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# Biology and Impacts of Leech Infestation in Largemouth Bass (Micropterus Salmoides) in Back Bay, Virginia

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BIOLOGY AND IMPACTS OF LEECH INFESTATION

IN LARGEMOUTH BASS (*MICROPTERUS SALMOIDES*)

IN BACK BAY, VIRGINIA

by

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ABSTRACT

BIOLOGY AND IMPACTS OF LEECH INFESTATION
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Back Bay is an oligohaline coastal bay in Southeast Virginia, USA. The Oregon Inlet along the Outer Banks of North Carolina is the only significant salinity source to the Bay via the Pamlico and Currituck Sounds of North Carolina. Today Back Bay is considered a brackish ecosystem with salinity of <1 psu. Since 2004, leeches have been observed in the oral cavities of Largemouth Bass (*Micropterus salmoides*) in Back Bay. Leeches (*Myzobdella lugubris*) have previously been documented in the oral cavities of largemouth bass in the Currituck Sound. In 2009, the Virginia Department of Game and Inland Fisheries (VDGIF) conducted an experimental Largemouth Bass restocking effort in hopes of increasing the Largemouth Bass population. The stocking was deemed successful and as a result VDGIF continued to restock in 2012, 2013, and 2014. After four years of stocking Largemouth Bass, VDGIF has indicated the population has not stabilized in the system as judged by yearly community fish sampling in Back Bay. This is worrisome because there may be a factor that is preventing growth of the Largemouth Bass population, and this raises the issue of whether restocking of Largemouth Bass should be continued. The purpose of this study is to determine the biology and impacts of oral leech infestations on the Largemouth Bass. From this study, we intend to determine if the leeches are inflicting harm on the Largemouth Bass.

I dedicate my thesis work to my family. I want to give a special thank you to my parents, David and Cristy who were there for me in love, supported me in everything I did, always encouraged me to follow my passion, and told me I could be anything I wanted. I would also like to thank my sister, Heidi. She is not only my little sister, but my best friend, my motivator, and my biggest support system.

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I. INTRODUCTION

HISTORY OF BACK BAY

Back Bay is an inland oligohaline, coastal Bay located in extreme Southeast Virginia (Figure 1). The only significant salinity source is Oregon Inlet located seventy miles south along the Outer Banks of North Carolina. There have been several ecological changes since the natural closing of the Old Currituck Inlet, located along the Virginia-North Carolina line, in 1830 (Norman and Southwick 1990). Before the inlet closed, the Bay was brackish and supported an ecosystem rich in marine fishes, oysters, clams and shrimp (Cortell and Mann 1984). Once Old Currituck Inlet closed, the Bay transitioned from a brackish to an oligohaline ecosystem, and the United States Wildlife Fish Service records indicated a substantial Largemouth Bass (*Micropterus salmoides*) fishery was established in Back Bay from 1901-1951 (Roseberry 1952; Norman and Southwick 1990). Another fish survey was conducted on the Bay from 1959-1962, and freshwater species including Largemouth Bass dominated the Bay.

Salinities of the Bay have been documented since the 1880s. The average salinity of the Bay from the 1880s-1950s averaged from 0-5 parts per thousand (ppt) (Norman and Southwick 1990). During this time, gamefish dominated the Bay. However, due to its close proximity to the Atlantic Ocean, Back Bay has been susceptible to ocean over-wash during extreme weather events. A powerful storm in 1962, known as the "Ash Wednesday storm", caused several breaks in the thin stretch of land that separates the Atlantic Ocean and Back Bay, allowing the introduction of saltwater into the Bay (Norman and Southwick 1990). The storm raised the salinity of the Bay up to 26 ppt only in portions directly adjacent breaches (Norman and Southwick 1990). Following the storm, rainfall and mixing due to prevailing winds gradually allowed the salinity to decrease to an average of 4.2 ppt (Norman and Southwick 1990). In areas of the Bay where the salinity averaged 3.5 ppt or higher, there was little to no Largemouth Bass reproduction (Johnston and Davis 1962).
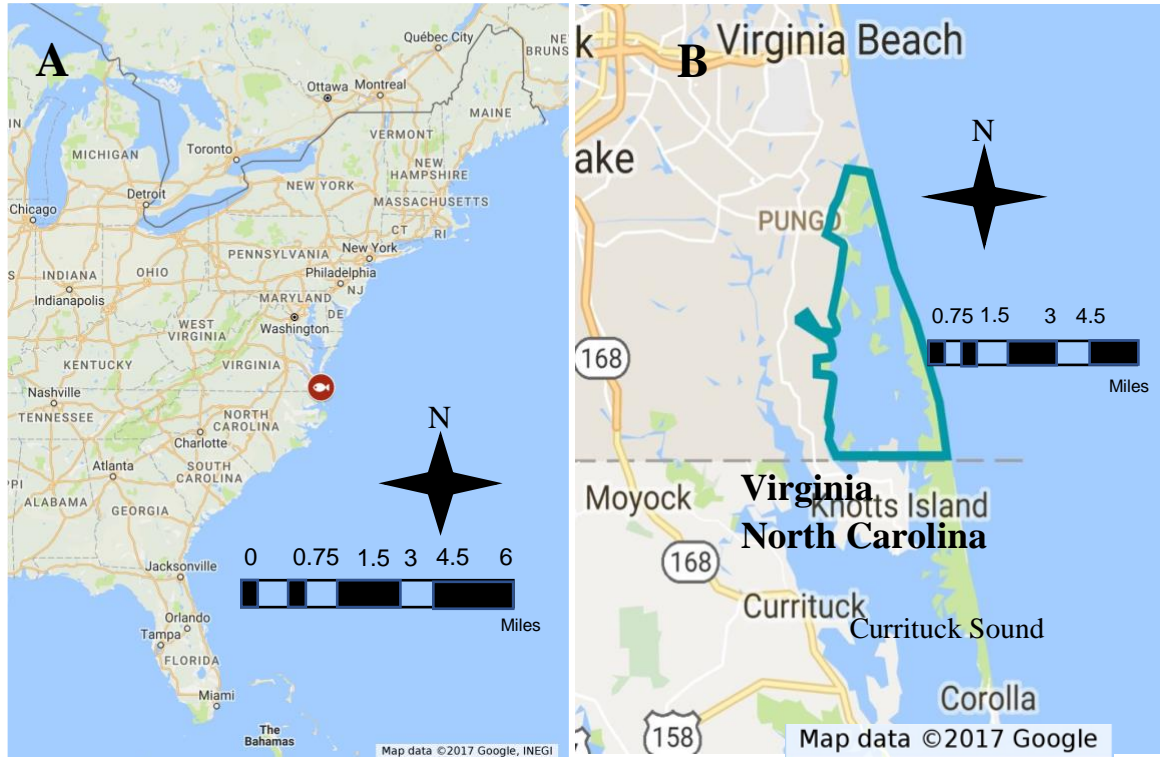
FIGURE 1. Map of Back Bay, Virginia Beach. **(a)** The location of Back Bay, Virginia along the US East Coast. The fish pinpoint indicates the location of Back Bay. **(b)** Detail of southeastern part of the City of Virginia Beach. Back Bay is enclosed by the blue line. Back Bay borders the North Carolina border, and connects to the Currituck Sound, NC.

The submerged aquatic vegetation (SAV) coverage throughout the Bay also decreased after the storm (Norman and Southwick 1990). Eurasian water milfoil (a non-native SAV) was first found in the Bay in 1966, and was likely over washed into the Bay with the Ash Wednesday storm (C. Boyce, Virginia Department of Game and Inland Fisheries, personal communication). SAV frequency was at 81% coverage in 1962 and dropped to 14% in 1964 (Schwab et al. 1991). SAV dropped because the salinity in the Bay decreased from 26 ppt after the storm in 1962 to 0.7 ppt in 1965, allowing the turbidity to increase in the Bay (Norman and Southwick 1991). Because the SAV conditions following the 1962 storm were so poor, local hunting groups proposed that the City of Virginia Beach should pump saltwater into the Bay in an attempt to mimic the conditions created after major ocean over-wash events, thus improving waterfowl habitat by reducing turbidity and allowing more tolerant SAV to grow (Norman and

Southwick 1990). The first salt water pumping efforts by the City of Virginia Beach occurred during 1965-1973 (Norman and Southwick 1990). In 1973 the pump was destroyed by fire, postponing the pumping (C. Boyce, Virginia Department of Game and Inland Fisheries, personal communication). The SAV rapidly increased and the high percent of SAV in the late 60s and mid 70s was mainly milfoil (C. Boyce, Virginia Department of Game and Inland Fisheries, personal communication). Figure 2 shows a graph of percent SAV vs. Largemouth Bass citations from 1965-1990. SAV increased from 1966 to 1973 during the first saltwater pumping. SAV stabilized in the Bay from 1973 to 1978, and decreased from 1978 to 1989 during the second saltwater pumping because it had become fragmented (C. Boyce, Virginia Department of Game and Inland Fisheries, personal communication). Largemouth Bass citations increased beginning in 1980. This was due to both SAV growth explosion and Largemouth Bass becoming a new popular recreational fishery in Back Bay. The spikes in the graph for citations are seen because Largemouth Bass congregated in specific areas of SAV, and there were more angler surveys to know where to catch bigger Largemouth Bass (C. Boyce, Virginia Department of Game and Inland Fisheries, personal communication). A reason for the decreased trend in Largemouth Bass citations was a combination of decreased SAV and the nature of the fishery, as anglers kept fishing for bigger Largemouth Bass (C. Boyce, Virginia Department of Game and Inland Fisheries, personal communication).

The salinity of the Bay averaged 3 ppt after the first pumping (Norman and Southwick 1990). From 1965-1972 the Bay was a slightly brackish ecosystem with moderate vegetation, and from 1973-1978 the salinity decreased to 0.7 ppt, but with heavy vegetation (Norman and Southwick 1990). Freshwater fishes, including Largemouth Bass (*Micropterus salmoides*), made up 67.6% of the ecosystem of Back Bay in 1978 (Norman and Southwick 1990). The City of Virginia Beach resumed saltwater pumping in August 1978, and continued until August 1987 (Norman and Southwick 1990). Average salinity during this time increased to 3.4 ppt (Norman and Southwick 1990). To determine the effects of the saltwater pumping on the freshwater fishes in the system, the Virginia Department of Game and Inland Fisheries (VDGIF) conducted annual fisheries community sampling. This sampling demonstrated that

increased salinity had an adverse effect on freshwater fish populations (Norman and Southwick 1990), with freshwater species comprising 23.8% of the total species collected in 1986 (Norman and Southwick 1990). The major declines in the freshwater fishes were in Largemouth Bass (*Micropterus salmoides*), Pumpkinseed (*Lepomis gibbosus*), Yellow Perch (*Perca flavescens*), and Longnose Gar (*Lepisosteus osseus*) (Norman and Southwick 1990). Based on these results, VDGIF was able to show that freshwater fish were suffering under higher salinity, but that was not the reason the pumping was discontinued. Rather, SAV coverage was not responding to the increased salinity (C. Boyce, Virginia Department of Game and Inland Fisheries, personal communication) and as a result, saltwater pumping was discontinued in August of 1987. Gradually the salinity decreased and stabilized at <1 ppt in August of 1989 (Norman and Southwick 1990).

From 2007 to 2011, VDGIF observed that SAV growth had greatly improved in Back Bay. This growth was hypothesized to be due to a prolonged period of lower turbidity that allowed SAV germination (C. Boyce, Virginia Department of Game and Inland Fisheries, personal communication). This prompted an experimental stocking of surplus bass in 2009 to see if stocking would be an option in the future. Largemouth Bass had not previously been stocked in the Bay. These Largemouth Bass came from two different fish hatcheries, one a VDGIF hatchery in in King and Queen, Virginia and Watha Fish Hatchery in North Carolina, both hatcheries mated progeny from Virginia and North Carolina waters (C. Boyce, Virginia Department of Game and Inland Fisheries, personal communication). These fish are known as F1 Bass or Tiger Bass, which are a first generation hybrid of Florida Bass (*Micropterus salmoides floridanus*) and northern Largemouth Bass (*Micropterus salmoides salmoides*) (Maceina and Murphy 1988). These fish are commonly used for stocking (Maceina and Murphy 1988; Moore 2010) and are claimed to grow faster than wild-type Largemouth Bass (Moore 2010). The experimental stocking was a success as VDGIF determined the Largemouth Bass population numbers were increasing when sampling was conducted in 2010 and 2011 (C. Boyce, Virginia Department of Game and Inland Fisheries, personal communication). Based on the numbers of the Largemouth Bass population increasing in the Bay, three re-stockings of 125,000 fingerling Largemouth Bass each were

conducted in 2012, 2013, and 2014 (C. Boyce, Virginia Department of Game and Inland Fisheries, personal communication). The strain used for those three consecutive stockings were Tiger Bass from a commercial hatchery in Alabama (C. Boyce, Virginia Department of Game and Inland Fisheries, personal communication). VDGIF used the Tiger Bass because they are able to adapt and mate with the native Largemouth Bass in Back Bay (C. Boyce, Virginia Department of Game and Inland Fisheries, personal communication).

RESTOCKING CONCERNS

Since the restocking of Largemouth Bass in Back Bay, there has been concern regarding the population. According to VDGIF, there has been an increase in the Back Bay Largemouth Bass population since the late 1980s. Catch per unit effort (CPE) via electrofishing has improved since 2007: CPE = 7.7 f/h in 2007, 14.6 f/h in 2008, 28.9 f/h in 2011, 20.7 f/h in 2012, 37.5 f/h in 2013, 51.7 f/h in 2014, 21 f/h in 2015, and 45 f/h in 2016. The low catch rates in 2015 were seen as an anomaly (C. Boyce, Virginia Department of Game and Inland Fisheries, personal communication). However, larger bass are less abundant than expected. Stress of Largemouth Bass can negatively affect their reproductive success (Ostrand et al. 2004). The offspring of stressed Largemouth Bass are more likely to be smaller, and weigh less than those offspring of unstressed Largemouth Bass (Ostrand et al. 2004). Leeches that have been documented in Back Bay, which could be affecting the Largemouth Bass population. These leeches could potentially be inducing stress in the population, and could be affecting the reproductive status of the Largemouth Bass or otherwise limiting expansion in the Largemouth Bass population.

LEECH OBSERVATIONS IN NORTH CAROLINA

*Myzobdella lugubris* previously has been identified in the oral cavities of Largemouth Bass in the Currituck Sound, NC (Noga et al. 1990). When the leech attaches to its host, it causes an inflammatory response (Amin 1981). Observations of the oral cavity of Largemouth Bass show ulceration at the site of leech attachment (Noga et al. 1990; Faisal et al. 2011). Bacteria found at the lesions in the oral cavity

have been identified as *Pseudomonas fluorescens*, *Bacillus* sp., *Staphylococcus hemolyticus*, and *Psuedomonas putrefaciens* (Noga et al. 1990).
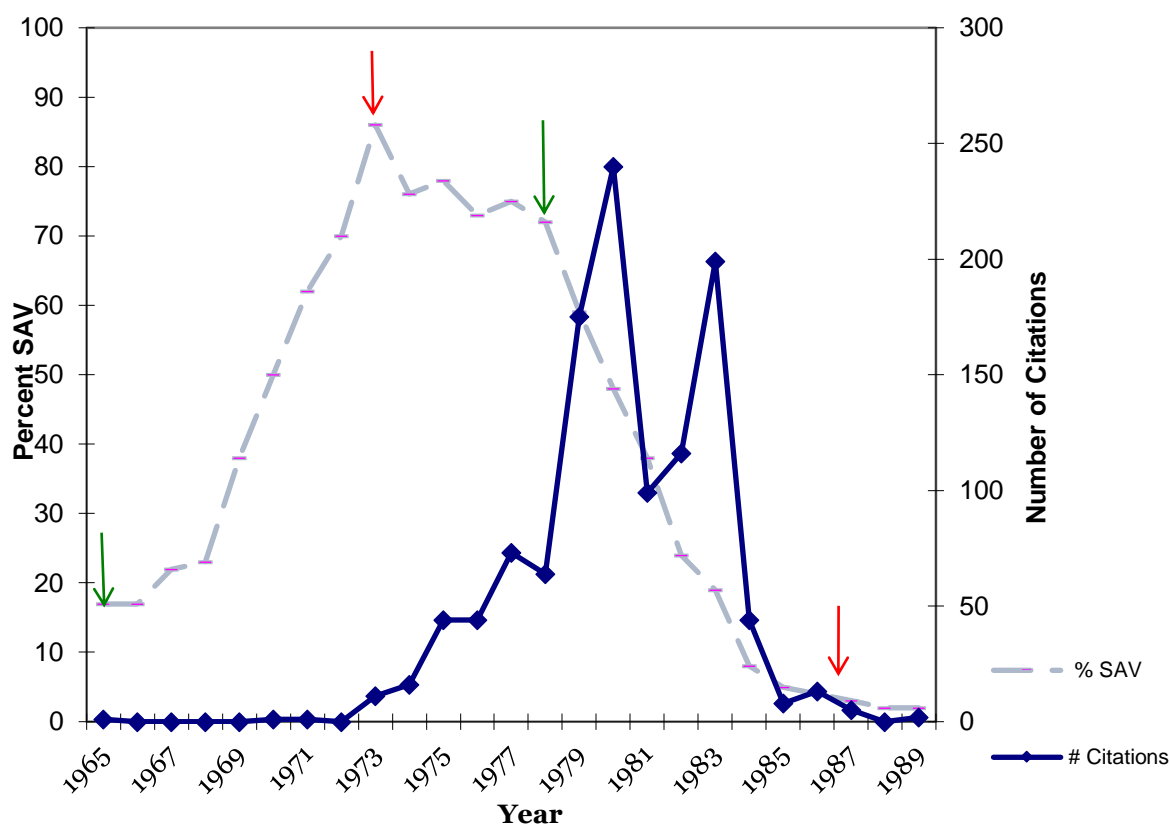


FIGURE 2. Percent SAV Coverage vs. Largemouth Bass Citations from 1965-1990. Percent SAV coverage (dashed line) and the number of Largemouth Bass citations (solid line) from 1965-1990. Green and red arrows indicate initiation and termination of saltwater pumping, respectively. An increase in SAV during this time could be due to the introduction of seeds into the Bay. SAV growth explosion and Largemouth Bass becoming a new popular recreational fishery are reasons for the two peaks in the Largemouth Bass citations. SAV declined during the second pumping due to fragmentation. By 1989, SAV and Largemouth Bass citations were low due to a combination of decreased SAV and fishing effort. During this time period, people kept fishing for bigger Largemouth Bass (C. Boyce, Virginia Department of Game and Inland Fisheries, personal communication).

