper Juniata (12/20 fish) compared to those from Tionesta Lake (1/12 fish). Further studies will be needed to definitively identify the pathogens, parasites or other factors leading to this response.

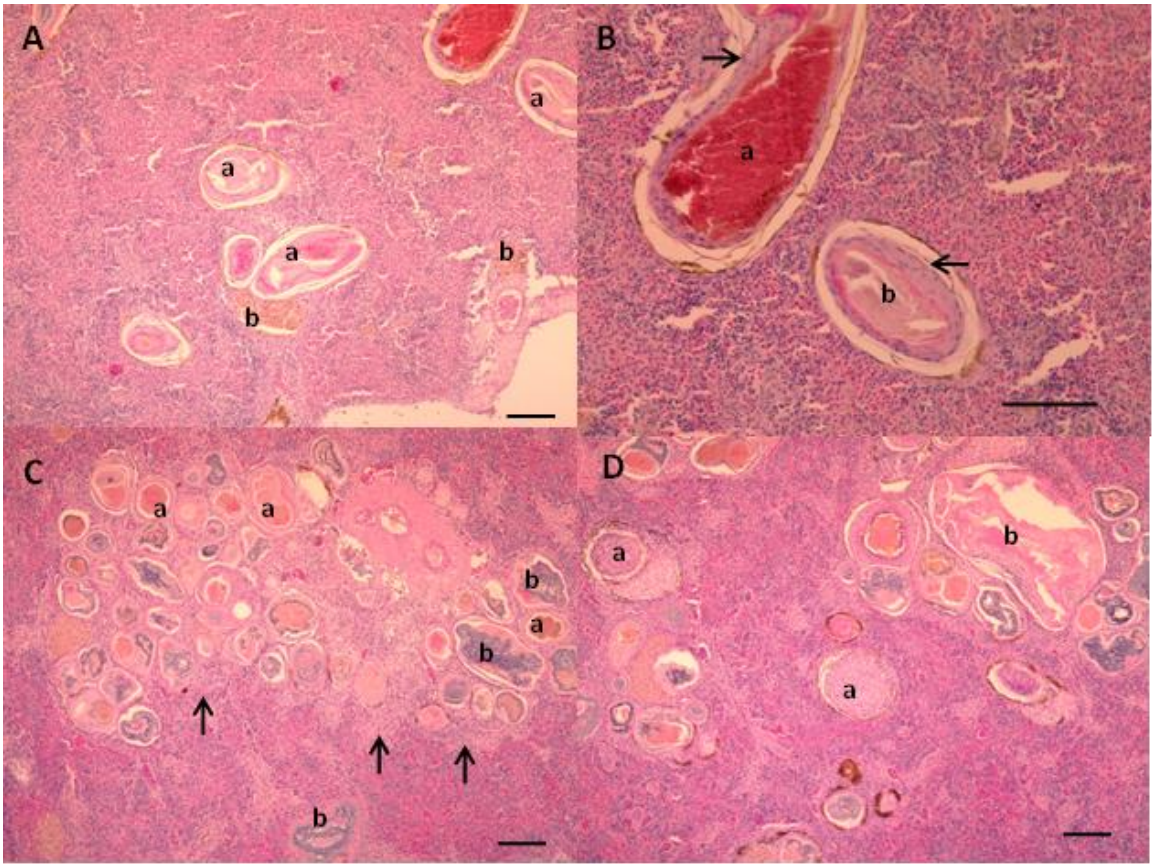

Figure 21. Microscopic pathology observed in smallmouth bass collected from Tionesta Lake and the upper Juniata River. A. Section of spleen from a Tionesta Lake bass illustrating granulomas with remnants of helminth parasites (a), probably trematodes and macrophage aggregates (b). Scale bar equals 50 µm. B. Section of spleen from a Tionesta Lake bass illustrating a granuloma containing unidentified material (a) and one most likely parasite-related (b). Both are surrounded by macrophages termed epitheliod-like (arrows). Scale bar equals 50 µm. C. Section of spleen from an upper Juniata bass illustrating an area of multiple granulomas some with yellowish-brown material that is lipofuscin or ceroid (a) and some that contain unidentified basophilic material (b). In the parenchyma around this area there is a chronic inflammatory response consisting primarily of macrophages (arrows). Scale bar equals 50 µm. D. Section of a spleen from an upper Juniata bass illustrating granulomas that do not appear to be parasite-related but are similar to those observed in response to Mycobacterial infections (a) and a parasite-associated granuloma (b). Scale bar equals 50 µm. Hematoxylin and eosin stain.

Macrophage aggregates (MA) were quantified in the spleen by counting the total number present in two 10X fields for each fish from both rivers. MA counts in the spleen of SMB from the Upper Juniata exhibited a biphasic relationship with background ROS activity based on insertion of a trendline (Figure 22A). There was a slight negative association between MA counts in the spleen and background ROS activity in SMB from Tionesta Lake based on trendline (Figure 22B), but it was not significant by Spearman's rank-order correlation.

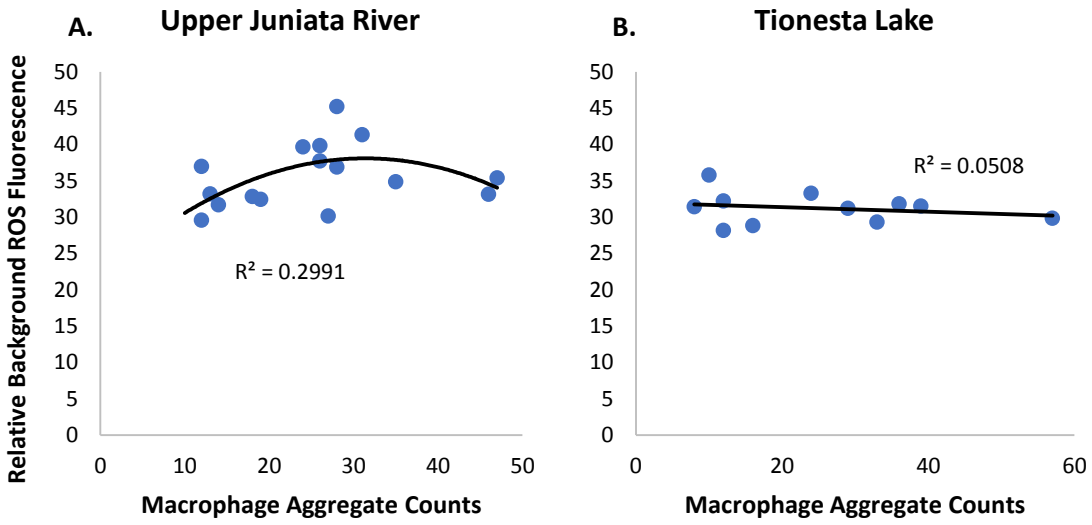

Figure 22. Relative background respiratory burst vs. macrophage aggregate counts in spleen tissue of SMB. (A) Upper Juniata River and (B) Tionesta Lake. Macrophage aggregates were quantified histologically by counting the total number present in two 10X fields. Relative background ROS fluorescence (a measure of ROS in cells unstimulated by LPS) was determined using the SpectraMax® M4 microplate reader.

Parasites were observed in the spleen of SMB from both the Upper Juniata River and Tionesta Lake. Individual SMB from each site were given a parasite rating, which ranged from 0 (not present) to 4 (abundant). Figure 23 shows associations of bactericidal activity and MA counts with parasite ratings in the spleen of SMB from the Upper

Juniata River (left) and Tionesta Lake (right). A Spearman's rank-order correlation was run to determine the relationships in Figure 23. Although there are slight trends based on trendline, there were no significant correlations. The relationship between bactericidal activity and parasite ratings at Tionesta Lake are not shown because bactericidal activity was 0% for all SMB collected (n=12). Mitogenesis responses in regards to parasite ratings in the spleen of SMB collected from the Upper Juniata River and Tionesta Lake are displayed in Figures 24 and 25, respectively.

