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An Evaluation of the Emerald Shiner (Notropis atherinoides) as a Bioindicator of Urban Water Pollution in the Upper Niagara River

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An evaluation of the emerald shiner (*Notropis atherinoides*) as a bioindicator of urban water pollution in the upper Niagara River

by

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An Abstract of a Thesis

in

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Abstract of Thesis

Using fishes as bioindicator species can be an effective method for detecting poor water quality in aquatic ecosystems. In the Niagara River, the emerald shiner (*Notropis atherinoides*) is a keystone species that is sensitive to ecosystem degradation and, therefore, fills the bioindicator role. Like other model bioindicators, emerald shiners are abundant and, when exposed to a persistent disturbance, exhibit individual signs of stress before the onset of population decline. This research evaluated the health of emerald shiners captured from the upper Niagara River, which is at times inundated with untreated sewage from combined sewer overflows (CSOs). Water samples were taken biweekly from seven sites in the upper Niagara River and one site in Lake Erie, to determine *Escherichia coli*'s most probable number (MPN)/100 mL from May-October 2016. Emerald shiners were captured from riverine sites and given an overall health score using the Health Assessment Index (HAI), which incorporates nine physiological parameters, plus their condition factor and liver bacterial infection. Most water samples were below the Environmental Protection Agency criteria for *E. coli* MPN in a class A stream. However, 35% of fish were positive for internal liver infection. Fulton's condition factor for emerald shiners reflected measured signs of severe stress such as hemorrhaging and high parasite loads. The most stressed fish were captured in the eastern branch of the river, which is highly urbanized and includes the cities of Buffalo, NY and Tonawanda, NY which produce high CSO effluent. In comparison, the emeralds shiners in the western branch of the river, which is less developed along both the USA and Canadian shorelines, had overall better health markers. These results provide supporting evidence that emerald shiners are exhibiting immunological stress and current water pollution levels are stressing the shiner population in the river, despite compliance with EPA regulations for *E. coli* input. The future of this keystone species and the health of the ecosystem hinge on solving the issue of the excessive anthropogenic contamination in the Niagara River.

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Dedication

This work is dedicated with love to my niece Miriam Shulman, who possesses seemingly limitless amounts of courage and potential. She has persevered through a difficult illness during my graduate career with immense bravery and I look forward to witnessing her future accomplishments.

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Introduction

Aquatic environments change in response to pollution. To assess the changes associated with the degradation of water quality, biomonitoring has been developed as a tool to gauge the condition of an aquatic ecosystem by evaluating the health of the organisms restricted to that waterway. For example, high input of sewage in a waterway can cause changes in oxygen, nutrient and bacterial levels that affect aquatic life. Although aquatic organisms, such as fish, have some ability to flee polluted areas, they are generally susceptible to continuous and cumulative pollution levels. Typically, the more degraded the environment becomes, the more biological stress will occur, with increased potential for lethal effects (Goede and Barton 1990, Shreck and Moyle 1990, Adams et al. 1993, Cazenave et al. 2009, Iwanowicz et al. 2012). Current standardized water quality indices are not sensitive enough to detect functional decreases in ecological integrity (Cazenave et al. 2009), therefore, by evaluating a model organism or bioindicator species it is possible to ascertain whether or not the environment is functionally suitable for other organisms (Khan and Billiard 2007, Cazenave et al. 2009, Watson et al. 2012, Colin et al. 2016).

To be considered a model bioindicator, a species should be widespread, easy to obtain and have a strong role in linking trophic levels (Cazenave et al. 2009). The bioindicator species should also exhibit discernable responses to degraded environments to warrant its use over multi-metric indices such as species diversity or abundance (Colin et al. 2016). If possible, the bioindicator species should also have economic or cultural value to humans (Khan and Billiard 2007). Biomarkers are the changes that are tested in the model species used as a bioindicator. These biomarkers are the physical responses to stress measured in a population before lethal toxicity levels are reached. In this way, the bioindicator population health can be evaluated

before population extirpation occurs due to environmental degradation (Goede and Barton 1990, Adams et al. 1993, Van Der Oost et al. 2003). Many biomarker responses can be grossly determined through necropsies, such as discoloration of an organ, hemorrhaging or lesions, or changes in blood parameters (Colin et al. 2016). Other, more sophisticated biomarkers include histology and cellular changes within organs (Cazenave et al. 2009, Iwanowicz et al. 2012, Colin et al. 2016). Additionally, it is best to test more than one biomarker for verification of biological stress to avoid falsely detecting, or failing to detect, a response (Goede and Barton 1990, Adams et al. 1993, Van Der Oost et al. 2003, Cazenave et al. 2009).

The emerald shiner (*Notropis atherinoides*) is a native minnow in the Great Lakes region with high trophic importance as a forage fish species that fits the role of a model bioindicator. It is a planktivorous fish, important for regulating zooplankton populations in Lake Erie (Pothoven et al. 2009). The shiner's preferred food sources are copepods and cladocerans, however, it also consumes chironomids, algae, detritus, and some small invertebrates (Hartman et al. 1992). Emerald shiners serve as a food source to avian species such as common terns (*Sterna hirundo,* a threatened species in New York State), ring billed gulls (*Larus delawarensis*) (Horner et al. 2010), double-crested cormorants (*Phalacrocorax auritus*) (DeBruyne et al. 2013), mergansers (*Mergus merganser*) (Bur et al. 2008) and common loons (*Gavia immer*), among others. They are also a main prey fish for walleye (*Sander vitreus*), steelhead trout (*Oncorhynchus mykiss*), smallmouth bass (*Micropterus dolomieu*), among others (Bur et al. 2008).

A decrease in the health of emerald shiners in the Niagara River ecosystem would have cascading negative effects on the numerous predators that depend on them. In addition to the emerald shiners' role in the food web, the presence of this fish has economic importance to the Great Lakes. It is used as bait for sport-fishing, and is harvested and sold in bait shops all around

the region. The relative abundance, trophic importance (Bur et al. 2008, Pothoven et al. 2009, Horner et al. 2010), and economic value of emerald shiners justify their use as a bioindicator species in the Niagara River ecosystem which is the main connecting channel between Lakes Erie and Ontario. Although the abundance of emerald shiners in a polluted river such as the Niagara can give an idea of their ability to endure some level of stress, it may not necessarily reflect their health status. Therefore, an investigation into various biomarkers of health for emerald shiners in conjunction with widely accepted water-borne bacterial testing procedures was warranted, and was the purpose of this study.

The Niagara River is classified as an Area of Concern (AOC) by the International Joint Commission (IJC) in the Great Lakes (DEC 2010). Contaminants contributing to this AOC status are polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and mercury contaminants which affect the health of fishes (DEC 2010). Nonetheless, the health status of the emerald shiners in the Niagara River is likely more directly linked to their exposure to a high-sewage-input environment due to the routine intermittent levels of effluent that enter the upper river. Fecal coliform and *Escherichia coli* levels in the water also are a concern in light of the high volume of city sewage input along the river's length and the established correlations that exist among sewage, water quality, and biological stress in fish (Dragun et al. 2013, Joh et al. 2013).

Sewage Impacts on Water Quality

The overall quality of an urban waterway depends on factors such as human population density, water velocity, diluting capacity of the river, and sewage input (Ouattara et al. 2014). The Niagara River, which connects lakes Erie and Ontario, has combined-sewer-overflow (CSO) infrastructure along the entirety of its length (DEC 2010). In CSO infrastructure both household water waste and storm run-off go through the same pipes on their way to wastewater treatment plants (WWTPs). When the volume of water is higher than the level that the WWTP can treat, the untreated mixed sewage (run-off and sewage) overflows into the Niagara River and tributaries. In times of dry weather, all the sewered waste goes to the WWTP. However, when there are heavy rains (which are common in the Buffalo area), the CSOs are inundated with water, and the mixture of storm water and untreated sewage spills over into a natural body of water (Mailhot et al. 2015). Wet weather events of 25.4 mm or more in Buffalo causes approximately 700,000 gallons of untreated mixed sewage to be released through CSOs into the Niagara River (Buffalo Sewer Authority 2017).

There is greater density of CSOs, WWTPs and other non-sewage industrial discharges along the eastern branch of the Niagara River compared to the western branch on the Canadian side (Figure 1). These high sewage inputs can lead to water quality issues such as low oxygen levels, high phosphate, nitrogen and ammonia levels, increased turbidity, heavy metal contamination, and high bacterial levels (Ouattara et al. 2014). Fecal coliforms and *E. coli* are used as surrogate indicators of water quality, because they are almost always found in tandem with the other bacteria derived from feces and are thus referred to as “fecal indicating bacteria” (FIB) (Cabral 2010, Gronewold et al. 2011). Some of the most common pathogenic bacteria released into natural waterways include *Vibrio, Salmonella, Shigella,* and *E. coli,* which are

always found in the feces of warm blooded animals, particularly livestock and humans (Cabral 2010). It is important to recall that the presence of one FIB (such as *E. coli*) is not an isolated bacterium, but in fact suggests that fecal matter containing many types of bacteria are contaminating a water source.

High fecal bacteria levels can affect a fish's immune health both directly through bacterial invasion, which can lead to diseases, hemorrhaging or lesions, and indirectly, such as through supporting ciliate parasites that feed on bacteria and can impair a fish's immune response if their densities are very high (Fattal et al. 1992, USFWS 2004, Sures 2008, Joh et al. 2013, Kavanagh et al. 2013). Additionally, the effluent in CSOs contains other emerging contaminants, such as pharmaceuticals and personal care products (PPCPs). PPCPs are known to impact the immune system of aquatic organisms and bioaccumulate in aquatic systems (Li et al. 2015), and this phenomenon is occurring within the Niagara River ecosystem (personal communication, Prapha Arnnok).

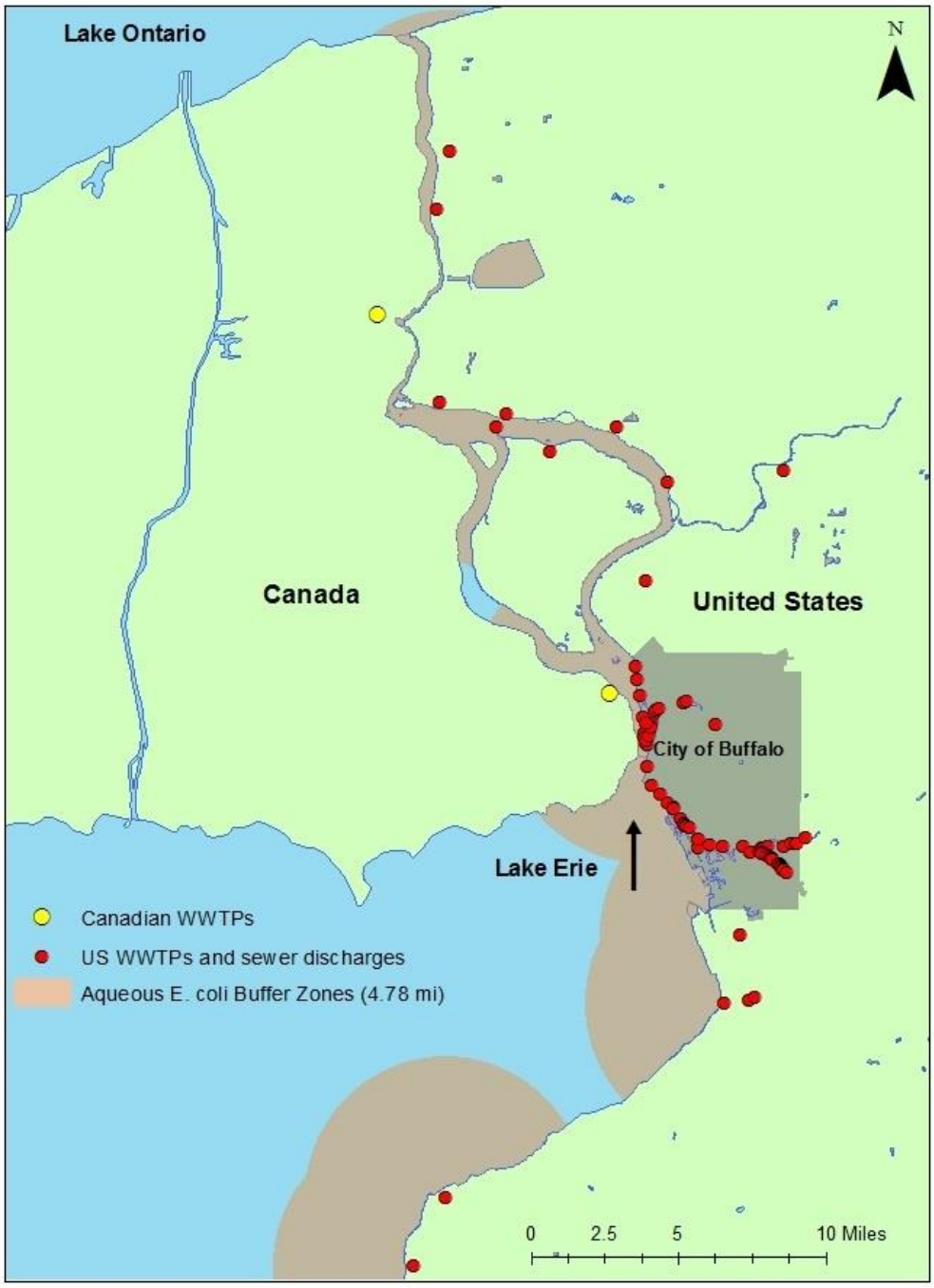

Figure 1
This map shows the distance that *E. coli* can potentially travel in aquatic systems before a one-$\log_{10}$ reduction in bacterial load occurs (for example, from 100 *E. coli*/100 mL to 10 *E. coli*/100 mL) indicated by the brown buffer zones surrounding CSO and WWTP discharge points. However, this buffer distance was determined from a study in Europe (Lee and Glover 1998) so it does not account for the Niagara River water velocity, or any other site-specific features. It does however, provide a basic visual context of the potential capacity for aquatic transport of fecal bacteria.

Hematological Indices

Hematological values, or measurements of the blood components, are easy to obtain and are good indicators of fish health (Goede and Barton 1990, Adams et al. 1993, Luskova 1998, Watson et al. 2012). Previous experiments have shown that hematological values in fish respond significantly and rapidly to stress induced by water quality changes, such as thermal or biological pollution (Tierney et al. 2004). Hematocrit (packed red-blood cell volume), leukocrit (packed white-blood cell volume) and plasma proportions can be determined by centrifuging a single sample of blood (Tierney et al. 2004). After centrifugation the red blood cells pack at the bottom, the white blood cells form a packed buffy white layer in the middle, and the plasma proteins are contained in the clear fluid on the top. The proportions of these blood components in a blood sample can help evaluate a fish's immune response.

There are numerous studies supporting the correlation between altered leukocrit values and a compromised immune system (Wedemeyer et al. 1983, Sun et al. 1995, Luskova 1998, Tierney et al. 2004, Cazenave et al. 2009, Milukaite et al. 2010, Watson et al. 2012, Lepak et al. 2013, Fazio et al. 2015). In general, leukocrit is more indicative of bacterial exposure than hematocrit, since hematocrit can reflect quick responses to handling and short-term stress. A low leukocrit value in fish, as in most other animals, is the result of long-term immune stress which causes an increase of corticosteroids, which in turn suppresses white blood cells (Goede and Barton 1990). Another cause of very low leukocrit values is leukocytosis, a bacterial infection that causes destruction of white blood cells. Conversely, extremely elevated leukocrits suggest acute bacterial stress, in which white blood cells are elevated to suppress a recent infection (Goede and Barton 1990). Thus, to reduce variability in hematocrit and leukocrit values, it is imperative to reduce the stress of sampled fish and normalize handling conditions.

Although baseline hematology values for commonly studied fish, such as coho salmon (*Oncorhynchus kisutch*) or Pacific herring (*Clupea pallasii*) can be found in the literature (Tierney et al. 2004), the emerald shiner has historically been understudied and their hematology values have not been previously published. Therefore, it was necessary for this project was to determine the physiological range of leukocrit values for emerald shiners in the upper Niagara River to properly assess whether they are in health-distress. To support the hematological data, necropsy and other immune-response testing are normally performed as part of a health evaluation in fish.

Fish Necropsy and the Health Assessment Index

Adams et al. (1993) modified a previous fish necropsy method (Goede and Barton 1990) to create a scaled index that scores 16 different vital organs and physiological factors based on appearance. This necropsy method, the Health Assessment Index (HAI), assumes that the affected organs will exhibit visual differences under stress (e.g., discoloration of the liver) before mortality occurs (Goede and Barton 1990). The HAI is intended as a screening detection for stress before extirpation of the population, and has been successfully used to evaluate the health of free-living fish populations (Iwanowicz et al. 2012, Watson et al. 2012, Lee et al. 2013, McHugh et al. 2013) . Typically, the HAI is used for larger fish, where all organs are discernible. However, emerald shiners are generally under 120 mm and some organs such as the spleen are hard to find and observe without damaging them, the HAI scoring system was further adapted by omitting impractical scoring factors in this species (spleen, kidney, thymus, hindgut, and protein analysis of blood plasma) (Table 1).

Table 1
Summary of the adapted Health Assessment Index scoring system for the emerald shiner. A higher score indicates a fish in poorer condition, with a maximum possible score of 270.

Individual HAI Scores	**0**	**10**	**20**	**30**
Leukocrit	0.6-3%	0-0.6%	3-5%	>5%
Hematocrit	30-45%	>45%	19-29%	<18%
Eyes	Not impacted			Hemorrhaging or missing
Gills	Not impacted (red with black markings)			Mucous covering; swollen; hemorrhaging; discoloration
Psuedobranch	Flat and white			Discolored; concave; convex; or swollen
Skin	Not impacted	Some scales missing	Many scales missing; small lesions; faint hemorrhaging	More than half scales missing; severe lesions; severe hemorrhaging
Fins	Not impacted	Minor fraying	Moderate fraying or tears	Severe fraying or tears; hemorrhaging; missing
Total number of parasites	0	1-6	7-14	>15
Liver	Dark-bright red			Paleness, any aberrations, cysts or nodules; fatty or cream coloration

It can be argued that the necropsy HAI approach may be considered less informative than more sophisticated biomonitoring techniques. However, its use is justified in determining overall health of a fish population when sampling and personnel biases are kept to a minimum. In a study of sharptooth catfish (*Clarias gariepinus)* exposed to DDT, researchers found that HAI was able to detect poor health status of fish in ways that histological analysis could not (McHugh et al. 2013). For example, although microscopic examination of gills, liver and spleen can elucidate damage on a cellular level, it does not account for "overall" physical degradation such as external lesions on a fish's body, reduced condition factor, or the parasitic load in the entire fish.

Of all biomarkers in the HAI, parasite number may be particularly useful (Watson et al. 2012). Parasites can cause behavioral changes in fish such as scraping against substrate for removal, which leaves the fish susceptible to fungal infections. A high parasite load can also lower swimming ability, leading to exhaustion and subsequent stress (Barber et al. 2000). Parasite load is a concern for the health of the emerald shiners in the upper Niagara River. The emerald shiners studied in previous research (2014-2015) had many species of parasites (confirmed by Dr. Rodman Getchell, College of Veterinary Medicine, Pathology Laboratory, Cornell University) including trematodes, intestinal worms, myxosporeans and *Ichthyophthirius multifiliis* (common name: white-spot disease, hereafter *Ich*). *Ich* is a ciliate parasite that feeds on fish tissue during the attached parasitic stage of its life cycle. When it detaches and enters the free-swimming stage, the fish is susceptible to secondary infections which can cause mortality in severe cases (Forwood et al. 2015). Recently, *Ich* has been found to lower the innate immune response by altering the expression of pathogen-receptor genes, which are necessary for a fish's immune system to recognize pathogens (the pathogen-recognition receptors) (Frank et al. 2017).

Liver Infection

The internal organs of healthy fish are sterile (with the exception of the gastrointestinal tract) and when bacterial infection is found, it suggests a compromised immune system (Fattal et al. 1992, Joh et al. 2013). Generally, the liver in fish is the first organ to become infected when continually exposed to high bacterial loads. For instance, in a study conducted on 621 eels (*Anguilla japonica*) raised in poor aquaculture conditions with bacterially degraded water, 60% of all bacteria recovered in eels (muscle, kidney, visceral cavity, and liver sampled) were located in the liver. Many of the infected eels had three or more different types of bacteria within an individual organ (Joh et al. 2013). Most of these bacteria found were opportunistic pathogens that only attacked eels with weakened immune systems. Also, European chub (*Squalius cephalus*) had bacterial infections in their livers, reduced condition factor and immune stress-induced histological changes in a Croatian river system even when sewage pollution was considered legally acceptable for resident fish (Dragun et al. 2013).

Summary

Because they are a critical species in the aquatic food web of the Niagara River (Bur et al. 2008, Pothoven et al. 2009, Horner et al. 2010), the importance of the emerald shiner cannot be understated. To determine whether these fish tolerate current levels of water pollution, it was necessary to establish a reference for the current immune health of emerald shiners, to better monitor their population health in future conservation efforts. It was expected that if emerald shiners exhibited immune stress in the Niagara River ecosystem due to continuous exposure to sewage, this would support necessary sewage mitigation. Conversely, if emerald shiners

appeared unstressed in the Niagara River, the ecosystem health may be adequate despite sewage input.

Objectives and Hypotheses

The objective of this study was to evaluate the health status of emerald shiners living in a sewage-impacted river subject to routine intermittent sewage pollution from CSOs and regular effluent input from WWTPs. There has been no previous formal effort to assess the health condition of this forage fish species in the Niagara River.

Hypothesis 1: *Escherichia coli* levels in river water will increase according to proximity to WWTPs and CSOs

Water samples collected at the eastern branch of the upper Niagara River (sites: Sandy Beach, **SB,** Gratwick Park, **GP**, Isle View, **IV**, Rich's Marina, **RM** and Black Rock Canal, **BR**; Figure 2) were expected to have higher *E. coli* levels than those on the western branch (Big Six Mile Creek, **B6** and Beaver Island, **BI**; Figure 2). There are more CSOs and WWTPs on the USA, or eastern branch of the river than on the Canadian, or west branch, which contribute to higher FIB input. *E. coli* levels from Lake Erie (**LE**) were predicted to be near zero due to its offshore from CSOs and WWTPs. Therefore, the lake was used as a local baseline or "control".

Hypothesis 2: Leukocrit values in emerald shiners will reflect higher immunological stress in more polluted sites

Emerald shiners exhibiting other signs of immunological stress, such as hemorrhaging, high parasite loads, reduced condition factor or bacterial infections were predicted to have irregular leukocrit values, either abnormally high or low, compared to healthy fish. It was hypothesized that fish captured from the eastern branch in more urban environments (SB, GP, IV, RM and BR) would differ in leukocrit values from those captured at the cleaner sites (B6 and BI). Additionally, the physiological range of a healthy leukocrit was expected to be determined in emerald shiners, which had not been reported to date.

Hypothesis 3: The HAI score for emerald shiners from sites with more pollution will be higher compared to shiners from less polluted sites

Emerald shiners from the more urban eastern branch sites with higher concentrations of waste outflows (CSOs, WWTPs) (SB, GP, IV, RM and BR) were predicted to have the highest HAI scores, and shiners from western branch sites (B6 and BI) were expected to have lower (but not perfect) HAI scores, since the Niagara is a highly developed river. The parasite load of fish was anticipated to be an important parameter for the HAI method. Older emerald shiners were also predicted to have higher HAI scores than young-of-year fish due to cumulative effects of exposure to stressors in the older fish.

Hypothesis 4: Emerald shiners' liver infection will be associated to other signs of immune stress

Emerald shiners with bacterial infection in their livers would also exhibit other signs of poor health, such as irregular leukocrit, poor condition factor, high parasite loads and poor HAI scores.