STUDY OBJECTIVES

        According to VDGIF, leeches were first observed in Back Bay in 2004, and since 2012, VDGIF has reported a subjective increase in leeches in the oral cavities of

Largemouth Bass (C. Boyce, Virginia Department of Game and Inland Fisheries, personal communication). Stocking fish can be an expensive investment, especially if it is continued every year. The 2012, 2013, and 2014 stockings were $62,000 per year, which is $186,000 in the three years combined (C. Boyce, Virginia Department of Game and Inland Fisheries, personal communication). Due to an increase in leech observations in the oral cavities of Largemouth Bass, there is great concern whether or not the Largemouth Bass stocking of Back Bay should continue. The purpose of this project is to study the effects of the leech, *Myzobdella lugubris*, in Largemouth Bass, *Micropterus salmoides*, by examining the stress response, bacteriology, hematology, virology, and relative survival, of infested versus non-infested fish.

Largemouth Bass Virus (LMBV) is also a concern and preliminary testing will be conducted for presence of the virus. Viral Hemorrhagic Septicemia Virus (VHSV) has been found in *M. lugubris*, and preliminary presence/absence assays will be conducted on a small number of leeches. Leech cocoons have been observed on the Blue Crabs in Back Bay, and the Blue Crabs are a possible source of leech infestation in Largemouth Bass. To examine this relationship, an agent-based model will be generated to model leech infestations in Back Bay Largemouth Bass.

LARGEMOUTH BASS

Largemouth Bass are top predators in freshwater ecosystems and in artificial ponds. Their diet includes crayfish, aquatic insects, and small-bodied fishes (Heidinger 1976; Braun and Walser 2011). Sexual maturity of a Largemouth Bass is dependent on size (Heidinger 1976). Female Largemouth Bass reach sexual maturity at 25 cm in length and males reach sexual maturity at 22 cm in length (James 1946; Bennett 1948; Moorman 1957; Holvik 1970; Heidinger 1976). Therefore, when sampling a mature Largemouth Bass population, females will be larger than males. Males begin building a nest in the spring when the water temperature ranges from 15-24°C (Kramer and Smith 1960; Heidinger 1976). Females will spawn once a year, but their spawning can be prolonged allowing them to lay their eggs in more than one nest (Heidinger 1976). In Back Bay, Largemouth Bass spawning begins in mid-April and continues through June (Norman and Southwick 1990). Salinity has an effect on the survival of Largemouth

Bass fry. The maximum concentration of saltwater Largemouth Bass eggs and fry can tolerate is between 10 and 15 ppt (Wollitz 1962; Tebo Jr and McCoy 1964). In salinities above 15 ppt there is no survival (Wollitz 1962; Tebo Jr and McCoy 1964).

THE LEECH *MYZOBDELLA LUGUBRIS*

Leeches are ectoparasites that are commonly found in freshwater and estuarine habitats. Leeches can be semi-permanent or intermittent. A leech that is semi-permanent attaches to only one host for the greater part of its adult life (Faisal et al. 2011). Intermittent leeches attach to several hosts throughout their lifetime (Faisal et al. 2011). *M. lugubris* have suckers at both ends of the body requiring the leech to develop coordination to distinguish between the front and rear ends (Sawyer 1981). They are unique in that they do not swim but rather oscillate, creating a rhythmic motion (Friesen and Kristan 2007; Zheng et al. 2007; Chen et al. 2008). Leeches are hermaphrodites and have one male pore and one female pore (Mann 1962).

*Myzobdella lugubris* is an intermittent leech and a member of the Piscicolidae family (Amin 1981; Utevsky et al., 2004; Williams and Burreson 2006; Faisal et al. 2011). Members of this family are parasitic to many fish species (Uteveksy et al. 2004, Williams and Burreson 2006), and are found in fresh, brackish, and marine water ecosystems (Sawyer et al. 1975). *M. lugubris* is a hematophagous parasite that attaches to both freshwater and estuarine fishes including Largemouth Bass (Daniels and Sawyer 1975; Sawyer et al. 1975; Amin 1981; Schramm et al. 1981; Muzzall et al. 1987; Morrison et al. 1993; Choudhury et al. 2004; Faisal et al. 2011; Schulz et al. 2011). Host responses associated with the leech range from mild epithelial hyperplasia, erosion, and ulceration (Appy and Cone 1982), to massive gross ulceration (Paperna and Zwerner 1974). *M. lugubris* commonly affects the fins and other skin surfaces of host fishes (Daniels and Sawyer 1975; Sawyer et al. 1975; Amin 1981). Buccal ulcerations have been documented in Largemouth Bass that could have been the site attachment of *M. lugubris* (Noga et al. 1990; Faisal et al. 2011). *M. lugubris* has an elongated body that is slightly flattened and smooth, an oral sucker, a caudal sucker, and one pair of eyespots (Sawyer et al. 1975). *M. lugubris* has been reported in different areas across the United States, including New Jersey (Moore 1946), the Colorado River

and Grand Canyon in Arizona (Linder et al. 2012), the Currituck Sound, NC (Noga et al. 1990), the Kentucky River, KY (Flotemersch et al. 2012), Florida (Ruiz-Carus et al. 2006), Hawaii (Font 1998), northern Arkansas (Moser et al. 2006), West River, Connecticut (Harris and Vogelbein 2006), the Laurentian Great Lakes (Faisal and Schulz 2009; Faisal et al. 2011; Schulz et al. 2011), Wisconsin (Amin 1981), and in South Carolina (Sawyer and Shelley 1976). *M. lugubris* can potentially vector Viral Hemorrhagic Septicemia virus (VHSV) (Faisal and Schulz 2009; Faisal et al. 2011). VHSV is a deadly fish virus that has been documented in the Great Lakes Basin (Faisal and Schulz 2009).

*Myzobdella lugubris* lays its cocoons on Blue Crabs (*Callinectes sapidus*) and other crustaceans once the leech has reached maturity (Daniels and Sawyer 1975; Sawyer et al. 1975). Cocoons are dark brown, found on the crab carapace, and are equally distributed dorsally and ventrally among both males and female crabs (Daniels and Sawyer 1975; Sawyer et al. 1975). The cocoons hatch on average 35 days from being laid (Sawyer et al. 1975), and juvenile leeches can survive for 13-15 days at 1.1 ppt (Sawyer et al. 1975). The juvenile leeches can tolerate low salinity environments, but cannot survive at more than 15 ppt (Sawyer et al. 1975). After the leech has taken a full bloodmeal, it leaves the host and returns to the Blue Crabs to lay cocoons (Daniels and Sawyer 1975). It is unknown how long the leech parasitizes a fish and if the leech feeds on the crab host.  The related species *Myzobdella platensis* does feed on the hemolymph of Blue Crabs and uses the crabs as a site for cocoon deposition (Zara et al. 2009). Therefore, *M. lugubris* could genuinely parasitize (i.e. feed upon) on Blue Crabs as well.

BLUE CRABS (*CALLINECTES SAPIDUS*)

Blue Crabs (*Callinectes sapidus)* have a complex life history that involves both marine and estuarine ecosystems (Tankersley et al. 1998; Carr et al. 2004). Mating occurs in the estuaries in the spring and continues through the fall (Carr et al. 2004; Aguilar et al. 2005). Females move from high salinity to low salinity to mate, and after mating they return back to the high salinity ecosystems (Turner et al. 2003; Carr et al. 2004). Back Bay contains predominantly male crabs. The female crabs migrate north

from the Currituck Sound to begin the mating process in the late winter and early spring months. It is unknown if the female crabs mate once and leave, or if they are able to mate more than once and stay in the Bay during the summer. It is during these months that crab fishermen lay their pots at the southern end of the bay, and continue to disperse the crab pots north during the spring and summer months. By summer, crabs are dispersed throughout the entire bay. Then in the late summer and early fall months the crabs begin to migrate back south, so by early winter they are dispersed at the southern end of the bay.

CORTISOL AND GLUCOSE AS STRESS INDICATORS IN FISHES

Cortisol is a steroid hormone that is produced by fishes and a wide variety of both vertebrates and invertebrates in response to stressors (Barton 2002; Davis and McEntire 2009). In vertebrates, the stress response is well-studied and relies on the hypothalamic-pituitary-adrenocortical (HPA) axis and the final glucocorticoids (GC) (Yeh et al. 2013) (Figure 3). In stressful situations, corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) are released by the neurons in the hypothalamic paraventricular nucleus (PVN) into the hypothalamo-pituitary portal circulation (Yeh et al. 2013). These hormones act on the pituitary gland and trigger the release of adrenocorticotropic hormone (ACTH) into the blood, and induce the release of GCs from the adrenal cortex (Yeh et al. 2013). Glucocorticoids are the final effectors of the HPA axis (Charmandari et al. 2005). Glucocorticoids serve multiple functions, including the regulation of the HPA-axis activity, immune activities, and moderation of cellular metabolism under both stressful and basal conditions (Sapolsky et al. 2000; Yeh et al. 2013). Glucocorticoids, once bound to the glucocorticoid receptor, can prevent transcription of genes for pro-inflammatory molecules such as cytokines, by preventing the translocation of transcription factors from the cytosol to the nucleus (Drouin et al. 1993; Barnes 1998). In the blood, there are cortisol-binding proteins (CBP), including endogenous serum proteins that bind to one molecule of cortisol (Brock et al. 1978; Andersen 2002). Free cortisol, or cortisol not bound to a CBP, initiates a biological response or is cleared through metabolic pathways (Rosner 1990; Andersen 2002). Total cortisol is defined as both the protein-bound cortisol and unbound cortisol

(Andersen 2002). Enzyme-linked immunosorbent assay (ELISA) or RadioImmune Assay (RIA) are two methods commonly used to measure free cortisol in fishes and other animals. ELISA is used to detect the binding of unconjugated antigen in a standard known solution that is compared to antigen in an unknown sample (Engvall and Perlmann 1971). ELISA can also be used as a competitive assay for the detection of antigen. Enzyme-labeled antigen is mixed with an unknown sample containing antigen, and those two antigens compete for antibody binding sites (Voller et al. 1978). The bound antigen is then separated from the free material by washing, and the enzyme activity is measured by the addition of a substrate (Voller et al. 1978) detected via spectrophotometer.
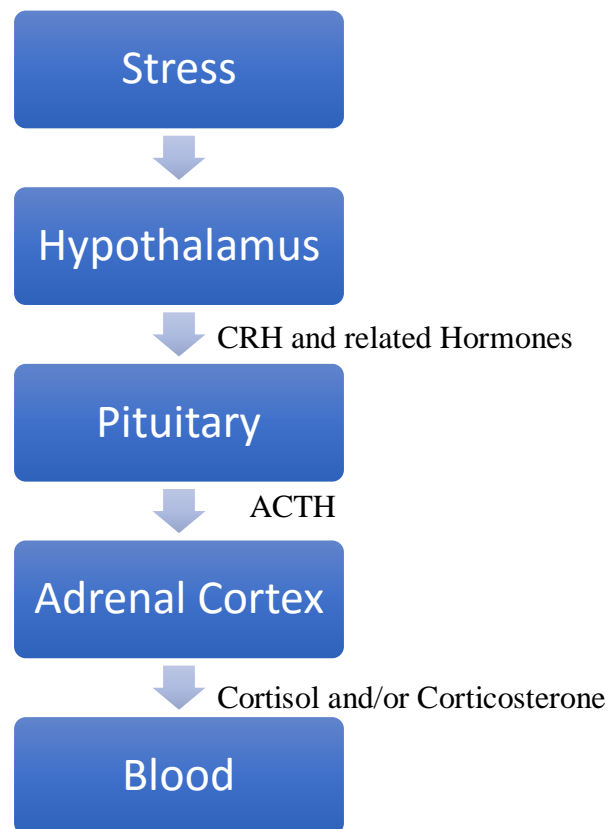


FIGURE 3. Hypothalamic-pituitary-adrenocortical (HPA) axis in vertebrates. The hypothalamic-pituitary-adrenal (HPA) axis of vertebrates. Adapted from (Sheriff et al. 2011).

Fishes synthesize cortisol in response to both acute and chronic stressors, and blood plasma cortisol levels are commonly used to indicate stress level in fishes and other animals (Jeney et al. 1992; Pickering 1992; Mommsen et al. 1999; Ellis et al. 2004). After exposure to an acute stressor for a few minutes, plasma cortisol levels rise rapidly (Bonga 1997). Fish can respond to rapid increases in cortisol by mechanisms designed to maintain physiological function by compensating for the stress for the short amount of time, and then when the stress is removed the fishes can return to their previous physiological state (Helfman et al. 2009). In fishes exposed to chronic stressors, cortisol levels will remain elevated but below peak levels (Bonga 1997). Chronically elevated levels of cortisol can suppress immune function and eventually diminish disease resistance and survival (Helfman et al. 2009).

In response to an increase in cortisol levels, glycogen and glycogenesis pathways are stimulated, and glucose is released into the bloodstream (Davis and McEntire 2009). Glucose is used as an indicator of stress in fishes; however, it is not considered to be as reliable as cortisol, due to its variability and reaction to dietary status (Silbergeld 1974; Barton and Iwama 1991; Wells and Pankhurst 1999; Barton 2000; Barton 2002; Davis and McEntire 2009; Santos et al. 2010; Davis and Gaylord 2011).

Glucose concentrations in fishes are commonly measured by glucose oxidase assays (Eames et al. 2010). Glucose oxidase oxidizes glucose to D-glucono-1,5-lactone and hydrogen peroxide, which then interacts with peroxidase to oxidize o-Dianisidine to a colored product. It is an endpoint assay, in which the samples are incubated with a substrate for a fixed period of time and then the reaction is stopped with the addition of sulfuric acid and detected via absorbance using a spectrophotometer. This assay is similar in chemistry, glucose oxidase and oxidation of glucose, to those used by diabetics to determine their blood glucose levels.

VIRAL HEMORRHAGIC SEPTICEMIA VIRUS (VHSV)

Viral Hemorrhagic Septicemia (VHS) is a deadly infectious fish disease caused by the rhabdovirus Viral Hemorrhagic Septicemia Virus (Einer-Jensen et al. 2004). It is an enveloped negative strand-RNA virus that belongs to the *Novirhabdovirus* genus and

the family *Rhabdoviridae* (Einer-Jensen et al. 2004). The VHSV genome is approximately 11,200 nucleotides long and encodes six genes in the order of 3'-N-P-G-Nv-L-5' (Schütze et al. 1999; Einer-Jensen et al. 2004). The five structural proteins are nucleoprotein (N), polymerase-associated phosphoprotein (P), matrix protein (M), glycoprotein (G), RNA polymerase (L) protein, and a non-structural protein (NV) with an unknown function proteins of VHSV (Schütze et al. 1999; Einer-Jensen et al. 2004; Pereiro et al. 2016). There are four genotypes of this virus that have been documented worldwide (Einer-Jensen et al. 2004; Snow et al. 2004; Panel 2010). Genotype I has been isolated from freshwater fishes, primarily genotypes from rainbow trout in Europe (Panel 2010). Genotype I has also been isolated from rainbow trout found in brackish water around Finland, and in marine fishes around Europe (Panel 2010). Genogroups II and III are found in marine fishes in Europe and are considered endemic (Panel 2010). In Greenland, genogroup III has been isolated from halibut caught at the Flemish Cap and in rainbow trout in Norwegian waters (Dopazo et al. 2002; Panel 2010). Genogroup IV has a sublineage, now referred to as genogroup IVa (Elsayed et al. 2006). Genogroup IVa is found in wild marine fishes and anadromous fishes along the Pacific coast (Winton et al. 1991; Meyers and Winton 1995; Marty et al. 1998; Hedrick et al. 2003; Panel 2010), and in Japan and Korea (Nishizawa et al. 2002; Kim et al. 2003; Panel 2010). Another sublineage of genogroup IV, genotype IVb, was isolated from the Great Lakes of the United States and Canada (Elsayed et al. 2006; Panel 2010). This sublineage of genogroup IV isolated from the Great Lakes is closely related to that of IVa, but is sufficiently distinct to suggest a different sublineage (Elsayed et al. 2006; Lumsden et al. 2007; Ammayappan and Vakharia 2009; Faisal et al. 2012).

VHSV is transmitted horizontally with attachment at the gills or skin of a host (Chilmonczyk et al. 1995; Harmache et al. 2006; Imanse et al. 2014). Once inside the host, VHSV targets the endothelial lining of blood vessels (Faisal et al. 2012). As a result, hemorrhaging occurs in locations such as the dermal, periocular, peritoneal, and visceral regions, and the fin bases (Cornwell et al. 2012). Darkening of the skin, anemia resulting in gill and hepatic pallor, exophthalmia, and serous or serosanguineous abdominal ascites are other pathologies associated with VHSV (Yasutake 1975; Al-Hussinee et al. 2011; Cornwell et al. 2012; Imanse et al. 2014).

LARGEMOUTH BASS VIRUS (LMBV)

Largemouth Bass virus (LMBV) is a DNA iridovirus that affects Largemouth Bass (*Micropterus salmoides*) (Grizzle et al. 2002). LMBV is closely related to 2 other iridoviruses, guppy virus (GV) and doctorfish virus (DFV) (Mao et al. 1999). It was first isolated in Largemouth Bass collected from Lake Weir, Florida in 1991 (Grizzle et al. 2002). Disease associated with LMBV occurs more frequently in summer months, and it affects fishes that are greater than 30 cm (Grizzle and Brunner 2003). Fish kills associated with LMBV can last several weeks after LMBV has been detected (Grizzle and Brunner 2003). LMBV infection in Largemouth Bass can cause a loss of equilibrium, enlarged or reddened swim bladders, and floating near the surface (Plumb et al. 1996; Grizzle et al. 2002). LMBV has only been found in the eastern part of the United States, and prevalence is low where LMBV has been documented (Grizzle and Brunner 2003).

Transmission of LMBV can occur through contaminated water (Plumb and Zilberg 1999) or by consumption of infected prey (Woodland et al. 2002). There is no evidence, from either experimental studies or observations from fish hatcheries, of vertical transmission of the virus (Woodland et al. 2002). The virus can be transmitted horizontally via contact with infected fishes, as the virus sometimes can be present in the cutaneous mucus (Woodland et al. 2002; Grizzle and Brunner 2003). When detecting LMBV, samples are taken from the swim bladder, anterior kidney, and spleen (Grizzle et al. 2003) followed by PCR techniques. There are no records of LMBV being found in the Back Bay ecosystem, however tests for LMBV have not been performed previously (C. Boyce, Virginia Department of Game and Inland Fisheries, personal communication).


MODELING OF BLUE CRABS AND LARGEMOUTH BASS

Agent-based models (ABM), or individual-based models, are stochastic simulations based on the global consequences of local interactions of members of a population. An advantage to using ABMs is that the models can represent a system of individuals and their environment, where system behavior arises from traits of the individuals and characteristics of the environment (Grimm and Railsback 2005).

Individuals can be plants and animals in a system, people in crowds, and cars in traffic. ABMs consist of an environment or framework in which the interactions occur, and where a number of individuals are defined by their behaviors and characteristic parameters (Grimm and Railsback 2005). ABMs are able to track the characteristics of each individual through time.

An aim of this project was to construct an agent-based model to test two questions about the Back Bay system:

- Does increased occurrence of crabs near the banks of Back Bay lead to increased prevalence of infections in Largemouth Bass?
- Does increased infestation of Blue Crabs lead to higher prevalence of leeches on Largemouth Bass?