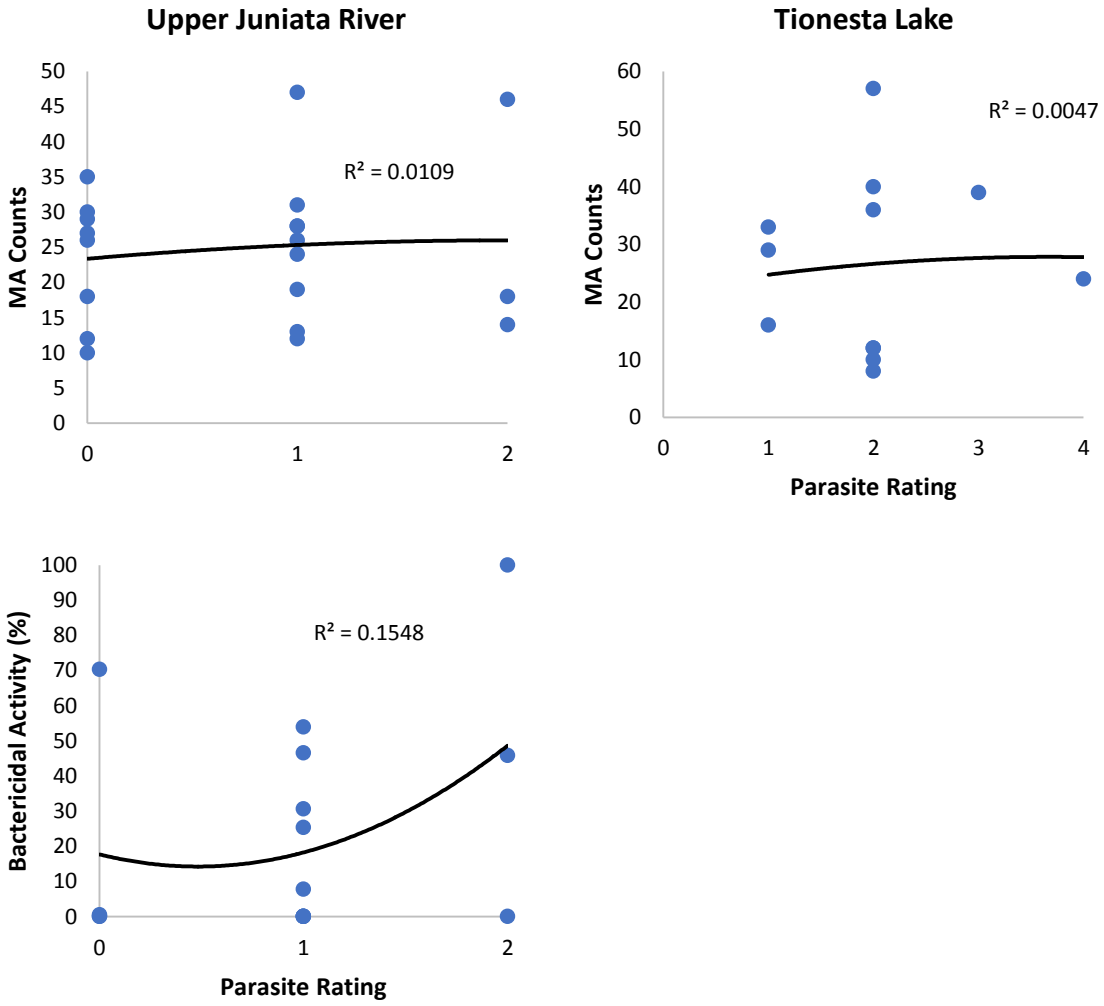

Figure 23. Parasite ratings, bactericidal activity, and MA counts in wild SMB. Upper Juniata River (left) and Tionesta Lake (right). No bactericidal activity is shown for Tionesta Lake because all activity was 0%. Parasite ratings ranging from 0 (not present) to 4 (abundant) were determined histologically from spleen tissue. Macrophage aggregates were quantified histologically by counting the total number present in two 10X fields.

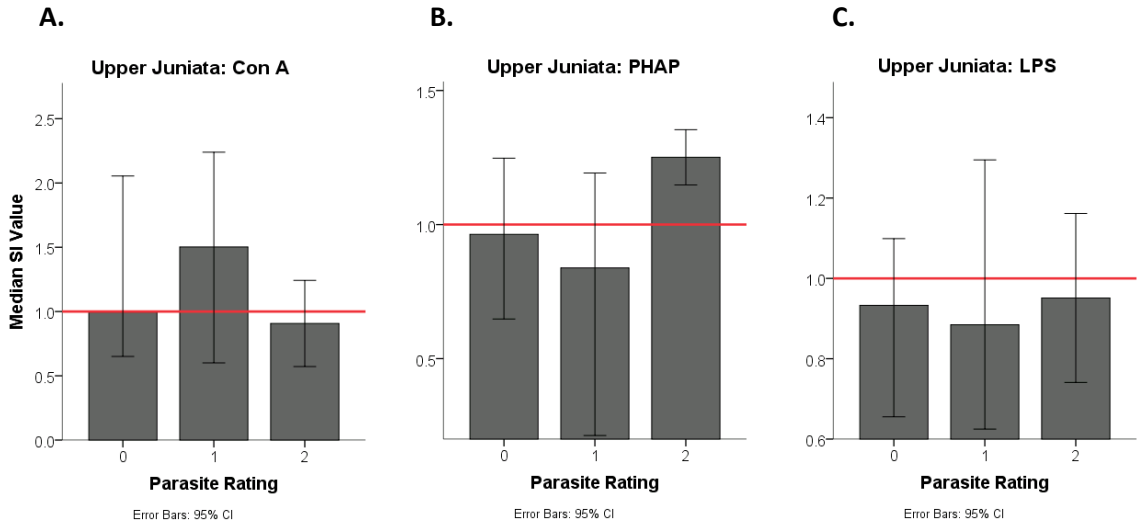

Figure 24. Upper Juniata median SI values for (A) Con A, (B) PHAP, and (C) LPS categorized by splenic parasite rating. The red line represents a positive SI value of 1 or greater. Error bars = 95% CI. (A) Positive responses to Con A were only seen for parasite rating of 1 (B) Positive responses to PHAP were only seen for parasite rating of 2 (C) No positive responses to LPS for any parasite rating.

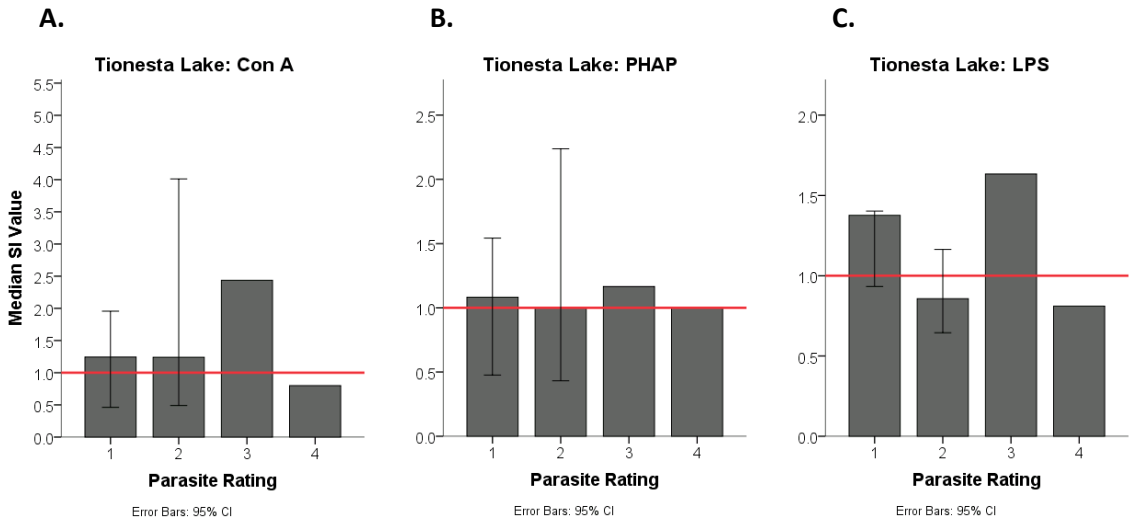

Figure 25. Tionesta Lake median SI values for (A) Con A, (B) PHAP, and (C) LPS categorized by splenic parasite rating. The red line represents a positive SI value of 1 or greater. Error bars = 95% CI. (A) Responses to Con A were positive for parasite ratings of 1-3 (B) Responses to PHAP were only positive for parasite ratings of 1 and 3 (C) Responses to LPS were positive for parasite ratings of 1 and 3. There are no error bars for parasite ratings of 3 or 4 because n=1 for these ratings.

Discussion

My study primarily focused on developing and assessing the feasibility of techniques to evaluate immune function in wild smallmouth bass (SMB). The techniques evaluated included *in vitro* assays for bactericidal activity, respiratory burst activity and mitogenesis. Multiple aspects of immune function were tested due to the complexity of the immune response. The immune network is so elaborate that often a single test is inadequate for evaluating immune response and therefore; multiple tests are recommended (Dunier and Siwicki 1993). The immune assays in this study were evaluated by comparing a) two sites with different surrounding land use and potential contaminant exposures and b) wild versus lab fish.

Responses to all three assays were decreased in fish collected at both field sites compared to the lab fish. Responses of the lab fish leukocytes in the EdU mitogenesis assay were surprising for the mitogens Con A and PHAP (Figure 12, bottom). Both of these mitogens should stimulate T lymphocytes. However, the highest responses to Con A were observed in the smaller cell subpopulation, while the highest response to PHAP was in the larger cell subpopulation.

When comparing field sites, the Upper Juniata site was predicted to have different immune function responses than the out-of-basin site, Tionesta Lake, due to differences in surrounding land use and potential contaminant exposures. Land use surrounding the Upper Juniata site is more agricultural with some development and is also located within the Susquehanna River basin where YOY SMB have suffered disease and mortalities over the past two decades. Land use surrounding Tionesta Lake is mostly forested. Both bactericidal and respiratory burst activity were higher at the more disturbed site, Upper

Juniata. A negative correlation was observed between bactericidal activity and background ROS at the Upper Juniata site (Figure 20). This was unexpected. ROS is a mechanism for bactericidal activity, so it would make more sense to see a positive correlation. Mitogenesis responses were not significantly different in total gated cells between field sites (Figure 13) but different trends were observed when looking at subpopulations of cells of different sizes (Figures 14-16).