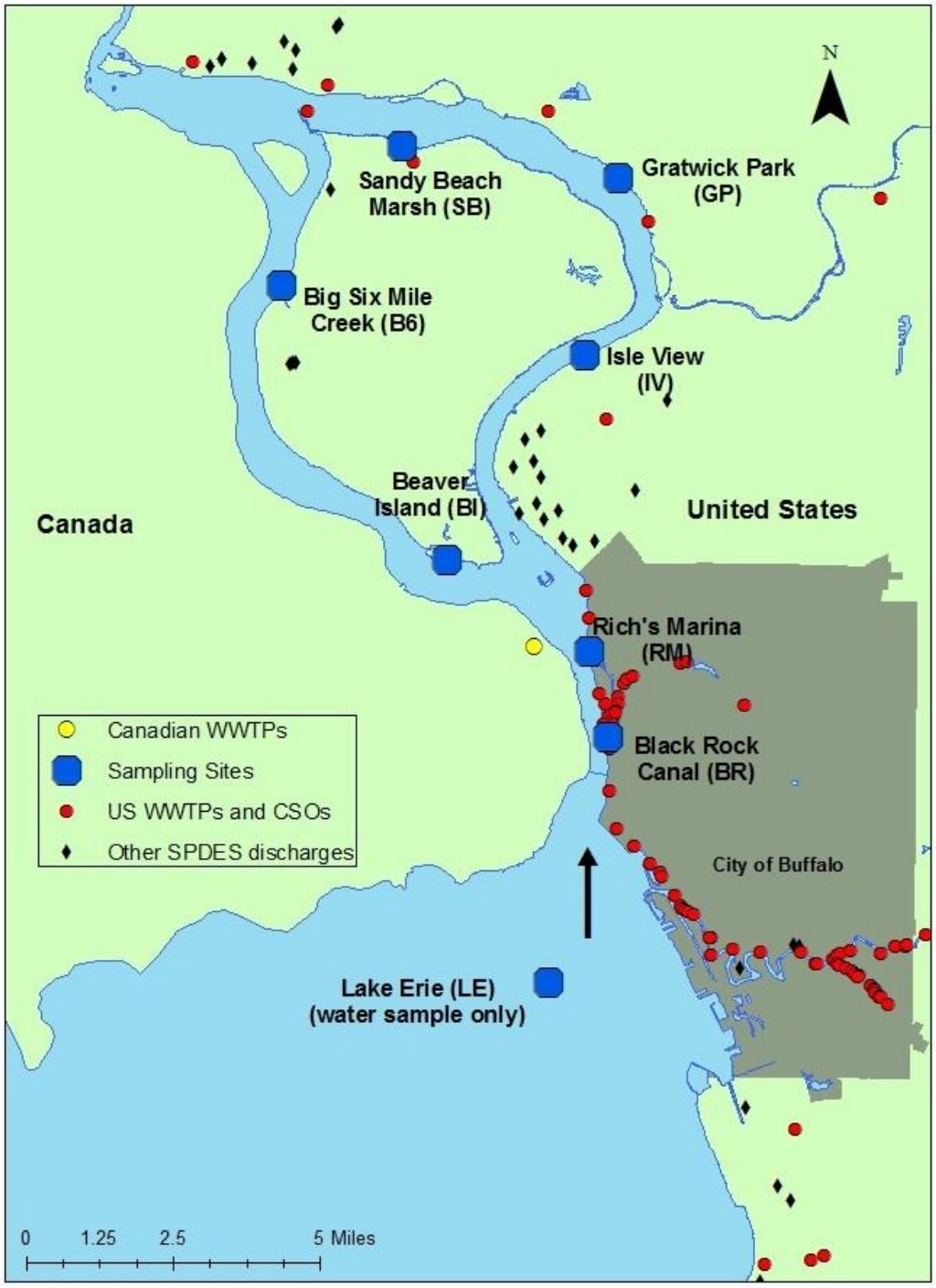

Figure 2
Map of the sampling sites and all nearshore waste water treatment plants (WWTP), combined sewer overflow (CSO) discharges, and other State Pollutant Discharge Elimination System (SPDES) points (industrial point sources that release non-sewage effluent). The only Canadian sewage GIS data available online was WWTPs, so there may be Canadian CSOs not depicted in this map.

Methods and Materials

Study Site Description

The upper Niagara River flows north for 24.8 miles from Lake Erie to Niagara Falls and is a binational waterway, situated between Canada and the United States (42-43°N and 78-79°W). The Niagara River is designated as a class "A" water source by the Department of Environmental Conservation (DEC), which requires it to be clean enough for recreational and drinking purposes (DEC 2010). However, as previously mentioned, the Niagara River is an Area of Concern (AOC) and has a Remedial Action Plan (RAP) established for rectifying its water quality issues (DEC 2010). Water quality issues listed in the RAP include: CSO input, storm-water and urban run-off, contaminated sediments and fish consumption advisories.

Six sites were sampled for water and emerald shiners on a biweekly basis from May through October 2016: B6, SB, GP, IV, RM and BR (Figure 2). The seventh site, BI, was added on the fourth sampling event due to a shortage of emerald shiners at the other sites. All sites are located in the upper Niagara River except BR which is a canal separated from the main river by a break wall with culverts that allow some water connectivity. The LE site (Lake Erie) was sampled for reference water samples only. There is a higher concentration of CSOs and WWTPs on the eastern branch of the river (sites SB, GP, IV, RM and BR) coming from the cities of Buffalo, Tonawanda, North Tonawanda and Grand Island. The western branch is adjacent to Canada, which has fewer sewage discharge points (sites BI and B6).

Water Sampling

Dissolved oxygen (mg/L), conductivity (µS/m) and water temperature (°C) were recorded using a YSI 556 MPS™ water quality meter at 0.5 m depth at each site. Additionally, three 250 mL water samples were taken at 0.5 m depth from each site using a subsurface grab sampling pole (Ben Meadows 6000 Series Jar Sampler™). Three samples were taken to account for spatial heterogeneity at each site, which can be quite extreme in aquatic settings. Bottles were autoclaved and the rim of the sampling pole was sterilized with ethanol prior to use. Water samples were stored on ice packs in Rubbermaid® totes and covered from sunlight until returned to the laboratory.

Fecal coliforms and *E. coli* were incubated within six hours of collection for most probable number (MPN) using the EPA-approved Quanti-Tray® method, which is significantly less labor intensive compared to serial dilution methods. The MPN by nature is not an exact count of bacteria cells, but rather it has an associated set of 95% confidence intervals in which the count lies. Serial dilution methods were previously a necessary step for environmental water MPN enumeration, because the MPN in a completely overcrowded, undiluted sample cannot be ascertained; it would just be positive for complete presence of bacteria. In serial dilution, MPN of each sample would be analyzed at several concentrations: undiluted, 1:10 dilution, 1:100 dilution and 1:1,000 dilution. This process is extremely labor-intensive. However, the Quanti-Tray® method involves aseptically adding water to trays that hold three different well sizes, functioning as three serial dilutions, without requiring manual dilution steps.

After returning to the laboratory, the three water samples at each site were combined to create one composite sample representing a site. The composite sample was thoroughly mixed and then subsampled into a 100 mL sterile plastic jar containing sodium thiosulfate, which

reduces WWTP chlorination impacts. Bacteria are killed when exposed to chlorine for extended amounts of time, and sodium thiosulfate will remove any residual chlorine in the sample so that it does not reduce MPN during the incubation period. Subsequently, Colilert®-18 medium was added to the 100 mL composite sample. The composite subsample was poured into a Quanti-Tray®, sealed, and incubated in a circulating water bath for 22 hours at 44.5°C ± 0.5°C (IDEXX Laboratories Inc. 2013).

After incubation, fecal coliforms and *E. coli* MPN were enumerated by counting the large and small wells that changed color. Each well was compared to a control comparator provided by the manufacturer to assess presence/absence. The comparator is a tray used to compare the intensity of the color change and fluorescence of each sample. For instance, if the sample in question had wells equal or greater in yellow intensity than the comparator, those wells were considered positive for fecal coliforms. Each well that fluoresced under UV light in equal or greater intensity than the comparator was considered positive for *E. coli*. The number of wells positive for *E. coli* were compared to a corresponding table to determine MPN per 100 mL of sample, with manufacturer's 95% confidence limits (IDEXX Laboratories Inc. 2013). On days following rainfall events, 10% dilutions of samples taken from potentially high bacteria sites (GP, IV, RM and BR) were used preventatively so that no overcrowded samples would have to be discarded from statistical analysis. However, all undiluted samples could be adequately censused in this study, therefore, the diluted datasets were not used for statistical analyses. For reference, the New York State *E. coli* geometric mean cut-off for a Class A stream is 126 Colony Forming Units (CFU)/100 mL when at least five samples are taken in a month, or 235 CFU/100 mL for a single grab sample at any given time (DEC 2015). The standards for a Class A stream are more stringent than for streams best suited for fish and wildlife, which require a 5-sample

geometric mean of 548 CFU/100 mL over 30 days (DEC 2015). Precipitation data was downloaded from Weather Underground (2017), a weather service with accessible datasets to support observed *E. coli* and water quality data.

Fish Sampling

Emerald shiners were sampled at the same time as water collection from all sites except Lake Erie. Fish were captured using either a shad trawl-net towed behind the boat or seining, depending on site depth. Fish were kept in aerated buckets, returned to the laboratory and dissected immediately. To reduce the amount of time fish spent in buckets before sacrifice, fish were captured from only two sites in a given day.

Necropsy and Health Assessment Index

For necropsy and health assessment, an assistant randomly selected one fish for analysis without revealing the location of its capture. When possible, both young-of-the-year (YOY) and adults were used for necropsies. Fish were fully anaesthetized with buffered tricaine methanesulphonate (MS-222) and their weight (in grams) and length (in millimeters) were recorded. The fish's tail was severed with a clean blade posterior to the anal vent to reveal the caudal vessel. Blood was extracted by touching a heparinized capillary microcentrifuge tube to the caudal vessel, then sealing the other end with hematocrit clay. The tubes had been pre-labeled to correspond with fish identification numbers. The tubes were stored in the refrigerator to prevent cell-bursting damage which can occur if left at room temperature. Afterward, fish were returned to water containing MS-222 for sacrifice via overdose.

Fish were then placed in a dish and examined under a stereo microscope and a score was given for each HAI trait in the order shown in Table 1. Example photographs of the process are given in Figure 3. After noting all external parasites, fish were opened on the ventral side with a scalpel and the liver was examined. Internal parasites were included in total parasite count. Total HAI score and Fulton's condition factor (CF) were calculated according to the following formulas, where W=weight in grams and L=length in centimeters.

$$\textbf{Total HAI} = \Sigma \textbf{ individual HAI scores}$$

$$\textbf{CF} = W/L^3 * 100$$

Within 45 minutes of collection, blood samples were centrifuged until the blood components were fully separated and packed (10-12 minutes, Clay Adams Micro-Hematocrit Centrifuge). Leukocrit, hematocrit and plasma percentage were all measured in the microcentrifuge tube using a stereo microscope and Olympus DP21 imaging software. The tube length occupied by all three blood portions was summed, and the percentage of each component in the tube was determined to be the packed volume of that blood component (e.g., the white blood cell portion of the tube length in µm would be divided by the tube length in µm of all blood portions in the tube) (Figure 3). Preliminary HAI and blood centrifugation trials were conducted on 40 emerald shiners prior to collecting data for this project to ensure familiarity with the organs' healthy and unhealthy appearances. Photographs of the fish examined were taken throughout the project for disease reference.

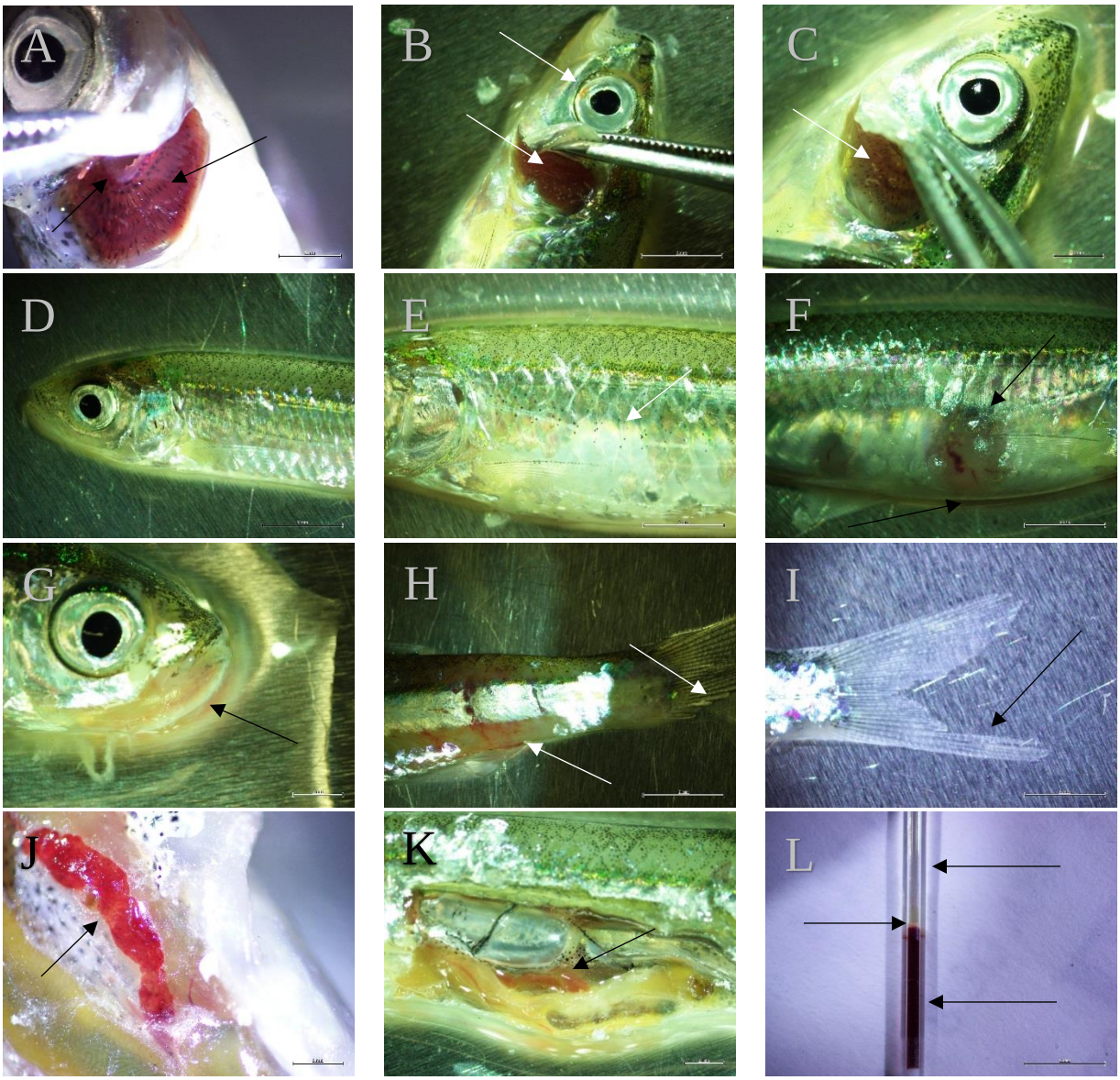

Figure 3
Example photographs of HAI process in emerald shiners. Scale differ among photographs. **A:** Healthy gills and pseudobranch. **B:** Gills infested with *Ich* parasites, hemorrhaged eye. **C:** Pale gills coated with mucous. **D:** Healthy skin and typical streamlined body shape. **E:** Moderate skin damage. **F:** Severe skin damage with lesion, bloated body. **G:** Severe fungal infection on mouth. **H:** Severe hemorrhaging and caudal fin damage. **I:** Severe caudal fin damage. **J:** Healthy liver. **K:** Pale liver (diseased). **L:** Centrifuged blood sample. The bottom portion is the hematocrit, middle small buffy portion is the leukocrit, and the top clear portion is the plasma component.

Microbiology Tests

A subsample of emerald shiners (266 out of 322 fish [83%]) were tested for bacterial infection in their livers. After noting the visual appearance of the liver for the HAI, it was removed with sterile forceps and the outer surface of the liver was sterilized with an ethanol pad. A sterile cotton swab and forceps were used to puncture the liver and streak the tissue onto a blood agar plate (95% Difco™ Tryptic Soy Agar, 5% defibrinated sheep's blood). The blood agar was cultured at room temperature for 24-48 hours. As a positive control, a sample from the gastrointestinal tract was streaked each day to ensure the agar medium was adequate to culture bacteria. The absence or presence of bacterial growth and any corresponding hemolytic activity in the agar was noted. Some bacterial colonies were hemolytic, meaning that they lysed the red blood cells of the agar media, which caused a faint halo surrounding the colony. This behavior is not typical of all bacteria, and in fact is an indicator of some pathogenic species such as *Aeromonas salmonicida*, so this information was used as an identifying feature to group bacteria. Example photographs of the microbiology tests are given in Figure 4.

Each colony contains a group of cells referred to as a colony forming unit (CFU), meaning that they are one distinct type of bacteria. However, when streaking colonies from a piece of liver tissue, the CFUs sometimes overlapped on the agar. Before conducting further identification tests, steps were taken to isolate a colony and guarantee that it contained only one bacterial strain. The colony was then transferred with a sterile loop to an anti-freeze solution (20% glycerol, 80% Difco™ Nutrient Broth) and vortexed to ensure cells were evenly distributed. The solution was then pipetted into a sterile Eppendorf® tube containing approximately 10 three mm glass beads and cryopreserved at -80°C. This preservation method

ensures that each coated bead has thousands of cells adhered to it, so that one bead may be taken out at a time for later biochemical tests.

After all of the field work was completed, the preserved bacteria were resuscitated by aseptically placing one glass bead from each sample into tryptic soy broth and incubating for 24-48 hours at room temperature. After incubation, turbid tryptic soy broth tubes were subsampled and streaked for isolation on tryptic soy plates. This step ensured that the colony was pure for subsequent identification tests. An isolated colony was obtained from the tryptic soy agar plate and transferred onto a tryptic soy slant for storage. Each culture was then gram-stained and examined using oil immersion microscopy to determine the organism's Gram reaction, size and shape. Bacteria were also tested for glucose fermentation, with or without gas formation (0.5% D (+) Glucose solution in phenol red broth base, using a Durham tube for gas formation) and the presence of cytochrome oxidase (1% solution oxidase reagent using a platinum loop). Positive and negative controls were necessary for gram-staining and cytochrome oxidase tests to ensure quality control. The controls for gram-staining were known stocks of *Staphylococcus spp.* (gram-positive) and *E. coli* (gram-negative). The cytochrome oxidase controls were *Pseudomonas putida* (positive) and *E. coli* (negative).

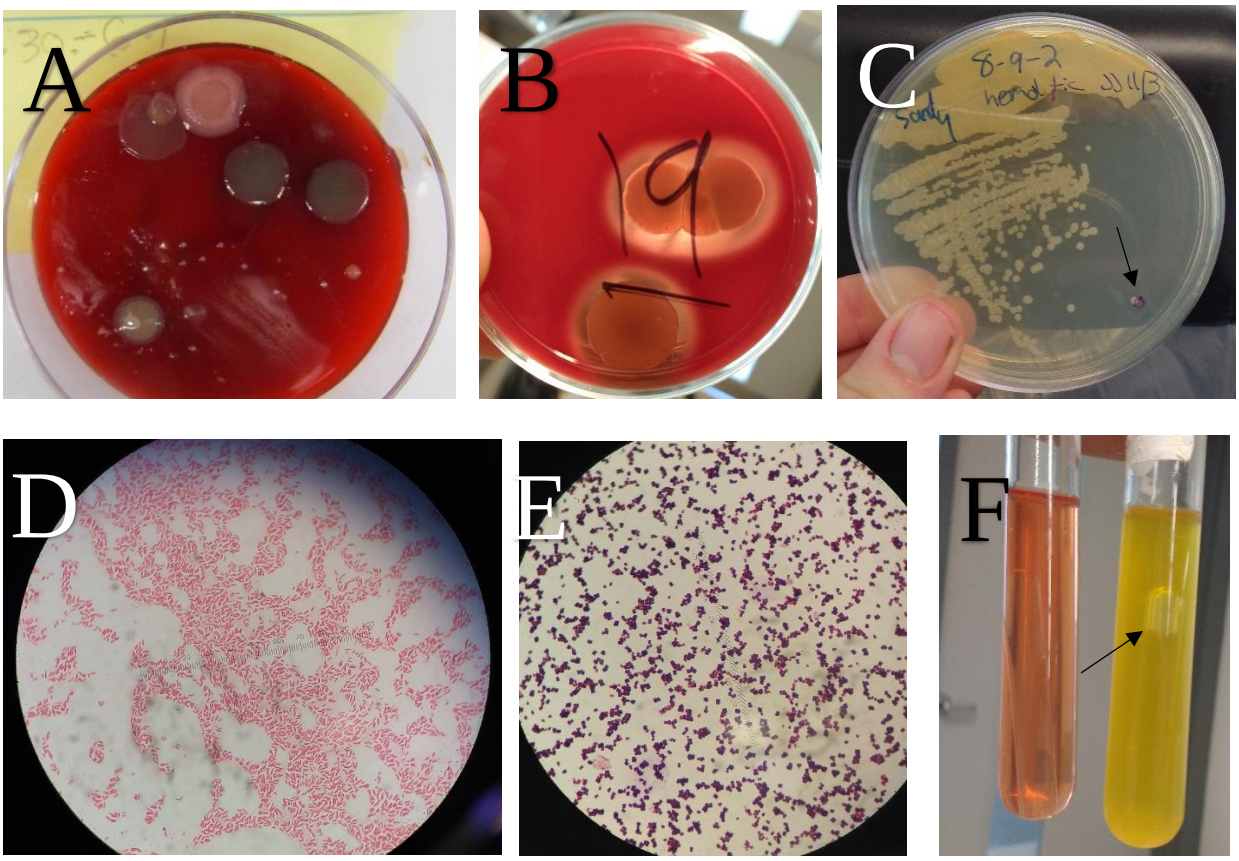

Figure 4
Photographs from the microbiology tests conducted on bacteria recovered from emerald shiner livers. **A.** Multiple bacteria types, all taken from one liver sample in an individual fish. **B.** Beta hemolysis of blood agar. This is a unique characteristic of pathogens such as *A. salmonicida.* **C.** Isolation streaking of bacteria that had been preserved from glass beads. The arrow indicates the colony that was chosen because it was completely isolated and considered to be derivative of one cell. **D.** Gram-negative, rod shaped bacteria from a liver. **E.** Gram-positive cocci shaped bacteria from a liver. **F.** Results from glucose fermentation tests. The phenol broth in the tubes contains a pink color phenol indicator which changes to yellow when glucose is fermented due to a pH change. The yellow tube is positive for glucose fermentation with a gas bubble present in the Durham tube, and the pink test tube is negative for glucose fermentation.

Statistical Analysis

All figures and statistical analysis were conducted using the open-source software R 3.3.1 with 'ggplot2' and 'lattice' packages; maps were created using ArcGIS 10.22**.** Linear regression was used to determine whether there was a relationship between *E. coli* MPN and water temperature. One-way analysis of variance (ANOVA) and Tukey's Honest Significance Difference (HSD) were used to detect differences in *E. coli* MPN grouped by site.

Leukocrit percentages were arcsine transformed before statistical analysis. Differences among pairs of comparisons (YOY/adult, hemorrhaged/not hemorrhaged, with/without liver infection, high/low parasite loads and eastern/western river branch sites) were examined using two-tailed t-tests after testing the data for homogeneity of variance. Fulton's condition factor was also analyzed using two-tailed t-tests for each of the above pairs of comparisons. One-way ANOVA and Tukey's HSD were used to detect differences in leukocrit value, condition factor, and number of parasites in shiners by collection site. Linear regression was used to determine whether there was a relationship between condition factor and number of parasites, condition factor and water temperature, and for number of parasites and fish length.

Because the physiological range for leukocrit in emerald shiners had not previously been reported, a conservative estimate was determined by examining the leukocrit values of fish that appeared healthy according to the health assessments used in this study (Supplemental, Figure 1, 2 and 3). For this purpose, blood parameters were excluded from the HAI scoring system, then analyzed the leukocrit observations in fish that were grouped based on obvious symptoms of stress (liver infection, parasite load and hemorrhaging). In this way, a range was calculated to evaluate affected/non-affected leukocrit values as described in Table 1. The healthy leukocrit range was 0.6 – 3.0, and all other leukocrit values were considered a reflection of disease.

Descriptive statistics (mean, range, coefficient of variation) were calculated for HAI scores as a pooled group for all emerald shiners, and among sites. The non-parametric Kruskal-Wallis test was used to detect differences in HAI scores grouped by site and the Mann-Whitney U test was used to detect differences between the eastern and western branch sites of the river. HAI trends across the season are reported graphically.

Multiple regression was used to evaluate multiple predictors affecting the leukocrit values of shiners. Two different models were created. In one of the models, *E. coli* MPN, water temperature, observed rainfall from the previous day, and dissolved oxygen were used as predictors for leukocrit values. In another model, condition factor and number of parasites were used as predictors for leukocrit values. A line of best fit was determined for statistically significant predictors in these models.

Results

Water Sampling Results

Black Rock Canal, which is partially separated from the river, had the highest observed *E. coli* levels (Figure 5) followed by three other eastern branch sites (GP, IV and RM). SB was the only eastern branch site to not exceed EPA thresholds for single grab samples (all sites are depicted in Figure 5; for individual results see Supplemental, Figure 4 and 5). Western branch sites (B6 and BI) had lower *E. coli* counts, and with the exception of one sampling event at BI, all observations were well below EPA thresholds. As predicted, Lake Erie had *E. coli* levels near zero per 100 mL at all sampling events, therefore providing a good local *E. coli* baseline to compare with the river data.

Because emerald shiner health status was examined as a pooled population, the riverine *E. coli* MPN observations were pooled for comparison with Lake Erie. The Niagara River as a whole was below the EPA criteria for a single grab detection for each sampling event (Figure 6). It was also below EPA criteria for the geometric mean when five or more samples are taken in one month. Mean *E. coli* MPN (log_{10}) was significantly different among sites (one-way ANOVA, df=7, F=15.62, p<0.001, Figure 7). The geometric mean (antilog value of logged averages) for each site is provided in Supplemental, Table 1, which reflects what is graphically depicted in Figure 7.