Blue Crabs are acting as a phoretic host, and act to transport *M. lugubris* cocoons. The Blue Crabs in Back Bay migrate north from the Currituck Sound, NC during late winter into early spring, and are dispersed throughout the bay during the summer months. The crabs then begin to migrate south, back into the Currituck Sound, NC in the fall, so by the winter months they are at the very southern end of the Bay. Spatial relations including distance between Blue Crabs and Largemouth Bass were also tested using the model. Blue Crabs are predominantly found in the middle channel of the bay, and they can be scattered along the banks of the bay. Largemouth Bass are found on the banks of the bay. These models will help better predict the Largemouth Bass and Blue Crab interaction for the future. Such information is useful to agencies such as VDGIF in determining if they should continue to restock Largemouth Bass in Back Bay, and whether costs outweigh the benefits if VDGIF were to restock. ABMs have been used to model fisheries, including parasitism of sea lice in Atlantic salmon, parasitism of monogeneans in wild salmon, and the relationship between reefs and fish density (Letcher et al. 1996; Werner et al. 2001; Campbell et al. 2011; Ramírez et al. 2012; Groner et al. 2013; DeCelles et al. 2015).

ABMs are not only applied in ecology and biology (DeAngelis and Gross 1992; Shugart et al. 1992; Van Winkle et al. 1993; Grimm 1999; Huse et al. 2002; DeAngelis and Mooij 2005; Grimm and Railsback 2005), but in the social sciences (Epstein and Axtell 1996; Gilbert and Troitzsch 2005), economics (Tesfatsion 2002), demography

(Billari and Prskawetz 2003), geography (Parker et al. 2003), and political sciences (Axelrod 1997; Huckfeldt et al. 2004). ABMs allow researchers to study the system level properties and how they emerge from the adaptive behavior of individuals (Railsback 2001; Strand et al. 2002), and how the system affects the individuals (Grimm et al. 2006). The processes addressed with ABMs are the movement through space, the formation of patterns among individuals, effects of diffusion and mixing, management and control measures, and evolutionary processes. ABMs are beneficial because no one agent is the same, every agent has different characteristics, similar to that in real world systems. Disadvantages of ABMs are that they are more complex than analytical models and therefore are more difficult to analyze, communicate and understand than analytical models (Grimm et al. 1999; Grimm et al. 2006).

## II. METHODOLOGY

FIELD STUDIES

Data Collection 2012-2015 VDGIF

   Annual fish surveys were conducted by Virginia Department of Game and Inland Fisheries to determine population numbers in Back Bay. Largemouth Bass were collected by Virginia Department of Game and Inland Fisheries throughout Back Bay Virginia, using boat-based pulsed DC electroshocking from September-October 2012, September-October 2013, and September-October 2014. Length and weight of each fish was recorded in millimeters (mm) and grams (g) respectively. Presence of leeches in the oral cavity was documented. Sex of fish was also determined.


Largemouth Bass Blood Collection Nonlethal Sampling

      We began our data collection of nonlethal sampling for the fish health survey of Largemouth Bass in Back Bay. Largemouth Bass were collected from nine sampling sites throughout Back Bay, Virginia (Figure 4) using boat-based pulsed DC electroshocking from the Fall of 2015 through the Fall of 2017. Fieldwork was completed twice a week, every other week per month giving four sampling days per month. The target sample was 5 fish from each site. Blood was drawn from the caudal vessels with a 20-gauge hypodermic needle and 3 mL syringe within a 3 minute time frame, transferred into a heparinized Vacutainer tube, and stored on ice. Any fish from which blood could not be obtained within the 3 minute time frame was removed from the data analysis. Largemouth Bass were not anesthetized because they are often caught as food fish, and therefore could not be treated with anesthetic drugs (e.g. MS-222) and released. After blood collection, the length and weight of each fish was recorded in millimeters (mm) and grams (g) respectively.  All field and associated laboratory procedures were performed under an approved ODU Institutional Animal Care and Use Committee (IACUC) protocol (approval #16-004).

   A thorough exterior analysis of each fish was conducted that included the pathology, scarring, number of leeches, and oral perforation of the maxillary membrane. Pathology observed in the oral cavity was determined using a numbered system 0-3 (Figure 5).

- None (0): no observations of pathology

- Mild (1): shallow erosion, and no bleeding

- Moderate (2): ulcers and/or erosion with bleeding, <50% oral cavity affected

- Severe (3): ulcers and/or erosion with bleeding, >50% oral cavity affected
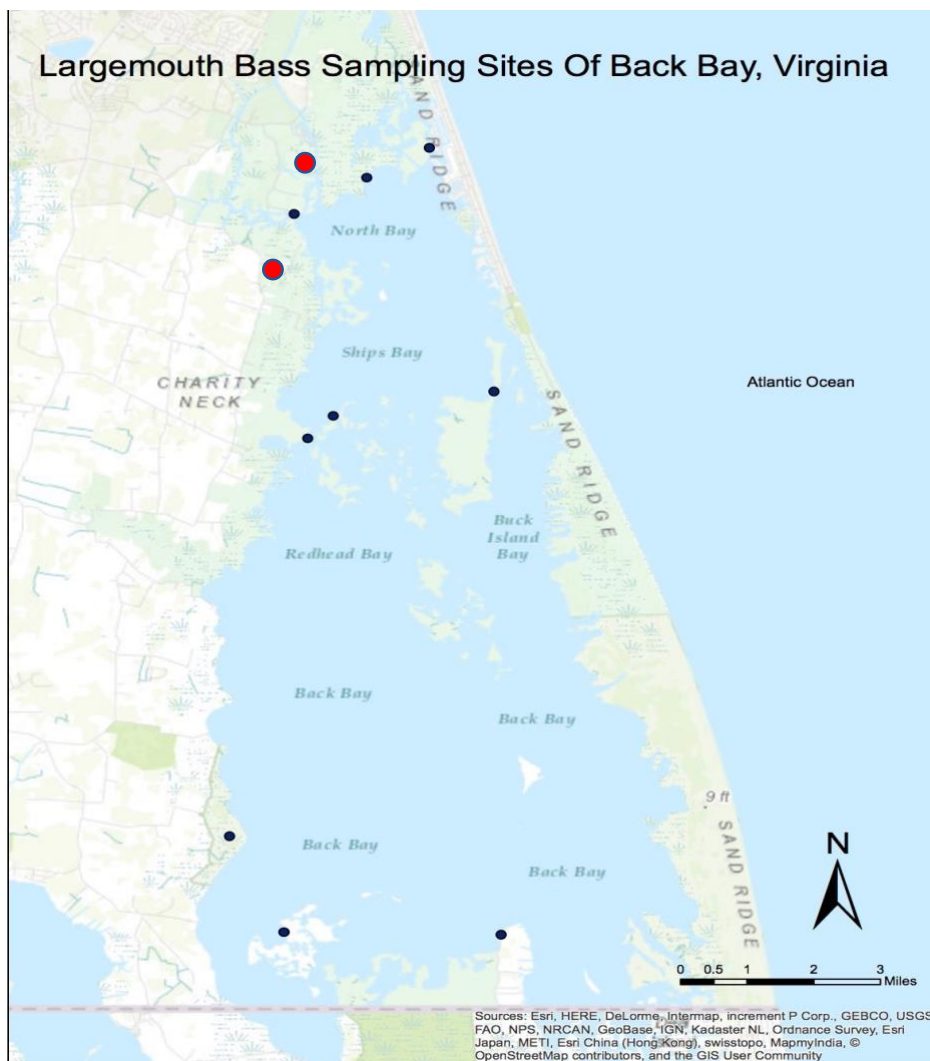


FIGURE 4. Map of Sampling Sites 2016 & 2017. Sampling site locations for 2016 are indicated by black dots. There are three northern sites (from left to right: Tabernacle Creek, Big Bend, Sandbridge Pond), three middle sites (from left to right: West Channel, Kemps Creek, Boy Scout Bridge), and three southern sites (from left to right: Back Bay Landing Canal, Great Cove, Tire Hell). For 2017, two additional sites were added to the original nine sampling sites of 2016. The additional two sites are represented by the red dots.

Scarring was determined as yes or no (Y/N), and was defined as presence of shallow depressions (scars) in the oral cavity (Figure 6A). Leeches were counted and categorized as small (S; <1cm), medium (M; 1-2 cm) or large (L; >2cm). Oral perforation was determined as yes or no (Y/N), and was defined as perforations or holes in the maxillary membrane (Figure 6B).

The sex of the fish was determined without lethal sampling when the fish were spawning by expressing milt or eggs. If the sex could not be determined in the field it was recorded as unknown.

Largemouth Bass Blood Collection Lethal Sampling

Lethal sampling was conducted for sex determination of fish, bacteriology and virology analysis. The same blood collection protocol for nonlethal sampling was conducted for lethal sampling from the Fall of 2015 through the Fall of 2016. Of those four sampling days, one was designated for lethal sampling. Five uninfested fish, and five infested fish were euthanized with a lethal dose of MS-222 prior to necropsy.

Water Quality Collection

Water quality data were collected and recorded during as part of the field studies protocol. At each sampling site, a YSI Professional Plus (YSI, Yellow Springs, OH) instrument was used to measure temperature (°C), dissolved oxygen (mg/L), conductivity (µS/cm), and salinity (psu).

Largemouth Bass Tag and Recapture Study

Tag and recapture study was initiated in order to determine the relative survival of infested fish, and to determine the seasonality and behavior of the leeches. Tag and recapture of Largemouth Bass started in Fall of 2016. Tagging was conducted at the original nine sampling sites. An additional two sampling sites, one in the north and one in the middle of the bay, were added due to high Largemouth Bass populations residing at those sites, increasing the chance of recapturing tagged fish (Figure 4). Hallprint (Hindmarsh Valley, South Australia) dart tags were used for this process. Methods for tagging and recapturing of Largemouth Bass were adapted from Renfro et al. (1995),

using PDAT-type tags.  In pond trials, these tags caused the least mortality among

Largemouth Bass had a mean retention rate of 98% for larger dart tags and 78%

retention rate for smaller dart tags (Renfro et al. 1995).
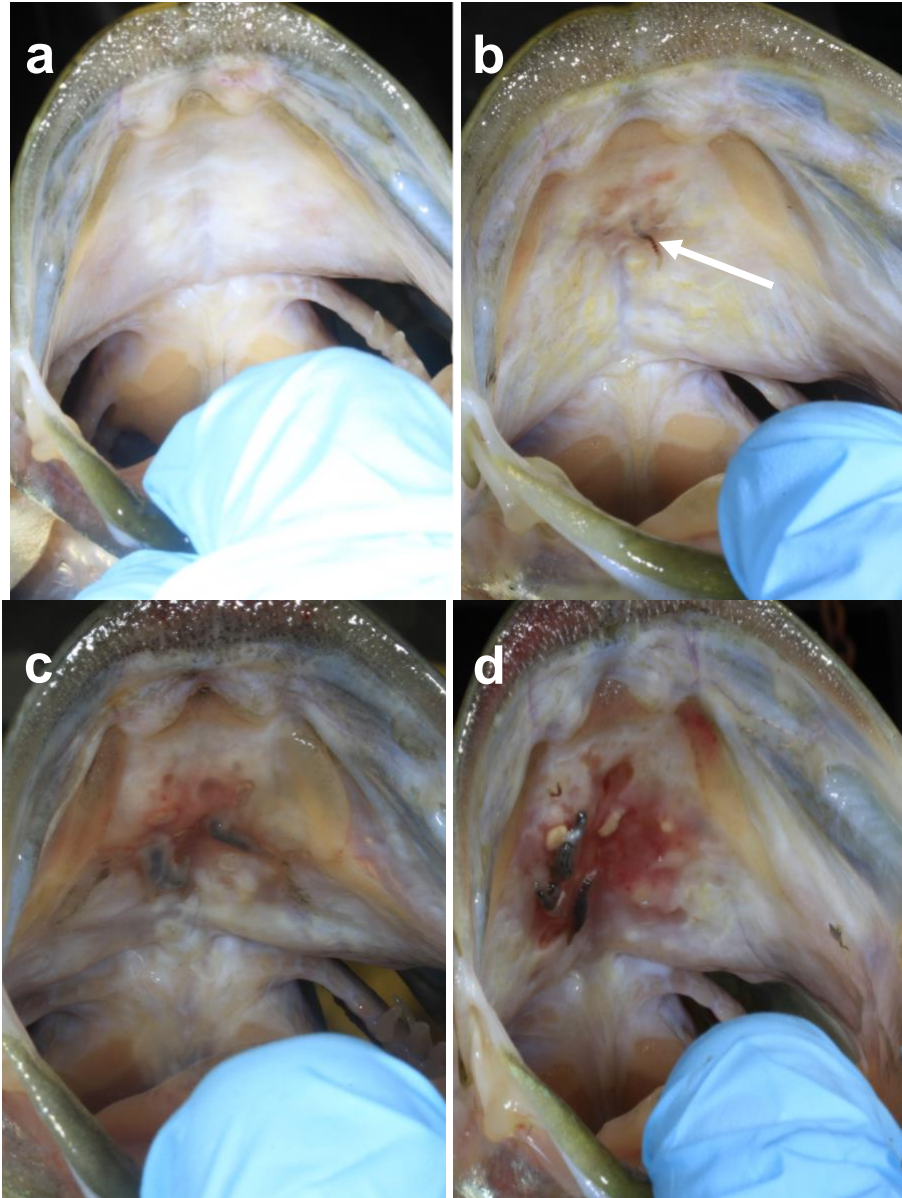


FIGURE 5. Pathology 0-3. Pathology of *M. lugubris* (arrow) in oral cavity of Largemouth Bass. **(a)** Pathology 0: no erosion, bleeding or ulcers. **(b)** Pathology 1: shallow erosion with no frank bleeding. **(c)** Pathology 2: ulcers and/or erosion with bleeding, <50% oral cavity affected. **(d)** Pathology 3: ulcers and/or erosion with bleeding, >50% oral cavity affected.
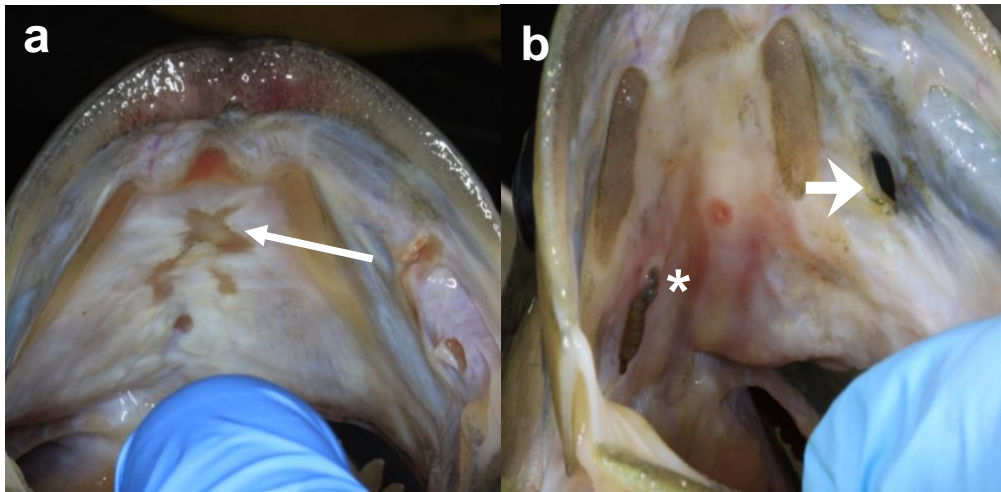
FIGURE 6. Scarring and Oral Perforation. **(a)** Scarring (arrow) from previous leech infestation.  Small circular erosions are present, and may be overlapping. **(b)** Maxillary membrane perforation (arrowhead) 2 (oral perforation). Attached leech (arrowhead) is present in 6b.

Upon capture, the fork length (to the nearest centimeter), weight (g), the number and size of the leeches in the oral cavity, the pathology (0, 1, 2, 3), scarring, oral perforation, and body leeches were recorded. Tags were attached to in the dorsal musculature, engaging pterygiophores of the hard dorsal fin. After each fish was tagged, photographs were taken of the tag and mouth for documentation and for comparison when the fish is recaptured. Photography settings used were AV mode, ISO 100 (Cannon EOS 50D, United States). The flash settings used were midexposure ½, and E-TTL 0 (Cannon Macro Ring Lite MR-14EX II, United States). All field and associated laboratory procedures were performed under an approved ODU Institutional Animal Care and Use Committee (IACUC) protocol (approval #16-020).

The purpose of tagging the Largemouth Bass is to determine survival of the fish and seasonality of the leeches. Comparisons of photographs from before tagging and then at recaptured will show when leeches are more prevalent, and their dynamics of attaching to/leaving their host. Relative survival rates of infested versus non-infested fish will be analyzed once sufficient recapture data is available.

Hemoglobin Analysis

Hemoglobin analysis was conducted in order to determine if Largemouth Bass with leeches were anemic compared to Largemouth Bass without leeches. Hemoglobin analysis was conducted beginning in the Spring of 2017. Blood was collected from 100 fish at the nine sampling sites in Back Bay, Virginia. Of those 100 fish, 65 were uinfested and 35 were infested. Hypodermic needles (20-gauge) and 3 mL syringes were used to withdraw blood. The blood was then filled into a HemoCue® microcuvette (HemoCue, Ängelholm, Sweden). The microcuvette was then read in a HemoCue® Hb 201+ (HemoCue, Ängelholm, Sweden), and the hemoglobin concentration was recorded (g/dL).

LABORATORY STUDIES

Sex Determination of Largemouth Bass From Lethal Sampling

For the majority of the Largemouth Bass collected, sex was difficult to determine in the field. As part of the lethal sampling protocol, sex was determined from the gonads. Males had milky white testes, and females had yellow ovaries. The sexual maturity of the male and female Largemouth Bass was categorized as immature, developing, spawning capable, regressing and regenerating based on observational characteristics (Brown-Peterson et al. 2011).

PCR and Sequencing for Leech and Cocoon Identification

PCR and Sanger sequencing were performed to identify oral leeches in Largemouth Bass and cocoons on Blue Crabs. DNA was extracted from leeches or cocoons by column-based DNeasy QIAGEN Kit (QIAGEN DNeasy, Valencia, CA). PCR was performed in a C1000 Thermal Cycler (Bio-Rad, Hercules, CA) in a 15 µL final volume containing 1 µL of extracted DNA template. Each reaction contained 0.2 mM dNTPs, 1 µM each of forward and reverse primer, PCR buffer containing 1.5 mM $MgCl_2$ (QIAGEN), and 0.05 U *Taq* polymerase (Top- Taq, QIAGEN). PCR was performed as follows: initial denaturation of 3 min at 94°C; 34 cycles of denaturation at 94 degrees for 30 s, annealing at 49°C (18S rDNA) for 45 s, and extension at 72°C for 60 s; and a 7

min final extension at 72°C. Primer pairs used for amplification can be found in Appendix 1. Amplified DNA was electrophoresed on a 1.5% agarose gel, stained with SybrSafe (Invitrogen), and imaged with 360 nm UV light.