Histopathology and the immune response

To better understand the immune assays, histopathology was also assessed for each site. The laboratory fish did not exhibit any major signs of disease or infections. SMB collected from the Upper Juniata exhibited active granulomas that did not appear to be parasite-related while SMB collected from Tionesta Lake, the more forested site, had higher parasite ratings and granulomas which contained encysted trematodes and other killed parasites surrounded by macrophages and macrophage aggregates. Land use can impact the prevalence of parasites in multiple ways. Parasitism may decrease at sites where densities of intermediate hosts declines or if parasites themselves are more susceptible to contaminants than their host (Lafferty 1997). Parasitism may increase at sites where host resistance is down from other stressors or where densities of intermediate hosts increase (Lafferty 1997). In addition to granulomas and parasites, histological analyses of the fish collected in the field also showed inflammation and high numbers of macrophage aggregates in the splenic tissues.

Macrophage aggregates are groups of macrophages and lymphocytes that can be used as a biomarker of exposure to environmental degradation (Fournie et al. 2001; Blazer 2002) as multiple studies have found positive correlations between the presence of

macrophage aggregates and environmental contamination particularly in the liver and spleen of aquatic organisms (Wolke et al. 1985; Spazier et al. 1992; Wolke 1992; Blazer et al. 1994; Couillard and Hodson 1996; Meinelt et al. 1997; Facey et al. 1999) but also in ovarian tissue (Stott et al. 1981; Johnson et al. 1988; Barry et al. 2001). Macrophage aggregates respond relatively rapidly in aquatic organisms that are stressed due to exposure to environmental contamination (Fournie et al. 2001). Therefore, the presence of macrophage aggregates can be used as an indicator or early warning sign of environmental disturbance.

Macrophage aggregates play an important role in the immune response of fishes. They localize tissue damage and accumulate one or more easily observable pigments, including lipofuscin or ceroid (oxidized lipid), hemosiderin (iron-containing) or melanin (Fournie et al. 2001; Blazer 2002). Lipofuscin accumulates with age and tissue destruction (mainly tissue necrosis and starvation of post-mitotic cells), ceroid accumulates in response to a variety of stressors including disease, hemosiderin accumulates from hemolysis of red blood cells, and melanin is suspected to be involved in removing substances that have been phagocytized by macrophages (Wolke et al. 1985; Fournie et al. 2001; Blazer 2002). Macrophage aggregates, especially in the spleen and liver, have also been compared to germinal centers in mammals for their ability to trap and store both particulate and soluble antigen extracellularly as a place where lymphocytes can migrate through to become stimulated (Ellis et al. 1976; Ellis 1980).

My results suggest that combinations of biomarkers are needed to more comprehensively understand fish health in the field, especially because land use alone does not tell the whole story. There are many factors other than contaminants, such as

parasites and bacterial infections, which may influence the immune response. The idea of multiple stressors on aquatic organisms is an increasing area of concern. Interactions between contaminants and disease decrease the fitness of an organism, which affects survival and reproductive potential of both the individual and the population (Morley et al. 2006). Exposure to multiple contaminants and disease can compromise immune function, making an organism more susceptible to subsequent stressors.

Parasites and bacterial infections

Specific effects of certain parasites and bacteria on the fish immune response have been documented. They have been found to both stimulate and suppress immune function. Trematodes, nematodes, myxozoan parasites and mycobacteria are all commonly observed in wild fishes. Extracellular infections of mycobacteria have been shown to induce inflammation of infected tissues (Chen et al. 1998), increase respiratory burst and phagocytic activity in Nile tilapia and rainbow trout (Chen et al. 1996; Chen et al. 1998), increase antibody production to *Mycobacterium* spp. in rainbow trout (Chen et al. 1996) and have an immunosuppressive effect on rainbow trout macrophages cultured *in vitro* with media supplemented with high levels of mycobacteria (Chen et al. 1998).

Contaminant exposure and parasites can interact to affect host species in multiple ways. Numerous types of contaminants and trematode parasites can affect immunological and physiological processes and have direct and indirect effects on disease and survival (Morley et al. 2006). For instance, pollutants were found to cause a decrease in circulating eosinophils which are important in the defense against macroparasite infections (Kiesecker 2002), a decrease in LPS-induced lymphocyte mitogenesis in the marine toad *Bufo marinus* (Linzey et al. 2003), an increase in anterior kidney (AK)

leukocyte mitogenesis in carp *Cyprinus carpio* (Schuwerack et al. 2001; Schuwerack et al. 2003), and a decrease in the hemolytic plaque-forming cell response in AK leukocytes of juvenile Chinook salmon (Jacobson et al. 2003).

Nematode parasites have been found to induce inflammation and macrophage phagocytic activity in minnows. Encapsulated nematode parasites in the liver and pancreas of minnow *Phoxinus phoxinus* (L.) were shown to induce a host immune response driven most abundantly by mast cells (inflammatory cells), but also rodlet cells, macrophages, and neutrophils (Dezfuli et al. 2009). Macrophages were the only active phagocytes (Dezfuli et al. 2009).

In a review by Sitjà-Bobadilla (2008), myxozoan parasites were found to increase respiratory burst in terms of ROS (Muñoz et al. 2000a; Sitjà-Bobadilla et al. 2006; Alvarez-Pellitero et al. 2008) and reactive nitrogen intermediates produced by activated phagocytes (Golomazou et al. 2006), decrease phagocytic activity (Chilmonczyk et al. 2002; Cuesta et al. 2006b), decrease lymphocyte activity (Densmore et al. 2004; Bermúdez et al. 2006; Cuesta et al. 2006b; Alvarez-Pellitero et al. 2008), and increase cell-mediated cytotoxicity of non-specific cytotoxic cells (Cuesta et al. 2006b). Myxozoans were also shown to affect nonspecific humoral factors including peroxidases, lysozyme and complement both positively and negatively (Muñoz et al. 2000b; Foott et al. 2004; Karagouni et al. 2005; Cuesta et al. 2006a; Cuesta et al. 2006b; Golomazou et al. 2006; Sitjà-Bobadilla et al. 2006; Kaltner et al. 2007; Muñoz et al. 2007; Alvarez-Pellitero et al. 2008) and to increase the presence of specific antibodies in the adaptive immune response (Furuta et al. 1993; Saulnier and Kinkelin 1996; Hedrick et al. 1998; Bartholomew 2001; Sitjà-Bobadilla et al. 2004).

Exposure to herbicides

One of the major differences in surrounding land use between the two field sites was the total kilograms of pesticide applied to cultivated fields between 2001 and 2015, which enter into aquatic systems through surface run-off. Chronic exposure to pesticides or other xenobiotics is suspected to have immunosuppressive effects putting fish more at risk for disease (Dunier and Siwicki 1993). During those 14 years, the total applied pesticide surrounding the Upper Juniata Site was over 350,000 kg and just a little over 9,000 kg applied to the watershed surrounding the Tionesta Lake site. Pesticides applied consisted of more than 99% herbicides at both sites. Various evaluations have been employed to assess immune function in response to environmental chemicals, particularly herbicides (Dunier and Siwicki 1993). Specific effects of herbicides to the fish immune response have been documented.

In a review by Dunier and Siwicki (1993), herbicides were found to cause death (Belamie and Giroud 1986), an increase in macrophage aggregates in liver of mugliads *Liza ramada* and *Liza aurata* (Biagianti-Risbourg 1990), decreases in circulating leukocytes of multiple fish species (Berry 1975; Walsh and Ribelin 1975; Kreutz et al. 2011), atrophy of lymphoid organs (Walsh and Ribelin 1975), decreased numbers of lymphocytes in the spleen of lake trout and coho salmon (Walsh and Ribelin 1975), and increased phagocytosis and suppressed hematopoiesis in rainbow trout (Spitzbergen et al. 1986). While Spitsbergen et al. (1986) found increased phagocytosis, Kreutz et al. (2010) found decreased phagocytic activity in fish following herbicide exposure. This could be due to a multi-phased immune response, where the immune response is stimulated for low-dose contaminant exposures but later becomes suppressed as dose increases (Sharma

1988). This type of multi-phased immune response was seen in my study where I found increased immune responses for low parasite ratings but decreased responses for higher parasite ratings (Figures 23a and 24a).

Herbicides have also been found to reduce resistance of fish to pathogen invasion (Fatima et al. 2007; Kreutz et al. 2010), enhance lysozyme activity in goldfish (Fatima et al. 2007), increase respiratory burst of phagocytes in spleen, kidney and liver of goldfish (Fatima et al. 2007), increase the number of immature circulating leukocytes (Kreutz et al. 2011), increase oxidative stress by disrupting oxidant-antioxidant balance in phagocytes (Ferreira et al. 2010), and induce hepatic cell injuries including hepatocyte degeneration and bile stagnation in silver catfish (Ferreira et al. 2010).