Water temperature, conductivity and dissolved oxygen followed typical seasonal trends (Figure 8). Dissolved oxygen and temperature were very similar between Lake Erie and the riverine sites. Water temperature was highest from July-September, and dissolved oxygen was inversely related to temperature and reached 6.41 mg/L in the river at its lowest level in August at Beaver Island (BI). Conductivity was higher in the riverine sites than in Lake Erie at all sampling events and it was significantly different among the river sites (one-way ANOVA, df=7, F=10.23, p<0.001 Supplemental, Figure 6) with SB and RM representing the lowest and highest values respectively. *E. coli* MPN levels were elevated in the days following rain events, but also in days without recent rain (Figure 9). *E. coli* MPN (log_{10}) tended to increase in warmer water temperatures (regression coef.=0.050, SE=0.021, t=2.401, p=0.018, R^2=0.06, Figure 10).

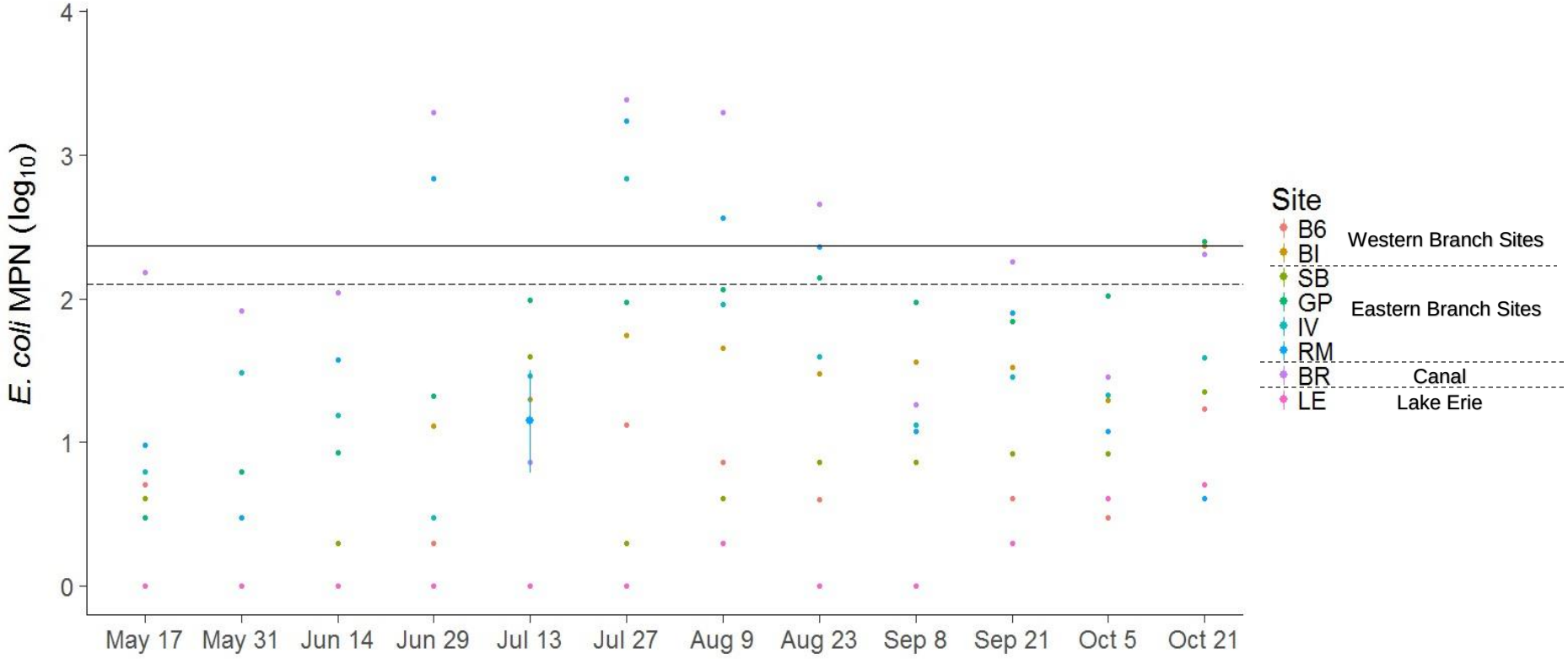

Figure 5
All *E. coli* MPN (log_{10}) observations and the mid-sampling date for each week in 2016. Each data point represents a composite sample (three grabs) for each site to account for spatial variability within sites. The dashed horizontal line illustrates the acceptable geometric mean *E. coli* MPN (log_{10}) according to EPA standards in recreational waters when five or more samples are taken in a month. The solid horizontal line illustrates the acceptable *E. coli* MPN (log_{10}) for a single grab sample. Four of the eastern branch sites (GP, IV, RM and BR) exceeded that value at one or more sampling events. Rich's Marina on July 13th shows the mean (± SE) of two samples: one taken from a plume of sediment that a barge kicked up, and the clear water sample adjacent to it. Note: Beaver Island was not included in the sampling regime until the week of June 29th.

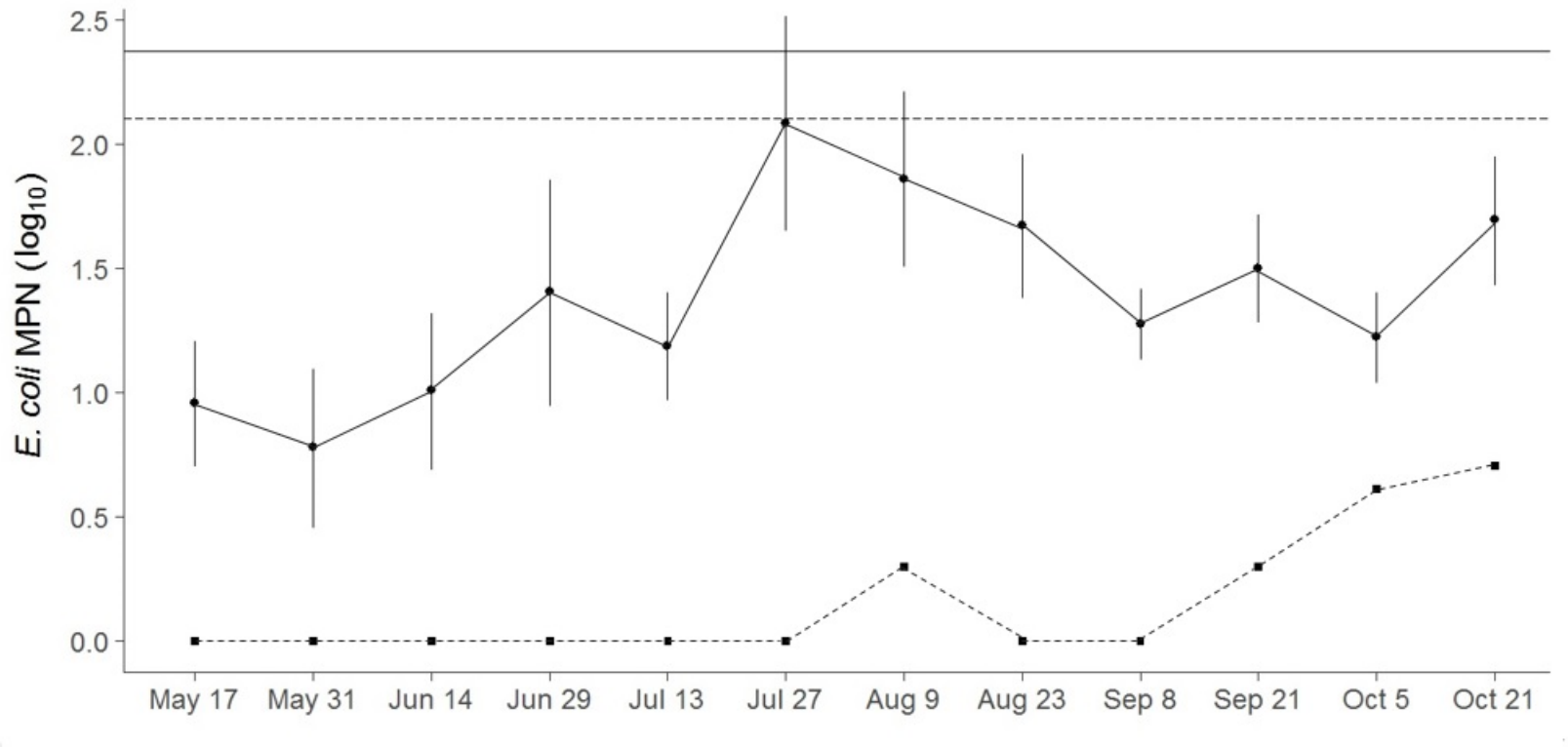

Figure 6
The mean (± SE) *E. coli* MPN (log_{10}) of all river sites pooled together (circles with standard error bars) in 2016. Lake Erie's samples are plotted as a reference (solid squares). The dashed horizontal line illustrates the acceptable geometric mean *E. coli* MPN (log_{10}) according to EPA standards in recreational waters when five or more samples are taken in a month. The solid horizontal line illustrates the acceptable *E. coli* MPN (log_{10}) for a single grab sample.

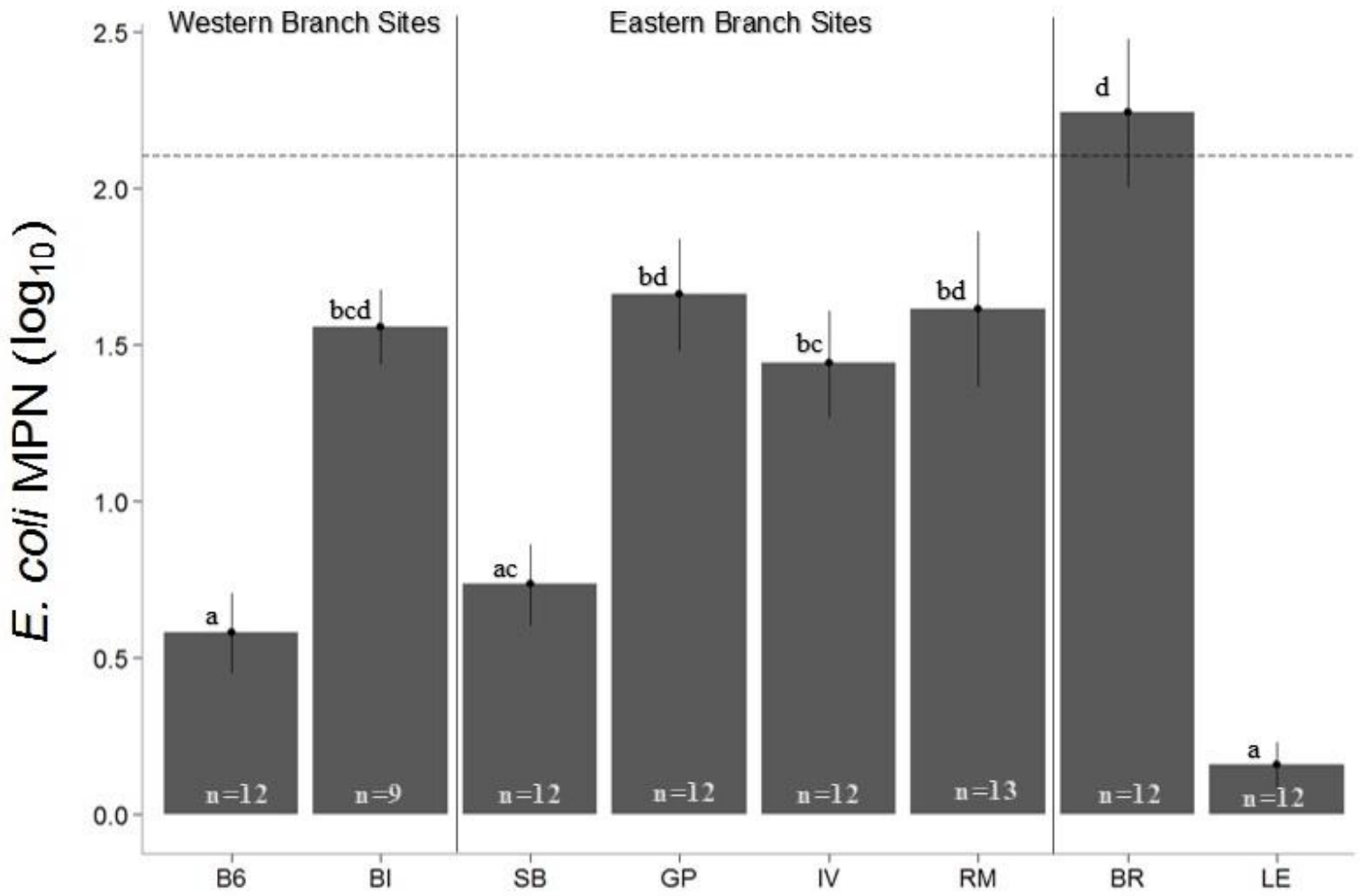

Figure 7

The mean ± SE *E.coli* MPN (log_{10}) for each site for the six month sampling season. Significant differences among sites are indicated by the letters above the bars. The dashed horizontal line illustrates the acceptable geometric mean *E. coli* MPN (log_{10}) according to EPA standards in recreational waters when five or more samples are taken in a month for reference, although the samples shown here reflect a twice-a-month sampling.

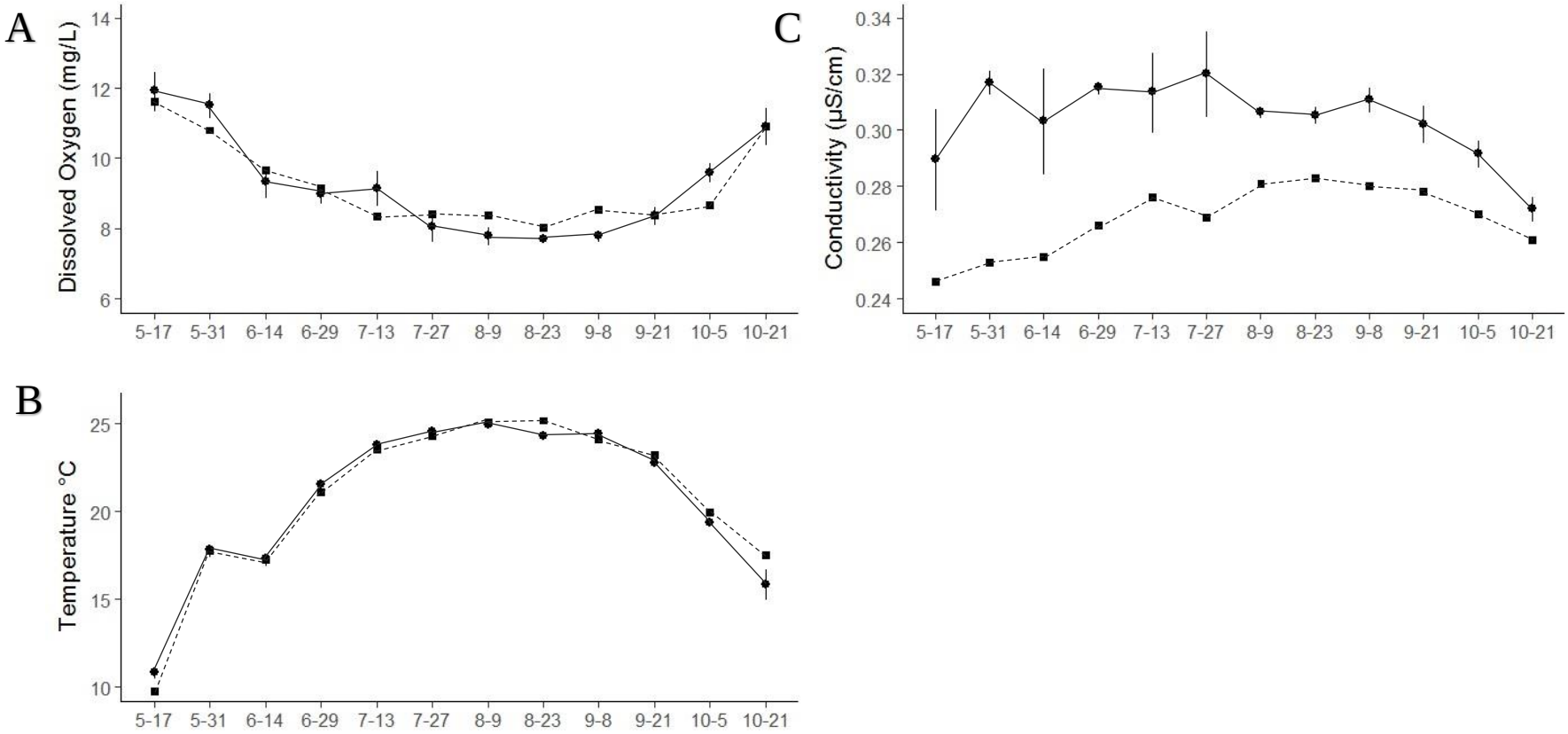

Figure 8
Water parameters for each site throughout the sampling season in 2016. **A.** dissolved oxygen **B.** water temperature and **C.** conductivity. The closed circles are the mean (± SE) of all Niagara River sampling sites, and closed squares are Lake Erie measurements for reference.

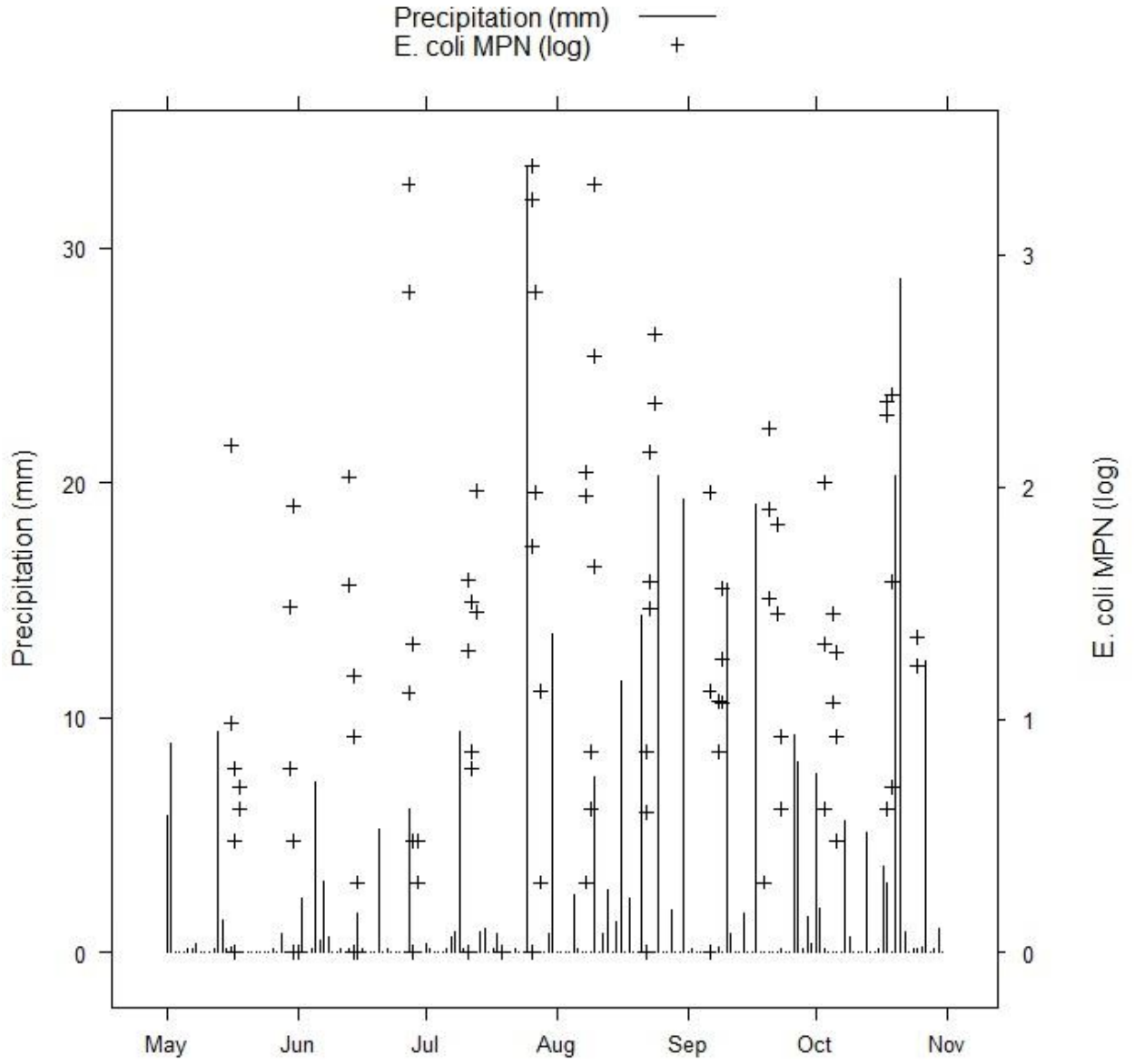

Figure 9
Precipitation (mm) and *E. coli* MPN (log_{10}) data from all sites including LE in 2016. Precipitation data are shown from May 1st until November 1st; *E. coli* sampling data are from May 16th-October 26th. Note: The precipitation values are the mean rainfall occurring in Buffalo, Tonawanda, and Grand Island for each date. These data were obtained from Weather Underground. Approximately 700,000 gallons of untreated water are released from CSOs when 25.4 mm of precipitation occurs.

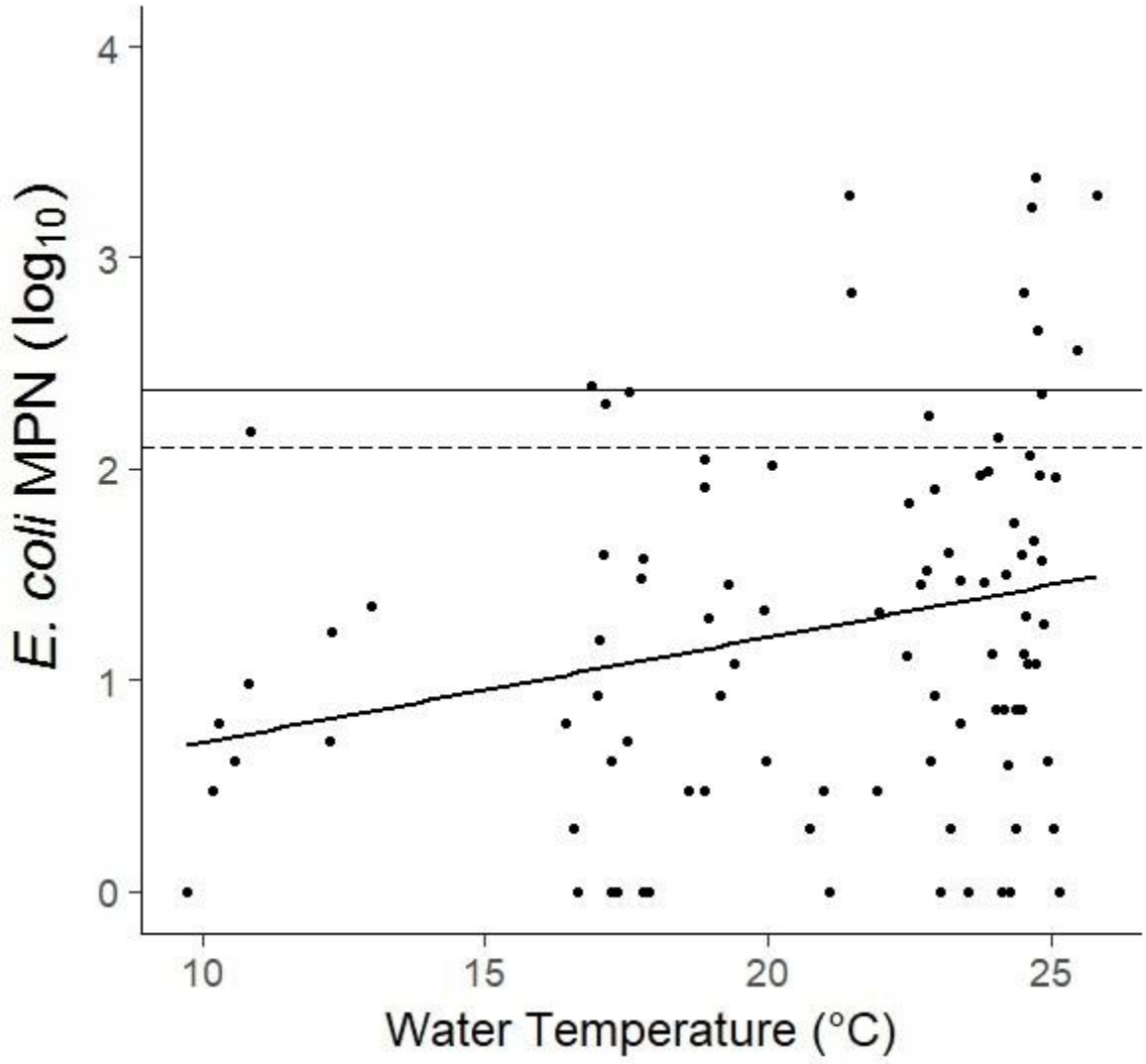

Figure 10
E. coli MPN (log_{10}) and water temperature. The dashed horizontal line illustrates the acceptable geometric mean *E. coli* MPN (log_{10}) according to EPA standards in recreational waters when five or more samples are taken in a month. The solid horizontal line illustrates the acceptable *E. coli* MPN (log_{10}) for a single grab sample.