PCR products were purified with Exo-SAP-IT (USB Scientific, United States) according to the manufacturer's directions. Direct sequencing was performed on PCR products bi-directionally using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, United States) and the primers used for initial amplification. At least 2 separate PCR reactions were sequenced for each sample.

Sequence editing and alignment was performed with Geneious software (version 8.1.6, Biomatters Ltd). BLAST was used to determine closest matches in the Genbank database (Altschul et al. 1990).

Cortisol Assay

Cortisol assays were used to assess the amount of cortisol in the blood as an indirect measure of stress. Two hundred ninety-six fish samples were used for the cortisol assays. Of those 296 samples, 84 were uninfested fish, and 212 were infested fish. Anti-cortisol Mab (1:5000 dilution of 3.1 mg/mL, Mouse Monoclonal anti-Cortisol-3-BSA, CalBioreagents, Foster City, CA) was coated on to 96-well microtiter plates (Nunc, Maxisorp) in coating buffer (0.05M carbonate buffer, pH 9.6) overnight at 4°C. Coated plates were warmed to room temperature and washed 3X with wash buffer (Dulbecco's Phosphate Buffered Saline (DPBS) + 0.05% Tween-80, pH 7.6). The samples or standards (2 µL) were then added to wells in 100 µL assay buffer [DPBS, 0.01% w/v Bovine Serum Albumin, 1:2000 dilution of 300 units/µL horseradish peroxidase-conjugated cortisol (C-HRP; CalBioreagents)]. Two µL of charcoal-stripped pooled bass plasma (Brock et al. 1978) was also added to wells containing standards. Assay plates were incubated for 4 hours at room temperature with rocking, then washed 3X with wash buffer. Bound labeled cortisol (C-HRP) was detected with TMB (3,3', 5,5' tetramethylbenzidine) substrate (KPL, Gaithersburg, MD). Plates were read at 3 min intervals at 650nm on an automated plate absorbance reader (FLUOstar OPTIMA, BMG LABTECH).

Data were analyzed for the plate read at which zero-cortisol standards reached ~80% of their maximum saturation value. The standard curves were generated by fitting a log function to triplicate known cortisol standards in Microsoft Excel. Standards were prepared from a first dilution of 7.5 mg hydrocortisone in 1 mL 100% ethanol, followed by twofold dilutions in DPBS (640 ng/mL, 320 ng/mL, 160 ng/mL, 80 ng/mL, 40 ng/mL, 20 ng/mL 10 ng/mL, 0 ng/mL). Cortisol data were analyzed using R software version 1.0.136 (R Core Team 2016).

Glucose Assay

Glucose assays were used to assess the amount of glucose in the blood as an indirect measure of stress. The same 296 fish samples used for the cortisol assays were used for the glucose assays; however, there were 23 samples that did not contain enough serum to conduct the assay to give a total of 273 samples used for the glucose assay. Of those 273 samples 79 were uninfested fish and 194 were infested fish. DPBS (5 µL) was added to 96-well microtiter plates (Nunc, Uncoated F-type). Samples of glucose standards diluted in DPBS (1250 mg/dL, 625 mg/dL, 312.5 mg/dL, 156 mg/dL, 78 mg/dL, 39 mg/dL, 19.5 mg/dL, 0 mg/dL) were then added to wells.  After the wells were plated with the standards, 5 µL of sample was added to the rest of the wells. One hundred microliters (100 µL) of Glucose oxidase/Horseradish Peroxidase/ o-Dianisidine reagent (GAR reagent) [(DPBS, 2.8 mg of 500 units/g glucose oxidase (Sigma G7141-10KU), 1 mg of 150 units/mg horseradish peroxidase (Sigma P8125-25KU), 5 mg of o-Dianisidine (Sigma D-9143-5G)], was added to each well. The plate was incubated at room temperature for 1 min. After 1 min, 100 µL 12N sulfuric acid was added to each well to stop the colorimetric reaction. Plates were read at one cycle at 570nm on an automated plate absorbance reader (FLUOstar OPTIMA, BMG LABTECH).

Standard curves were generated by fitting a linear function to triplicate known glucose standards (1250 mg/dL, 625 mg/dL, 312.5 mg/dL, 156 mg/dL, 78 mg/dL, 39 mg/dL, 19.5 mg/dL, 0 mg/dL) in Microsoft Excel. The data were then analyzed using R software version 1.0.136 (R CoreTeam 2016).

Blood Smears Hematology

Blood cell counts were used to assess immunological status of the fish. Preparation of two blood smears per fish blood sample for differential blood cell count was performed using the Camco Quik Stain II (CAMCO, United States) protocol. The characteristics for each cell type were based on previous studies from Largemouth Bass (Esch and Hazen 1980). Differential counts were performed at 1000X magnification on an Olympus AX70 compound microscope. Lymphocytes, thrombocytes, macrophages, and neutrophils were counted and analyzed as percentage of total leucocytes. Figure 7 illustrates the cell types identified.

- Lymphocytes: scant deep blue cytoplasm, small (5 μm)
- Thrombocytes: elongated cigar shaped, colorless and scant cytoplasm, small (5 μm), pink nucleus
- Monocytes: condensed nucleus, dark to light blue cytoplasm, large (>5 μm)
- Neutrophils: open nucleus, pale blue cytoplasm, large (>5 μm)

One hundred cells were counted on each of two slides. A total of 226 slides were counted, giving 113 fish samples. Of those 113 samples, 33 were unifested fish and 80 were infested fish. Data were then recorded in Microsoft Excel and data analysis was conducted using R software version 1.0.136 (R Core Team 2016).

Bacteriology

Bacteriology was used to determine the presence of systemic bacterial infections in the Largemouth Bass. After fish were brought back to the lab, each was dissected using aseptic technique. Stabs of liver, anterior kidney, and spleen were streaked on MacConkey Agar, Blood Agar, and Brain-Heart Infused Agar. MacConkey Agar was used for distinguishing lactose versus non-lactose fermenters. Blood Agar was used for growth of fastidious organisms, and for distinguishing hemolysis patterns $\beta$, $\gamma$, and $\alpha$. Brain-Heart Infusion Agar was used for general bacterial growth. A total of 27 fish were analyzed for the bacteriology study. Plates were placed in a plastic Ziploc bag to prevent the media from drying out and incubated at room temperature for at least 72 hours to allow for bacterial growth.

Preliminary external bacteriology was also conducted on the site of attachment in the oral cavity of the Largemouth Bass. The lesions were touched with a sterile cotton

tipped applicator (Hardwood Products Compant LLC, Guliford, ME) and were immediately inoculated on MacConkey Agar, Blood Agar, and Brain-Heart Infused Agar. The plates were kept at room temperature (about 25°C). Bacterial colonies were taken and DNA was extracted from those colonies by column-based methods (QIAGEN DNeasy, Valencia, CA).
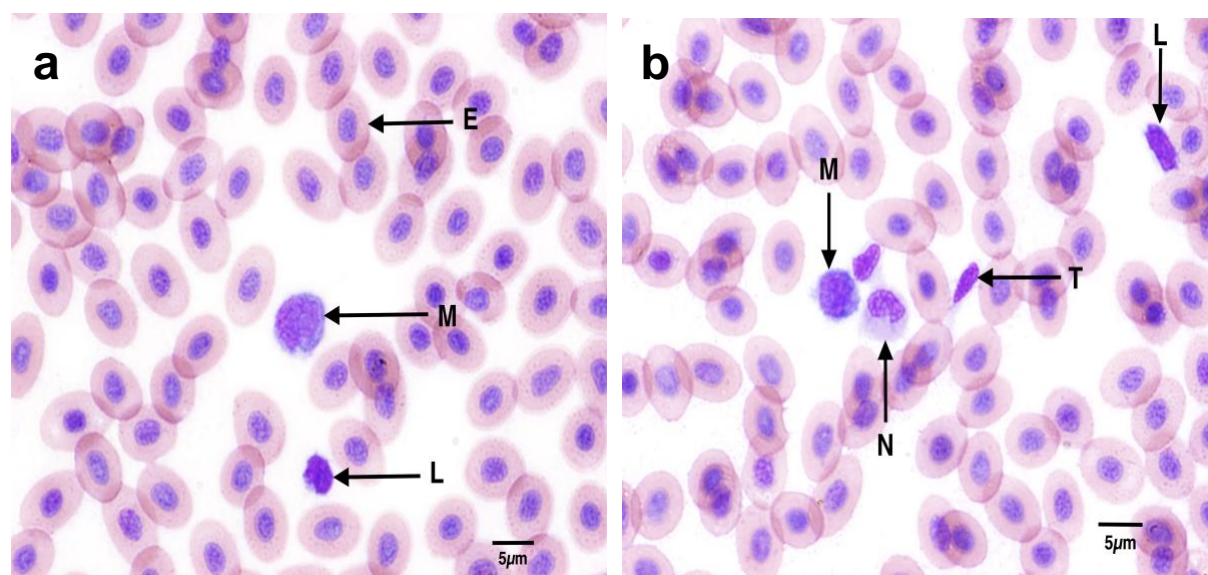


FIGURE 7. Hematology Smear. An example photomicrograph of a largemouth bass blood smear. **(a)** Erythrocytes (E) are abundant and typical in appearance for fishes. Macrophages (M) are characterized by relatively large size and basophilic cytoplasm. Lymphocytes (L) are characterized by smaller size and scant, basophilic cytoplasm. **(b)** Macrophages (M) are differentiated from similarly sized neutrophils (N) by pale cytoplasm and more open-faced nuclear presentation in the latter. Thrombocytes (T) are small and oblong with scant pale cytoplasm. Scant basophilic cytoplasm identifies a probable lymphocyte (L).  (1000X)

PCR was performed as for leech identification, with the following exceptions: Cycling conditions were: initial denaturation of 3 min at 95°C; 60 cycles of denaturation at 94 degrees for 30 s, annealing at 49°C (16S rDNA gene) for 45 s, and extension at 72°C for 60 s; and a 7 min final extension at 72°C. Primer pairs used for amplification of

16S can be found in Appendix 1. Amplified DNA was electrophoresed on a 1.5% agarose gel, stained with SyberSafe (Invitrogen), and imaged with 360 nm UV light.

PCR products were purified with Exo-SAP-IT (USB Scientific, United States) according to the manufacturer's directions. Direct sequencing was performed on PCR products bi-directionally using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, United States) and the primers used for initial amplification. At least 2 separate PCR reactions were sequenced for each bacterial sample. Sequence editing and alignment, as well as BLAST (Altschul et al. 1990) searches, was performed with Geneious software (Biomatters; Drummond et al. 2010).

Virology of Leech Homogenates

Virology of leech homogenates was done to detect viruses and to any potential cytopathic effects (CPE) of those viruses. Five leeches were collected during the Fall 2015 sampling to be analyzed for viruses. The experiment was conducted in a sterile hood. Each leech sample was homogenized in a Tenbroek homogenizer in sterile Hanks' Balanced Salt Solution (HBSS). The samples were transferred to centrifuge tubes. The samples were kept on ice for the duration of the experiment. The samples were centrifuged at 3,000x g. The supernatant was then passed through a Nalgene Syringe Filter 0.45 µm PES (Thermo Scientific, Nazareth, PA). The samples were then shipped to the Animal Health Diagnostic Laboratory at the New Jersey Department of Agriculture for further virology analysis, where they were treated with antibiotics in case of possible bacterial contamination. Two cell lines, Epithelium Papulosum Cyprini (EPC) and Chinook salmon embryo (CHSE), were used for cell culture assays to detect any potential viruses via observation of cytopathic effect (CPE).

LMBV Detection

Largemouth Bass Virus was tested for because it should always be tested for when working with Largemouth Bass. Liver, spleen, and swim bladder samples were taken from 10 fish giving a total of 30 samples. Total genomic DNA was extracted by a column-based technique (QIAGEN DNeasy, Valencia, CA).

The LMBV positive control for PCR techniques was a gift from Luke Iwanonwicz (USGS, Kearneysville, WV). PCR was performed in a C1000 Thermal Cycler (Bio-Rad) in a 15 µL final volume containing 1 µL of extracted LMBV template. Samples were run in duplicate, giving a total of 60 samples. Each reaction contained 0.2 mM dNTPs, 1 µM each of forward and reverse primer, 10x PCR buffer containing 3.0 mM $MgCl_2$ (QIAGEN), and 0.05 U Taq polymerase (Top-Taq, QIAGEN). PCR was performed as follows: initial denaturation of 3 min at 94°C; 60 cycles of denaturation at 94 degrees for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 60 s; and a 7 min final extension at 72°C. Primer pairs used for amplification can be found in Appendix 1, and those primers amplified a 460 base pair (bp) region of the targeted gene DNA polymerase (Iwanowicz et al. 2013). Amplified DNA was electrophoresed on a 1.5 % agarose gel, stained with SyberSafe (Invitrogen), and imaged with 360 nm UV light.

VHSV Detection

Viral Hemorrhagic Septicemia Virus was tested for because *M. lugubris* has been shown to vector the virus (Faisal and Schulz 2009; Faisal et al. 2012). Eleven leeches, sampled for presence of VHSV. RNA extractions were completed for each leech sample. Samples were homogenized in 1 mL of TRIzol Reagent in a Tenbroek homogenizer (Ambion by Life Technologies, Carlsband, CA). After homogenization, the samples were transferred to tubes and incubated for 5 minutes are room temperature. Each sample received 0.2 mL of chloroform (Alfa Aesar, Chloroform, Molecular Biology Reagent, Ward Hill, MA) per 1 mL of TRIzol Reagent used for homogenization. The tubes were then vigorously shaken for 15 seconds and incubated for 3 minutes at room temperature. The samples were centrifuged at 12,000 x *g* for 1 min in 4°C. The aqueous phase of the solution was removed avoiding the interphase and placed into a new tube. Then 0.5 mL of 100% ice cold isopropanol was added to aqueous phase per 1 mL of TRIzol Reagent used for the homogenization. The samples were incubated on ice for 10 min, and then centrifuged at 12,000 x *g* for 10 minutes 4°C. After centrifugation, the supernatant was removed from the tubes leaving the RNA pellet. The pellet was washed with 1 mL 75% ice-cold ethanol per 1 mL of TRIzol Reagent used in the initial homogenization. The samples were mixed briefly and centrifuged at 7500 x *g* for 5 min

at 4°C. The wash was discarded and the RNA pellet was allowed to air dry for 1.5 hours. Fifty µL of RNase-free water was then added to the samples. The samples were mixed and then placed in a hot water bath at 55°C for 15 minutes in order to resuspend RNA.

RT-qPCR was performed in a CFX96 Real Time System (Bio-Rad, United States) in a 15 µL final volume containing 2 µL of extracted VHSV RNA template from Michigan State University. Samples were run in duplicate, giving a total of 22 samples. Runs included no-template and no-reverse transcriptase controls. Each RT reaction contained SYBR® Green (Bio-Rad, United States) (7.5 µL), Iscript (Bio-Rad, United States) (0.1875 µL), forward primer (0.3 µM), reverse primer (0.3 µM), and water (5.22 µL), and 2 µL of sample giving a total volume of 15 µL. Each NRT reaction contained the sample master mix as the RT reaction using 0.1875 µL of water in place of Iscript, and 2 µL of sample giving a total volume of 15 µL. The RT-qPCR conditions were as follows: reverse transcriptase reaction 10 min at 50°C, denaturation 1 minute at 95°C; the amplification process was denaturation at 95°C for 10 s for 40 cycles, annealing 30 s at 60°C, and finally a temperature increase from 65°C-95°C in increments of 0.5°C for 5 s for the melting curve step. The primers used were recommended by the Faisal laboratory at Michigan State University and can be found in Appendix A.


Logistic Regression

A logistic regression was performed to measure independent variables (year, location, length) versus a binomial outcome (leech prevalence). A logistic regression was performed for data from 2012-2015 and a separate logistic regression was performed for 2016 data. Two logistic regression models were generated because VDGIF had a different sampling and observation protocol versus what we instituted in 2016. The logistic regression for both models was performed in R (R Core Team 2016). The best fit model for both data sets was implemented through the multi-model inference (MuMin) package (version 1.15.6, https://CRAN.R-project.org/package=MuMIn). Delta AIC was calculated using the MuMin R package for both the 2012-2015 data and the 2016 data. The best fit model for the 2012-2015 data only included length and year. The best fit model for the 2016 data included length,

latitude and their interaction. Latitude was separated into low, middle, and high for the different sampling sites throughout the Bay. Latitude sections were calculated in R using the equation of the latitude degrees plus the latitude hours divided by 60 plus the latitude minutes divided by 3600. The cutoffs were latitude decimals at 36.625 for low sections, 36.68 for middle sections, and 36.8 for high sections.

Condition Factor

Condition factor was calculated in order to determine the physical condition of the fish. Two separate condition factor analyses were conducted for the 2012-2015 data and the 2016 data. Condition factor (K) was calculated as $\frac{w}{l^3} * 100$ (Fulton 1904). The calculations were conducted in R (R Core Team 2016). Outliers were removed, and the cutoff values for condition factor were greater than 0.8 and less than 1.5. Normality of the data was tested using the Shapiro-Wilk test, and differences between means were tested via Student's T-test or one-way ANOVA.

Agent-Based Model

A model was designed using NetLogo 6.0 (Wilensky, U., 1999. NetLogo. http://ccl.northwestern.edu/netlogo/, Center for Connected Learning and Computer-Based Modeling, Northwestern University, Evanston, IL, United States). It represents the Back Bay system where Largemouth Bass grow, reproduce, die, and become infested with leeches. The model also represents Blue Crabs that migrate into and out of the bay seasonally. Blue Crabs migrate north in the spring, so by the summer they are dispersed throughout the bay. They then migrate back south during the fall months, so by the winter they are at the very southern end of the Bay. Simulations of leech infestation using this model were run using NetLogo, and data on total bass infestation were exported into Microsoft Excel spreadsheets.

MODELING-ODD TEMPLATE (Grimm et al. 2006)

This is an agent-based modeling template used to describe the model. The ODD is a first step for establish a more detailed common format of the description of ABMs (Grimm et al. 2006).

Purpose

This model represents the ecosystem in Back Bay, Virginia. It addresses the Largemouth Bass (*Micropterus salmoides*) leech infestation in Back Bay. This infestation is dependent on crabs, how they move into and out of the Bay, and how close they are to the banks of the Bay. The model specifically explores the role of the crabs influencing the amount of leech infestation in Largemouth Bass. If we assume there are more crabs in the Bay with more leeches, does that mean more Largemouth Bass will become infested? If we assume the crabs are within the banks of the Bay where the Largemouth Bass reside, does that mean more Largemouth Bass will become infested as opposed to crabs not coming to the banks of the Bay?

The model could also be modified to investigate seasonality of the crabs. If the crabs are dispersed throughout the entire Bay, does that mean there will be an increase in leech infestation in Largemouth Bass as opposed to crabs only at the southern end of the Bay?