Detecting mitogenesis via EdU labeling and image-based flow cytometry

One of the major goals of my study was to develop a mitogenesis method for smallmouth bass leukocytes using image-based flow cytometry and EdU labelling techniques to allow analysis of different populations of leukocytes in more detail than standard flow cytometry. Analyzing subpopulations of leukocytes is important because different populations of leukocytes have shown variable sensitivities to contaminants (Dunier and Siwicki 1993). For example, lymphocytes in general may be more susceptible to damage from the effects of xenobiotics than macrophages and even further, suppressor T cells may be more sensitive than helper T cells (Sharma 1988; Dunier and Siwicki 1993). Looking at subpopulations is also important because not all adherent cells during the mitogenesis assay are lymphocytes, but also include macrophages, granulocytes and immature cells. The technique developed in this study does not

differentiate the adherent cell populations, but it could with a few additions to the assay (see limitations below).

Various methods for mitogenesis have been applied to several fish species, not including smallmouth bass. Previously, mitogenesis methods utilized for fish have been for rainbow trout (*Oncorhynchus mykiss*; Lin et al. 1999; Scharsack et al. 2001; Gauthier et al. 2003), white perch (*Morone americana*; Gauthier et al. 2003), Atlantic salmon (*Salmo salar* L.; Espelid et al. 1996), brown bullhead (*Ameirus nebulosus*; Iwanowicz et al. 2009), largemouth bass (*Micropterus salmoides*; Iwanowicz et al. 2012), channel catfish (*Ictalurus punctatus*; Sizemore et al. 1984), and hybrid striped bass (female striped bass *Morone saxatilis* x male white bass *M. chrysops*; Harms et al. 2000). None of the studies mentioned above used the EdU detection method or image-based flow cytometry. No previous studies were found that divided their analysis into subpopulations based on cell size as in this study.

Iwanowicz et al. (2009) used standard flow cytometry for analysis and compared cell populations by forward and side scatter properties. Based on forward and side scatter properties alone, they defined cell populations as lymphocytes (small low-complexity), neutrophils (large-complex), and macrophages (large-low-complexity). Forward scatter gives an indication to cell size and side scatter tells how complex, or granular, a cell is (Basiji et al. 2007). Iwanowicz et al. (2009) reported lymphocytes as 50-60% of total gated cells, neutrophils as 30-40% and macrophages as 10-20%.

I defined cell populations based on cell diameter and found the smallest (4-7 μm) cells constituted 5-20% of total gated cells, 7-12 μm cells were 40-60%, 12-15 μm cells were 10-30% and the largest (15-20 μm) cells were 0-10%. It was impossible for me to

tease out cell types based on cell size alone. Lymphocytes in teleosts usually range from 5 to 10 μm (Catton 1951; Blaxhall and Daisley 1973; Williams and Warner 1976; Barber et al. 1981). Stage of maturation also affects cell size. This is important in my study because my cells were isolated from anterior kidney, the main hematopoietic tissue in fish, so various stages of development were inevitably present including blast cells which are larger than mature lymphocytes. Hematological assessments in trout have measured small lymphocytes from 5.4 to 8.1 μm ($\overline{x}$ = 7.0), large lymphocytes from 8.1 to 10.8 μm ($\overline{x}$ = 9.1) and blast cells were as big as 12.6 to 15.3 μm ($\overline{x}$ = 15.1; Blaxhall and Daisley 1973).

In an attempt to validate the cell sizes in my study, I looked at a subpopulation of cells microscopically during one of the mitogenesis assay runs. This validation has limitations in that I only sampled a small subset of fish and only included laboratory-reared fish. However, this gave me an idea of what the different cell size subpopulations could possibly be. This provided important information as I measured lymphocytes larger and smaller than 10 μm (Figure 26B-C). By looking at the cellular morphology of the cells collected with image-based flow cytometry, another issue with identifying cell types based on size alone became clear. Some of the single cell events that the flow cytometer collected appeared to be smaller cells going through mitosis (Figure 27). Considering Con A stimulation alone, there were clearly some small lymphocytes that were stimulated (Figure 28) but there were also some larger cells (Figure 29).

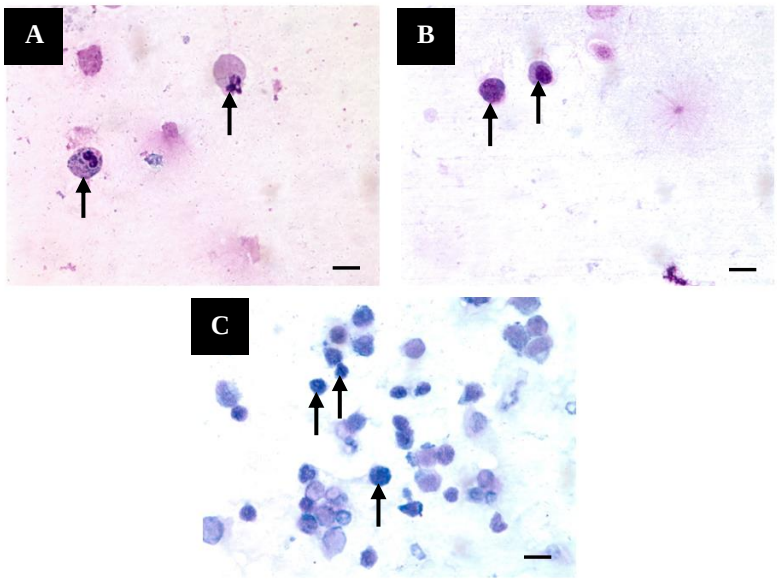

Figure 26. Leukocyte blood smears. Blood smears collected from laboratory fish in Kearneysville, WV. Cell suspensions were taken directly from the mitogenesis plate during assay and smeared on glass slides. Hema 3™ (A-B) and Giemsa (C) stain, bar equals 10 µm. (A) Neutrophils >10 µm (arrows), (B) Lymphocytes ≥10 µm (arrows), and (C) Lymphocytes <10 µm (arrows).

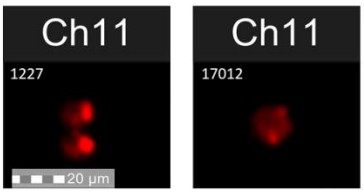

Figure 27. Leukocyte images taken on Amnis FlowSight® Image-based flow cytometer. Channel 11 is EdU fluorescence. Fluorescence of EdU indicates newly synthesized DNA in S phase cells. Dark red indicates positive response to Con A. Cells appear to be going through telophase (left) and anaphase (right) stages of mitosis. This affects reported cell size.

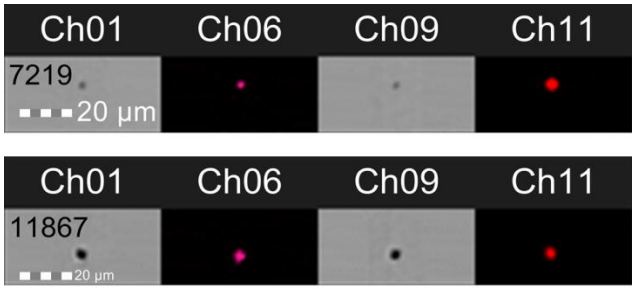

Figure 28. Con A positive lymphocytes. Channel 1 and channel 9 are brightfield, channel 6 is side scatter and channel 11 is EdU fluorescence. Fluorescence of EdU indicates newly synthesized DNA in S phase cells. Dark red indicates positive response to Con A.

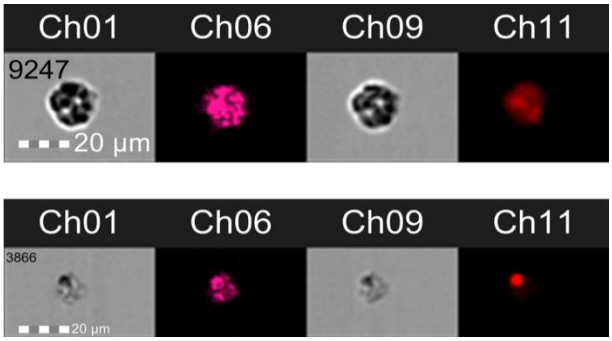

Figure 29. Larger Con A positive cells. Channel 1 and Channel 9 are brightfield, channel 6 is side scatter and channel 11 is EdU fluorescence. Fluorescence of EdU indicates newly synthesized DNA in S phase cells. Dark red indicates positive response to Con A.

The inability to further classify cell sizes is one of the limitations of my study, but the timeline and funding did not permit further analyses. In the future, a nuclear stain could easily be added to the end of the mitogenesis assay to separate cell types by nuclear morphology and then an antibody can be used to tease out B cells from T cells by targeting specific B cell receptors, both via image-based flow cytometry. Additionally, subgroups of cell sizes could be separated differently (versus 4-7 µm, 7-12 µm, 12-15 µm, and 15-20 µm). Although from this study it is not possible to tease out cell types based on size alone, it is still interesting to break the mitogenesis results down into

subgroups because it gives more information than just looking at the total population of cells.