Necropsy Results

There were no statistically significant differences in leukocrit values of emerald shiners among sites (one-way ANOVA), or in the t-tests between YOY/adults, with/without bacterial infection in the liver, with/without hemorrhaging, low/high parasite loads, or between eastern/western riverine sites. The literature suggests that any leukocrit value above 4% is, in general, considered unhealthy for fishes (Adams et al. 1993). For the emerald shiners from the Niagara River, it appeared that the healthiest individuals (based on low parasite load, no hemorrhaging and lacking bacterial infection in the liver, Supplemental, Figure 1, 2 and 3) had leukocrits in the range of 0.6 - 3%. Therefore, this was used as the range for healthy emerald shiner's leukocrit for the HAI scoring system as indicated in the Methods section (Table 1).

The emerald shiner's Fulton condition factor was significantly different among sites (one-way ANOVA, df=6, F=27.78, $p<0.001$, Figure 11) and also among eastern and western branch sites (Mann-Whitney U-test, w=3,551, $p<0.001$). The lowest condition factor in shiners was observed at IV in the eastern branch while fish captured from the western branch (B6 and BI) had the highest condition factor. Condition factor was also lowest in the first two sampling dates in May, but never dropped below a mean of 0.6 for any subsequent sampling week (Supplemental, Figure 7). In fact, condition factor significantly increased with increasing water temperatures (regression coef.=0.018, SE< 0.001, t=20.45, $p<0.001$, $R^2=0.38$, Figure 12). Condition factor was significantly higher in fish without bacterial infection in their liver than in those with bacterial infection (t-test, t=4.366, df=264, $p< 0.001$, Figure 13). It was also higher in shiners with low compared to high parasite loads (t-test, t= -6.464, df=120.34, $p<0.001$, Figure 13) and in YOY compared to adult fish (t-test, t = -6.996, df=320, $p<0.001$, Figure 13). Additionally, condition factor was highest in shiners lacking any parasites, then tended to

decrease with increasing number of parasites (regression coef = -0.002, SE=0.001, t= -3.823, $p<0.001$, $R^2=0.04$, Figure 14). However, there were no differences in Fulton's condition factor for shiners with/without hemorrhaging.

The number of parasites on a fish was significantly different among sites (one-way ANOVA, df=6, F=3.628, p=0.002, Figure 15). The greatest number of parasites were found on fish from SB in the north of Grand Island in the eastern branch of the river, and the lowest numbers of parasites on fish from BI, B6 (western branch sites) and BR in the canal. However, the sample size at BR was much smaller than at western branch sites (Black Rock Canal=8, Big Six=73, Beaver Island=86) therefore, the low parasite load on fish from the canal should be interpreted with caution. All of the shiners from the eastern branch sites had intermediate numbers of parasites. The number of parasites on a fish tended to increase with length (regression coef.=0.142, SE=0.063, t=2.283, p=0.023, $R^2=0.02$, Figure 16), particularly when fish were one-year and older, which generally occurs at ≥ 55 mm total length.

In total, 1,803 parasites were observed on 322 emerald shiners and 1,607 (89.1%) of those parasites were identified as *Ich*. Of the 322 shiners, 145 individuals (45%) were infested by at least one parasite and 136 (42%) of those individuals were infested with *Ich*. The remaining 196 parasite observations were trematodes, leeches, intestinal worms, and myxosporeans. Further investigation into the relationship between the most common parasite on fish, *Ich*, and water temperature revealed a polynomial relationship. Interestingly, the number of *Ich* parasites on fish was highest at intermediate water temperature, around 15-20°C (polynomial regression, df=691, SE=12.6, $p<0.001$, Supplemental, Figure 8) however the relationship was also very weak ($R^2=0.02$).

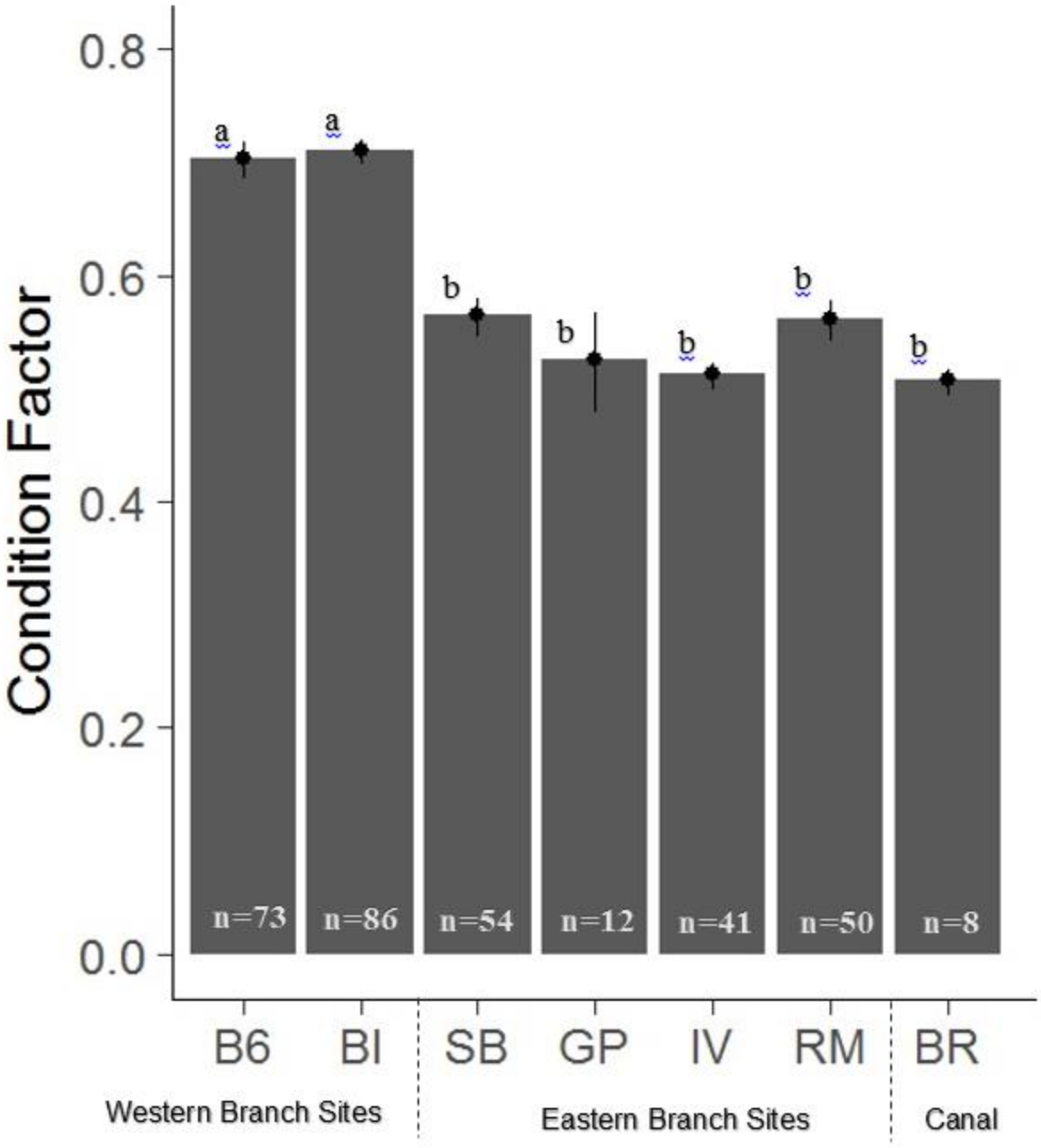

Figure 11
Mean (± SE) emerald shiner condition factor among sites. Significant differences among sites are indicated with letters above bars.

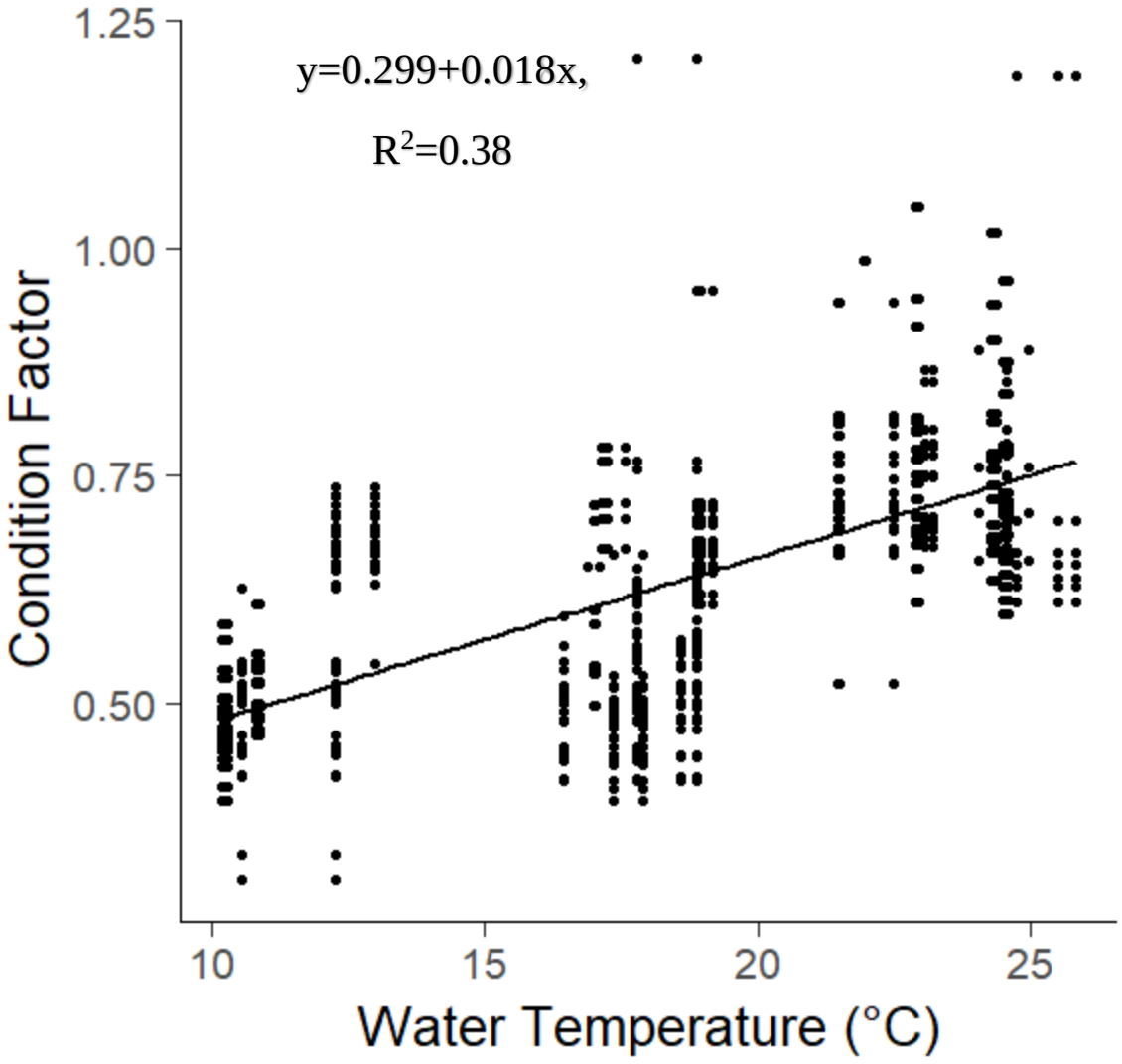

Figure 12
The emerald shiner condition factor increased with water temperatures.

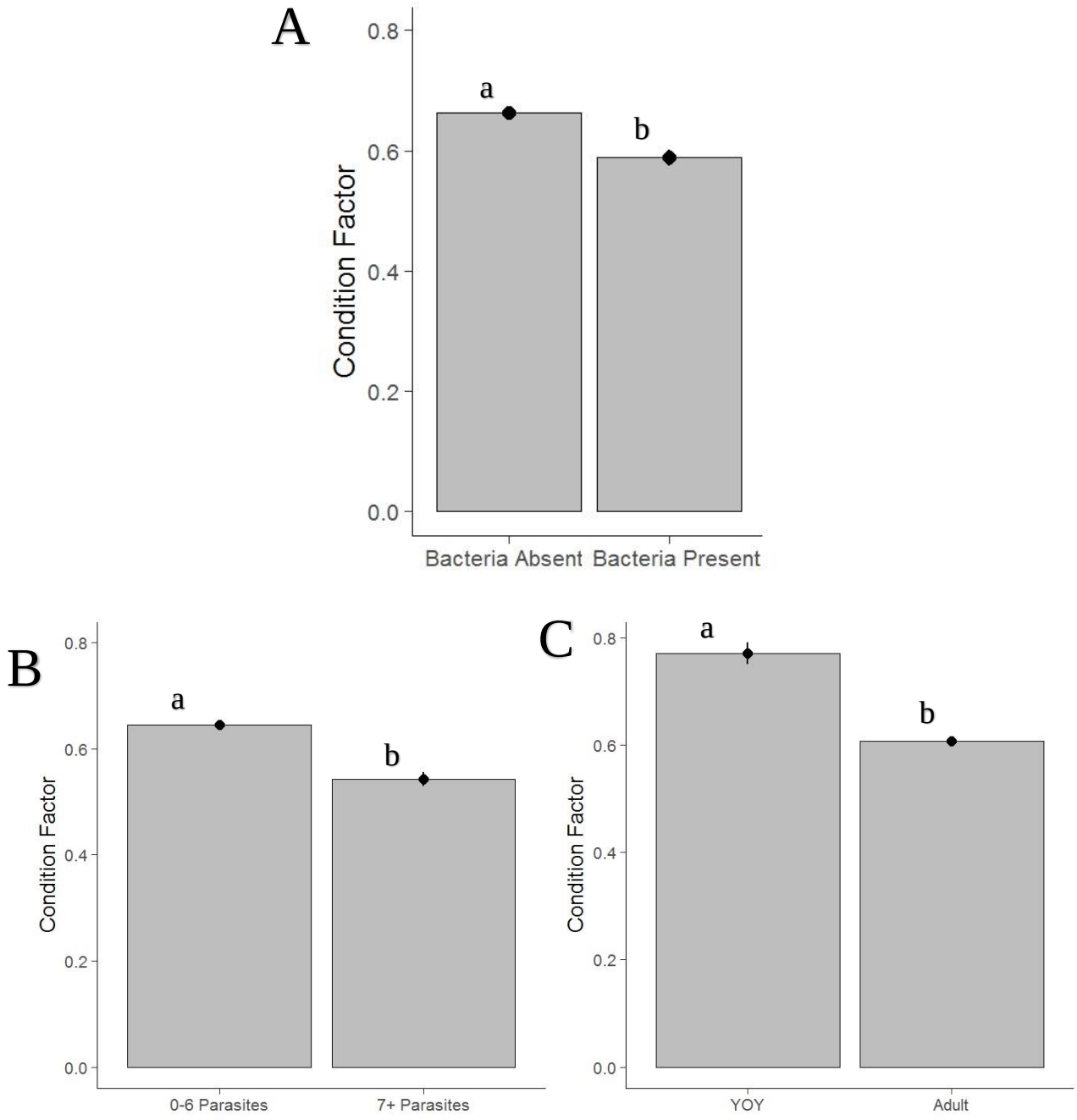

Figure 13
Mean (± SE) condition factor in emerald shiners when grouped by: **A.** Presence/absence of bacteria in the livers **B.** grouping of total parasite loads associated to HAI scores "none and few" (up to six parasites) and "moderate and high" (more than seven parasites) (see Table 1) and **C.** age class. Different letters indicate significant differences.

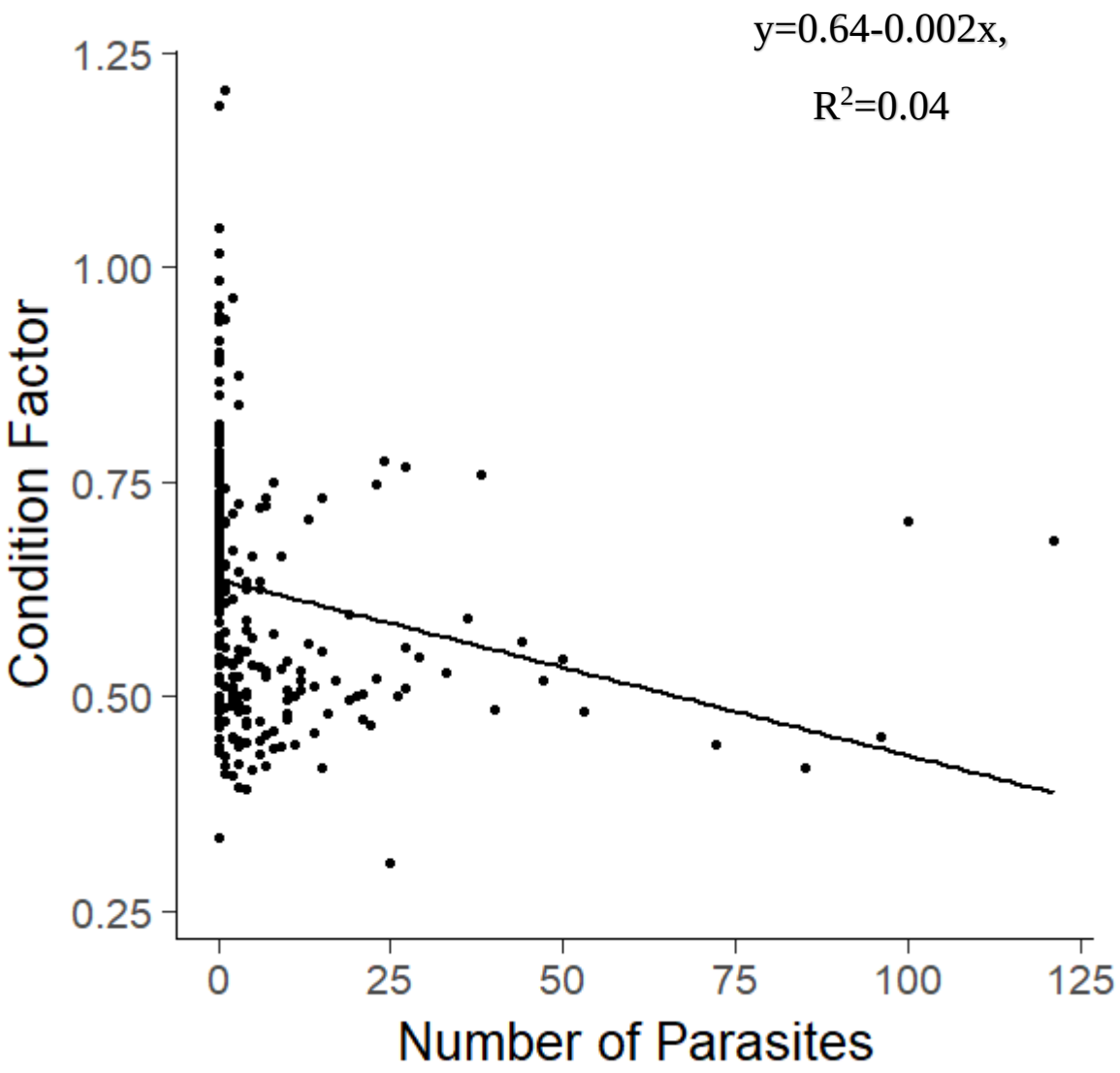

Figure 14
Condition factor and number of parasites in emerald shiners.

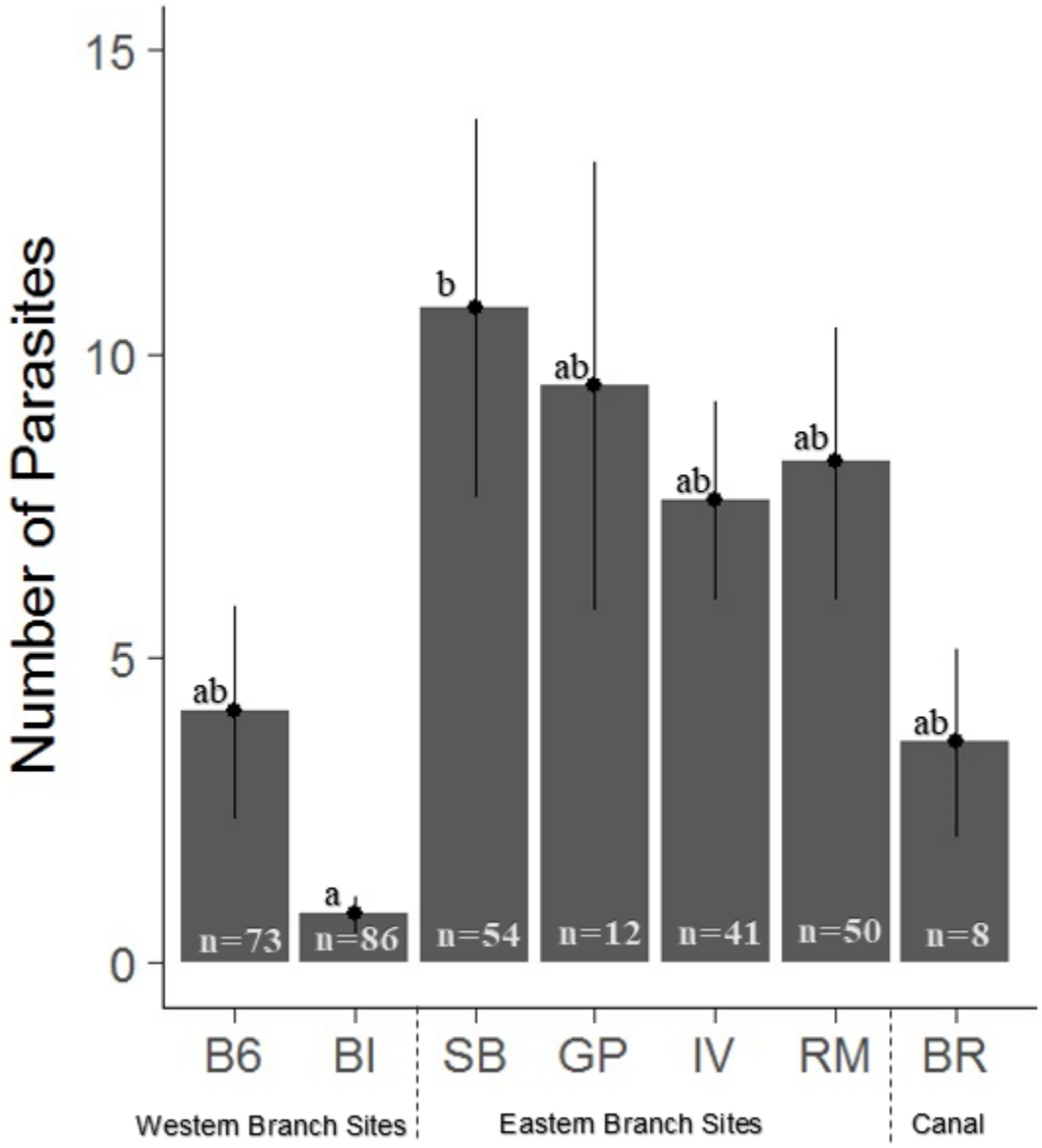

Figure 15
The mean (±SE) number of parasites on the emerald shiners was significantly different among sites. Significant differences among sites are indicated by letters above bars.

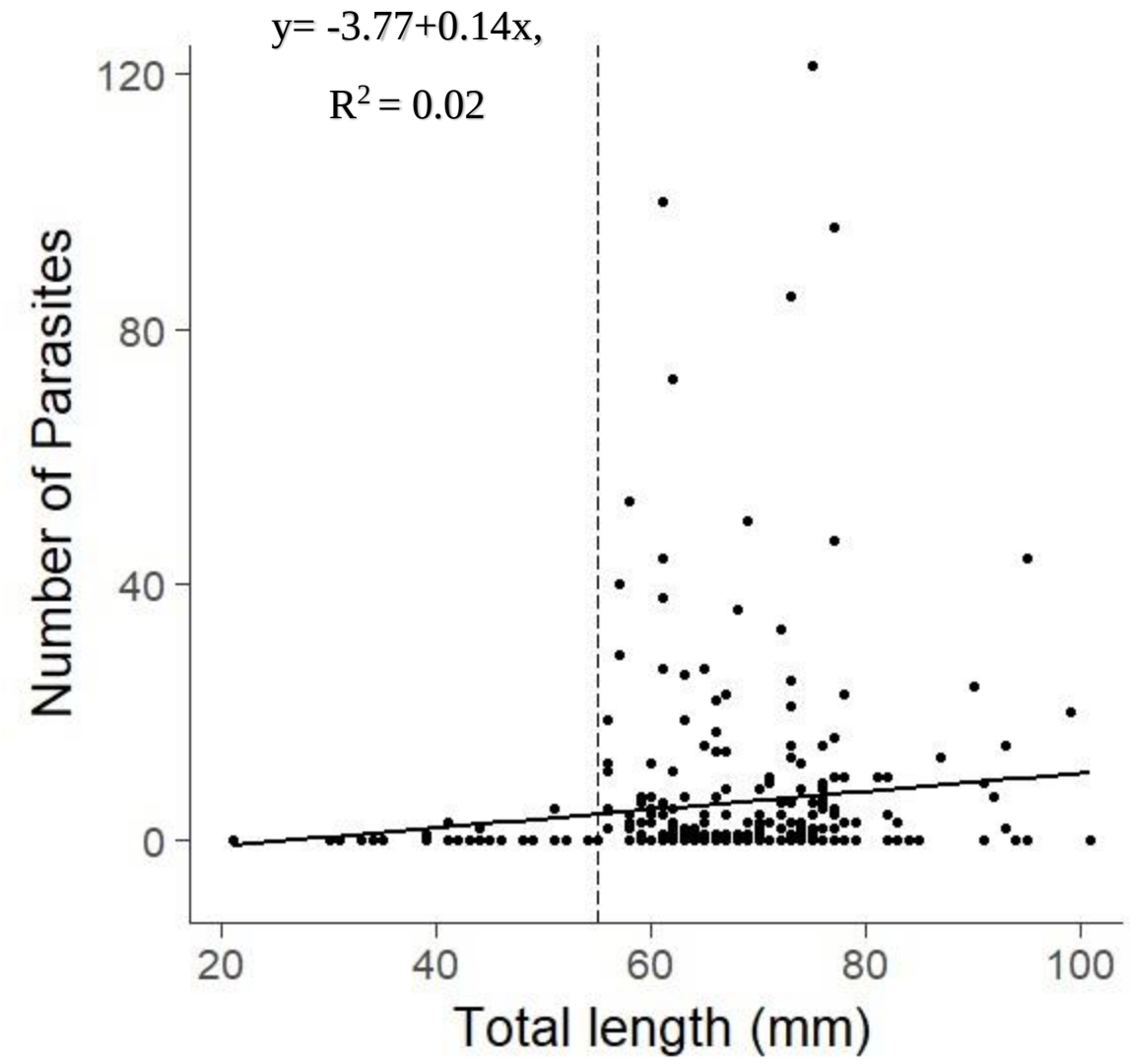

Figure 16
Number of parasites on emerald shiners of different lengths. As a general guideline, fish under ~55 mm were young-of-the-year (YOY), which is indicated by the dashed line.