Entities, State Variables, and Scales

The agents in this model represent Largemouth Bass and Blue Crabs. Largemouth Bass reside on the banks of Back Bay. Blue Crabs reside in the middle channel of Back Bay. Largemouth Bass have variables for their age, energy, and leech attack. Blue Crabs have the variable of leech coverage and distance to banks.

This model does not use explicit geographic space, but instead represents the Back Bay ecosystem as a two-dimensional space. A Largemouth Bass' location (where they live in the Bay) is described via their Cartesian coordinates (real numbers between -20 and -10 and real number between 10 and 20) on the plane. A Blue Crab's location (where they live in the Bay) is described via their Cartesian coordinates (real numbers between -10 and 10) on the plane.

The model runs for a year with daily time steps.

Process Overview and Scheduling

The model includes the following actions executed each time step.

Largemouth Bass

- Aging and death: The age of all Largemouth Bass is incremented. When energy exceeds 4 a juvenile bass becomes an adult. Largemouth Bass exceeding age 16 years die. Largemouth Bass that do not have any energy die.

- Leech Attack: The leech count on the bass is updated.

Crabs

- Migration: The movement of Blue Crabs is north for 6 months and then south for 6 months.

Design Concepts

Emergence

The model's primary output is the "Total Leech Infestation For All Bass" distribution, which emerges from leech density on crabs, leech attack probability, and distance to banks.

Adaptive Behavior

None

Fitness

Largemouth Bass need energy to survive. Swimming costs so much and eating gains so much. The Blue Crabs are a phoretic host, they are the transport of leeches into and out of the Bay. Blue Crab energetics are not included in the model.

Learning, Prediction

No learning or prediction is represented.

Sensing

Blue Crabs know when they are near a bass. This information is used for leech transfer from the Blue Crab to the Largemouth Bass.

Interaction

Attach a leech to a bass then the leeches move with that bass, but does not affect the bass behavior. Largemouth Bass do not interact differently with each other. The Blue Crabs have no interaction with themselves.

Stochastity

Stochastic functions are used to initialize Largemouth Bass life stage, Largemouth Bass age, Largemouth Bass energy, Largemouth Bass location in the banks, Largemouth Bass reproduction, Largemouth Bass leech recovery, Largemouth Bass old age death, grass patch growth rate, food patch growth rate, leech attack probability, and Blue Crab location in the Bay. Whether a leech attacks a bass, or how many leeches are on each bass is a stochastic function.

Collectives

Largemouth Bass do not school. There are no collective behaviors in the model because the Back Bay system does not have any.

Observation

The key model output is the leech density per bass. This data is collected at each time step, and is dependent on the distance to the banks, the leech density on the crabs, the leech attack rate on the bass, and the leech recovery on the bass.

Initialization

The population of 200 bass (100 juveniles and 100 adults) is initialized with life stage selected randomly with equal probability of life stage energy 1-10. The initial number of leeches per bass is set to 0. The placement of Largemouth Bass in the banks of the Bay is assigned randomly. There is a random probability Largemouth Bass of being attacked by a leech between 0 and 100. There is a random probability of a Largemouth Bass recovering from leech attack is between 0 and 100. There is a random number of crabs that can be set, and they are randomly distributed at the

southern edge of the Bay. A random number of those crabs can have a certain amount of leech density. The distance to the banks for the crabs to move is random from 0 to 20 patches. The initial parameters for each model are represented in Table 1 and Table 2.

Input Data

No time-series inputs are used.

Submodels

Process passage of time:

Each time step represented one day. Time of year influenced crab migration, with 6 months moving north and 6 months moving south. Each year was divided into 360 days, and each month (30 days) had a parameter that influenced crab migration.

Process Largemouth Bass life cycle:

Each time step, the Largemouth Bass had random probability of a leech attack. Largemouth Bass were able to move, reproduce, eat, and lose energy. Swimming caused largemouth bass to lose some energy, adult Largemouth Bass lost 5% of their energy from swimming, juvenile Largemouth Bass lost 2.5% of their energy to swimming. Largemouth Bass were given a maximum age to live of 192 months, which is equivalent to 16 years (Brown et al. 2009). Juvenile bass were able to switch to adults once they reached an energy greater than 4. A leech was added to a bass with probability equal to the leech attack parameter. Similarly, a bass could lose a leech with probability equal to the recovery rate parameter.

Crab movement:

Crabs migrate into and out of the Bay seasonally. The heading is north for 7 months and then south for 5 months. The speed at which the crabs move was determined by daily increments that added to the desired total length of movement into the Bay. The time, heading, and speed were item together to provide new parameters of direction and migrate for crabs.

TABLE 1. Modeling Parameters of Distance. These parameters were chosen for the agent-based model for the crab distance to the banks. The parameters were chosen from observational and seasonal data. A sensitivity analysis was conducted to determine the recovery chance parameter, and the attack rate parameter.

| Parameter | Description | Value |
|---|---|---|
| Switch stage slider | Allows juvenile bass to become adults when they hit that stage in the model | 3 |
| Offspring bass | The number of offspring per bass | 9 |
| Leech-attack-probability | The probability of a Largemouth Bass getting attacked by a leech | 20 |
| Fish gain from food | The amount of energy fish gain from eating food | 10 |
| Initial number crabs | The number of crabs in the system | 18 |
| Distance to banks | The distance the Blue Crabs travel from the center of the model to where the Largemouth Bass reside | Tested using 0, 10, 20 patches |
| Swim energy loss | The amount of energy Largemouth Bass lose by swimming | 1 |
| Leech Density | The amount of leech density on the Blue Crabs | 60 |
| Bass carrying capacity | The carrying capacity of Largemouth Bass | 200 |
| Recovery chance | The probability of a Largemouth Bass losing a leech | 50.25 |

TABLE 2. Modeling Parameters of Density. These parameters were chosen for the agent-based model for the amount of leech density on the crabs. The parameters that were used in this model were similar to the distance model parameters, with the exception of the distance and the leech attack probability. Those two parameters were chosen from the sensitivity analyses of the attack rate and distance.

| Parameter | Description | Value |
|---|---|---|
| Switch stage slider | Allows juvenile bass to become adults when they hit that stage in the model | 3 |
| Offspring bass | The number of offspring per bass | 9 |
| Leech-attack-probability | The probability of a Largemouth Bass getting attacked by a leech | 15 |
| Fish gain from food | The amount of energy fish gain from eating food | 10 |
| Initial number crabs | The number of crabs in the system | 18 |
| Distance to banks | The distance the Blue Crabs travel from the center of the model to where the Largemouth Bass reside | 10.5 |
| Swim energy loss | The amount of energy Largemouth Bass lose by swimming | 1 |
| Leech Density | The amount of leech density on the Blue Crabs | Varied 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 |
| Bass carrying capacity | The carrying capacity of Largemouth Bass | 200 |
| Recovery chance | The probability of a Largemouth Bass losing a leech | 50.25 |

III. RESULTS

PRELIMINARY TAGGING

   Since the tag and recapture study began in the Fall of 2016, there are currently 526 tagged Largemouth Bass in Back Bay. Forty-one of those 526 fish have been recaptured by ODU and VDGIF, and 30 of those 526 have been recaptured by anglers, giving a total recapture number of 71 and a recapture rate of 13%. Example observational Largemouth Bass tag and recapture data are depicted in Figure 8 and Figure 9. Figure 8 shows a Largemouth Bass that was tagged in November of 2016, and was recaptured in February of 2017. The before and after comparisons show another leech had attached during this time frame. Figure 9 shows a Largemouth Bass that was tagged in March of 2017, and was recaptured in June of 2017. The before and after comparisons show the leech had dropped off the fish during this time frame. There is also an additional scar on this fish, possibly indicating there was a leech attachment during this time, which had fallen off. The Largemouth Bass tag and recapture study is important for learning more about the Largemouth Bass leech relationship in Back Bay and the overall survival of infested fish.
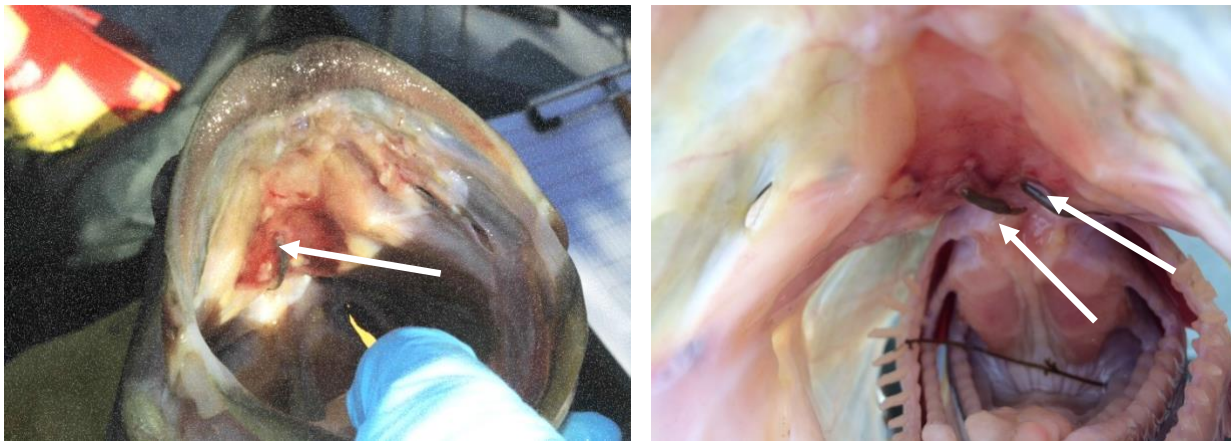


FIGURE 8. Tagging and Recapture T0065. A fish initially tagged in November 2016 (left). The fish had only one leech at the time, with bleeding and ulcers. The same fish was recaptured in February 2017, and gained another leech (right), with similar pathology.

FIGURE 9. Tagging and Recapture T0282. A fish initially tagged in March 2017 (left). The fish had only one leech at the time with bleeding. The same fish was recaptured in June 2017, and lost the leech (right) and did not have similar pathology. There appears to be a new scar depicted by the asterisk, which could be from a possible leech attachment, and the leech could have lost the leech during the March to June time frame.

HEMOGLOBIN

There was no significant difference in median hemoglobin (g/dL) levels between Largemouth Bass with and without leeches present (Mann-Whitney-Wilcoxon Test; W = 320.5, p = 0.9016) (Figure 10). The median hemoglobin value for Largemouth Bass with leeches was 7.6 g/dL, and the median hemoglobin value for Largemouth Bass without leeches was 7.7 g/dL. There was no significant difference among different pathology categories (Kruskal-Wallis Rank Test; $\chi^2$ = 1.1469, df = 3, p = 0.7658) (Figure 11). Median hemoglobin values for Largemouth Bass with Pathology of 0, 1, 2, and 3 were 7.8, 7.5, 7.5, and 7.5 g/dL respectively.
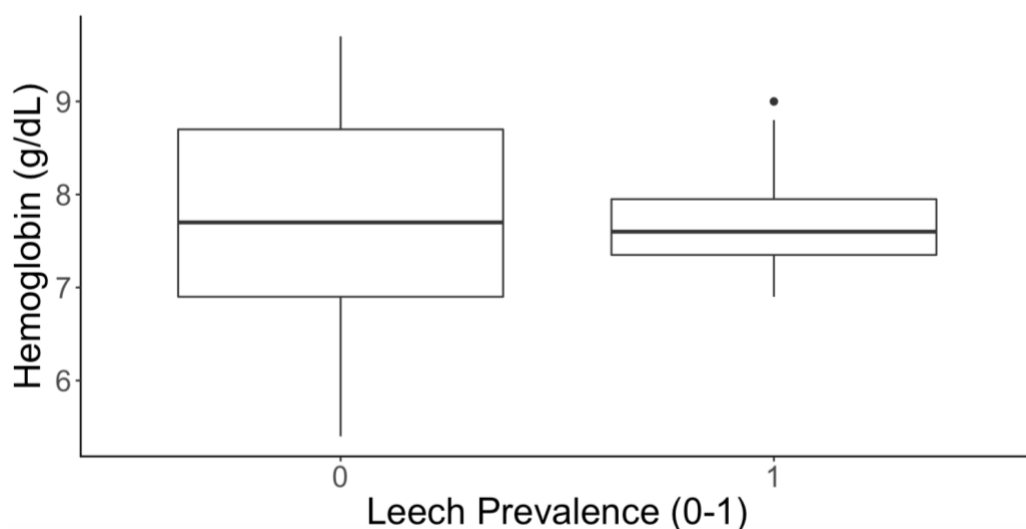
FIGURE 10. Prevalence vs. Hemoglobin. Data of leech prevalence vs. hemoglobin (g/dL) in Largemouth Bass (*Micropterus salmoides*; n = 100) from Back Bay, Virginia for 2017 (un-infested: n = 65, infested: n = 35). There was no significant difference in median hemoglobin levels between bass with and without leeches present (Mann-Whitney-Wilcoxon Test; W = 320.5, p = 0.9016).

## PCR IDENTIFICATION OF THE LEECH AND COCOONS

Consensus sequences were generated from 8 total forward and reverse sequences, and including sequences from at least 2 PCR reactions. 18S rDNA gene sequences from both samples were aligned, and manually curated including removal of primer sequences. 18S rDNA sequences from both leeches and cocoons yielded 100% pairwise matches to *Myzobdella lugubris* 18S small subunit rDNA (GenBank Accession #AF115994), coordinates 37-833. One sample originally thought to be a juvenile leech was identified as a brachiobdellidan, yielding 100% pairwise matches to *Cambarincola mesochoreus* 18S ribosomal complete RNA gene (GenBank Accession #JQ821503), coordinates 43-496.

## CORTISOL ASSAYS

Cortisol assays were conducted to determine fish stress levels and to compare the cortisol levels between fish with and without leeches. There was a small significant difference in median plasma cortisol (ng/mL) measurements between Largemouth Bass
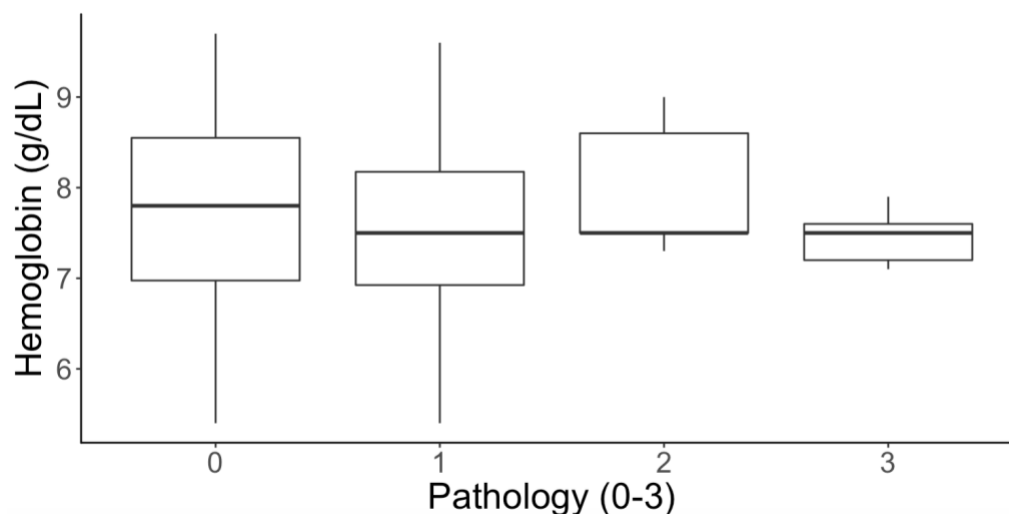
FIGURE 11. Pathology vs. Hemoglobin. Data of pathology (0,1,2,3) vs. hemoglobin (g/dL) in Largemouth Bass (*Micropterus salmoides*: n= 100) in Back Bay, Virginia for 2017 (un-infested: n = 65, infested: n = 35). Pathology parameters were defined as: none (0), no bleeding and no ulceration; mild (1), ulcers present, shallow erosion, and no bleeding; moderate (2), ulcers, bleeding, and significant erosion affecting less than 50% of the oral cavity; severe (3), ulcers, bleeding, and significant erosion affecting greater than 50% of the oral cavity. There was no significant difference between hemoglobin measurements for different pathology categories (Kruskal-Wallis Rank Test; $\chi^2$ = 1.1469, df = 3, p = 0.7658).

with and without leeches present (Mann-Whitney-Wilcoxon Test; W =5777, p = 0.005487) (Figure 12). The median cortisol values for Largemouth Bass with and without leeches was 20.5 and 15.7 ng/mL respectively.

There also was a small significant difference between median plasma cortisol (ng/mL) measurements for different pathology categories (Kruskal-Wallis Rank Test; $\chi^2$ = 22.194, df = 3, p < 0.05) (Figure 13). A Wilcoxon Rank Sum Test was used to determine which levels of pathology were statistically significant from one another. The post hoc test demonstrated Pathology 0 was statistically significant from Pathology 1 and Pathology 3. There was no statistical difference between Pathology 2 and Pathology 0 (Figure 13). The median cortisol values for Largemouth Bass with Pathology a of 0, 1, 2, and 3 were 13.9, 21.9, 19.2, and 25.9 ng/mL respectively.

FIGURE 12. Prevalence vs. Cortisol. Boxplot of leech prevalence (0-1) vs. plasma cortisol (ng/mL) in Largemouth Bass (*Micropterus salmoides*: n=296) from Back Bay, Virginia for 2016 (un-infested: n=84, infested: n = 212). There is a small significant difference in median plasma cortisol levels between bass with and without leeches present (Mann-Whitney-Wilcoxon Test; W =5777, p = 0.005487). The median cortisol value for Largemouth Bass with leeches was 15.7 ng/mL, and the median cortisol value for Largemouth Bass without leeches was 20.5 ng/mL.


GLUCOSE ASSAYS

There was no significant difference in median plasma glucose (mg/dL) measurements between Largemouth Bass with and without leeches present (Mann-Whitney-Wilcoxon Test; W =6440.5, p = 0.6353) (Figure 14). The median glucose values for Largemouth Bass with and without leeches was 92.6 and 87.6 mg/dL, respectively.

There was no significant difference in median plasma glucose (mg/dL) measurements for different pathology categories (Kruskal-Wallis Rank Test; $\chi^2$ = 5.6907, df = 3, p = 0.1277) (Figure 15). The median glucose values for Largemouth Bass with a Pathology of 0, 1, 2, and 3 were 87.6, 83.5, 102.6, and 98.7 mg/dL respectively.