For example, leukocytes isolated from SMB collected at Upper Juniata did not show positive responses to PHAP until the results were broken down into subpopulations (Figures 13 and 15). The same occurred with leukocytes isolated from SMB collected at Tionesta Lake. At Tionesta Lake, there appeared to be no positive responses to LPS until the results were broken into subpopulations (Figures 13 and 16). The cell populations most sensitive to Concanavalin A varied based on site (Figure 14). Generally, the largest cell groups (12-15 and 15-20 μm) were most sensitive to PHAP (Figure 15) while the smallest (4-7 μm) and largest groups (15-20 μm) were most sensitive to LPS stimulation (Figure 16). LPS is normally thought of as a B cell mitogen, but it has also been shown to target other cell types, including macrophages and other antigen-presenting cells in mice (Tough et al. 1997).

During the methods development for the mitogenesis assay, a few concerns became apparent. The first concern was that I was not centrifuging the mitogenesis plate in between/before wash steps during the assay, so I may have been losing high numbers of cells. This was addressed by performing a side study comparing plates side by side (one centrifuged plate and one non-centrifuged plate) with leukocytes isolated from leftover laboratory SMB. There were no significant differences in mitogenesis stimulation indices between plates, so it was determined that centrifugation between wash steps is not necessary for the method developed here. Another concern was whether or not to analyze the cells based on width or diameter. Diameter was chosen to single out

cells that are completely round, but this could also have eliminated cells actively going through mitosis, and therefore more elongated, in the analysis.

Limitations and conclusions

One limitation of my entire study is that although disease in the Susquehanna basin has affected mainly YOY, I was only able to sample adults because only adults had high enough cell yields to run the immune function assays. Towards the end of this study, however, I worked with other scientists at the USGS Fish Health Research Laboratory in Kearneysville, WV to develop immune function methods that could work effectively with fewer cells by running the assays in 384-well plates instead of 96-well plates, so this could be used in future studies to directly study smaller fish when needed.

Another limitation is that I was not able to do antibody titers to see if the wild fish in my study were already exposed to *Aeromonas sobria* due to time restraints. Previous exposure would be a confounding variable for the bactericidal results, although in a review by Secombes and Chappell (1996) it was noted by four previous studies that little direct relationship has been found to exist between infection intensity, degree of protection and antibody titer. Still, in the future, it would be helpful to perform antibody titers to directly determine if the fish were already exposed to *A. sobria* in the field. A third limitation to this study is the small sampling sizes and the use of bioassays, which are useful at predicting effects on the individual and population levels but can be limited in predicting effects on the community level (Lafferty 1997). Some of the results show slight differences but are not significant because sample sizes are too small. The high variability in the immune response of wild fish as seen in the results indicates that larger sample sizes would have been better.

A fourth limitation is that cell counts made in the laboratory were not normalized based on anterior kidney tissue size. In the future, it would be worthwhile to weigh anterior kidney tissue in the field after excision. This will help determine ratio of total isolated cells to total amount of tissue. Also, background ROS comparison could not be made for the laboratory-reared fish because a more efficient method was used to reduce time and increase sensitivity of the respiratory burst assay. Phorbol myristate acetate (PMA) was used as the stimulant for the laboratory-reared fish and the results were read with image-based flow cytometry. It would be useful to sample more laboratory fish, repeat the respiratory burst assay using LPS as the stimulant, and read the results on the microplate reader. This would allow comparison of background ROS in laboratory-reared fish to the wild fish in this study.

Ultimately, the products of my study are three immune function assays that have been validated for wild fish, specifically smallmouth bass, which will be used to help inform the U. S. Geological Survey's Chesapeake Science Strategy (Phillips et al. 2015) on relationships between land use, contaminants and wild fish health. These provide information on smallmouth bass leukocytes' ability to kill bacteria, produce ROS, and proliferate in response to mitogens. Whether or not smallmouth bass in the Susquehanna and Potomac Rivers are actually suffering from immunosuppression cannot be concluded from this study. However, the development of immune assays will allow more work to be done on the immune response in wild fish and integration with chemical contaminant data and other biological endpoints can be studied to provide an answer to that question.

References

Ahmed SA, Gogal RM, Walsh JE. 1994. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H] thymidine incorporation assay. Journal of Immunological Methods 170(2):211-224.

Alvarez-Pellitero P, Palenzuela O, Sitjà-Bobadilla A. 2008. Histopathology and cellular response in *Enteromyxum leei* (Myxozoa) infections of *Diplodus puntazzo* (Teleostei). Parasitology International 57(2):110-120.

Anderson MJ, Barron MG, Diamond SA, Lipton J, Zelikoff JT. 1997. Biomarker Selection for Restoration Monitoring of Fishery Resources. In: Dwyer JF, Doane TR, Hinman LM, editors. Environmental Toxicology and Risk Assessment: Modeling and Risk Assessment (Sixth Volume): American Society for Testing and Materials. p 333-359.

Baldridge CW, Gerard RW. 1932. The extra respiration of phagocytosis. American Journal of Physiology--Legacy Content 103(1):235-236.

Barber DL, Westermann MJE, White MG. 1981. The blood cells of the Antarctic icefish *Chaenocephalus aceratus* Lönnberg: light and electron microscopic observations. Journal of Fish Biology 19(1):11-28.

Barry CE, Wainscott MR, Foott JS, Patiño R. 2001. Gonadal macrophage aggregates: novel biomarker of contaminant exposure and effect in male fishes? Proceedings of the Society of Environmental Toxicology and Chemistry 2001 Annual meeting, Nov 11-15, Baltimore, MD. p 222.

Bartholomew JL. 2001. Parasites, their hosts and why disease occurs (or doesn't?). Proceedings of the 131st Annual Meeting of the American Fisheries Society(August):1104.

Basiji DA, Ortyn WE, Liang L, Venkatachalam V, Morrissey P. 2007. Cellular image analysis and imaging by flow cytometry. Clinics in Laboratory Medicine 27(3):653-670.

Beegle D. 2013. Nutrient management and the Chesapeake Bay. Journal of Contemporary Water Research & Education 151(1):3-8.

Belamie R, Giroud S. 1986. [Pollution connected with the use of pesticides by agriculture in France]. [French].

Bermúdez R, Vigliano F, Marcaccini A, Sitjà-Bobadilla A, Quiroga MI, Nieto JM. 2006. Response of Ig-positive cells to *Enteromyxum scophthalmi* (Myxozoa) experimental infection in turbot, *Scophthalmus maximus* (L.): a histopathological and immunohistochemical study. Fish & Shellfish Immunology 21(5):501-512.

Berry CRJ. 1975. The effects of herbicide treatment on a reservoir ecosystem. Ph.D. thesis: Virginia Polytechnic Institute and State University, Blacksburg.

Biagianti-Risbourg S. 1990. Contribution a l'etude du foie de juveniles de muges Teleosteens, (Mugilides) contamines experimentalement par l'atrazine (s-triazine herbicide): interet en ecotoxicologie. Ph.D. thesis: Academie de Montpellier, University of Perpignan, France.

Biller-Takahashi JD, Takahashi LS, Saita MV, Gimbo RY, Urbinati EC. 2013. Leukocytes respiratory burst activity as indicator of innate immunity of pacu *Piaractus mesopotamicus*. Brazilian Journal of Biology 73(2):425-429.

Blaxhall PC, Daisley KW. 1973. Routine haematological methods for use with fish blood. Journal of Fish Biology 5(6):771-781.

Blazer VS. 2002. Histopathological assessment of gonadal tissue in wild fishes. Fish Physiology and Biochemistry 26(1):85-101.

Blazer VS, Facey DE, Fournie JW, Courtney LA, Summers JK. 1994. Macrophage aggregates as indicators of environmental stress. In: Stolen JS, Fletcher TC, editors. Modulators of Fish Immune Responses, volume 1. Fair Haven, New Jersey: SOS Publications.

Blazer VS, Iwanowicz DD, Walsh HL, Sperry AJ, Iwanowicz LR, Alvarez DA, Brightbill RA, Smith G, Foreman WT, Manning R. 2014. Reproductive health indicators of fishes from Pennsylvania watersheds: association with chemicals of emerging concern. Environmental Monitoring and Assessment 186(10):6471-6491.

Blazer VS, Iwanowicz LR, Starliper CE, Iwanowicz DD, Barbash P, Hedrick JD, Reeser SJ, Mullican JE, Zaugg SD, Burkhardt MR, Kelble J. 2010. Mortality of centrarchid fishes in the Potomac drainage: survey results and overview of potential contributing factors. Journal of Aquatic Animal Health 22(3):190-218.

Blazer VS, Walsh HL, Braham RP, Hahn CM, Mazik P, McIntyre PB. 2017. Tumours in white suckers from Lake Michigan tributaries: pathology and prevalence. Journal of Fish Diseases 40(3):377-393.

Borden WC, Stepien CA. 2006. Discordant population genetic structuring of smallmouth bass, *Micropterus dolomieu* Lacepede, in Lake Erie based on mitochondrial DNA sequences and nuclear DNA microsatellites. Journal of Great Lakes Research 32(2):242-257.

Brewer SK, Orth DJ. 2015. Smallmouth Bass *Micropterus dolomieu* Lacepède, 1802. American Fisheries Society Symposium 82:9-26.