Health Assessment Index Results

The mean emerald shiner Health Assessment Index (HAI) score significantly differed among sites (Kruskal-Wallis, $p < 0.001$, Figure 17). The unhealthiest shiners as determined by the HAI came from four of the eastern branch sites (GP, IV, RM and BR), whereas fish from SB in the northern part of the eastern branch had healthier HAI scores similar to fish from the western branch sites (B6 and BI). Shiners from SB did have a higher numbers of parasites compared to other sites; however, that only accounts for 30 out of the total possible score of 270. Emerald shiners sampled in May and August were the least healthy overall based on their HAI scores (Figure 18). The western branch sites had significantly lower HAI scores (indicating healthier shiners) than the eastern branch sites (Mann-Whitney U-test, $w=15{,}380$, $p<0.001$). The mean (±SE) HAI score for all fish sampled was 33.22 ± 1.603. The highest observed HAI score was 150, out of a total possible score of 270 (Figure 19; for individual site results, see Supplemental, Figure 9).The coefficient of variation (CV) for the entire emerald shiner sample from the upper Niagara River population throughout the sampling season was 84.56. This high value suggests that there was a lot of variability in the HAI scores of the emerald shiners from the upper Niagara River in 2016. Frequently encountered factors contributing to poor health were: abnormal white blood cell values, hemorrhaging in body or eyes, high numbers of parasites, and mucous-covered gills. Occasionally, fish were observed with extreme abnormalities such as liquefaction of internal organs, a missing eye, drastic hemorrhaging in the fins, or severe fungal infections (Figure 20). There was a positive trend between HAI score and length, suggesting that larger (older) fish were in poorer health than the smaller juveniles (Supplemental, Figure 10).

A multiple regression was used to detect relationships among water quality (*E. coli* MPN, water temperature, dissolved oxygen and observed rainfall from the day prior to a sampling

event) and the leukocrit of the least healthy shiners (those with an HAI score ≥50). Only water temperature had a significant effect on leukocrit and the line of best fit was a polynomial (df=148, SE=0.048, $p<0.001$, $R^2=0.19$, Figure 21). In a multiple regression using condition factor and number of parasites as predictors for leukocrit, only condition factor had a significant effect and the line of best fit also was a polynomial (df=148, SE=0.05, $p=0.002$, $R^2=0.08$, Figure 22). Thus, in the sickest fish, the extremely high leukocrit values observed (10-30%) occurred when the water temperature was very low (10-13°C) or high (>20°C). The highest leukocrit measurements occurred in shiners with very low condition factors, and the leukocrit was lowest in shiners with intermediate condition factors (0.6 – 0.8).

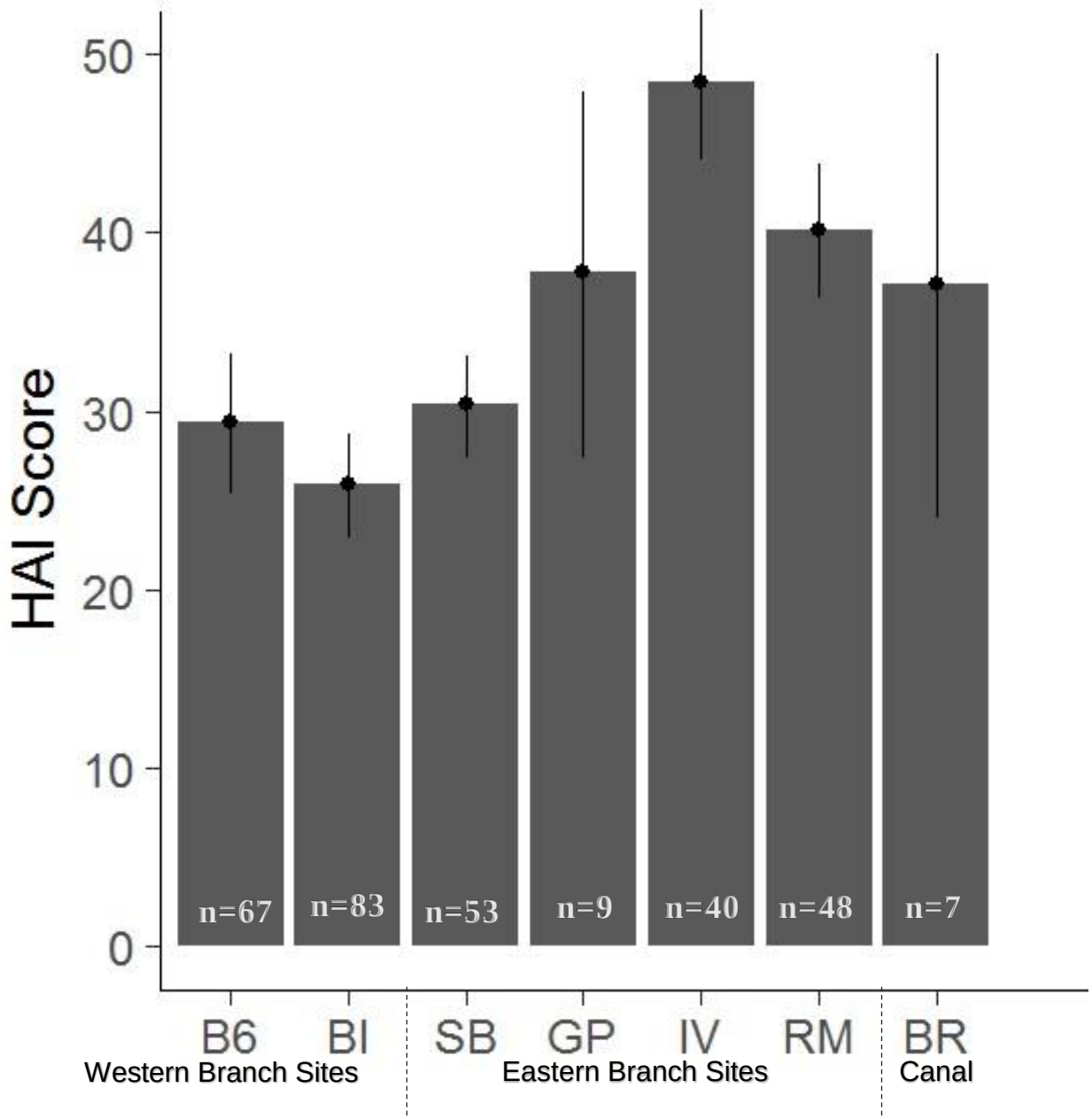

Figure 17
Mean (± SE) emerald shiner Health Assessment Index (HAI) score from sites from the eastern and western branches of the upper Niagara River. A higher HAI score indicates poorer health.

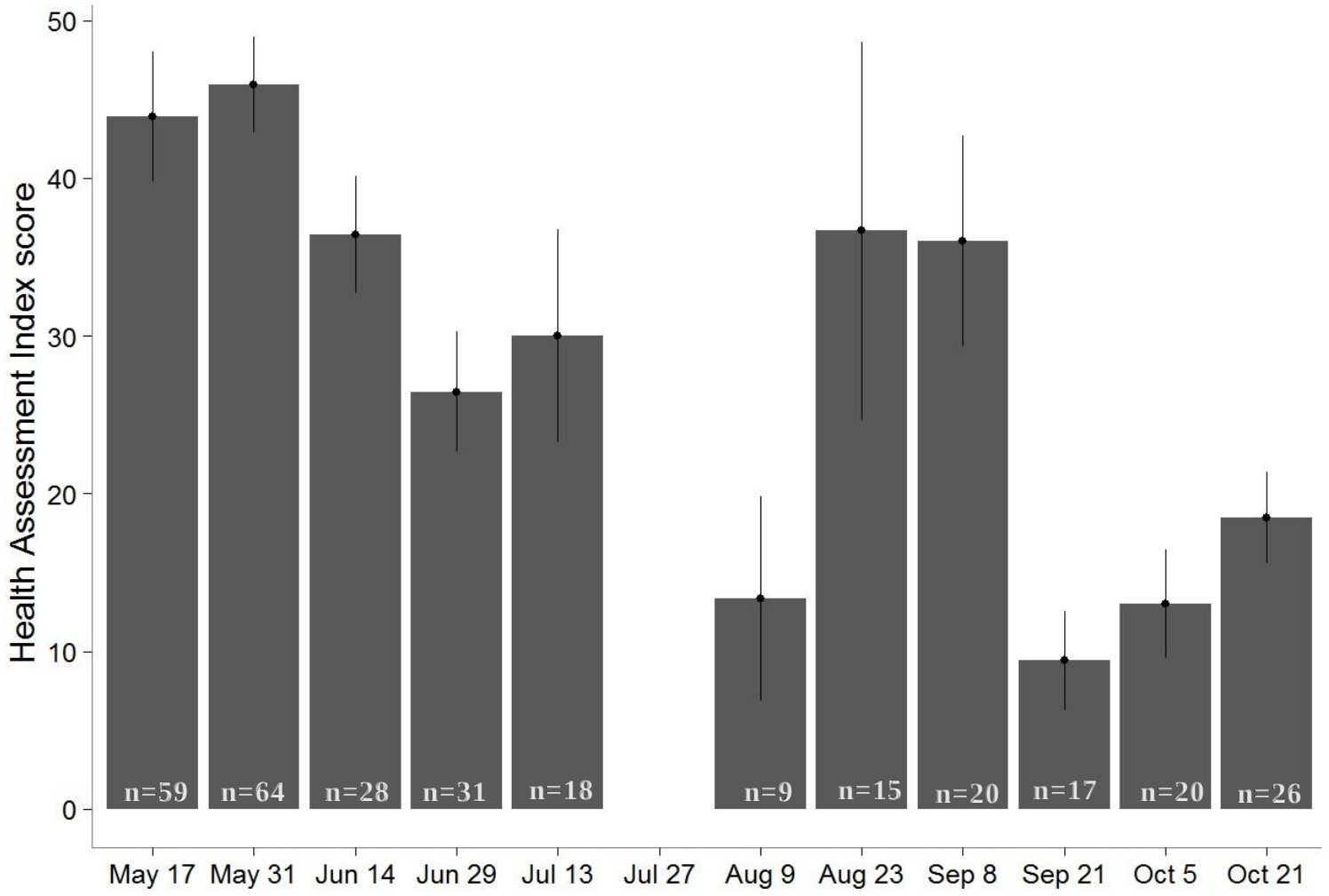

Figure 18
The mean (±SE) HAI score for emerald shiners per sampling week in 2016, with the median sampling date shown. No fish were captured the week of July 27th, although the same fishing effort was exerted.

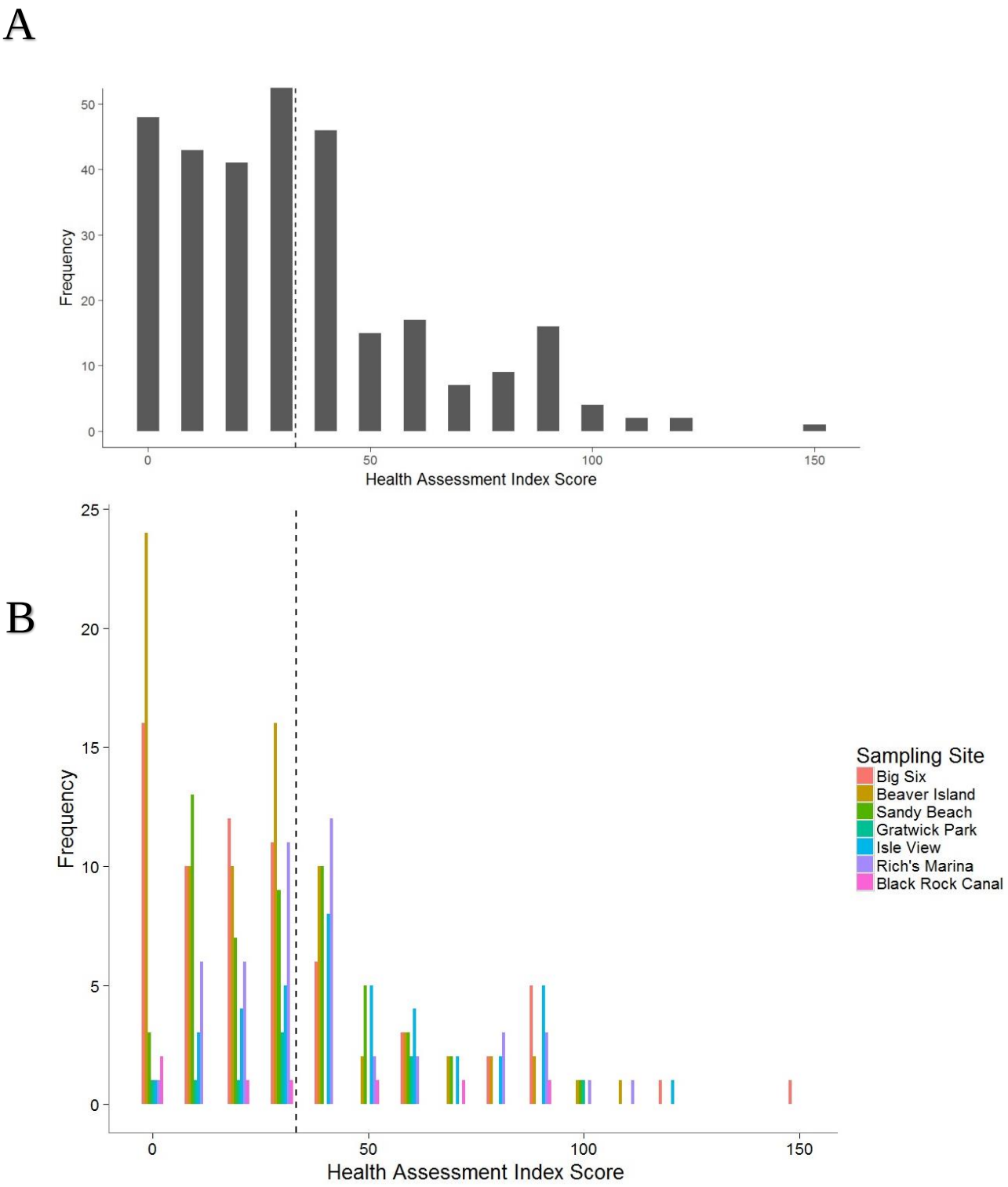

Figure 19
Frequency distribution of HAI scores for **A.** all emerald shiners sampled and **B.** for the emerald shiners sampled at each site. A higher HAI number indicates poorer health. The dashed vertical line illustrates the mean HAI score for all fish, 33.22 (± 1.603). Although the maximum possible score is 270, the highest score observed in emerald shiners in the upper Niagara River was 150.

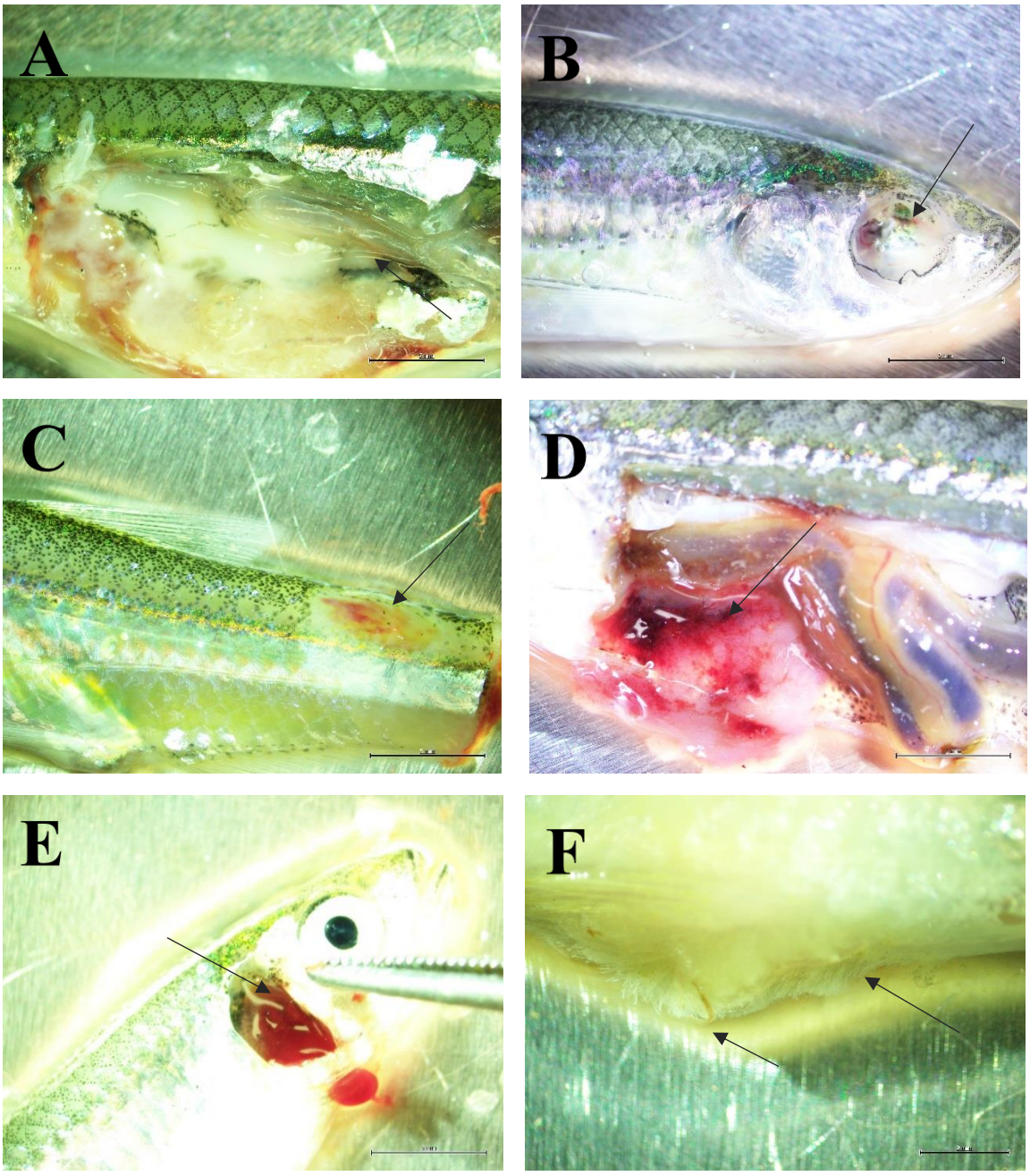

Figure 20
Photographs of some extreme abnormalities; scale bars differ among photographs. These occurrences were rare, but the affected fish were likely moribund. **A.** Liquefied internal cavity. This particular fish had a hemolytic bacterial invader in the liver, which was consistent with *A. salmonicida* characteristics, but did not have any other signs of stress except for an external lesion. **B.** Missing eye. **C.** Lesion consistent with furunculosis. **D.** Ruptured liver. **E.** Ruptured, mucous-covered gills. **F.** Severe fungal infection, which covered entire abdomen and had a protruding parasite.

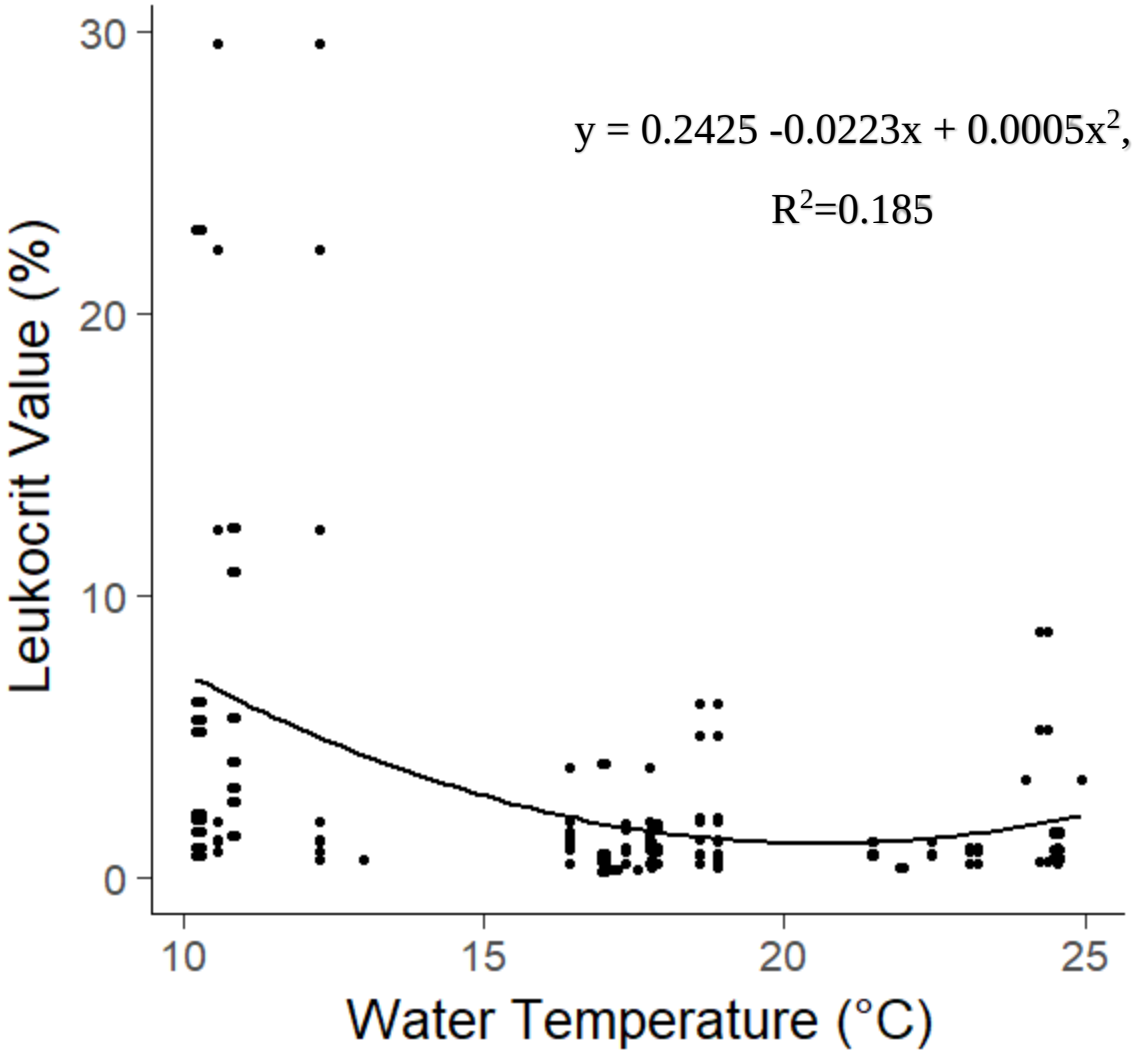

Figure 21
The leukocrit values of shiners with an HAI score ≥ 50 tended to decrease with increasing water temperature, then increase around 20°C.