FIGURE 13. Pathology vs. Cortisol. Boxplot of plasma cortisol (ng/mL) vs. pathology (0,1,2,3) in Largemouth Bass (*Micropterus salmoides*: n=296) in Back Bay, Virginia for 2016. Pathology parameters were defined as: none (0), no bleeding and no ulceration; mild (1), ulcers present, shallow erosion, and no bleeding; moderate (2), ulcers, bleeding, and significant erosion affecting less than 50% of the oral cavity; severe (3), ulcers, bleeding, and significant erosion affecting greater than 50% of the oral cavity. There was a small significant difference between plasma cortisol measurements for different pathology categories (Kruskal-Wallis Rank Test; $\chi^2 = 22.194$, df = 3, p < 0.05). A post hoc test showed Pathology 0 was statistically significant (indicated by letter A) from Pathology 1 and Pathology 3 (indicated by letter B). Letter AB indicates that Pathology of 0 was not statistically significant from Pathology 2. The median cortisol values for Largemouth Bass with a Pathology of 0, 1, 2, and 3 were 13.9, 21.9, 19.2, and 25.9 ng/mL respectively.
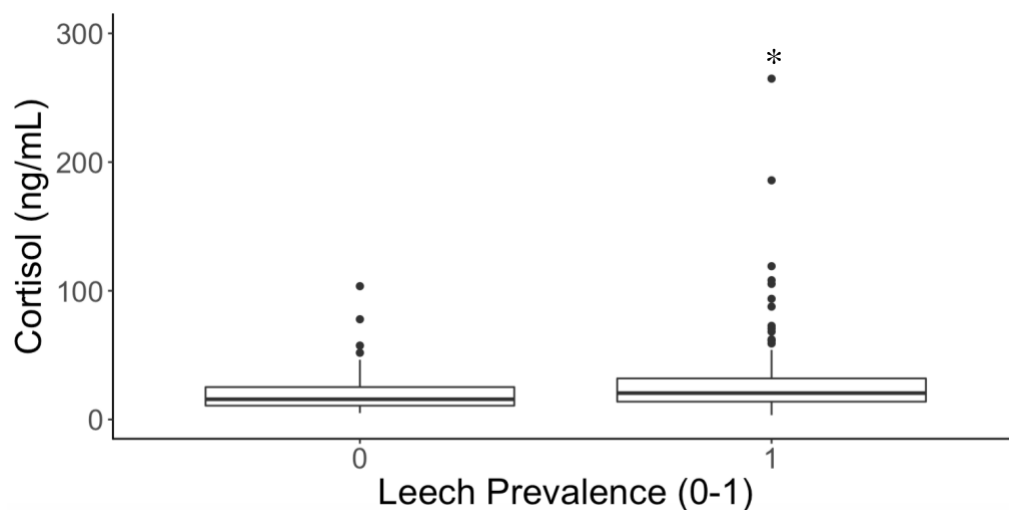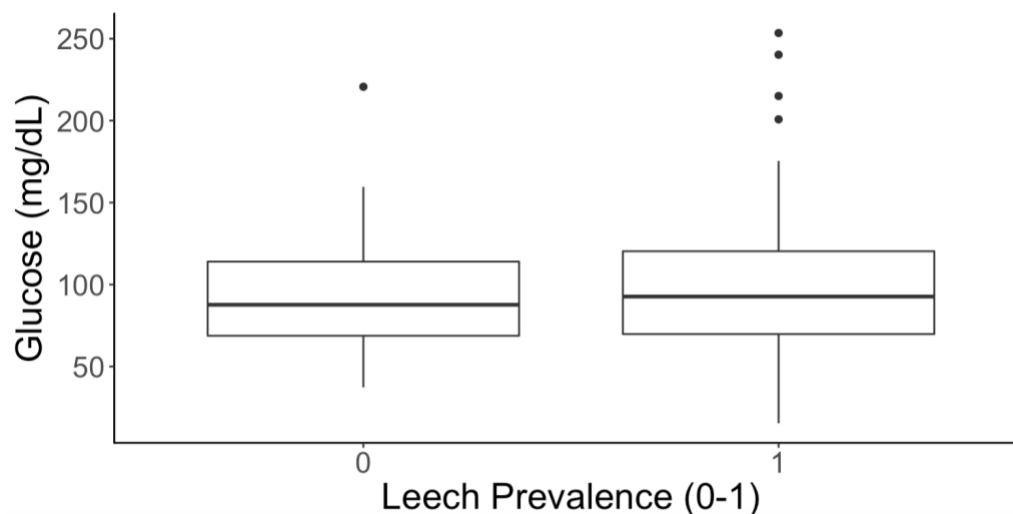
FIGURE 14. Prevalence vs. Glucose. Boxplot of plasma glucose (mg/dL) vs. leech prevalence in Largemouth Bass (*Micropterus salmoides*: n=273) from Back Bay, Virginia for 2016 (un-infested: n = 79, infested: n = 194). There was no significant difference in median plasma glucose levels between bass with and without leeches present (Mann-Whitney-Wilcoxon Test; W =6440.5, p = 0.6353).
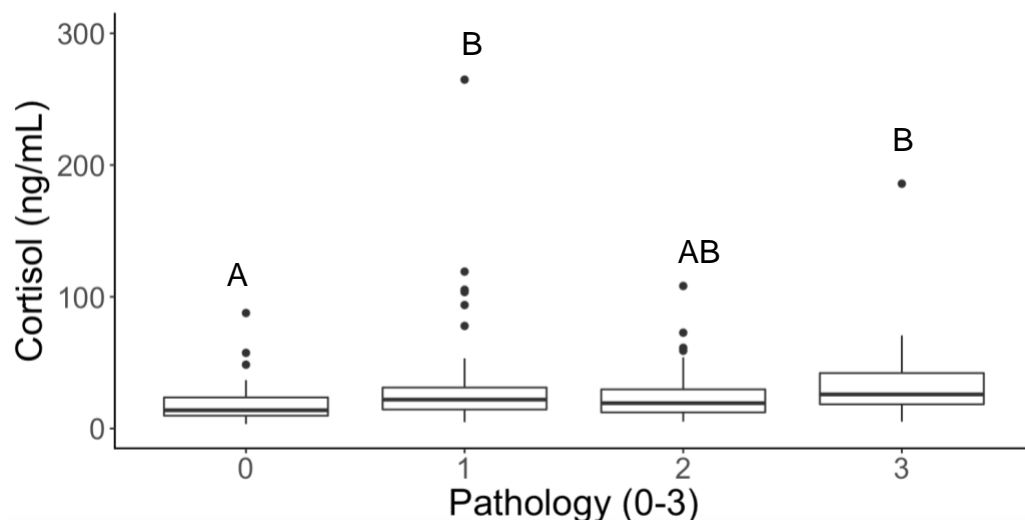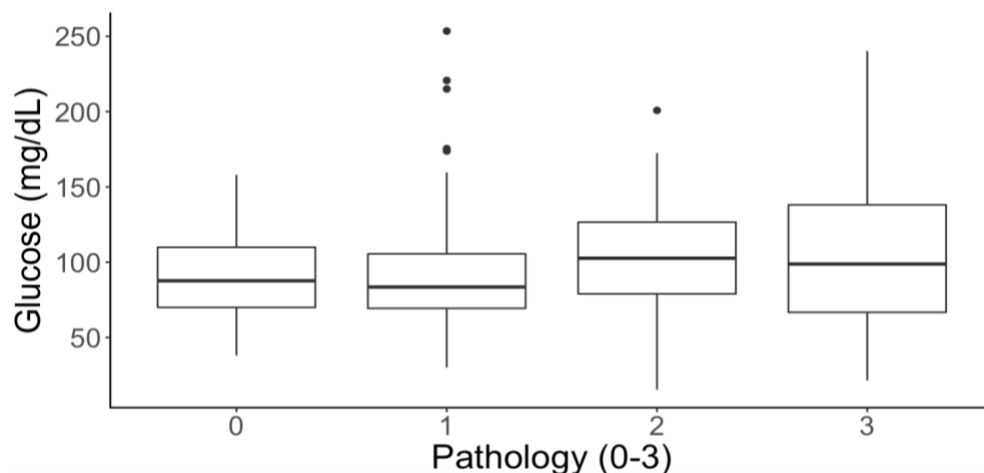


FIGURE 15. Pathology vs. Glucose. Boxplot of plasma glucose (mg/dL) vs pathology (0,1,2,3) in Largemouth Bass (*Micropterus salmoides*: n = 273) in Back Bay, Virginia for 2016. Pathology parameters were defined as: none (0), no bleeding and no ulceration; mild (1), ulcers present, shallow erosion, and no bleeding; moderate (2), ulcers, bleeding, and significant erosion affecting less than 50% of the oral cavity; severe (3), ulcers, bleeding, and significant erosion affecting greater than 50% of the oral cavity. There was no significant difference between plasma glucose measurements for different pathology categories (Kruskal-Wallis Rank Test; $\chi^2$ = 5.6907, df = 3, p = 0.1277).

HEMATOLOGY

There was no significant difference in percent leukocytes counted between Largemouth Bass with and without leeches for lymphocytes (Mann-Whitney-Wilcoxon Test; W =5191), thrombocytes (Mann-Whitney-Wilcoxon Test; W = 4404, p = 0.5962), neutrophils (Mann-Whitney-Wilcoxon Test; W = 4025, p =0.0982), or monocytes (Mann-Whitney-Wilcoxon Test; W = 4578.5, p = 0.9178) (Figure 16).

There was no significant difference in percent of leukocytes types counted between pathology levels for lymphocytes (Kruskal-Wallis Rank Test; $\chi^2$ = 43.22, df = 53, p = 0.8288), thrombocytes (Kruskal-Wallis Rank Test; $\chi^2$ = 58.341, df = 51, p = 0.2236), neutrophils (Kruskal-Wallis Rank Test; $\chi^2$ = 15.991, df = 16, p = 0.4536), or monocytes (Kruskal-Wallis Rank Test; $\chi^2$ = 13.278, df = 18, p = 0.7742) (Figure 17).

BACTERIOLOGY

From 27 fish sampled, the 81 stabs of liver, anterior kidney, and spleen plated on MacConkey Agar, Blood Agar, and Brain-Heart Infused Agar showed either no colony growth or 1-2 colonies on the plates. There was not enough colony growth on the plates to pursue further evaluation. Individual "positive" fish showed colonies on only one of the three plates. No individual fish demonstrated colony growth on all three plates.

Consensus sequences from the oral swabs were generated from one large colony total forward and reverse sequences, and including sequences from at least 2 PCR reactions. 16S rDNA gene sequences from the oral swabs were aligned, primer sequences removed, and manually curated. 16S rDNA sequences from the oral swabs yielded 100% pairwise matches to *Aeromonas hydrophila* strain SR578-V 16S small subunit rDNA (GenBank Accession #MF138110), coordinates 645-320.

LMBV

Largemouth Bass Virus was not detected in any sample. PCR for the positive control produced appropriate band size at 460 bp. The negative control had no bands. Largemouth Bass samples had no amplifications at the appropriate band size.
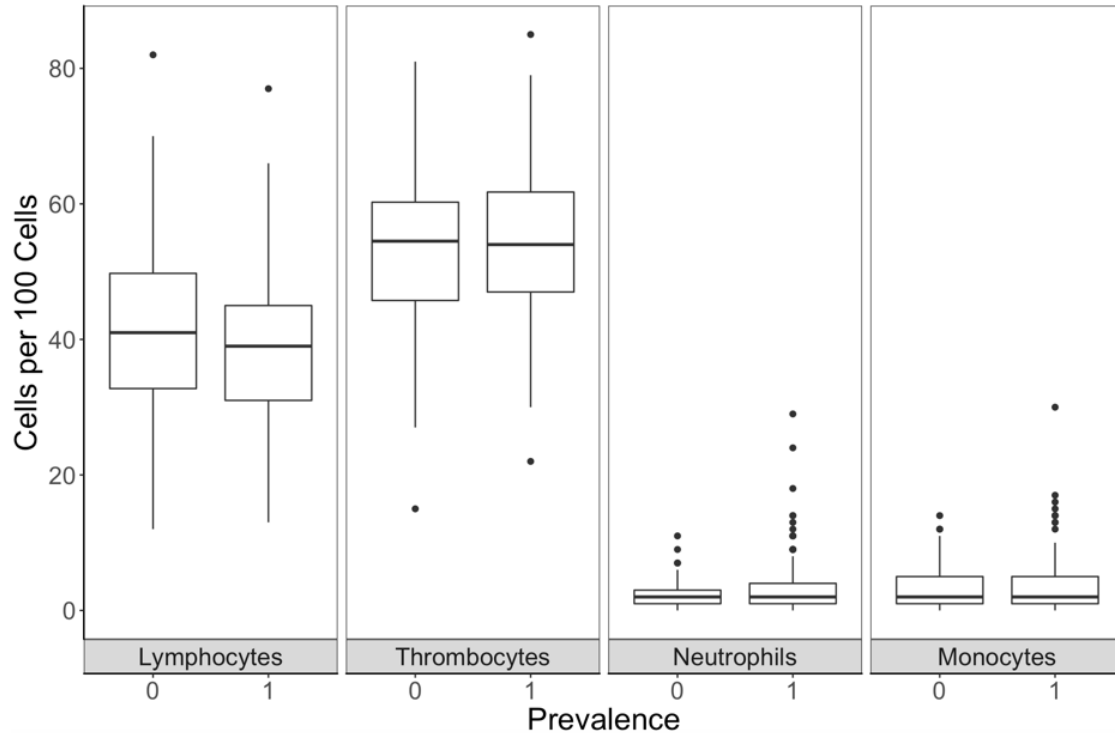
FIGURE 16. Differential Cell Counts Prevalence. Percent of leukocyte types counted per 100 cells and percent of lymphocytes, thrombocytes, neutrophils, and monocytes per 100 cells in Largemouth Bass (*Micropterus salmoides*; n=113) from Back Bay, Virginia for 2016 (un-infested: n = 33, infested: n = 80). There was no significant difference in percentage of total leukocytes nor in the percentage of each leukocyte type between bass with and without leeches. (Mann-Whitney-Wilcoxon Test; W =5191, p = 0.1606; W = 4404, p = 0.5962; W = 4025, p =0.0982, W = 4578.5, p = 0.9178).

VHSV

The positive control for VHSV had a melt curve of 81.5°C. In some leech samples there were no amplifications, in cases where there were weak cases of amplification the melt curves were not consistent with the positive control. VHSV was not detected in any of the 11 leech samples.

VIROLOGY OF LEECH HOMOGENATES

None of the five leech homogenates tested for cytopathic effect on EPC and CHSE cells demonstrated detectable virus after 7 days in culture.
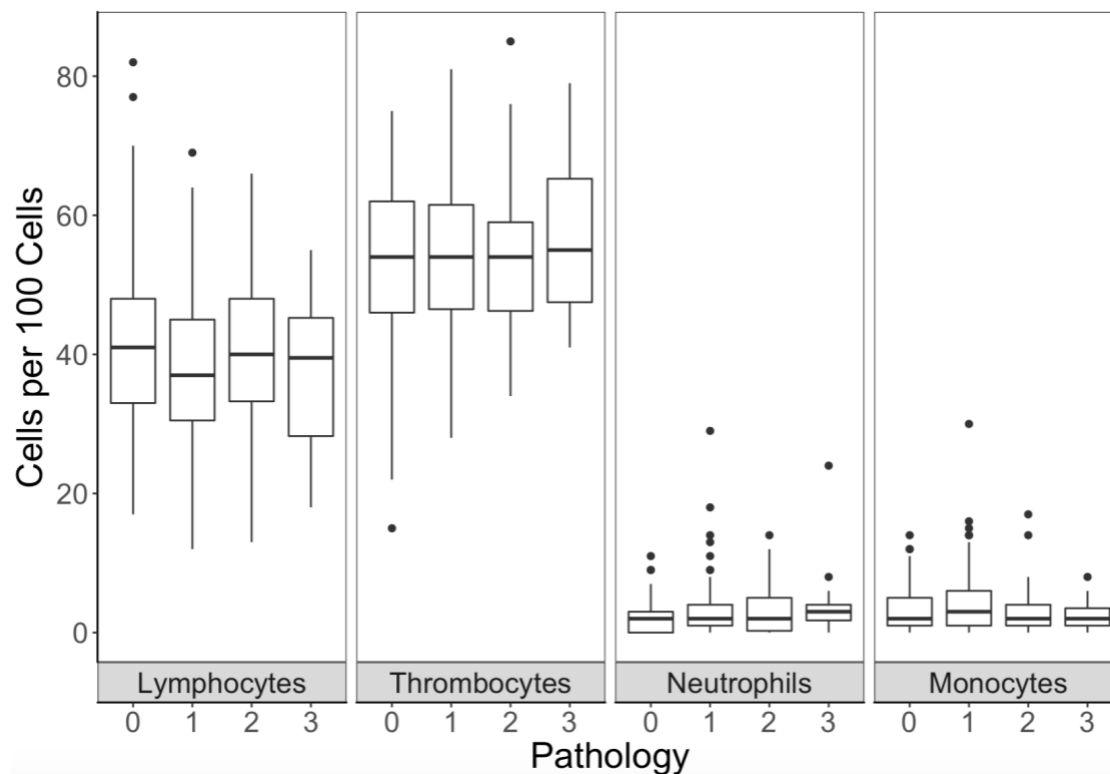
FIGURE 17. Differential Cell Counts Pathology. Percent of leukocyte types counted per 100 cells vs. pathology in Largemouth Bass (*Micropterus salmoides*; n=113) from Back Bay, Virginia for 2016 (un-infested n: = 33, infested n: = 80). Pathology parameters were defined as: none (0), no bleeding and no ulceration; mild (1), ulcers present, shallow erosion, and no bleeding; moderate (2), ulcers, bleeding, and significant erosion affecting less than 50% of the oral cavity; severe (3), ulcers, bleeding, and significant erosion affecting greater than 50% of the oral cavity. There was no significant difference in percent of leukocytes counted between bass with and without leeches for each leukocyte category respectively. (Kruskal-Wallis Rank Test; $\chi^2$ = 43.22, df = 53, p = 0.8288; $\chi^2$ = 58.341, df = 51, p = 0.2236; $\chi^2$ = 15.991, df = 16, p = 0.4536; $\chi^2$ = 13.278, df = 18, p = 0.7742).

CONDITION FACTOR

Condition factor for 2016 was calculated as $\frac{w}{l^3} * 100$ (Fulton 1904). There was a small significant difference in the condition factor between bass with and without leeches (Figure 18). Median condition factor value for Largemouth Bass with leeches was 1.25 and median condition factor value for Largemouth Bass without leech was 1.29.

FIGURE 18. Prevalence vs. Condition Factor 2016. Boxplot of leech prevalence vs. condition factor $\frac{w}{l^3} * 100$ in Largemouth Bass (*Micropterus salmoides*: n = 574) from Back Bay, Virginia for 2016. Data were arbitrarily trimmed to the range of 0.8-1.5 due to extreme outlier samples. There was a small significant difference in the condition factor between bass with and without leeches present (T-test; t= 6.3117, df = 1146, p < 0.05). The median condition factor value for Largemouth Bass with leeches was 1.25 and the median condition factor value for Largemouth Bass without leech was 1.29.