Brown TG, Runciman B, Pollard S. 2009. Canadian Manuscript Report of Fisheries and Aquatic Sciences 2887: Biological synopsis of smallmouth bass (*Micropterus dolomieu*).

Cappella P, Gasparri F, Pulici M, Moll J. 2008. Cell proliferation method: click chemistry based on BrdU coupling for multiplex antibody staining. Current Protocols in Cytometry:7-34.

Casanova-Nakayama A, Wenger M, Burki R, Eppler E, Krasnov A, Segner H. 2011. Endocrine disrupting compounds: Can they target the immune system of fish? Marine Pollution Bulletin 63(5):412-416.

Catton WT. 1951. Blood cell formation in certain teleost fishes. Blood 6(1):39-60.

Cavanagh BL, Walker T, Norazit A, Meedeniya ACB. 2011. Thymidine analogues for tracking DNA synthesis. Molecules 16(9):7980-7993.

Chaplin JJ, Crawford KJ, Brightbill RA. 2009. Water-Quality Monitoring in Response to Young-of-the-Year Smallmouth Bass (*Micropterus dolomieu*) Mortality in the Susquehanna River and Major Tributaries, Pennsylvania: 2008. US Geological Survey.

Chen SC, Yoshida T, Adams A, Thompson KD, Richards RH. 1996. Immune Response of Rainbow Trout to Extracellular Products of *Mycobacterium* spp. Journal of Aquatic Animal Health 8(3):216-222.

Chen SC, Yoshida T, Adams A, Thompson KD, Richards RH. 1998. Non-specific immune response of Nile tilapia, *Oreochromis nilotica*, to the extracellular products of *Mycobacterium* spp. and to various adjuvants. Journal of Fish Diseases 21(1):39-46.

Chilmonczyk S, Monge D, De Kinkelin P. 2002. Proliferative kidney disease: cellular aspects of the rainbow trout, *Oncorhynchus mykiss* (Walbaum), response to parasitic infection. Journal of Fish Diseases 25(4):217-226.

Coteur G, Warnau M, Jangoux M, Dubois P. 2002. Reactive oxygen species (ROS) production by amoebocytes of *Asterias rubens* (Echinodermata). Fish & Shellfish Immunology 12(3):187-200.

Couillard CM, Hodson PV. 1996. Pigmented macrophage aggregates: a toxic response in fish exposed to bleached-kraft mill effluent. Environmental Toxicology and Chemistry 15:1844-1854.

Cuesta A, Muñoz P, Rodríguez A, Salinas I, Sitjà-Bobadilla A, Alvarez-Pellitero P, Esteban MA, Meseguer J. 2006a. Gilthead seabream (*Sparus aurata* L.) innate defence against the parasite *Enteromyxum leei* (Myxozoa). Parasitology 132(1):95-104.

Cuesta A, Salinas I, Rodriguez A, Muñoz P, Sitjà-Bobadilla A, Alvarez-Pellitero P, Meseguer J, Esteban MA. 2006b. Cell-mediated cytotoxicity is the main innate immune mechanism involved in the cellular defence of gilthead seabream (Teleostei: Sparidae) against *Enteromyxum leei* (Myxozoa). Parasite Immunology 28(12):657-665.

Daly JG, Moore AR, Olivier G. 1995. A colorimetric assay for the quantification of brook trout (*Salvelinus fontinalis*) lymphocyte mitogenesis. Fish & Shellfish Immunology 5(4):265-273.

Densmore CL, Ottinger CA, Blazer VS, Iwanowicz LR, Smith DR. 2004. Immunomodulation and disease resistance in postyearling rainbow trout infected with *Myxobolus cerebralis*, the causative agent of whirling disease. Journal of Aquatic Animal Health 16(2):73-82.

Dezfuli BS, Manera M, Giari L. 2009. Immune response to nematode larvae in the liver and pancreas of minnow, *Phoxinus phoxinus* (L.). Journal of Fish Diseases 32(4):383-390.

Dunier M, Siwicki AK. 1993. Effects of pesticides and other organic pollutants in the aquatic environment on immunity of fish: a review. Fish & Shellfish Immunology 3(6):423-438.

Ekman DR, Ankley GT, Blazer VS, Collette TW, Garcia-Reyero N, Iwanowicz LR, Jorgenson ZG, Lee KE, Mazik PM, Miller DH, Perkins EJ. 2013. Biological effects–based tools for monitoring impacted surface waters in the Great Lakes: A multiagency program in support of the Great Lakes Restoration Initiative. Environmental Practice 15(4):409-426.

Ellis AE. 1980. Antigen-trapping in the spleen and kidney of the plaice *Pleuronectes platessa* L. Journal of Fish Diseases 3(5):413-426.

Ellis AE. 1999. Immunity to bacteria in fish. Fish & Shellfish Immunology 9(4):291-308.

Ellis AE, Munroe ALS, Roberts RJ. 1976. Defence mechanisms in fish. Journal of Fish Biology 8(1):67-78.

Ellsaesser CF, Clem LW. 1987. Cortisol-induced hematologic and immunologic changes in channel catfish (*Ictalurus punctatus*). Comparative Biochemistry and Physiology Part A: Physiology 87(2):405-408.

Espelid S, Løkken GB, Steiro K, Bøgwald J. 1996. Effects of cortisol and stress on the immune system in Atlantic salmon (*Salmo salar* L.). Fish & Shellfish Immunology 6(2):95-110.

Facey DE, Leclerc D, Dunbar D, Arruda L, Pyzocha, Blazer V. 1999. Physiological indicators of stress among fishes from contaminated areas of Lake Champlain. In:

Manley TO, Manley PL, editors. Lake Champlain in Transition: From Research Toward Restoration. Washington, D.C.: American Geophysical Union. p 349-359.

Fatima M, Mandiki SNM, 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. Aquatic Toxicology 81(2):159-167.

Ferreira D, da Motta AC, Kreutz LC, Toni C, Loro VL, Barcellos LJG. 2010. Assessment of oxidative stress in *Rhamdia quelen* exposed to agrichemicals. Chemosphere 79(9):914-921.

Fofonoff PW, Ruiz GM, Steves B, Hines AH, Carlton JT. 2003. National Exotic Marine and Estuarine Species Information System: Chesapeake Bay Introduced Species Database: http://invasions.si.edu/nemesis/chesapeake.html.

Foott JS, Harmon R, Stone R. 2004. Effect of water temperature on non-specific immune function and ceratomyxosis in juvenile Chinook salmon and steelhead from the Klamath River. California Fish and Game 90(2):71-84.

Fournie JW, Summers JK, Courtney LA, Engle VD, Blazer VS. 2001. Utility of splenic macrophage aggregates as an indicator of fish exposure to degraded environments. Journal of Aquatic Animal Health 13(2):105-116.

Furuta T, Ogawa K, Wakabayashi H. 1993. Humoral immune response of carp *Cyprinus carpio* to *Myxobolus artus* (Myxozoa: Myxobolidae) infection. Journal of Fish Biology 43(3):441-450.

Gauthier DT, Cartwright DD, Densmore CL, Blazer VS, Ottinger CA. 2003. Measurement of in vitro leucocyte mitogenesis in fish: ELISA based detection of the thymidine analogue 5 -bromo-2 -deoxyuridine. Fish & Shellfish Immunology 14(4):279-288.

Goldsborough EL, Clark HW. 1908. Fishes of West Virginia (No. 631). U. S. Government Printing Office.

Golomazou E, Athanassopoulou F, Karagouni E, Tsagozis P, Tsantilas H, Vagianou S. 2006. Experimental transmission of *Enteromyxum leei* Diamant, Lom and Dykova, 1994 in sharpsnout sea bream, *Diplodus puntazzo* C. and the effect on some innate immune parameters. Aquaculture 260(1):44-53.

Gratzner HG. 1982. Monoclonal antibody to 5-bromo-and 5-iododeoxyuridine: a new reagent for detection of DNA replication. Science 218(4571):474-475.

Halliwell B, Gutteridge JM. 2007. Free Radicals in Biology and Medicine. USA: Oxford University Press.

Harms CA, Ottinger CA, Kennedy-Stoskopf S. 2000. Correlation of transforming growth factor- messenger RNA (TGF- mRNA) expression with cellular immunoassays in triamcinolone-treated captive hybrid striped bass. Journal of Aquatic Animal Health 12(1):9-17.

Haugland R. 2005. Assays for Cell Viability, Proliferation, and Function. The Handbook: A Guide to Fluorescent Probes and Labeling Technologies, 10th ed. USA: Invitrogen Corp. p 699-776.

Hedrick RP, Adkison MA, El-Matbouli M, MacConnell E. 1998. Whirling disease: re-emergence among wild trout. Immunological Reviews 166(1):365-376.

Hoar WS, Randall DJ, Farrell AP. 1997. Fish Physiology: Vol. 15. The fish immune system: organism, pathogen, and environment. Iwama G, Nakanishi T, editors. San Diego: Academic Press.