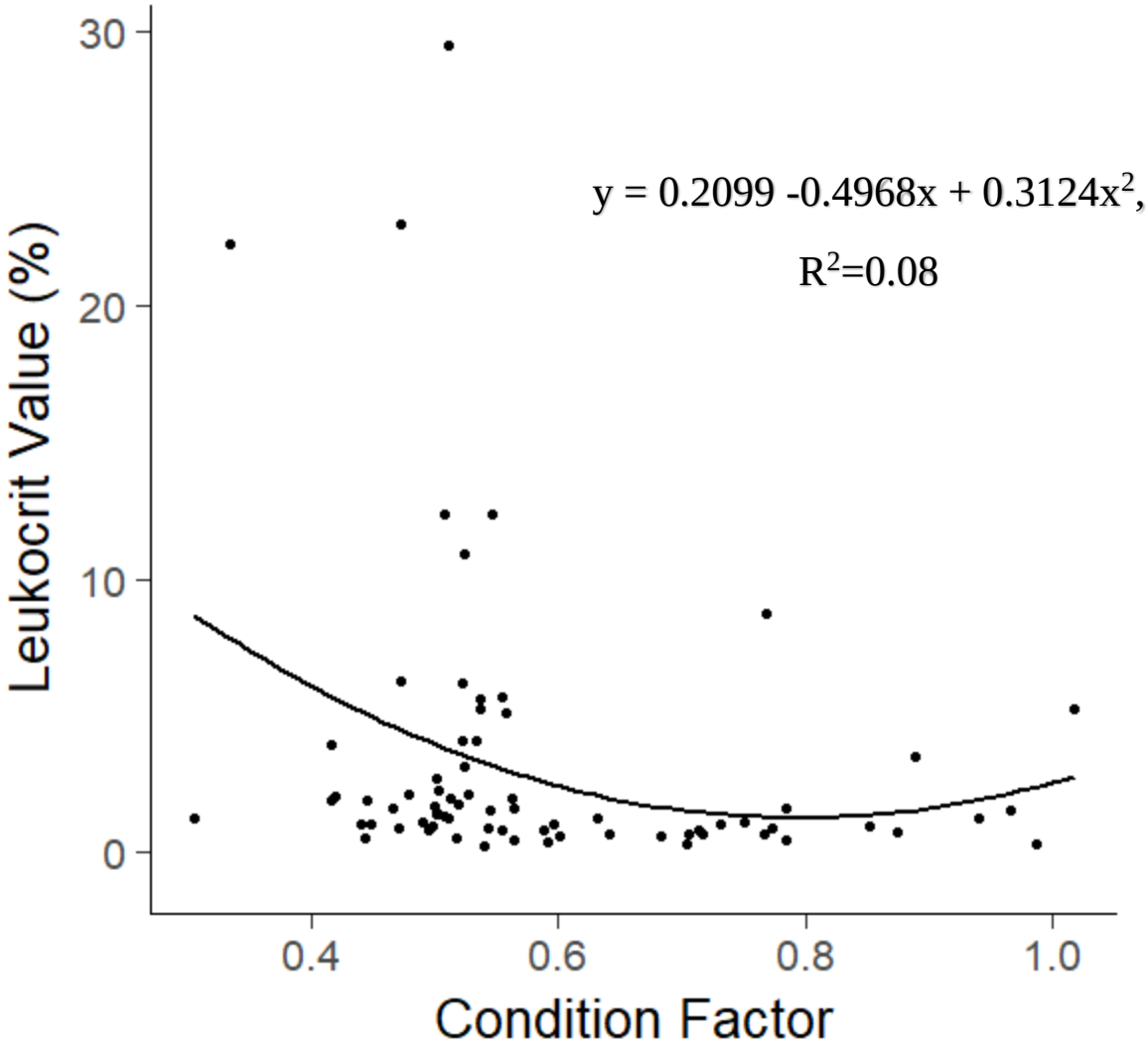

Figure 22
The leukocrit values of shiners with an HAI score ≥ 50 tended to decrease with increasing condition factor, then increased after 0.8.

Microbiology Results

Of the 266 emerald shiners sampled for bacterial infection in their livers, 94 (35.3%) were positive. This subsample included 24 YOY and 242 adults. Only three of the YOY were positive, thus, most emerald shiners with liver infection were adults. It was initially hypothesized that shiners with infected livers would also have irregular leukocrit values, reduced condition factor, increased parasites and higher HAI scores. However, only condition factor significantly changed with liver infection.

Not all of the cryopreserved bacterial cultures could be resuscitated for secondary growth, so those could not be used for further biochemical analysis. Forty-seven bacteria cultures from 32 individual fish were resuscitated and subjected to further testing. The microbiology tests revealed that these bacteria were diverse and could be separated into 12 distinct groups (Figure 23 and 24). Fourteen individual fish (14.9% of the 94 positive for infection) had more than one bacterial type in their liver which is consistent with studies on fish with compromised immune systems in which opportunistic bacteria are able to invade the fish (Joh et al. 2013). Several bacterial colonies exhibited beta-hemolysis on tryptic soy agar supplemented with sheep's blood (Figure 4). Beta-hemolysis is a typical characteristic of *A. salmonicida* and *A. hydrophila* (Austin and Austin 2012) which are known opportunistic pathogens that can lead to hemorrhaging, lesions, reduced feeding and a lowered immune system (Khan and Thulin 1991, Austin and Austin 2012).

Most bacteria in the livers were gram-negative which have a secondary outer membrane protecting a reduced peptidoglycan cell wall. Most antibiotics target the peptidoglycan layer and, thus, gram-negative bacteria are often harder to treat with antibiotics. The responses for cytochrome oxidase and glucose fermentation were varied, again confirming the diversity of

bacterial invaders. Although identification of the bacteria species was not possible in the current study, some of the observed biochemical test results are consistent with many well-studied fish pathogens such as *A. salmonicida* (furunculosis), *A. hydrophila, Edwarsiella tarda* (fish gangrene) and *Flavobacterium psychrophilum* (bacterial cold water disease). For instance, *A. salmonicida* and *A. hydrophila* are both gram-negative, rod-shaped, and positive for oxidase and glucose fermentation with the presence of gas, but can be either hemolytic or non-hemolytic (Austin and Austin 2012). Each of these bacteria are opportunistic pathogens, and are often associated with increased water pollution (Khan and Thulin 1991). Perhaps of greater importance, *A. hydrophila* is found in the fecal matter of humans and thus, is possibly released through CSO effluent (Cabral 2010). However, further investigation of the cultures would be required to confirm this, as many other bacteria are also consistent with the identification schemes depicted in Figures 23 and 24.

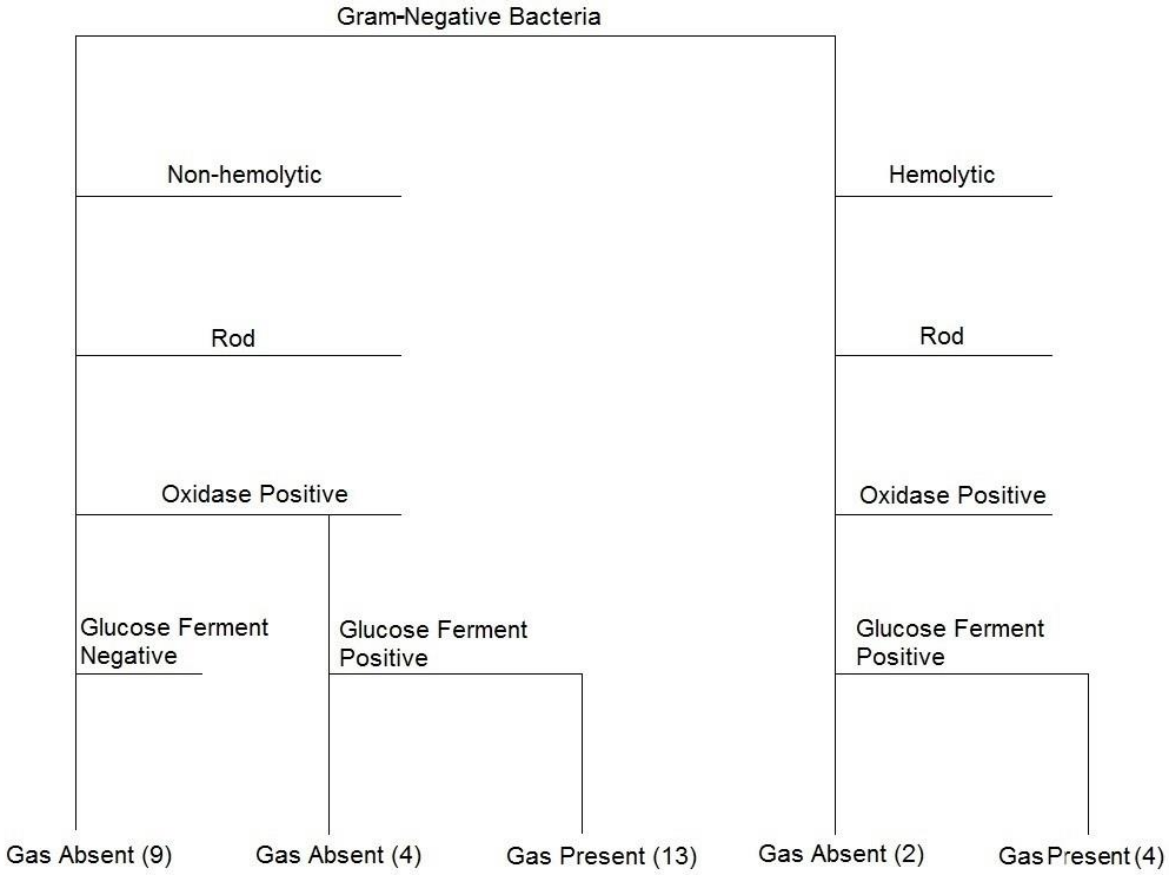

Figure 23
Identification schemes of biochemical tests conducted on gram-negative bacteria.

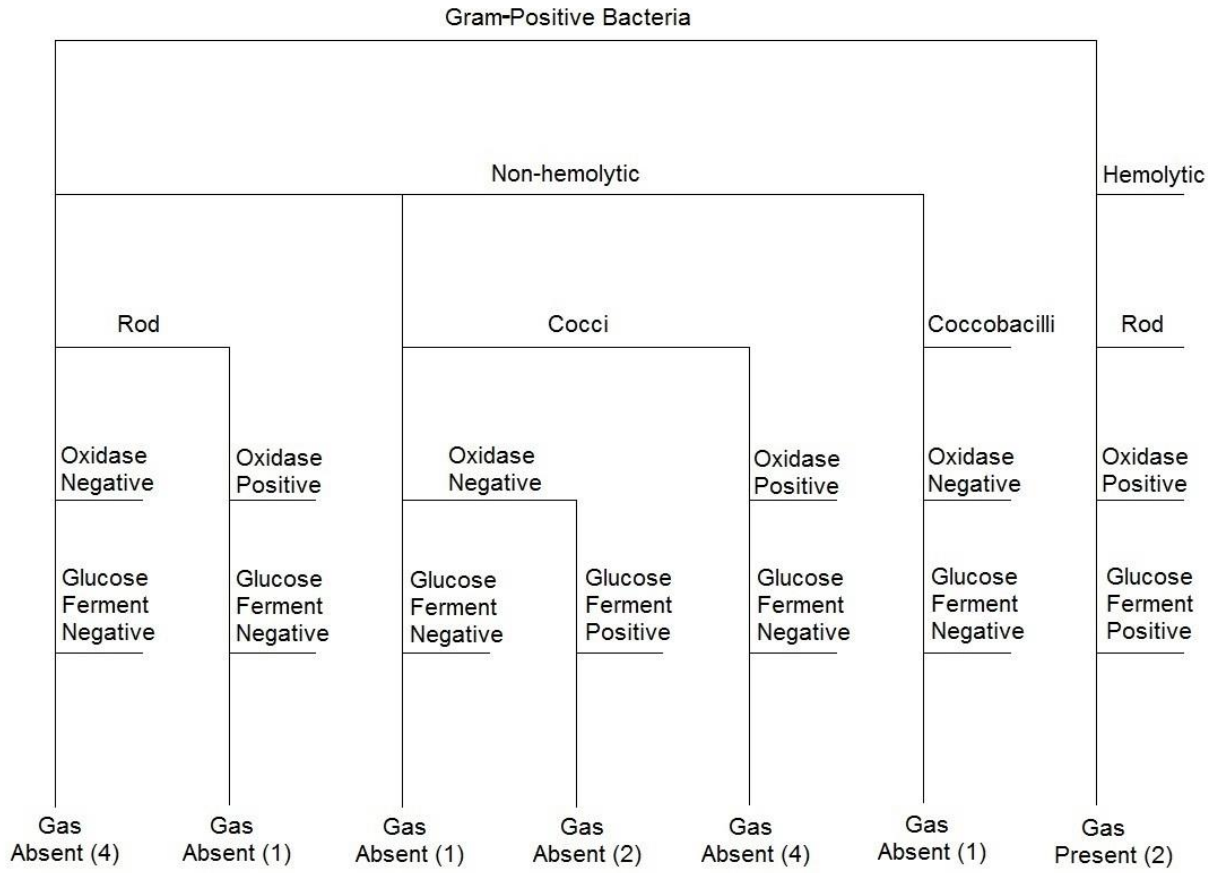

Figure 24
Identification schemes of biochemical tests conducted on gram-positive bacteria.

Discussion

There were distinct water quality differences among the sites at the eastern and western branches of the river, as determined by fecal indicator bacteria in water samples. The western branch sites (Big Six and Beaver Island) were below EPA limits for a monthly *E. coli* MPN geometric mean value; in fact, Beaver Island had only a single incident of elevated *E. coli* MPN. In contrast, all but one (Sandy Beach) eastern branch sites exceeded EPA limits in 2016 for single grab samples. Black Rock Canal exceeded the monthly geometric mean threshold, while Gratwick Park, Isle View and Rich's Marina had intermediate *E. coli* levels that were in compliance with the EPA standard. Sandy Beach was the only eastern branch site with low *E. coli* levels despite its proximity to a WWTP. Sandy beach was also the only eastern branch site on the Grand Island shoreline, so it was further away from the high density of CSOs and urbanization on the US mainland waterfront. These results suggest that the waters on the mainland US shoreline are more contaminated than the Grand Island shore, which receives less fecal input. Big Six and Sandy Beach were very similar to Lake Erie, with geometric means of less than 10 MPN/100 mL. Surprisingly, these results were similar to observations taken along 15 beaches along Lake Superior shores, where most beach sites were below 100 MPN/mL for a season-long geometric mean (Sampson et al. 2006), suggesting that Niagara River *E. coli* observations are fairly low. Similar to the Niagara River observations, Sampson et al. (2006) found little correlation between rainfall and *E. coli* input, despite common assumptions regarding rainfall and CSO event incidence.

In the 2016 sampling season, there was overall less precipitation in the region compared to previous years. From May to November 2016 there were only two rain events exceeding 25.4 mm (one inch), the amount at which at least 700,000 gallons of untreated mixed sewage are

released (Buffalo Sewer Authority 2017). The amount of effluent released in 2016 was not available at the time of this writing. However, in the upper Niagara during the same six-month period in 2014 and 2015, there were five and six of those heavy rain events, respectively (Weather Underground 2017). Therefore, these data may represent a best-case scenario for the upper Niagara River. Long-term monitoring of these sites may capture even more drastic differences between the western and eastern branch sites in the upper Niagara. There were several elevated *E. coli* observations in mid-summer when rainfall events were below ten mm. Similar results were found by Passerat et al. (2011), and the author determined this was caused through resuspension of fecal matter build up in the pipes. Many fecal bacteria such as *E. coli* form biofilms, which lay dormant in the pipe infrastructure. When treated water evacuates through these pipes, the bacteria are released into the waterway (Passerat et al. 2011). Because there was a positive relationship between water temperature and *E. coli*, resuspension of fecal build up may have more deleterious effects in the receiving aquatic system in periods of warm weather, and the effects would be more drastic in the eastern branch sites where a high density of effluent pipes occur. In a Lake Michigan harbor that is protected with a break wall, similar to the Black Rock Canal site in Buffalo, NY, there was significantly poorer water quality conditions within the break wall in a year with abnormally high rainfall and CSO input (McLellan et al. 2007). Studies in Lake Superior found that *E. coli* prevalence was not always correlated to temperature (Sampson et al. 2005) or rainfall (Sampson et al. 2006), particularly in seasons with below average precipitation. These studies support the notion that *E. coli* does not always follow expected trends, and continuous monitoring is essential for elucidating water quality conditions.

It was expected that emerald shiners would exhibit a biological stress response to sewage pollution in several ways: irregular leukocrit, reduced condition factor, high parasite loads,

bacterial infection of the liver and a high Health Assessment Index score. These biological stress responses were also predicted to be strongest at the eastern branch sites, which are more urbanized and closer to CSOs. Interestingly, although the leukocrit was most impacted in shiners with signs of systemic stress, it was not markedly changed in fish that exhibited lower levels of immune stress. For example, the shiners that were positive for bacterial infection in their liver but lacked other signs of stress (i.e., those with low HAI scores), had similar leukocrit values to uninfected, unstressed fish (Supplemental, Figure 1). However, when additional stress symptoms were observed (increased HAI scores), the leukocrits of fish with clean livers increased whereas the leukocrits of fish with infected livers decreased to near 0%. These observed trends are consistent with bacterial-induced leukocytosis (Wedemeyer et al. 1983, Goede and Barton 1990). Leukocytosis occurs when white blood cells are exposed to pathogenic bacteria for an extended amount of time, and the bacteria cause the white blood cells to lyse. This may explain why some fish had leukocrits scarcely visible at all in the microcentrifuge tube. Conversely, in fish without bacterial infection in the liver, an increase in stressors may also cause white blood cell counts to increase (Wedemeyer et al. 1983, Goede and Barton 1990) and this would explain why uninfected fish had high leukocrits with increasing HAI scores, which in this case would be an adaptive immune response.

However, the above described leukocrit responses to stressors were not universal for all the emerald shiners with bacterial infection, most likely because of the high diversity of bacteria found in their livers, which may induce different physiological responses. Previous studies have shown that not all pathogens affect leukocrit values; different species of bacteria can induce different immune responses (Wedemeyer et al. 1983). For example, infected salmonids exposed to *Aeromonas salmonicida* or *Yersinia ruckeri* (enteric red-mouth) had significantly lower

leukocrits than those exposed but remained uninfected, whereas fish infected with *Renibacterium salmoninarum* (bacterial kidney disease) had no difference in leukocrit values between infected and uninfected fish. Additionally, some pathogens did not induce leukocytosis or affect leukocrit values in this study until the fish were nearly dead (Wedemeyer et al. 1983). This explains why leukocrit values are important supporting data, but should always be complemented with other health biomarkers for proper interpretation.

The differential effect of fish exposure to bacterial pathogens also could explain why no significant differences were detected in leukocrits of emerald shiners when grouped into different categories. In general, the Niagara River emerald shiners had a leukocrit of 0.6-3%, regardless of number of parasites, life stage, hemorrhaging, or the site they were captured from. However, in some shiners, the extremely elevated leukocrit observations (10-30%) may have been fish that were moribund and just happened to be captured before death. In individuals with abnormally high leukocrits, hematocrit did not respond similarly. There were instances when hematocrit was elevated while plasma was considerably low, suggesting that fluid loss may have been a factor. Conversely, there were individuals with atypically low hematocrit and typical plasma proportion. Even though the leukocrit value was not always concurrent with other symptoms of biological stress in emerald shiners, it was quick and easy to sample and should still be used in future studies.

Fulton's condition factor was one of the most significant measurements of well-being in this study. Reduced condition factor typically suggests that a fish is not consuming enough calories (Shreck and Moyle 1990).The condition factor was significantly reduced in emerald shiners that had liver bacterial infection, in adult shiners and in shiners with seven or more parasites. Additionally, Fulton's condition factor was significantly reduced in fish captured from

the eastern branch sites, which are more highly urbanized and had higher *E. coli* levels. Condition factor is a good indicator of the health status in other fishes, such as hulafish (*Trachinops taeniatus,* in New South Wales, Australia) and spottail shiners (*Notropis hudsonius,* in Montreal, Canada) that were captured close to CSOs and urban centers (Smith and Suthers 1999, Menard et al. 2010). These small forage fish species, which are comparable in size to emerald shiners, had slow growth and reduced health status associated with proximity to urbanized areas. The reduced condition factor of fish captured close to CSOs and bacterially degraded waterways is well correlated with increased parasite susceptibility, reduced feeding (Shreck and Moyle 1990, Khan and Thulin 1991), reduced white blood cell phagocytic function (Menard et al. 2010) and high mortality (Smith and Suthers 1999). Therefore, the condition factor of the emerald shiners in this study was a good indicator of their health status and these results concur with the literature on the use of this index for fish health assessments.

The emerald shiner condition factor was lowest in May (CF = 0.5) and then it consistently scored between 0.6 – 0.8 for the remainder of the sampling season. Potentially, after June, emerald shiners were not consuming enough food to maintain adequate condition factor and fight disease, independently of food availability. Reduced feeding associated with bacterial infection and high parasite loads is well documented (Khan and Thulin 1991, Austin and Austin 2012), and this may be a serious threat for emerald shiners in bacterially-degraded waterways. Several pathogens that cause reduced feeding, such as *Aeromonas hydrophila* are commonly found in human feces and thus released through CSOs (Cabral 2010). Furthermore, this opportunistic pathogen has been reported to be correlated to *Ich* parasitism in the gills of channel catfish (*Ictalurus punctatus*) (Austin and Austin 2012).

Consistent with previous studies, the number of parasites in emerald shiners was one of the best predictors of fish health in the HAI method (Watson et al. 2012, McHugh et al. 2013). Most often, emerald shiners were highly infested with *Ich*, a ciliate which is known to alter gene expression in fish and also alter their innate immune responses (Frank et al. 2017). The high concentration of *Ich* in fish may have led to the observed excessive mucous production in the gills. Potentially, *Ich* infestation may interfere with gas exchange and create hypoxic conditions for the fish, even though the dissolved oxygen in the surrounding water was adequate and never dropped below 6.41 mg/L at any site during the six-month sampling season.

Five types of parasites were observed in this study: *Ich*, myxosporeans, flukes, tapeworms and leeches. None of the parasites occurred on any emerald shiner under 39 mm, and the number of parasites was positively correlated to shiner length (Figure 16). In a previous study on emerald shiners from three sites in Michigan (St Mary's River, Raber Bay and Lake Manuscong), non-infested individuals were even smaller than in the upper Niagara River, with a mean length of 27 mm (Muzzall and Peebles 1987). The parasites observed in that study spanned 14 taxonomic groups, most of which were internal Digenea or Monogenea flatworms (Muzzall and Peebles 1987). A study in the St. Louis River (Wisconsin) revealed that emerald shiners were systemically infested with myxosporeans, an internal parasite in the phylum Cnidaria, which causes extreme bloating of the abdomen (Horner et al. 2010). Myxosporeans are known to affect swimming behavior and lower a fish's immune system (Barber et al. 2000). Although this was not the dominant parasite observed in the present study, they were detected in several emerald shiners in September and October, and those fish also had abdominal swelling. These observations, in conjunction with parasite data from the literature supports the notion that

parasite infestation of emerald shiners spans many taxa, varies temporally and regionally, and is more prevalent in older (larger) emerald shiners.

In 2016, *Ich* was by far the most dominant parasite on emerald shiners where transfer of *Ich* most likely occurs because of close proximity during schooling, as seen in other schooling fish (Khan and Thulin 1991). The parasite consumes fish tissue for two stages of its life cycle (theront to tomont) until it falls off the fish and prepares to reproduce through binary fission, i.e., asexual division into two daughter cells (Forwood et al. 2015). The newly divided daughter cells, called tomites, are free-swimming and seek out a new host. The tomites then burrow into the skin tissue, develop into a theront and the process starts again. This process is accelerated in warmer water with a range of 4°C to 30°C, which are favorable temperatures for *Ich* reproduction (Forwood et al. 2015). In 2016 the abundance of *Ich* on shiners was greatest between 16^{o}C and 19^{o}C (Supplemental, Figure 8). This result should be interpreted with caution, however, because the relationship was statistically weak and all of the observations fell within *Ich*'s temperature tolerance range of 4 - 30°C.

Fish that were positive for bacterial infection in the liver tended to have on average more parasites (although not statistically significant). *Ich* has also been identified as a synergistic stressor with the bacterial pathogens *Aeromonas hydrophila* (Xu et al. 2012) and *Edwardsiella tarda* (Shoemaker et al. 2012*).* In two laboratory studies, fish exposed to both *Ich* and one type of bacteria (either *A. hydrophila* or *E. tardia*) experienced significantly more mortality than those exposed to only one pathogen or none at all. Additionally, both the bacterial load in organ tissues and the parasite load were higher in fish exposed to both stressors (parasites and bacteria) (Shoemaker et al. 2012, Xu et al. 2012). Because the ciliate *Ich* was so prevalent in the Niagara

River emerald shiners, the synergistic effect of this parasite and exposure to pathogenic bacteria may have been a driver of mortality and affected the health of the emerald shiners.

On average, shiners captured from Sandy Beach (SB, on northern Grand Island) had the most parasites. Even though *E. coli* levels at Sandy Beach were low, this was the only site immediately adjacent to a WWTP, so shiners captured there were still exposed to sewage and the whole suite of bacteria that may increase parasite susceptibility. A laboratory study conducted on goldfish found that physiological changes occurred in fish exposed to 5% diluted treated sewage, even when dissolved oxygen levels were above 6.5 mg/L (Kakuta 1997). The author believes this occurs because untreated sewage has very high biological oxygen demand due to the high amount of microbial respiration, and consequently reduces oxygen availability for fish. The author also cited that other contaminants such as metals and nutrients present in sewage can impair a fish's immune response. These effects were amplified when fish were inoculated with *A. salmonicida,* and most goldfish died even in the 5% diluted sewage exposure treatment (Kakuta 1997).