Condition factors for 2016 were not significantly different among pathology levels of 0, 1, 2, and 3. From these results, fish that have ulcers, bleeding, and erosion are not more likely differ in condition factor than fish with no pathology in the oral cavities (Figure 19). Median condition factor values for Largemouth Bass with Pathology of 0, 1, 2, and 3 were 1.29, 1.27, 1.25, and 1.24 respectively.

Condition factor for preliminary data from 2012-2015 was calculated as $\frac{w}{l^3} *$ 100 (Fulton 1904). This condition factor was only graphed with leech prevalence because pathology was not recorded for this time period. There was a small significant difference in the condition factor between bass with and without leeches (Figure 20). Median condition factor for Largemouth Bass with leeches was 1.31, and the condition factor for Largemouth Bass without leeches was 1.24. The condition factor was higher in non-infested fish for 2016. Fish with leeches were more likely to have a lower condition factor in 2016. Outliers were removed from the data due to small fish being sampled and recorded, which skewed the analysis of the condition factor because those

fish did not fit the condition factor curve. An arbitrary cutoff range for condition factor was set at 0.8-1.5.
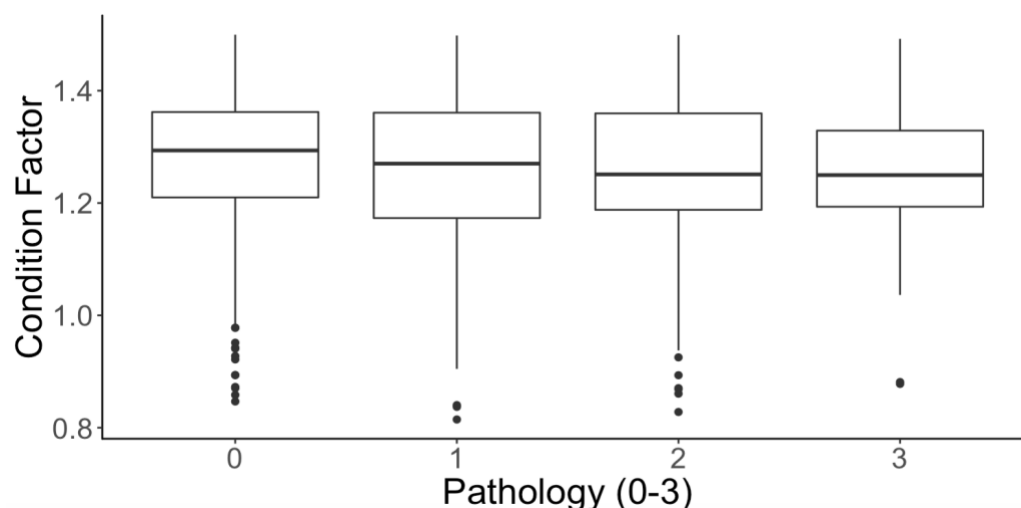


FIGURE 19. Pathology vs. Condition Factor 2016. Boxplot of pathology (0,1,2,3) vs. condition factor $\frac{w}{l^3} * 100$ in Largemouth Bass (*Micropterus salmoides*: n= 574) in Back Bay, Virginia for 2016. Pathology parameters were defined as: none (0), no bleeding and no ulceration; mild (1), ulcers present, shallow erosion, and no bleeding; moderate (2), ulcers, bleeding, and significant erosion affecting less than 50% of the oral cavity; severe (3), ulcers, bleeding, and significant erosion affecting greater than 50% of the oral cavity. There was no significant difference between pathology levels and condition factor (ANOVA; df = 3, F = 1.13, p = 0.336).

LOGISTIC REGRESSION

In the 2012-2015 model (Table 4), there was a positive association (OR = 1.26, 95% CI=1.2-1.33) between fish length and leech prevalence. There was a negative association between leech prevalence and year 2014 (OR =0.22, CI=0.0705-0.66). This best fit model had the lowest AIC of 181.974 and delta AIC of 0. Other candidate models had delta AIC fits of 182.7062, 184.4938, 184.8100, 185.2900, 185.3964, 185.6922, and 185.9623 (Table 3).

In the 2016 analysis, there was a negative association between high latitude and leech prevalence (OR=0.204, CI=0.05-0.848). There was also a positive association for

the interaction term, length*latitude section high, for the year 2016 (OR=1.6, CI=1.01-1.11) (Table 6). This best fit model had the lowest delta AIC of 564.5336. Other candidate models had delta AIC fist of 567.2937 and 570.9906 (Table 5).
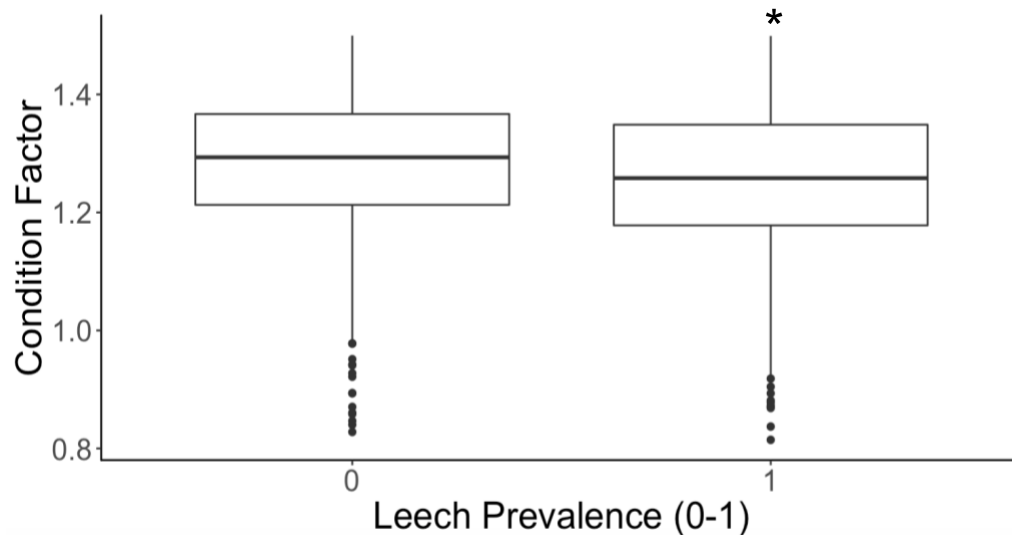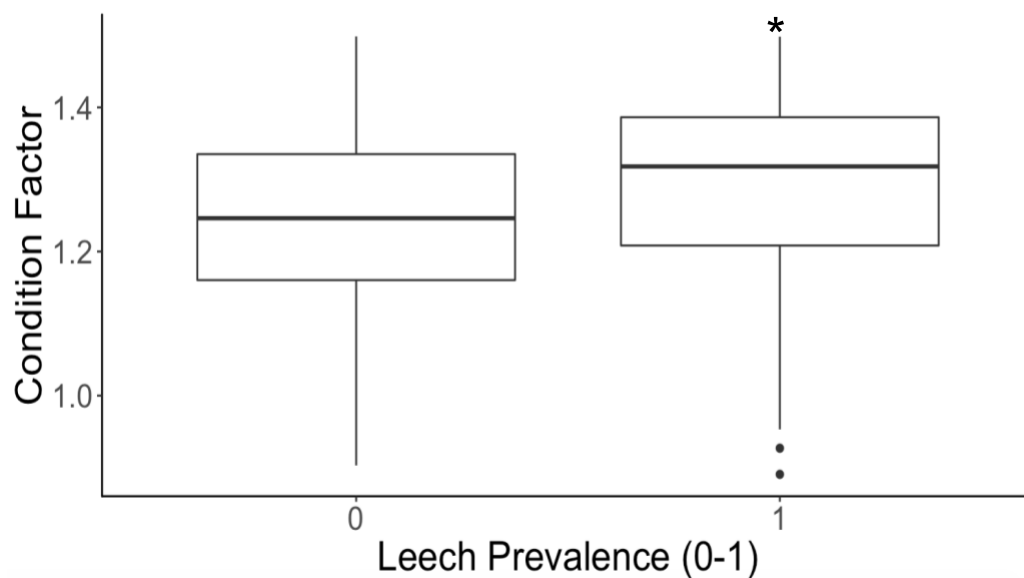


FIGURE 20. Prevalence vs. Condition Factor 2012-2015. Boxplot of leech prevalence vs. condition factor $\frac{w}{l^3} * 100$ in Largemouth Bass (*Micropterus salmoides*: n = 582) from Back Bay, Virginia for 2012-2015. There was a small significant difference in the condition factor between bass with and without leeches present (T-test; t= 6.1599, df = 1162, p < 0.05). The median condition factor for Largemouth Bass with leeches was 1.31, and the condition factor for Largemouth Bass without leeches was 1.24.

TABLE 3. Candidate Logistic Regression Models (2012-2015). Model parameters were generated using Rstudio. The top ten models are displayed. The parameters and interaction terms that were recommended to be used in the model are depicted with (+). Two best fit models included length and year.

| Model | Latitude Sections | Length | Year | Latitude Sections* Length | Latitude Sections* Year | Length* Year | df | Log lik | AIC | Delta |
|---|---|---|---|---|---|---|---|---|---|---|
| 7 | | + | + | | | | 5 | -85.97 | 181.94 | 0 |
| 39 | | + | + | | | + | 8 | -83.35 | 182.7 | 0.75 |
| 48 | + | + | + | + | | + | 12 | -80.24 | 184.5 | 2.5 |
| 8 | + | + | + | | | | 7 | -85.40 | 184.81 | 2.86 |
| 40 | + | + | + | | | + | 10 | -82.64 | 185.29 | 3.34 |
| 64 | + | + | + | + | + | + | 18 | -74.69 | 185.39 | 3.44 |
| 16 | + | + | + | + | | | 9 | -83.84 | 185.69 | 3.74 |
| 3 | | + | | | | | 2 | -90.98 | 185.96 | 4.01 |
| 56 | + | + | + | | + | + | 16 | -77.13 | 186.27 | 4.32 |
| 4 | + | + | | | | | 4 | -89.24 | 186.48 | 4.54 |

TABLE 4. Logistic Regression Model Outputs for 2012-2015 Data.  Odds ratios were determined from the confidence intervals. OR > 1 indicates positive correlation, OR < 1 negative correlation, and OR = 1 no association. From this table, it can be determined that Length (cm) has a positive correlation with leech prevalence, while year 2014 had a negative correlation with leech prevalence. There is no association between year 2013 and year 2015 and leech prevalence since the confidence intervals contain 1.

| | OR | 95% CI | p-value |
|---|---|---|---|
| (Intercept) | 0.006 | 0.001-0.02 | p < 0.05 |
| Length (cm) | 1.263 | 1.204-1.337 | p <0.05 |
| YEAR 2013 | 0.916 | 0.268-3.120 | 0.889 |
| YEAR 2014 | 0.223 | 0.070-0.663 | 0.008 |
| YEAR 2015 | 0.388 | 0.128-1.129 | 0.086 |

TABLE 5. Candidate Logistic Regression Models for 2016. Model parameters were generated using Rstudio. The top five models are displayed. The parameters and interaction terms that were recommended to be used in the model are depicted with (+). The two best fit models include latitude and length. The full model was used for this logistic regression.

| Model | Latitude Sections | Length | Latitude Sections*Length | df | Log lik | AIC | Delta |
|---|---|---|---|---|---|---|---|
| 8 | + | + | + | 8 | -245.4 | 506.98 | 0 |
| 4 | + | + | | 5 | -249.5 | 509.12 | 2.14 |
| 2 | + | | | 4 | -263.0 | 534.0 | 27.0 |
| 3 | | + | | 2 | -283.4 | 570.9 | 64.0 |
| 1 | | | | 1 | -301.1 | 604.3 | 97.3 |

TABLE 6. Logistic Regression Model Outputs for 2016 Data. Odds ratios were determined from the confidence intervals. Odds ratios were determined from the confidence intervals. OR > 1 indicates positive correlation, OR < 1 negative correlation, and OR = 1 no association. From this table, it can be determined that Length(cm) has a negative association for leech prevalence, and there is positive association with high latitude sections (the top three sampling sites for 2016 in Back Bay) and length together for leech prevalence.

| | OR | 95% CI | p-value |
|---|---|---|---|
| (Intercept) | 0.382 | 0.139-0.729 | 0.032 |
| Latitude Sections (Mid) | 0.547 | 0.153-2.239 | 0.380 |
| Latitude Sections (High) | 0.204 | 0.054-0.848 | 0.023 |
| Length (cm) | 1.016 | 0.997-1.055 | 0.305 |
| Latitude Sections (Mid)*Length (cm) | 1.050 | 0.997-1.098 | 0.051 |
| Latitude Sections (High)*Length(cm) | 1.068 | 1.015-1.116 | 0.006 |

PHENOLOGY OF LEECHES

Leech prevalence at each sampling site location (high, middle, low and tributaries) is depicted in Figure 21. Red lines for the tributaries and some sampling dates indicate no sampling was performed at those sites/dates. Zero prevalence when sampling was performed is represented by the numeral zero. Subjectively, fish appeared more likely to be infested by leeches during the spring and summer months as opposed to the fall and winter. We did not begin sampling tributary sites until the end of September.

MODELING

The model results for distance of Blue Crabs to Largemouth Bass showed that there was less than a 10% infestation rate at 10 patches, but then approached 100% at 20 patches (Figure 23). The individual spatial divisions within the model are known as patches (e.g. grid squares (Figure 22)). To further evaluate the model, to see what other factors were causing a high increase in leech infestation, a sensitivity analysis was conducted on attack rate of Largemouth Bass. This analysis indicates that once Blue Crabs are greater than 11.5 patches from the central channel, infestation prevalence is insensitive to attack rate. Therefore, the model was more sensitive to where the Blue Crabs were in relation to the Largemouth Bass, and indicate that as there is closer contact between Blue Crabs and Largemouth Bass, prevalence will rise.