Iwanowicz LR, Blazer VS, Hitt NP, McCormick SD, DeVault DS, Ottinger CA. 2012. Histologic, immunologic and endocrine biomarkers indicate contaminant effects in fishes of the Ashtabula River. Ecotoxicology 21(1):165-182.

Iwanowicz LR, Blazer VS, McCormick SD, VanVeld PA, Ottinger CA. 2009. Aroclor 1248 exposure leads to immunomodulation, decreased disease resistance and endocrine disruption in the brown bullhead, *Ameiurus nebulosus*. Aquatic Toxicology 93(1):70-82.

Iwanowicz LR, Ottinger CA. 2009. Estrogens, estrogen receptors and their role as immunoregulators in fish. Fish Defenses 1:277-322.

Jackson DA. 2002. Ecological effects of *Micropterus* introductions: the dark side of black bass. American Fisheries Society Symposium 31:221-232.

Jacobson KC, Arkoosh MR, Kagley AN, Clemons ER, Collier TK, Casillas E. 2003. Cumulative effects of natural and anthropogenic stress on immune function and disease resistance in juvenile Chinook salmon. Journal of Aquatic Animal Health 15(1):1-12.

Jaworski NA, Lear DW, Villa O. 1971. Nutrient management in the Potomac estuary. Report nr 45. 69 p.

Jenkins RE, Burkhead NM. 1994. Freshwater fishes of Virginia. Bethesda, Maryland: American Fisheries Society.

Johnson LL, Casillas E, Collier TK, McCain BB, Varansi U. 1988. Contaminant effects on ovarian development in English sole (*Parophrys vetulus*) from Puget Sound, Washington. Canadian Journal of Fisheries and Aquatic Sciences 45:2133-2146.

Kaltner H, Stippl M, Knaus M, El-Matbouli M. 2007. Characterization of glycans in the developmental stages of *Myxobolus cerebralis* (Myxozoa), the causative agent of whirling disease. Journal of Fish Diseases 30(11):637-647.

Karagouni E, Athanassopoulou F, Tsagozis P, Ralli E, Moustakareas T, Lytra K, Dotsika E. 2005. The impact of a successful anti-myxosporean treatment on the phagocyte functions of juvenile and adult *Sparus aurata* L. International Journal of Immunopathology and Pharmacology 18(1):121-132.

Kiesecker JM. 2002. Synergism between trematode infection and pesticide exposure: a link to amphibian limb deformities in nature? Proceedings of the National Academy of Sciences 99(15):9900-9904.

Kreutz LC, Barcellos LJG, de Faria Valle S, de Oliveira Silva T, Anziliero D, dos Santos ED, Pivato M, Zanatta R. 2011. Altered hematological and immunological parameters in silver catfish (*Rhamdia quelen*) following short term exposure to sublethal concentration of glyphosate. Fish & Shellfish Immunology 30(1):51-57.

Kreutz LC, Barcellos LJG, Marteninghe A, Dos Santos ED, Zanatta R. 2010. Exposure to sublethal concentration of glyphosate or atrazine-based herbicides alters the phagocytic function and increases the susceptibility of silver catfish fingerlings (*Rhamdia quelen*) to *Aeromonas hydrophila* challenge. Fish & Shellfish Immunology 29(4):694-697.

Lafferty K. 1997. Environmental parasitology: What can parasites tell us about human impacts on the environment? Parasitology Today 13(7):251-255.

Lin SH, Ellis AE, Davidson GA, Secombes CJ. 1999. Migratory, respiratory burst and mitogenic responses of leucocytes isolated from the gills of rainbow trout (*Oncorhynchus mykiss*). Fish & Shellfish Immunology 9(3):211-226.

Linzey D, Burroughs J, Hudson L, Marini M, Robertson J, Bacon J, Nagarkatti M, Nagarkatti P. 2003. Role of environmental pollutants on immune functions, parasitic infections and limb malformations in marine toads and whistling frogs from Bermuda. International Journal of Environmental Health Research 13(2):125-148.

Lushchak VI. 2011. Environmentally induced oxidative stress in aquatic animals. Aquatic Toxicology 101(1):13-30.

Mayes WM. 2013. Water Pollution Biology. In: Harrison RM, editor. Pollution 5th Edition: Causes, Effects and Control. 5 ed. p 80-114.

Meador MR, Cuffney TF, Gurtz ME. 1993. Methods for sampling fish communities as part of the National Water-Quality Assessment Program. Books and Open-File Reports (distributor): US Geological Survey.

Meinelt T, Kruger R, Pietrock M, Osten R, Steinberg C. 1997. Mercury pollution and macrophage centers in pike (*Esox lucius*) tissues. Environmental Science and Pollution Research 4(1):32-36.

Milla S, Depiereux S, Kestemont P. 2011. The effects of estrogenic and androgenic endocrine disruptors on the immune system of fish: a review. Ecotoxicology 20(2):305-319.

Milner JW. 1874. The progress of fish-culture in the United States. Washington, Government Printing Office. 524-536 p.

Morley NJ, Lewis JW, Hoole D. 2006. Pollutant-induced effects on immunological and physiological interactions in aquatic host–trematode systems: implications for parasite transmission. Journal of Helminthology 80(2):137-149.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65(1-2).

Muñoz P, Alvarez-Pellitero P, Sitja-Bobadilla A. 2000a. Modulation of the in vitro activity of European sea bass (*Dicentrarchus labrax* L.) phagocytes by the myxosporean parasite *Sphaerospora dicentrarchi* (Myxosporea: Bivalvulida). Fish & Shellfish Immunology 10(7):567-581.

Muñoz P, Cuesta A, Athanassopoulou F, Golomazou H, Crespo S, Padrós F, Sitjà-Bobadilla A, Albiñana G, Esteban MA, Alvarez-Pellitero P, Meseguer J. 2007. Sharpsnout sea bream (*Diplodus puntazzo*) humoral immune response against the parasite *Enteromyxum leei* (Myxozoa). Fish & Shellfish Immunology 23(3):636-645.

Muñoz P, Sitja-Bobadilla A, Alvarez-Pellitero P. 2000b. Cellular and humoral immune response of European sea bass (*Dicentrarchus labrax* L.)(Teleostei: Serranidae) immunized with *Sphaerospora dicentrarchi* (Myxosporea: Bivalvulida). Parasitology 120(5):465-477.

Nash RDM, Valencia AH, Geffen AJ. 2006. The origin of Fulton's condition factor-setting the record straight. Fisheries 31(5):236-238.

Neumann NF, Barreda DR, Belosevic M. 2000. Generation and functional analysis of distinct macrophage sub-populations from goldfish (*Carassius auratus* L.) kidney leukocyte cultures. Fish & Shellfish Immunology 10(1):1-20.

O'Halloran K, Ahokas JT, Wright PFA. 1998. The adverse effects of aquatic contaminants on fish immune responses. Australasian Journal of Ecotoxicology 4(1):9-28.

Page LM, Burr BM. 1991. Freshwater Fishes. New York, NY: Houghton Mifflin.

Paolisso M, Trombley J, Hood RR, Sellner KG. 2015. Environmental models and public stakeholders in the Chesapeake Bay watershed. Estuaries and Coasts 38(1):97-113.

Papadakis M. 2006. The Economic Impact of the 2005 Shenandoah River Fish Kill: A Preliminary Assessment. James Madison University: Center for Energy and Environmental Sustainability.

Pelton T, Blazer V, Mullican J, Smith G, Langland M, Phillips S, Lipton D. 2013. Angling for healthier rivers: the link between smallmouth bass mortality and disease and the need to reduce water pollution in Chesapeake Bay tributaries. Chesapeake Bay Foundation.

Phillips S, Blomquist JD, Bennett M, Berlin A, Blazer V, Claggett P, Faulkner S, Hyer K, Ladino C, Moyer D, Muir R, Noe G, Phillips PJ. 2015. U.S. Geological Survey Chesapeake Science Strategy, 2015-2025-Informing Ecosystem Management of America's Largest Estuary. US Geological Survey. https://doi.org/10.3133/ofr20151162.

Poljsak B, Šuput D, Milisav I. 2013. Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants. Oxidative Medicine and Cellular Longevity 2013.

Rieger AM, Hall BE, Barreda DR. 2010. Macrophage activation differentially modulates particle binding, phagocytosis and downstream antimicrobial mechanisms. Developmental & Comparative Immunology 34(44):1144-1159.

Ripley J, Iwanowicz L, Blazer V, Foran C. 2008. Utilization of protein expression profiles as indicators of environmental impairment of smallmouth bass (*Micropterus dolomieu*) from the Shenandoah River, Virginia, USA. Environmental Toxicology and Chemistry 27(8):1756-1767.

Salic A, Mitchison TJ. 2008. A chemical method for fast and sensitive detection of DNA synthesis in vivo. Proceedings of the National Academy of Sciences 105(7):2415-2420.

Saulnier D, Kinkelin P. 1996. Antigenic and biochemical study of PKX, the myxosporean causative agent of proliferative kidney disease of salmonid fish. Diseases of Aquatic Organisms 27(2):103-114.