An interesting environmental finding in the upper Niagara River was that water conductivity was highest at the eastern sites. The eastern branch of the river receives a greater amount of effluent input from CSOs and WWTPs that release human waste with salt ions that increase conductivity levels. Kavanagh et al. (2013) conducted a study on fathead minnows in a pond containing water that was used for the processing of oil sands from oil extraction. They found that excessive salts (Na^+ and Cl^-) in impacted water was positively correlated with ciliate-parasite presence and mucous build-up in the fish's gills. The minnows with the most impacted gills and opercula came from an oil sand pond with conductivity levels >1,000 µs/cm. Although the oil sand ponds had much greater conductivity levels than the Niagara River, the conductivity

levels in the eastern branch sites may play a role in *Ich* infestation and mucous build-up of the gills in emerald shiners. These symptoms were most often observed in May-July, which coincided with high conductivity levels (Figure 8).

The Health Assessment Index (HAI) scores for emerald shiners were highest in May and late August, suggesting that seasonality plays a role in their health status. Further analyses utilizing regression models revealed interesting trends among various health biomarkers and water temperature. In the sickest emerald shiners (HAI scores ≥ 50), abnormal leukocrit values (10-30%) were associated with low and high water temperatures. Poikilothermic organisms such as fish can have irregular leukocrits as a result of temperature changes, both when water temperatures are too low or too high (Luskova 1998, Tierney et al. 2004). The number of *Ich* parasites also followed a non-linear trend with water temperature, but *Ich* was most prevalent in intermediate water temperatures. Condition factor, however, increased with water temperature and this relationship was fairly strong (Figure 12), likely due to increasing food availability and improved metabolism. These results suggest that when water temperature is at either extreme, either too cool or too warm, leukocrit and overall health (HAI) are affected.

The use of the HAI scoring in emerald shiners was appropriate at visually determining which organs of a fish were most impacted. It was apparent from this study that older fish were more biologically stressed than YOY, most likely because of cumulative exposure to poor water quality and spawning stress, which affect condition factor. HAI scores were the lowest, or healthier, in shiners captured from the western branch than from the eastern branch of the river. In the western branch, most shiners examined had an HAI score of 0. In contrast, in the eastern branch the majority of the shiners had at least some disease markers reflected in their HAI score (Supplemental, Figure 4) and the worst HAI scores were from shiners captured at the Isle View

(IV) site, which is just downstream from a very high density of both CSOs and industrial effluent point-sources (Figure 2).

Some abnormalities observed using the HAI method were severe such as: liquefied internal organs, ruptured livers and gills, severe hemorrhaging, and severely eroded caudal fins. The emerald shiners that were extremely sick had signs of systemic stress, such as hemorrhaging or fungal infection. Hemorrhaging is a tell-tale sign of viral hemorrhagic septicemia virus (VHSV) infection, which is known to affect emerald shiners (Winton et al. 2008). However, it can also be a sign of systemic stress caused by most opportunistic bacterial pathogens. Hemorrhaging was found in emerald shiners quite frequently, in both the eyes and along the body. When fungal infections are found on a fish's skin, they usually indicate a stressed immune system because they are a secondary invader (Barber et al. 2000, Austin and Austin 2012). Emerald shiners with fuzzy abdomens or mouths were most likely suffering from severe fungal infections that invaded pre-existing lesions caused by another agent. As previously mentioned, these fungal infections may also take hold because of the scraping behavior against rough surfaces, such as rocks, that a fish uses to try to rub off parasites (Barber et al. 2000), or they may have been a secondary infection of ulcers caused by bacterial pathogens such as *Aeromonas spp.* (Austin and Austin 2012).

The coefficient of variation (CV) for the HAI scores of the whole Niagara River emerald shiner population was 85, which is very high compared to the ideal target CV of 15 that the creators of the HAI method suggest. Adams et al. (1993) suggest examining the CV because it is indicative of how similarly individuals in a population of fish respond to their environment. For example, if all of the fish in the population of interest had high HAI scores and the CV was low, there would be a strong relationship between degraded water conditions and immune stress.

Conversely, in a scenario where most fish had low HAI scores (suggesting good health status) and a low population CV, you could infer that water quality is adequate and the fish population is not stressed, nor are they responding in highly variable ways. However, in the upper Niagara River ecosystem, most fish had very low HAI scores, but the ones with high HAI scores were likely nearing death and may not have survived much longer in the wild. The CV for this population was 85, suggesting that their health status is highly variable and these fish are subject to many intermittent stressors. The water quality conditions were variable, and there were fluctuations in conductivity and *E. coli* observations. Consequently, the emerald shiners did not all respond to intermittent stressors uniformly; some were quite healthy while other individuals were very sick and suffered from bacterial infection in their livers. Fourteen out of 94 (14.9%) of the emerald shiners sampled had multiple types of bacteria in their livers.

The traits observed through microbiology tests (Figure 23 and 24) were diverse and could be attributed to a number of bacteria species. However, 17 of the recovered 47 (36.3%) bacterial cultures shared biological traits with *A. salmonicida* and *A. hydrophila*, which both cause furunculosis and have been found in fishes from the Great Lakes (Austin and Austin 2012). Some *Aeromonas* species cause mortality in just a few days, while others are easier for fish to recover from. Most *Aeromonas* infections cause disorientation, lethargy, reduced feeding, septicemia and hemorrhaging throughout the body, widespread lesions on skin, liquefaction of internal organs and swelling and discoloration of the liver, kidneys and/or intestines (Austin and Austin 2012). Each of these changes from a fish suffering from furunculosis could potentially contribute to a reduced condition factor. However, not all the fish that were positive for bacterial infection showed other signs of health distress. For example, one fish captured in mid-July had a bacterial infection in the liver, liquefied internal cavity (Figure 19, photo A), but few apparent

signs of stress as analyzed through the HAI methods (HAI scores= 30 for severe lesions on skin). This individual could have been suffering from *A. salmonicida* infection, but further testing is required.

Some bacterial pathogens of fish may remain dormant until the fish is stressed by environmental factors, and then the pathogen begins to cause disease (Austin and Austin 2012). Other bacterial cultures recovered from the livers of emerald shiner had the same biochemical test results as *Renibacterium salmoninarum* (bacterial kidney disease) which causes abdominal distention and ulcers, *Corynebacterium aquaticum* which causes hemorrhaging of the brain, loss of feeding, abnormal swimming and death, and several other pathogens such as *Aerococcus* spp., *Bacillus spp.* and *Nocardia spp.* (Austin and Austin 2012). Although these data should be interpreted with caution, and further confirmation is needed, each of the previously listed pathogens cause reduced feeding in fish, and many cause lesions and/or hemorrhaging, but they do not impact the leukocytes in the same manner. Thus, all pathogens which induce a reduced feeding response would be expected to reduce condition factor, one of the best observed signs of stress in emerald shiners, while changes in leukocrit values are not similarly unidirectional.

Conclusions

Emerald shiners are a keystone species in the Niagara River and this study provides clear evidence that they are a sensitive bioindicator species in the upper Niagara. There is some concern that the emerald shiner population in the Niagara River may be experiencing a low point in population size (Emerald Shiner Project, SUNY Buffalo State). Thus, the HAI scoring method provides some insight as to why their populations may be declining locally. Shiners captured

from the western branch of the river were healthier overall and in better condition than shiners from the eastern branch. These results suggest that although the western branch of the upper river is not pristine, it may offer refugia for fish as far as water quality conditions are concerned.

This study provided supporting evidence for three of four of the hypotheses, as well as providing baseline data from which future studies may build upon. There were differences in water *E. coli* levels between the western and eastern branch sites, which correlated with urbanization levels (Figure 7). Leukocrits did not reliably distinguish between healthy and unhealthy fish. Rather, the most apparent signs of failing health were condition factor and parasite infestation, particularly by the ciliate *Ichthyophthirius multifiliis.* To make a thorough assessment of the health of the upper Niagara aquatic ecosystem, it would be appropriate to repeat this study with shiners captured in more than one year, include other fish species and also assess the effects of chemical pollution on fish. Future studies may attempt to narrow the health assessment of fish to only a few of the important symptoms of stress such as parasite load, liver infection and condition factor.

The observed levels of *E. coli* in the Niagara River did not exceed the EPA limits in 2016, yet it is not a functionally safe environment for aquatic organisms confined to this system. Multiple bacteria and pathogens are associated with sewage and they can accumulate in fish, increase their parasite susceptibility and cause biological stress (Fattal et al. 1992). The Buffalo Sewer Authority is working to mitigate some of the effects of CSO input in the Niagara watershed through installing supporting infrastructure to contain stormwater (Buffalo Sewer Authority 2017). The data presented here suggests that there are still necessary improvements required to reduce sewage pollution, as emerald shiners exhibited immune stress at multiple levels. It cannot be assumed that the Niagara River will be below EPA bacterial pollution levels

in future years as it was in 2016, when precipitation was very low, since bacterial levels usually go up with increased precipitation and increased CSO input (Passerat et al. 2011, Mailhot et al. 2015). It is safe to assume, however, that there are synergistic effects between all the cumulative types of pollution entering the upper river and the many alterations to the river channel, including the hardening of shorelines. It is imperative to recall that the legally acceptable levels set by the EPA for *E. coli* levels are an artifact of human policy. In reality, the presence of *E. coli* suggests fecal contamination, which could indicate multiple sources of biological pollution. If important forage species such as the emerald shiner are not able to withstand these stressors, there will be irreversible effects on the rest of the food web in the Niagara River. The Niagara River is a valuable asset to this region and it should be restored and protected from ongoing habitat and water quality deteriorations.

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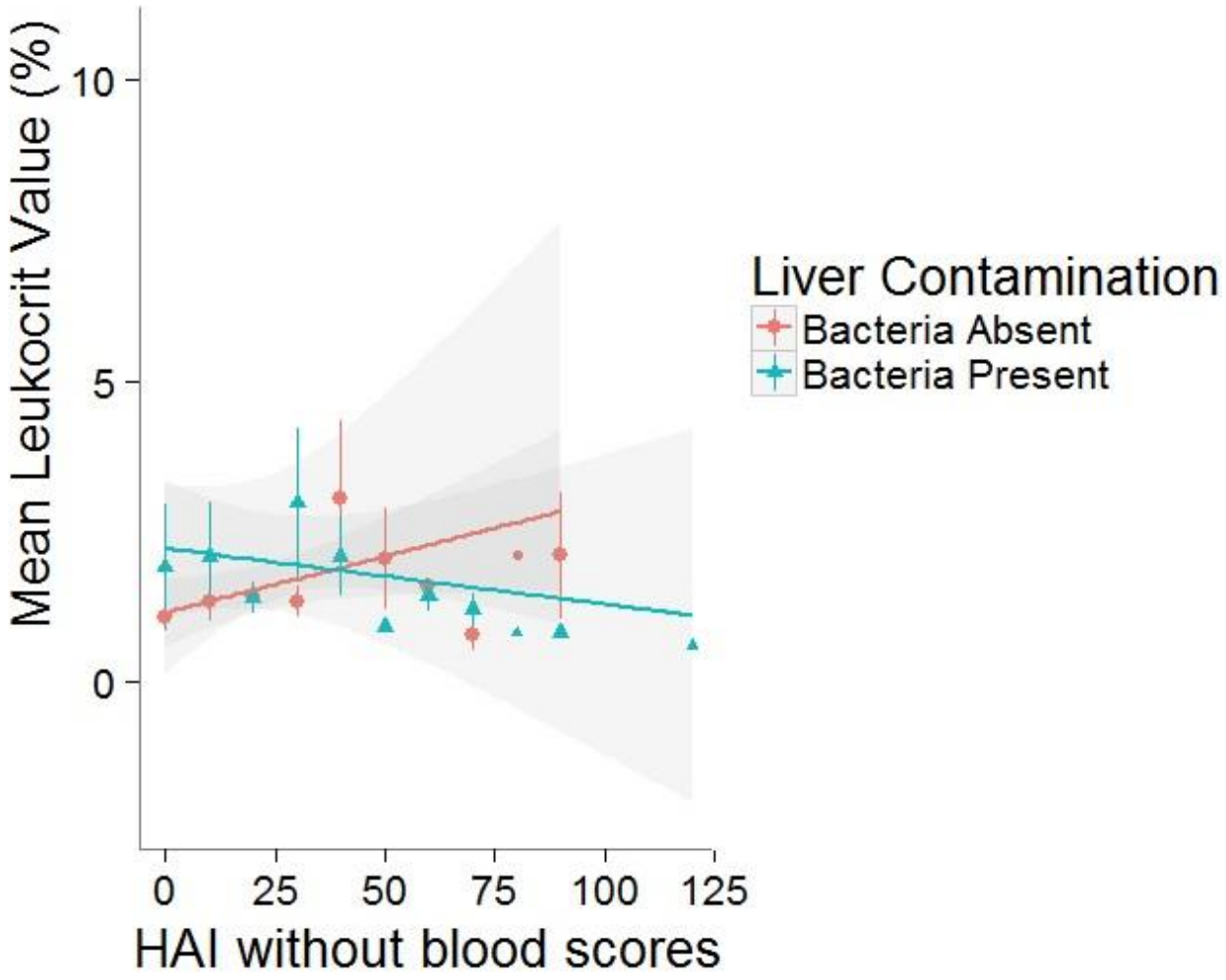
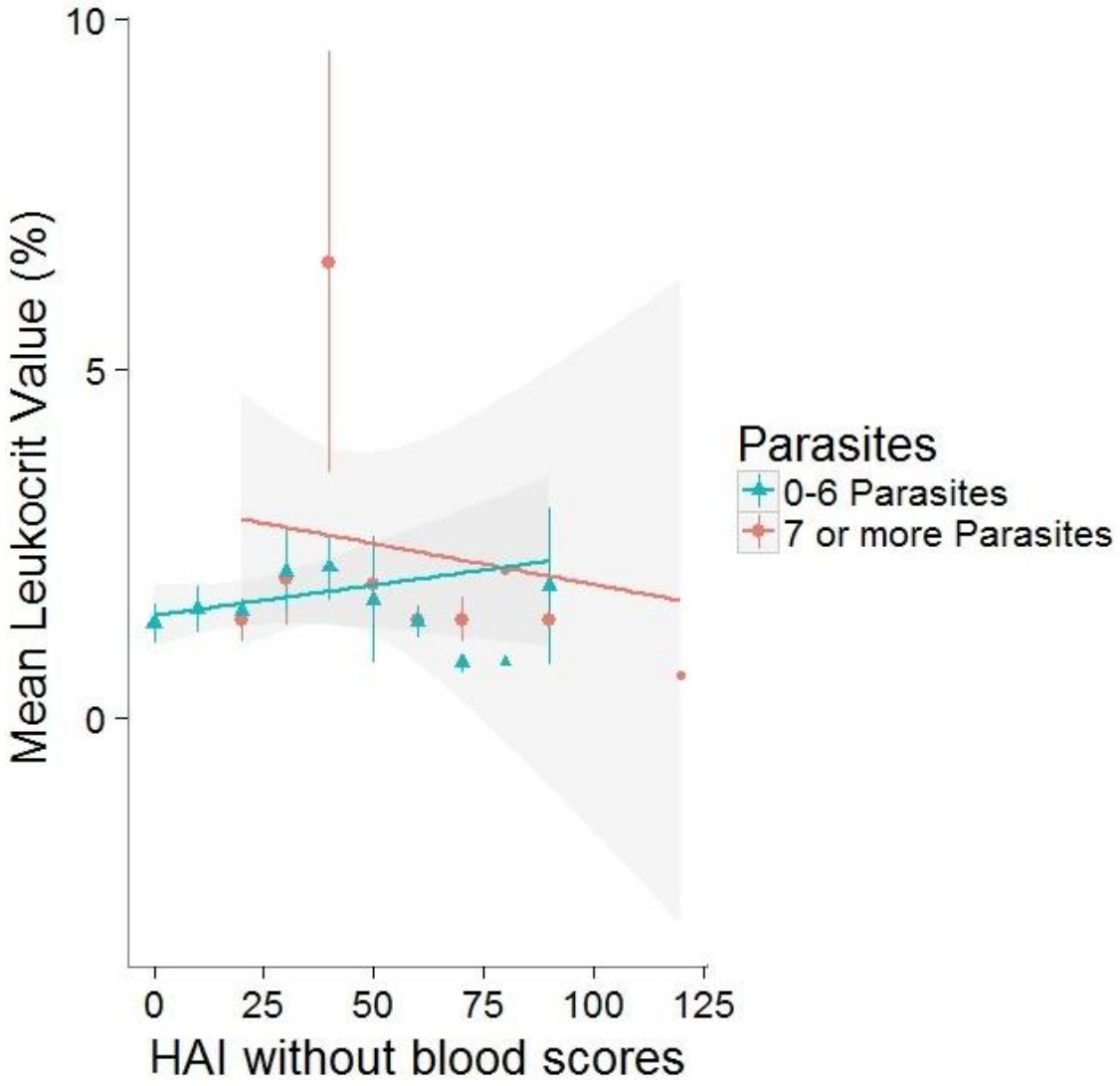
Supplemental Information

Supplemental, Table 1
Geometric means of *E. coli* observations for each study site. The geometric mean is calculated by taking the antilog of the average $\log_{10}$ observations. Each site includes 12 observations over the course of the sampling season, except Beaver Island (n=9) and Rich's Marina (n=13, where an extra sample was taken because of a visible plume). The threshold for class A recreational waters by the EPA is determined when at least five samples are taken in a month, however, in this study each site was sampled twice a month. The EPA recreational threshold value is 126 MPN/100 mL.

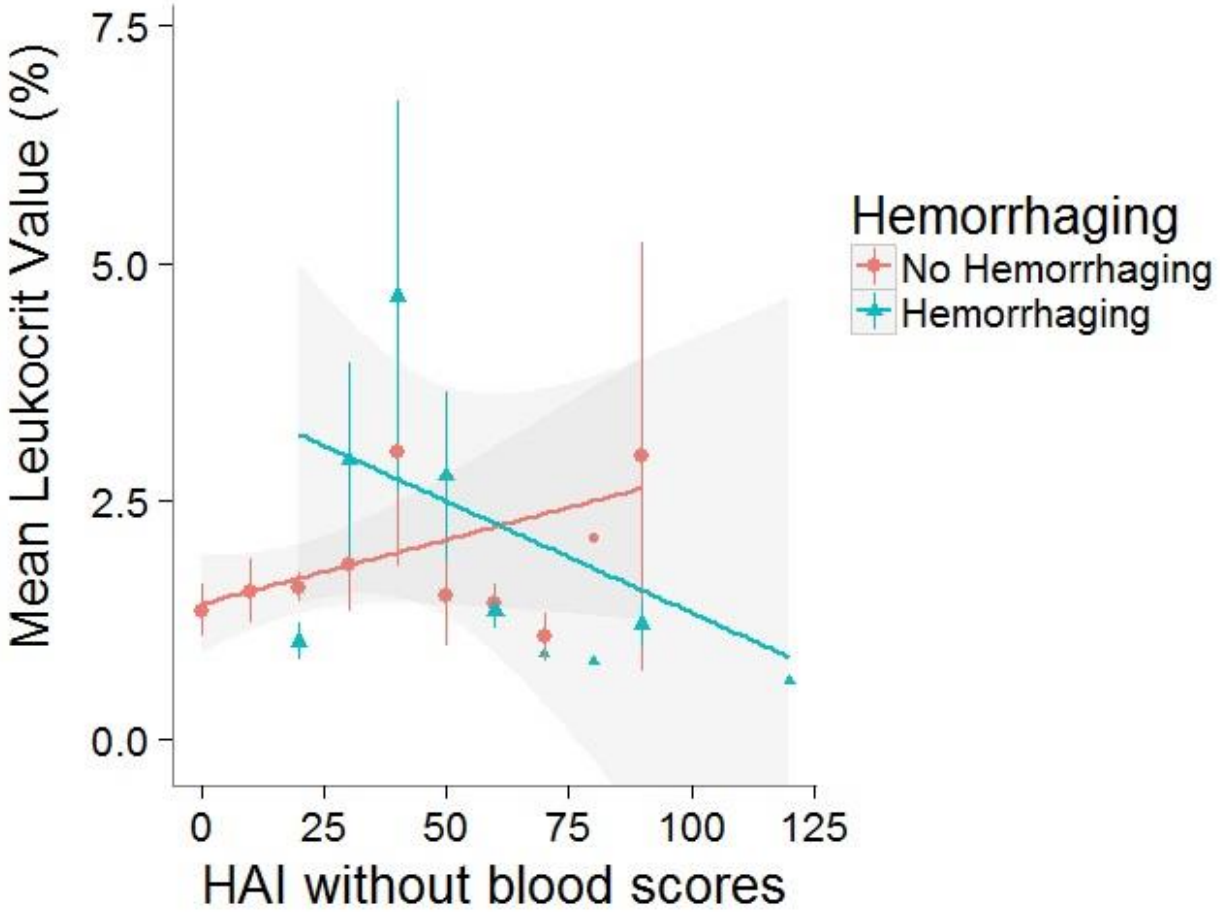
Site Name	Geometric mean *E. coli* for the season (MPN/100 mL)
Big Six Mile Creek	3.8
Beaver Island	36.2
Sandy Beach	5.4
Gratwick Park	45.7
Isle View Park	27.6
Rich's Marina	41.2
Black Rock Canal	175.0
Lake Erie	1.4



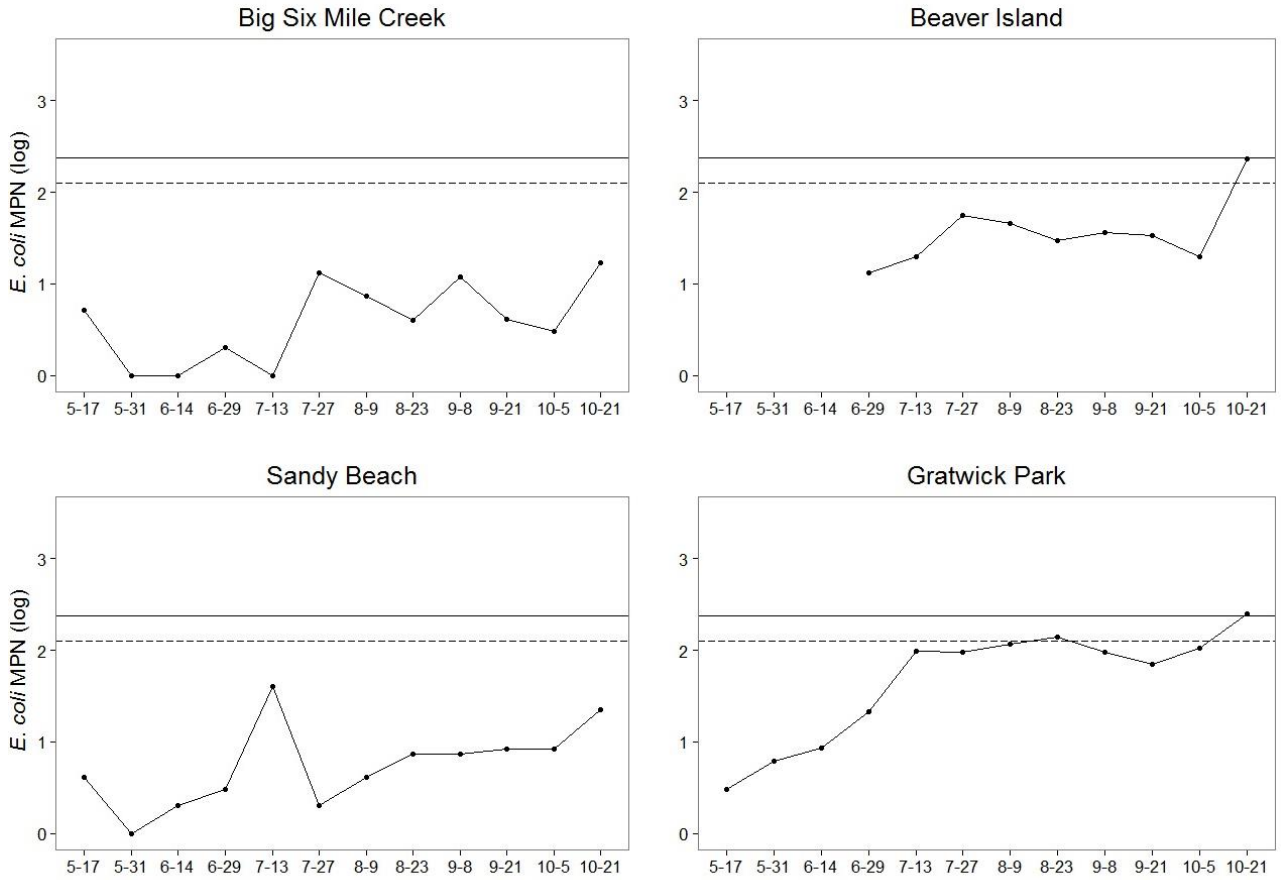
Supplemental, Figure 1
Examination of leukocrit values with HAI scores that do not include a score for blood. The emerald shiners were grouped into presence/absence of liver bacteria. Fish uninfected with bacteria in their livers had higher leukocrits with increasing HAI score and infected fish had lower leukocrits with increasing HAI score (poorer health).



Supplemental, Figure 2
Examination of leukocrit values with HAI scores that do not include a score for blood. The emerald shiners were grouped into low/moderate to high parasite loads. Fish with ≤ 6 parasites had higher leukocrits with increasing HAI score and fish with ≥ 7 parasites had lower leukocrits with increasing HAI score (poorer health).

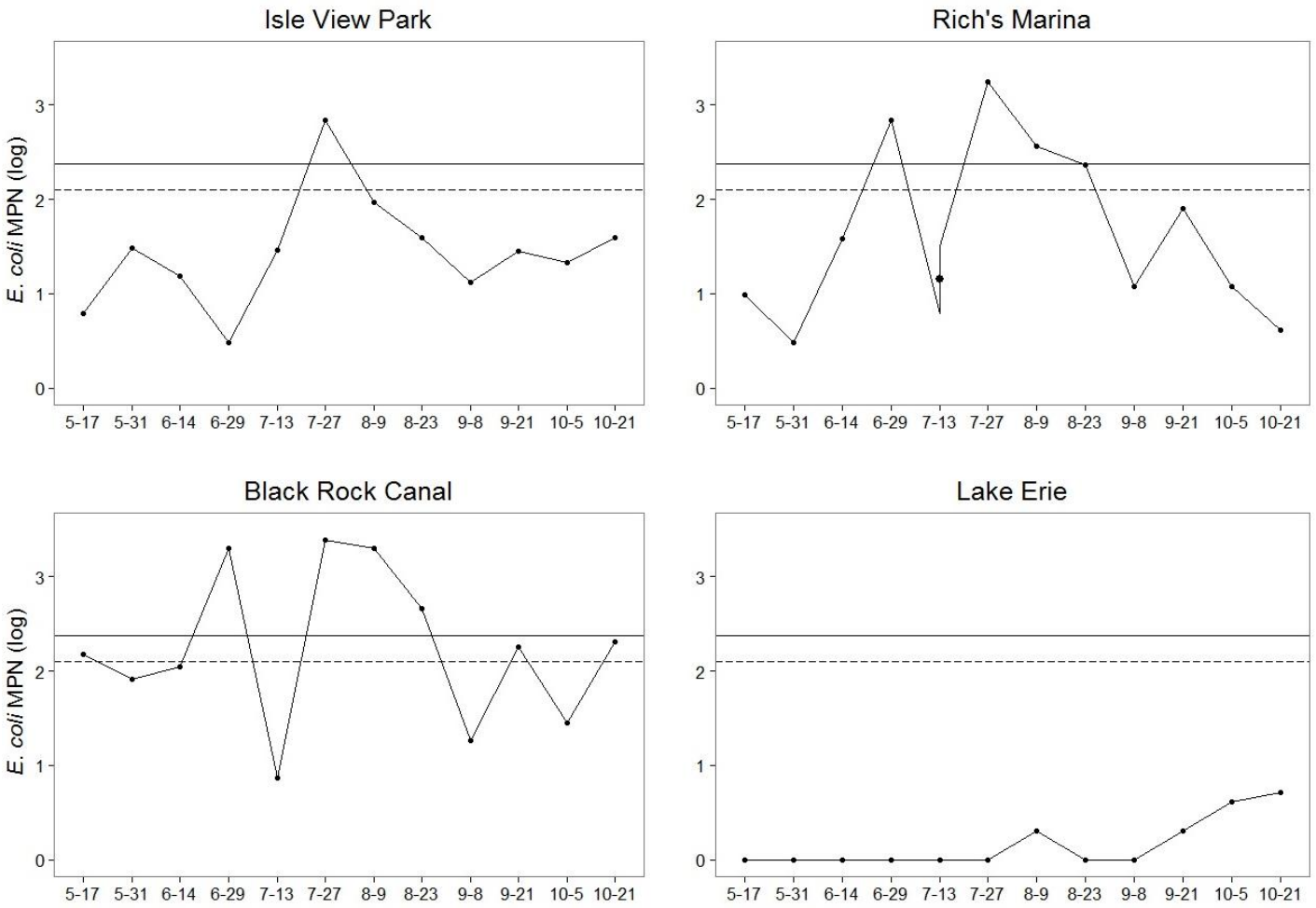


Supplemental, Figure 3
Examination of leukocrit values with HAI scores that do not include a score for blood. The emerald shiners were grouped by hemorrhaging/no hemorrhaging present. Fish without hemorrhaging had higher leukocrits with increasing HAI score and fish with hemorrhaging had lower leukocrits with increasing HAI score (poorer health).



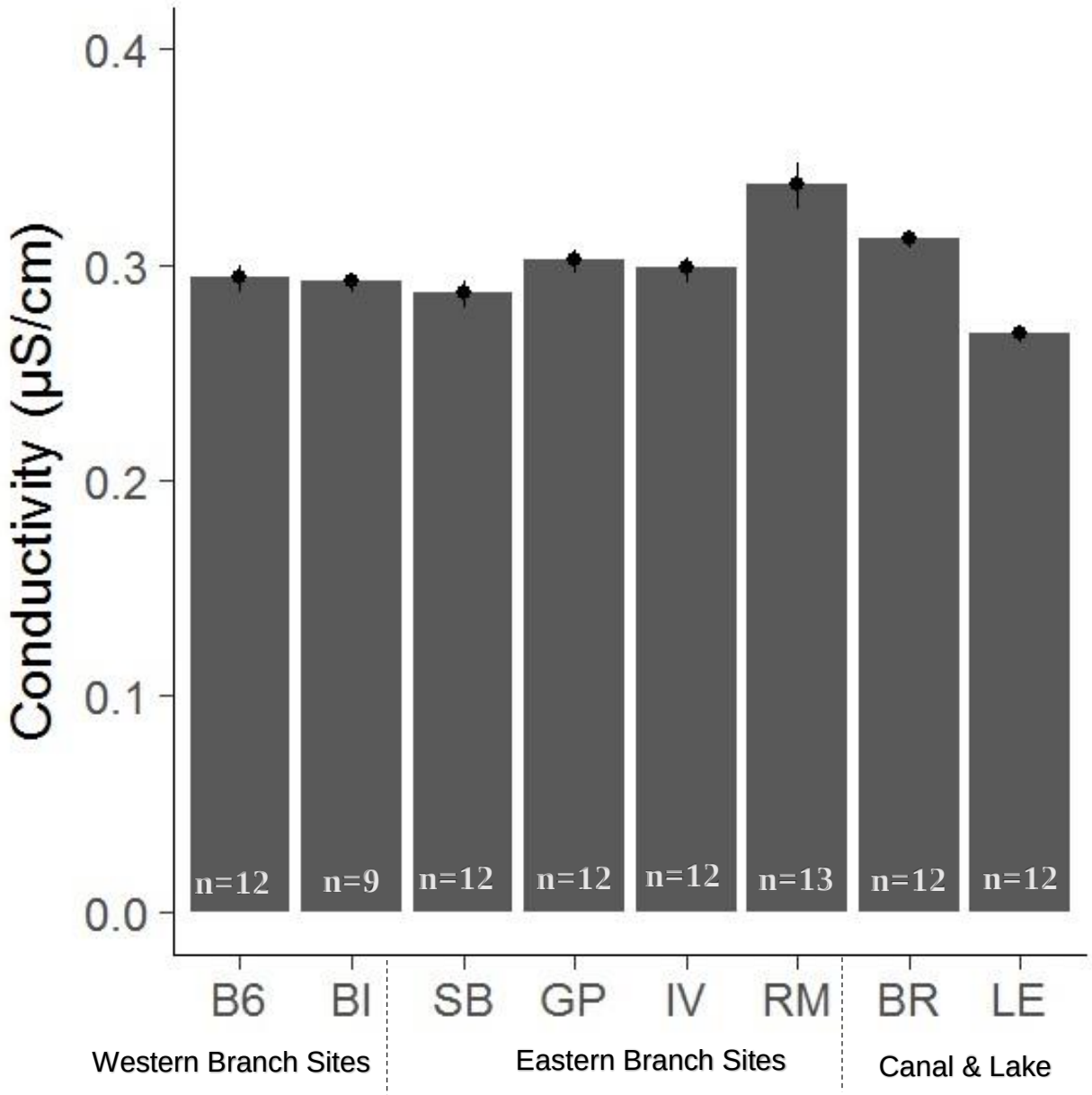
Supplemental, Figure 4

The *E. coli* MPN (log) observations by site with the median date for each week in 2016. Each data point represents a composite sample (three grabs) for each site to account for spatial variability within sites. The dashed horizontal line illustrates the acceptable geometric mean *E. coli* MPN (log_{10}) according to EPA standards in recreational waters when five or more samples are taken in a month. The solid horizontal line illustrates the acceptable *E. coli* MPN (log_{10}) for a single grab sample. Note: Beaver Island was not sampled until June 27th.

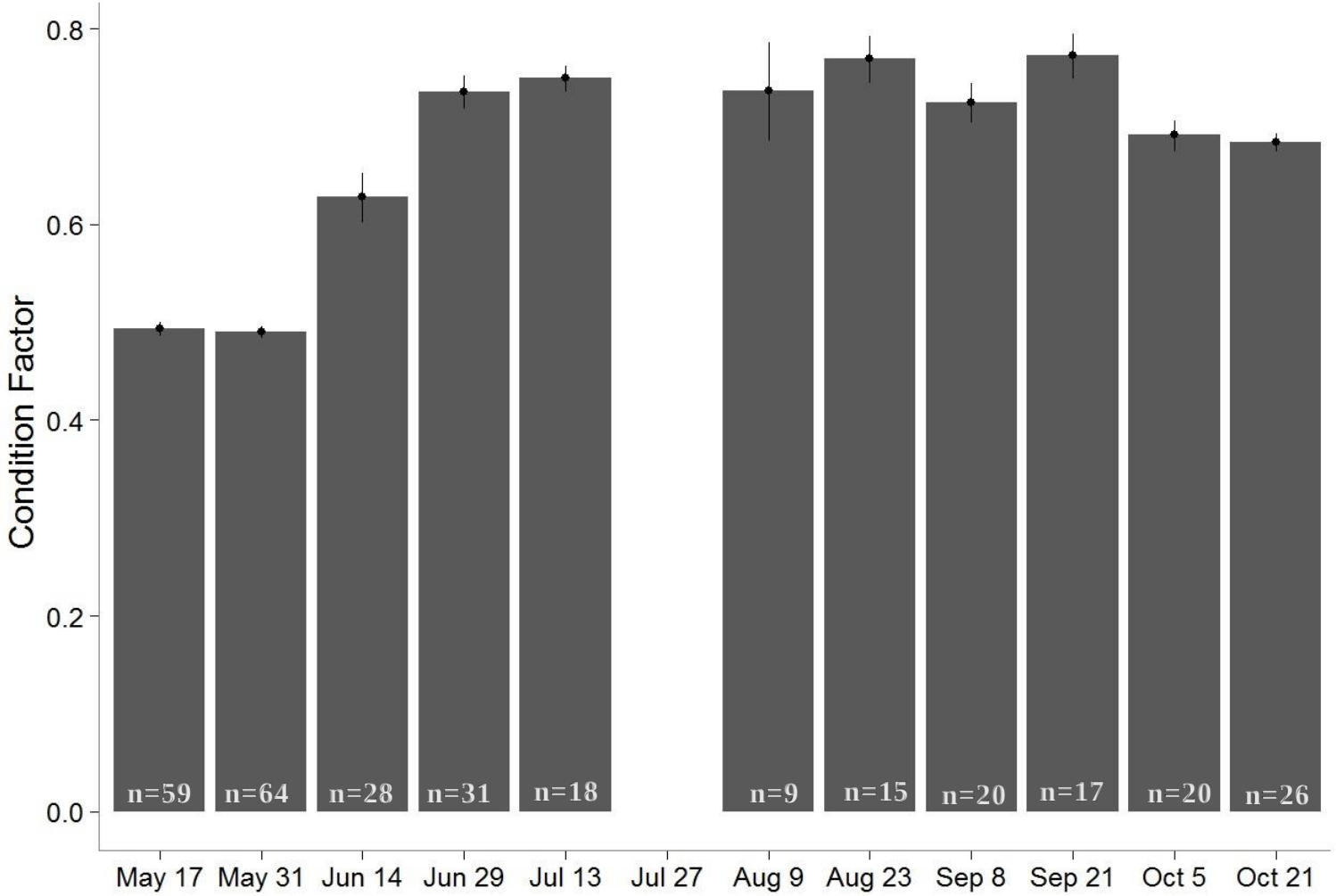


Supplemental, Figure 5

The *E. coli* MPN (log) observations by site with the median date for each week in 2016. Each data point represents a composite sample (three grabs) for each site to account for spatial variability within sites. The dashed horizontal line illustrates the acceptable geometric mean *E. coli* MPN (log_{10}) according to EPA standards in recreational waters when five or more samples are taken in a month. The solid horizontal line illustrates the acceptable *E. coli* MPN (log_{10}) for a single grab sample.

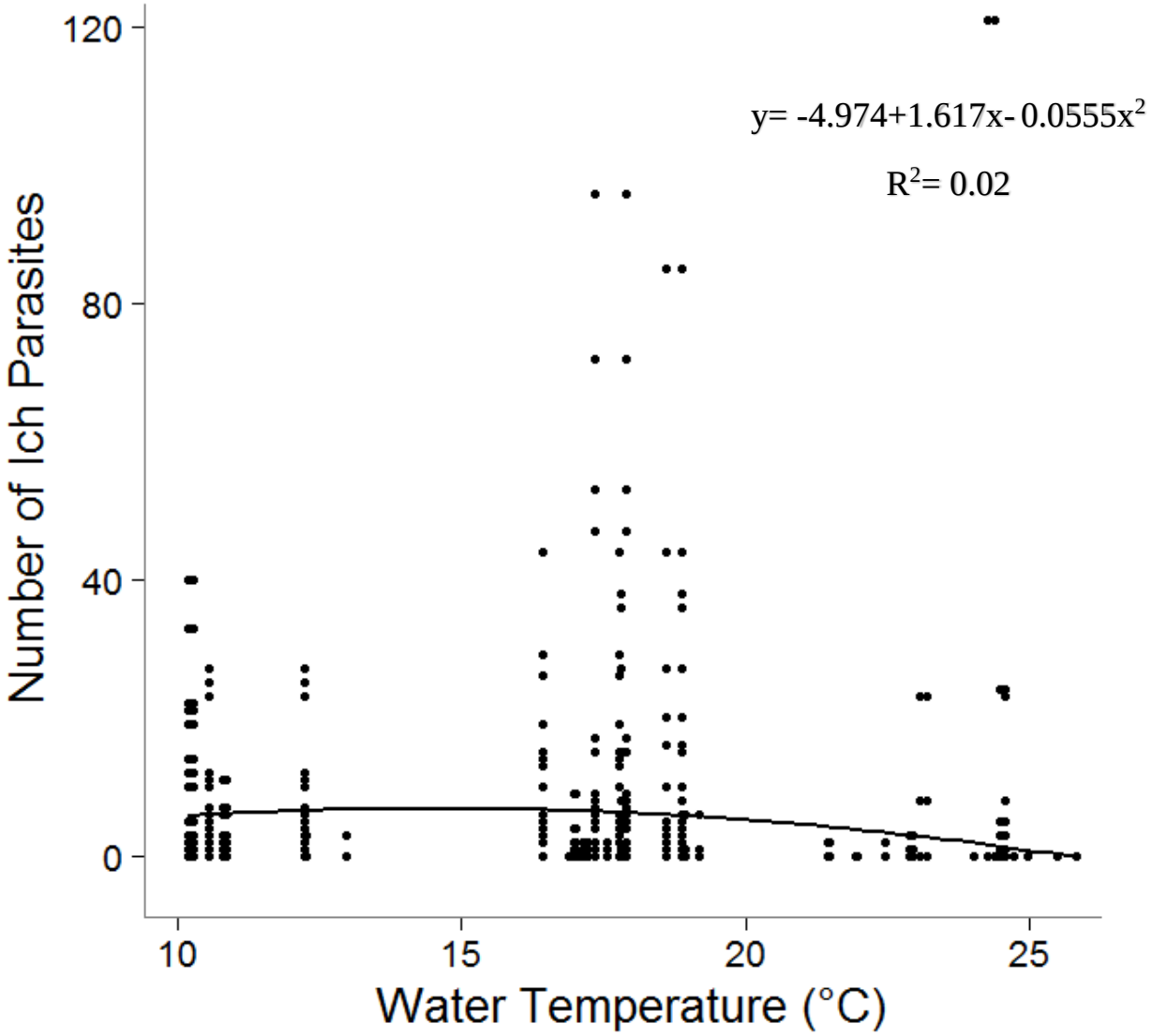


Supplemental, Figure 6
The mean (±SE) conductivity (µS/cm) for each riverine site for the six month sampling season.



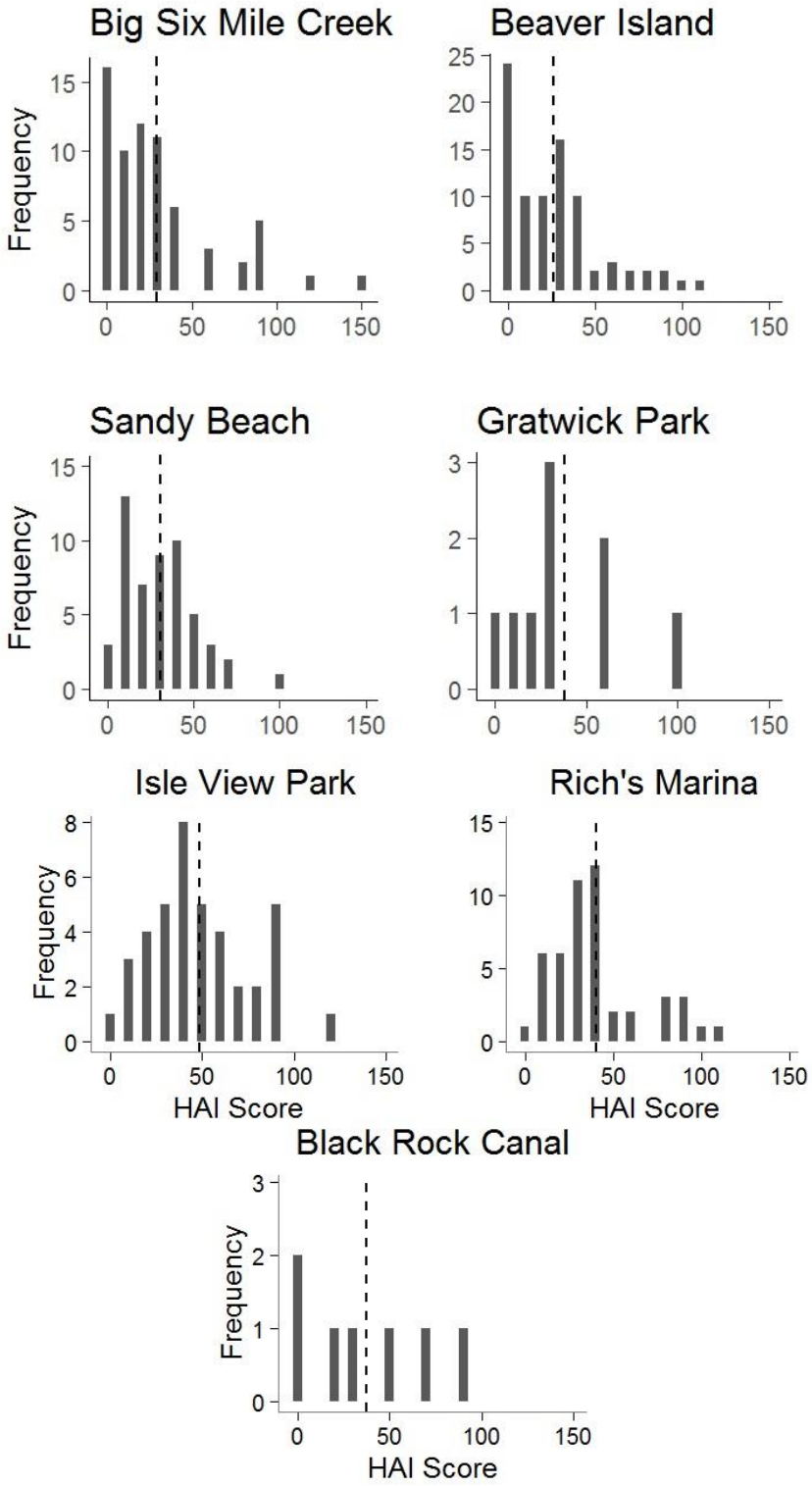
Supplemental, Figure 7

Condition factor (±SE) for each sampling week in 2016. Condition factor was lowest in May, when fish were likely still recovering from winter. By mid-June condition factor increased and appeared to stabilize. No fish were captured the week of July 27th, although the same fishing effort was exerted.

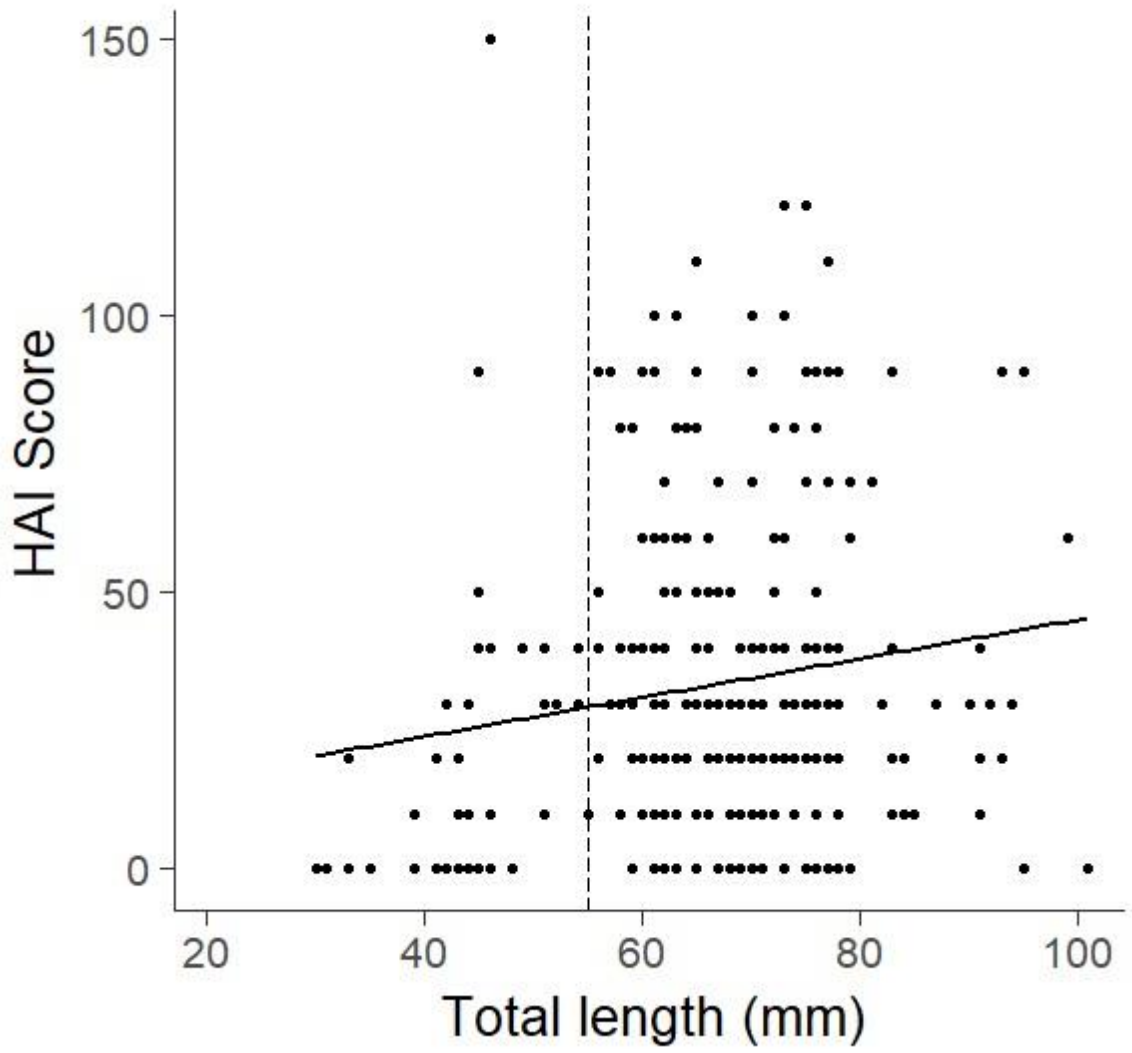


Supplemental, Figure 8

Polynomial relationship between *Ich* parasites attached to emerald shiners and water temperature. *Ich* tended to be most prevalent in intermediate water temperature conditions.



Supplemental, Figure 9
Frequency distribution of HAI scores for all emerald shiners collected from each site. A higher number indicates poorer health. The dashed vertical line illustrates the mean HAI score for the population captured from their respective sites. Note that the y-axes differ.



Supplemental, Figure 10
HAI score and total length of emerald shiners. HAI score tended to be higher in larger fish. The dashed vertical line at 55 mm is a guideline to differentiate between young-of-the-year and adult shiners.