Scharsack JP, Steinhagen D, Leibold W, Rabe U, Körting W, Schuberth HJ. 2001. Flow Cytometric Analysis of Mitogen-Induced Activation of Rainbow Trout (*Oncorhynchus mykiss*) Peripheral Blood Leucocytes. Journal of Veterinary Medicine, Series B 48(5):331-339.

Schmitt CJ, Blazer VS, Dethloff GM, Tillitt DE, Gross TS. 1999. Biomonitoring of Environmental Status and Trends (BEST) Program: field procedures for assessing

the exposure of fish to environmental contaminants. Columbia MO Biological Resources Div: US Geological Survey.

Schuwerack PM, Lewis JW, Hoole D. 2003. Cadmium-induced cellular and immunological responses in *Cyprinus carpio* infected with the blood parasite, *Sanguinicola inermis*. Journal of Helminthology 77(4):341-350.

Schuwerack PM, Lewis JW, Hoole D, Morley NJ. 2001. Ammonia-induced cellular and immunological changes in juvenile *Cyprinus carpio* infected with the blood fluke *Sanguinicola inermis*. Parasitology 122(3):339-345.

Scott WB, Crossman EJ. 1973. Freshwater fishes of Canada. Fisheries Research Board of Canada Bulletin 184.

Secombes CJ. 1996. The nonspecific immune system: cellular defenses. In: Iwama G, Nakanishi T, editors. The Fish Immune System: Organism, Pathogen and Environment. San Diego: Academic Press Inc. p 63-103.

Secombes CJ, Chappell LH. 1996. Fish immune responses to experimental and natural infection with helminth parasites. Annual Review of Fish Diseases 6:167-177.

Sharma RP. 1988. Evaluation of pesticide immunotoxicity. Toxicology and Industrial Health 4:3-25.

Sharp GJE, Pike AW, Secombes CJ. 1991. Leucocyte migration in rainbow trout (*Oncorhynchus mykiss* [Walbaum]): optimization of migration conditions and responses to host and pathogen (*Diphyllobothrium dendriticum* [Nitzsch]) derived chemoattractants. Developmental & Comparative Immunology 15(4):295-305.

Sitjà-Bobadilla A. 2008. Fish immune response to Myxozoan parasites. Parasite 15(3):420-425.

Sitjà-Bobadilla A, Redondo MJ, Bermúdez R, Palenzuela O, Ferreiro I, Riaza A, Quiroga I, Nieto JM, Alvarez-Pellitero P. 2006. Innate and adaptive immune responses of turbot, *Scophthalmus maximus* (L.), following experimental infection with *Enteromyxum scophthalmi* (Myxosporea: Myxozoa). Fish & Shellfish Immunology 21(5):485-500.

Sitjà-Bobadilla A, Redondo MJ, Macias MA, Ferreiro I, Riaza A, Alvarez-Pellitero P. 2004. Development of immunohistochemistry and enzyme-linked immunosorbent assays for the detection of circulating antibodies against *Enteromyxum scophthalmi* (Myxozoa) in turbot (*Scophthalmus maximus* L.). Fish & Shellfish Immunology 17(4):335-345.

Sizemore RC, Miller NW, Cuchens MA, Lobb CJ, Clem LW. 1984. Phylogeny of lymphocyte heterogeneity: the cellular requirements for in vitro mitogenic responses of channel catfish leukocytes. The Journal of Immunology 133(6):2920-2924.

Smith G. 2010. Susquehanna River smallmouth bass disease investigation: Annual Report to the Susquehanna River Policy Committee. Division of Fisheries Management: Pennsylvania Fish and Boat Commission.

Smith GD, Blazer VS, Walsh HL, Iwanowicz LR, Starliper C, Sperry AJ. 2015. The Effects of Disease-Related Mortality of Young-of-Year Smallmouth Bass on Population Characteristics in the Susquehanna River Basin, Pennsylvania and Potential Implications to Conservation of Black Bass Diversity. American Fisheries Society Symposium 82.

Smith HM. 1907. Our fish immigrants. National Geographic 18:385-400.

Spazier E, Storch V, Braunbeck T. 1992. Cytopathology of spleen in eel *Anguilla anguilla* exposed to a chemical spill in the Rhine River. Diseases of Aquatic Organisms 14:1-22.

Spitzbergen JM, Schat KA, Kleeman JM, Peterson RE. 1986. Interactions of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) with immune responses of rainbow trout. Veterinary Immunology and Immunopathology 12:263-280.

Stark WJ, Echelle AA. 1998. Genetic structure and systematics of smallmouth bass, with emphasis on Interior Highlands populations. Transactions of the American Fisheries Society 127(3):393-416.

Starliper C, Blazer V, Iwanowicz L, Walsh H. 2013. Microbial isolates in diseased fishes, primarily Smallmouth Bass (*Micropterus dolomieu*), within the Chesapeake Bay drainage in 2009-2011. Proceedings of the West Virginia Academy of Science 85(2):18-32.

Stillwell SB, Demuth HC, Streuber L, Ford HC, Dale JA, Corwin DP. 1895. Report of the State Commissioners of Fisheries, Year 1895. Harrisburg, PA: Fish Commissioners of Pennsylvania.

Stott GG, McArthur NH, Tarpley R, Jacobs V, Sis RF. 1981. Histopathologic survey of ovaries of fish from petroleum production and control sites in the Gulf of Mexico. Journal of Fish Biology 18:261-269.

Thorpe KL, Gross-Sorokin M, Johnson I, Brighty G, Tyler CR, Jobling S. 2005. Health effects in fish of long-term exposure to effluents from wastewater treatment works. Environmental Health Perspectives 114(1):81-89.

Tough DF, Sun S, Sprent J. 1997. T cell stimulation in vivo by lipopolysaccharide (LPS). Journal of Experimental Medicine 185(12):2089-2094.

Tripp RA, Maule AG, Schreck CB, Kaattari SL. 1987. Cortisol mediated suppression of salmonid lymphocyte responses in vitro. Developmental & Comparative Immunology 11(3):565-576.

Valavanidis A, Vlahogianni T, Dassenakis M, Scoullos M. 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicology and Environmental Safety 64(2):178-189.

Wagner U, Burkhardt E, Failing K. 1999. Evaluation of canine lymphocyte proliferation: comparison of three different colorimetric methods with the 3H-thymidine incorporation assay. Veterinary Immunology and Immunopathology 70(3):151-159.

Walsh AH, Ribelin WE. 1975. The pathology of pesticide poisoning. The Pathology of Fish (W E Ribelin and E Migaki, eds): University of Wisconsin Press. p 22.

Walsh HL, Blazer VS, Iwanowicz LR, Smith G. 2012. A redescription of *Myxobolus inornatus* from young-of-the-year Smallmouth Bass (*Micropterus dolomieu*). The Journal of Parasitology 98(6):1236-1242.

Wang R, Belosevic M. 1994. Estradiol increases susceptibility of goldfish to *Trypanosoma danilewskyi*. Developmental & Comparative Immunology 18(5):377-387.

Weyts FAA, Cohen N, Flik G, Verburg-van Kemenade BML. 1999. Interactions between the immune system and the hypothalamo-pituitary-interrenal axis in fish. Fish & Shellfish Immunology 9(1):1-20.

Willard DA, Bernhardt CE, Hupp CR, Newell WN. 2015. Coastal and wetland ecosystems of the Chesapeake Bay watershed: Applying palynology to understand impacts of changing climate, sea level, and land use. Field Guides 40:281-308.

Williams MS, Kwon J. 2004. T cell receptor stimulation, reactive oxygen species, and cell signaling. Free Radical Biology and Medicine 37(8):1144-1151.

Williams RW, Warner MC. 1976. Some observations on the stained blood cellular elements of channel catfish, *Ictalurus punctatus*. Journal of Fish Biology 9(6):491-497.

Winterbourn CC. 2008. Reconciling the chemistry and biology of reactive oxygen species. Nature Chemical Biology 4(5):278-286.

Wolke RE. 1992. Piscine macrophage aggregates: a review. Annual Review of Fish Diseases 2:91-108.

Wolke RE, Murchelano RA, Dickstein CD, George CJ. 1985. Preliminary evaluation of the use of macrophage aggregates (MA) as fish health monitors. Bulletin of Environmental Contamination and Toxicology 35(1):222-227.

Zelikoff JT. 1993. Metal pollution-induced immunomodulation in fish. Annual Review of Fish Diseases 3:305-325.

Zoschke DC, Messner RP. 1984. Suppression of human lymphocyte mitogenesis mediated by phagocyte-released reactive oxygen species: comparative activities in normals and in chronic granulomatous disease. Clinical Immunology and Immunopathology 32(1):29-40.

Zuba-Surma EK, Ratajczak MZ. 2011. Analytical Capabilities of the ImageStream Cytometer. In: Darzynkiewicz Z, Holden E, Orfao A, Telford WG, Wlodkowic D, editors. Recent Advances in Flow Cytometry, Part A. 5 ed: Elsevier Inc. p 207-230.

Zweifel RD, Hayward RS, Rabeni CF. 1999. Bioenergetics insight into black bass distribution shifts in Ozark border region streams. North American Journal of Fisheries Management 19(1):192-197.