The modeling results for the amount of leech prevalence on Blue Crabs showed that leech density on Blue Crabs does not affect the amount of leech infestation in Largemouth Bass (Figure 24). More leech density on Blue Crabs will not increase leech infestation in Largemouth Bass.
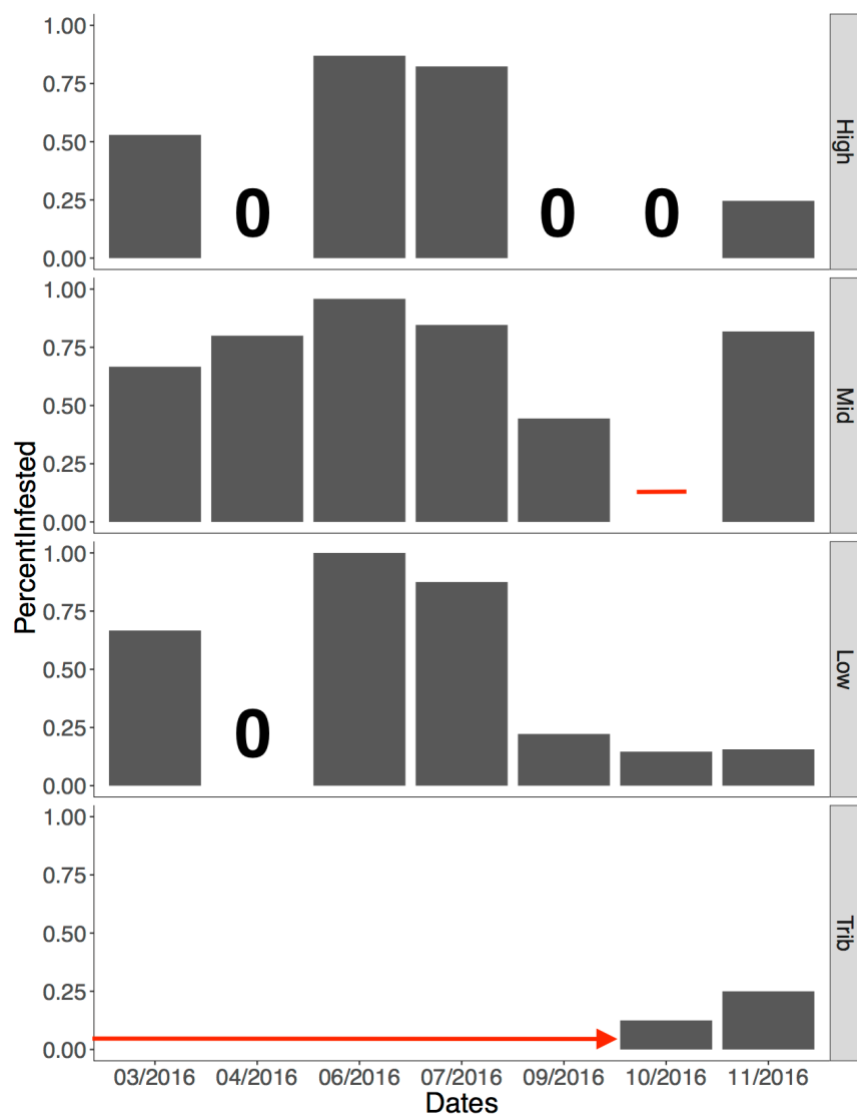
FIGURE 21. Phenology of Leeches. Phenology of leeches for the high (high), middle (mid), low (low) and tributaries (trib) for the sampling sites in Back Bay, Virginia for 2016. The red lines indicate that those sites were not sampled on those dates. The percent of fish infested vs. the date of sampling is graphed. The zeroes indicate that no leeches were observed in any fish sampled from those sites on those days.
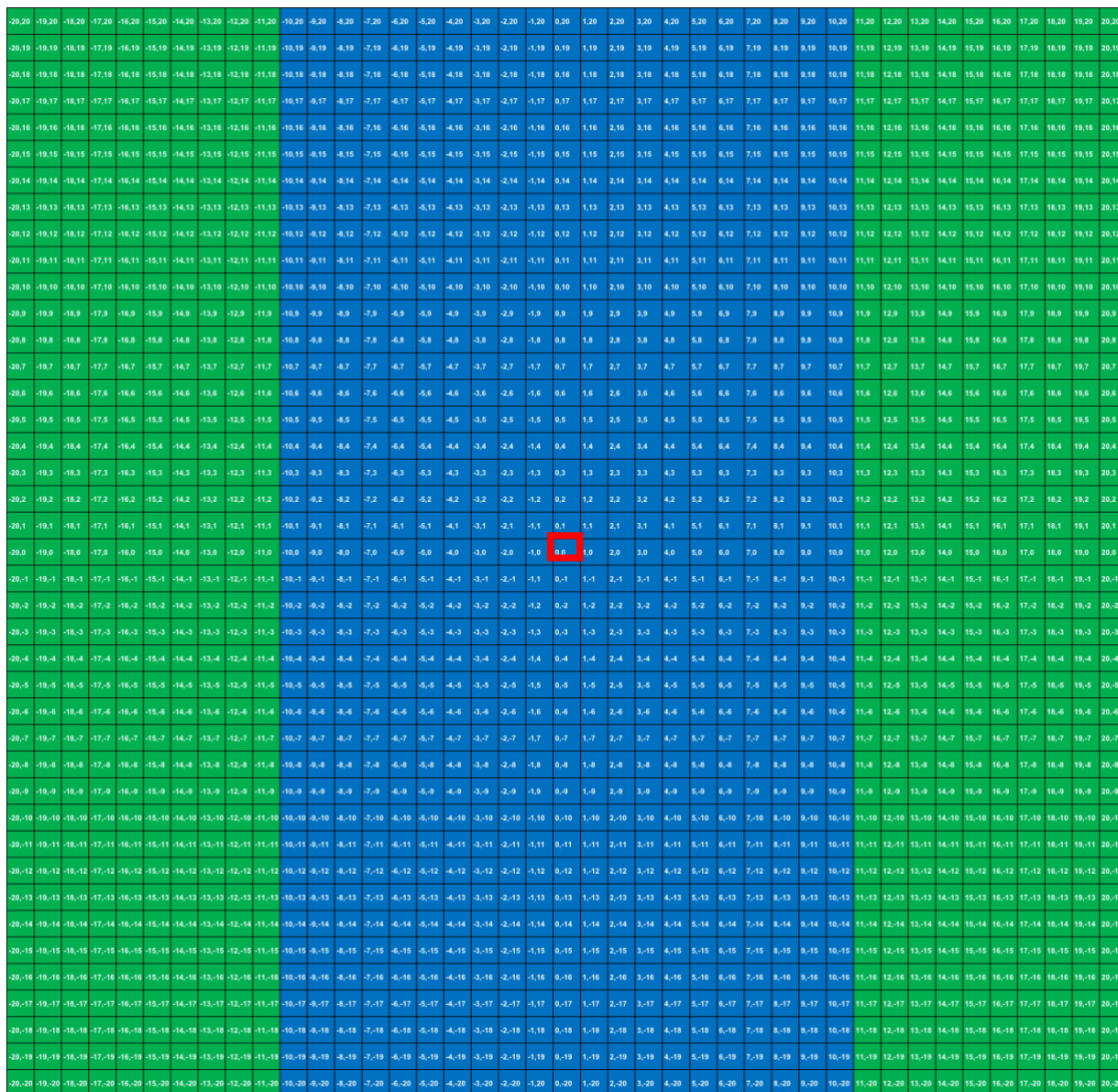
Figure 22. Grid of NetLogo. NetLogo grid used for the model. The numbers represent coordinates on the grid, and each square is a patch. The patch (0,0) is the middle of the entire grid (red box). The blue patches go from (0-10) and (-10, 0), which is the channel of the Bay where the crabs reside, while the (11-20) and (-20, -11) are green patches, which represents the banks of the Bay where the bass reside. The north arrow indicates the direction of migration for the crabs into the Bay.
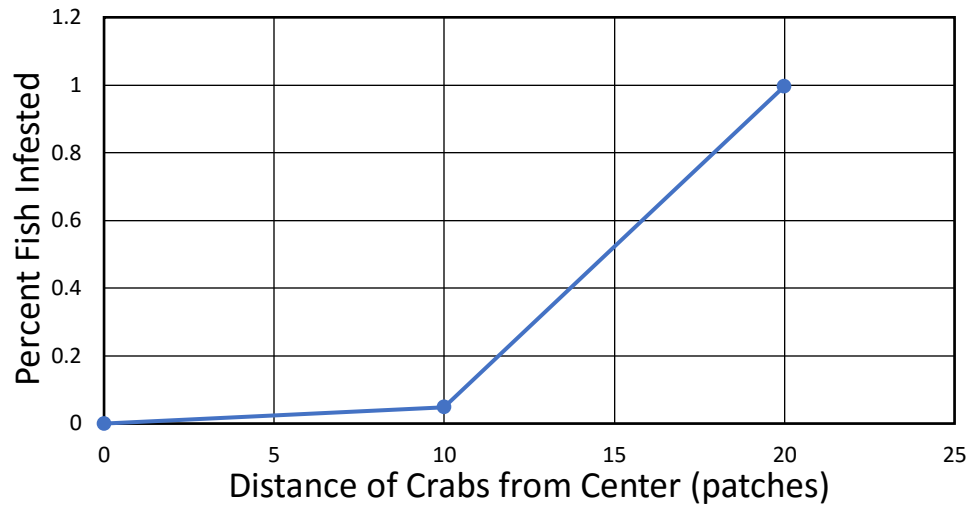
FIGURE 23. Modeling the Distance of Crabs from the Center of the Grid. Distance of crabs from the center of the model (0) to the banks of the Bay (20), and the percent of fish infested with leeches for those path distances. There is less than 10% infestation at 10 patches away from the center, and approximately at 100% infestation at 20 patches away from the center. There is an increase in fish infestation from 10 to 20 patches.
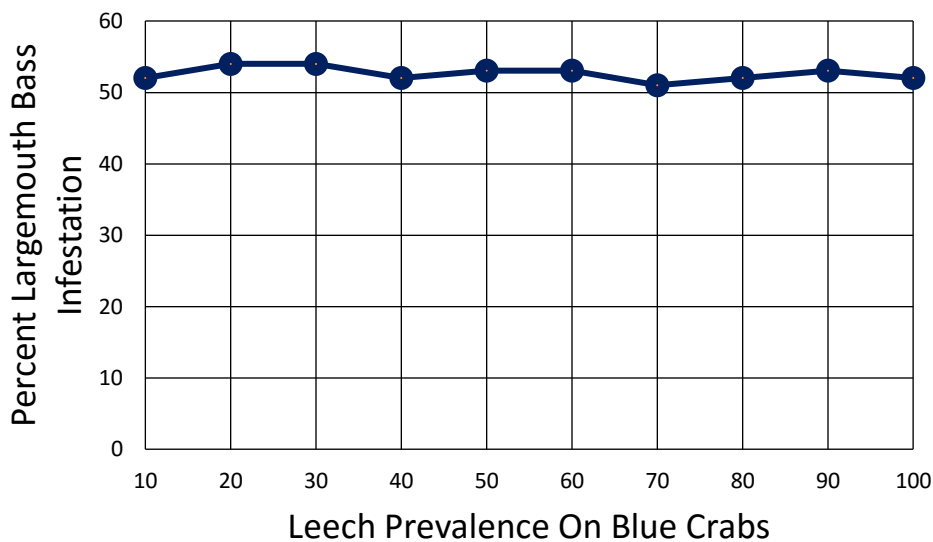


FIGURE 24. Modeling of Leech Density on Blue Crabs. The graphed results of leech prevalence on Blue Crabs and the percent of Largemouth Bass Infestation. The model parameters were determined from running the distance model, and running a sensitivity analysis. The parameters were set at 10.5 patches from the center and an attack rate of 15.

## IV. DISCUSSION

As anticipated from previous reports in adjacent Currituck Sound, NC (Noga et al. 1990), leeches infesting Back Bay Largemouth Bass were identified by 18S DNA sequencing as *Myzobdella lugubris*. Furthermore, cocoons on the Blue Crabs were identified with molecular methods as *M. lugubris*. The leech cocoons have previously only been putatively morphologically identified as *M. lugubris* (Daniels and Sawyer 1975; Sawyer et al. 1975). From these results, *M. lugubris* does lay its cocoons on Blue Crabs (*C. sapidus*) in Back Bay. We have also noted the presence of brachiobdellidan worms on Blue Crabs, the identity of which was confirmed morphologically and 18S PCR. Although brachiobdellidans generally used crayfish as hosts, they previously have been reported on Blue Crabs in the Chesapeake Bay (Gelder and Messick 2006).

Chronic stress of Largemouth Bass in response to leeches was measured using serum plasma to assess cortisol and glucose levels. There was a small significant difference in median plasma cortisol measurements between Largemouth Bass with and without leeches present. These results do not indicate the leeches are inducing a great amount of stress on the Largemouth Bass, but it does imply a host response to the leeches. It is questionable whether the impact is biologically significant. The cortisol results for the different levels of pathology also indicated a significant difference between pathologies of 1 and 3 but not pathology 2, suggesting an effect of outliers on the analysis. From these results, fish that have ulcers, bleeding, and erosion are more likely to have slightly higher cortisol levels than fish that have no pathology in the oral cavities. The majority of our Back Bay samples ranged in cortisol from 0-25 ng/mL. In literature in aquaculture settings, cortisol levels of control Largemouth Bass range from 20-60 ng/mL (Vanlandeghem et al. 2010). We know our samples are a close representation of natural cortisol levels because DC electrofishing does not cause cortisol, long-term or short-term, levels to rise (Awata et al. 2013).

Median glucose levels between Largemouth Bass with and without leeches present were not significantly different, nor did fish with differing pathology demonstrate differing glucose levels. Glucose levels did not indicate a stress response due to the leeches. Despite having ulcers, bleeding, and erosion, these fish are not more likely to have different glucose levels than fish that have no pathology in the oral cavities. This is

the first time testing has been conducted on fish from Back Bay, Virginia. The implications of these findings for the health of Back Bay Largemouth Bass are that leeches do not appear to be an important source of stress on the bass.

Internal bacteriology was conducted on Largemouth Bass in Back Bay with leech infestation to determine if any systemic bacterial infections were present. Negative bacteriology results indicate that *M. lugubris* does not vector the bacteria to cause systemic bacterial infections in Largemouth Bass. Low culture growth from all of the samples suggests that leech infestation does not make fish more susceptible to bacterial infections. Using PCR and 16S rDNA sequencing, bacteria from the oral ulcers swab samples were identified as *Aeromonas hydrophila*. *Aeromonas* spp. are short, gram-negative (Bergey et al. 1957; Popoff and VeEron 1976; Cipriano et al. 1984; Swann and White 1991), motile bacilli (Bergey et al. 1957; Popoff and VeEron 1976; Cipriano et al. 1984) with a single flagellum (Bergey et al. 1957; Popoff and VeEron 1976; Cipriano et al. 1984). They are commonly found in freshwater habitats, and are associated with disease among cultured and wild fishes (Cipriano et al. 1984). *A. hydrophila* is associated with Red-Sore Disease, a disease frequently found in Largemouth Bass (Esch et al. 1976; Esch and Hazen 1980; Cipriano et al. 1984; Swann and White 1991). Red Rot Disease and Scale Protrusion Disease (Cipriano et al. 1984), as well as Ulcer Disease and Motile Aeromonas Septicemia (MAS) (Swann and White 1991), and Hemorrhagic Septicemia are all diseases associated with *A. hydrophila* that affect fishes (Cipriano et al. 1984; Swann and White 1991). In the United States, *A. hydrophila* causes disease primarily in warm water fishes including minnows, baitfishes, Channel Catfish (*Ictalurus punctatus*), and Striped Bass (*Morone saxatilis*) (Cipriano et al. 1984). The pathogen may also affect a variety of cool water and cold water fishes as well (Cipriano et al. 1984). *A. hydrophila* has been previously misidentified as *Pseudomonas*, *Proteus*, *Bacillus*, *Aerobacter*, and *Achromobacter* in hemorrhagic septicemias in fishes (Cipriano et al. 1984). The genera *Pseudomonas* and *Aeromonas* are commonly isolated from normal healthy fish, but certain species such as *A. hydrophila*, can induce disease (Cahill 1990). Previous research identified at *Pseudomonas fluorescens*, *Bacillus* sp., *Staphylococcus hemolyticus*, and *Pseudomonas putrefaciens* from swabs of leech attachment sites in the oral cavity of

Currituck Sound Largemouth Bass (Noga et al. 1990). Upon further study, we identified the bacteria at the lesions in the oral cavity of Back Bay largemouth bass as *A. hydrophila*. No internal infection was detected, indicating that appropriate host or environmental factors were not favorable for disease to occur. Several authors have examined the role of leeches as a vector of bacterial infections, but there is no evidence of leeches as vectors of bacteria (Leatherland 2006).

Hematological evaluation can be useful in monitoring the health status of fishes (Clauss et al. 2008). In this study, leukocyte ratios were assessed to identify any immunological response Largemouth Bass may have to leeches or pathogens they vector. Neutrophils are a major phagocytic cell in fishes (Ellis 1977). They are the most common type of granulocyte in zebrafish and are part of the innate immune response (Lieschke and Trede 2009), where they immediately move to the site of initial infection (Ellis 1977; Lieschke and Trede 2009). Neutrophils were counted to see if there was any indication of a bacterial, viral, or parasitic infection. Monocytes were counted to indicate if there was any evidence of chronic inflammation or active infection. Monocytes are immature cells that are thought not to proliferate further (Gottlieb et al. 1972). They reach maturity when they enter tissues from the blood and develop into macrophages (Ellis 1977). Monocytes are actively phagocytic and move from the blood to tissues when stimulated by chemokines (Ellis 1977). Thrombocytes mediate clotting in fishes (Wardle 1971; Ellis 1977). Thrombocytes are important in the innate immune response of fishes because they work with coagulation pathways to maintain the integrity of the host (Lieschke and Trede 2009). Thrombocytes were counted to see if leeches were able to deplete the clotting ability of the Largemouth Bass. The percentages of different leukocyte types from fish with and without leeches were not significantly different. This indicates that the presence of leeches does not alter the hematological characteristics of Largemouth Bass. These results also imply that Largemouth Bass are not exhibiting an immune response to the leeches, the Largemouth Bass are not immunocompromised due to stress, and the Largemouth Bass are not more broadly challenged by other pathogens vectored by the leeches. Furthermore, leeches do not impact the clotting abilities of the Largemouth Bass.

Hemoglobin values are important for identifying anemia in fishes (Kawatsu 1966; Kawatsu 1968; Kawatsu 1969; Smith and Halver 1969; Kawatsu 1971; Blaxhall 1972; Kawatsu 1972; Blaxhall and Daisley 1973; Kawatsu 1977). In this study, there was no significant difference in hemoglobin levels between fish with and without leeches present. The range was 5.4-9.7 g/dL, with a mean of 7.7 g/dL. These data are consistent with the normal range of hemoglobin values of 3.0-8.7 g/dL, and mean of 6.2 g/dL (Clark et al. 1979), 8.1 g/dL (Black 1955), 5.8-8.5 g/dL (Hunn and Robinson 1966), and 5.32-12.3 g/dL (Denyes and Joseph 1956) that have been previously reported for Largemouth Bass. Data also show no significant difference in hemoglobin values among the different pathology levels. These results indicate Largemouth Bass are not anemic, and that leeches are not inducing anemia. This is the first time hemoglobin values have been measured for Largemouth Bass in Back Bay, Virginia.

Viral assays indicate that LMBV was not present in the fish that were sampled; however, further sampling needs to be conducted to support these findings. This is the first time LMBV has been studied in Back Bay, Virginia. Detection of Viral Hemorrhagic Septicemia Virus (VHSV) was also attempted for the first time. The results indicate VHSV was not present in the leeches that were sampled and that further sampling needs to be conducted to support these findings.

Condition factor data for 2012-2015 data show Largemouth Bass with leeches had a higher condition factor than uninfested fish. In contrast, the condition factor for the 2016 data show Largemouth Bass with leeches had a lower condition factor than uninfested fish. This could be confounded by the fact that more larger fish were being caught with leeches during the 2012-2015 sampling season. Also, even though there was a significant difference, it was very small for both the 2012-2015 data and the 2016 data.

Logistic regression results for the 2012-2015 data show a positive association with length and leech prevalence. The 2016 data did not show an association with length and leech prevalence. This discrepancy between the models could be due to a possible sampling artifact. The group who sampled from 2012-2015 were focused on leech prevalence in bigger Largemouth Bass, and were not looking for leeches in smaller Largemouth Bass. Whereas in 2016, we were looking at leech prevalence in all

Largemouth Bass. 2014 was seen as a significant low year for leeches, but not in 2016 because year was not a variable for that logistic regression model. High latitude sampling sites had a negative association with leech prevalence for the 2016 logistic regression due to different sampling and different sampling sites.

Blue Crabs migrate into Back Bay during the spring months, so by the summer they are present throughout the entire Bay. They then begin to migrate back toward the Currituck Sound, NC during the fall months so by the winter they are at the very southern end of the Bay. The phenology graph of leeches (Figure 20) shows that Largemouth Bass are more likely to be infested throughout the Bay during the summer months. The graph also shows that during spring months the leech infestation is more prevalent in the middle and southern ends of the Bay as the crabs begin to migrate north. During the fall months, the same is true: the middle and lower parts of the Bay have high leech counts as the crabs begin to migrate back south.

In Back Bay, the Blue Crabs reside in the middle channel of the Bay. There have been observations of Blue Crabs along the banks of the Bay. The model was able to show leech infestation was more sensitive to where Blue Crabs are in order for infestation to occur, and if close enough to a Largemouth Bass then infestation would occur. Whereas leech density on Blue Crabs did would not impact the amount of leech infestation in Largemouth Bass. This is important in understanding the Back Bay system, and answering the question how are the leeches getting from the Blue Crabs to the Largemouth Bass.

# V. CONCLUSIONS

## PRIMARY CONTRIBUTIONS

We have completed a large-scale fish health study on Largemouth Bass in Back Bay, Virginia, with focus on health impacts of infestations by the leech *Myzobdella lugubris*. We definitively identified the leech and cocoons as *M. lugubris* through molecular methods. The data from this study indicate that leeches are not substantially impacting stress, condition, hematological parameters, or bacterial infection status of the Largemouth Bass in Back Bay, VA. Instead, there is a commensal relationship between the Largemouth Bass and the leeches, in which the leech benefits from feeding but does not significantly harm the Largemouth Bass. This is important because leech infestations do appear to be limiting the expansion of the bass population. A more definitive answer regarding the impacts of leeches on expansion of the Largemouth Bass population will come from the ongoing tag and recapture study. Other findings of interest include that leeches appear to not be permanent in the oral cavities of Largemouth Bass, but instead may be intermittent.

We did not find any evidence of leeches vectoring viral or parasitic infections. The phenology of the leech infestation suggests higher infestation rates during summer months possibly due to increased presence of Blue Crabs. This was the first time where an agent-based model was made to simulate the Back Bay ecosystem with both Blue Crabs and Largemouth Bass as agents, and the model can be further expanded.

## FUTURE DIRECTIONS

Finally, this project lays important ground work for future studies. These future studies include ongoing field collection, tag and recapture studies, further LMBV testing, and continued expansion of the agent-based model. In 2012-2015, more leeches were observed in bigger fish. This has important implications since females tend to be larger than males. As such, more research needs to be conducted to determine if there is a strong correlation between size and leech infestation. To date there are 526 tagged fish in Back Bay, and tagging will continue for the remainder of the 2017 season and in the 2018 season. Tagging and recapture results will help determine the relative survival of the Largemouth Bass, as well as the seasonality and behavior of the leeches. It will also

help in determining attachment duration and seasonality, both of which can be built in to the existing model. LMBV testing could also be continued during the entire field season to determine if the virus is dependent on seasonality.  This larger sample size would provide stronger data as to whether the virus is found in Back Bay. Additional research is also needed to determine how leeches are vectored from Blue Crabs to Largemouth Bass. This information would help to improve the existing model presented here. Expansion of the model could include adding leeches as an agent. Another important offshoot of this project involves the biology of the leeches themselves.  We have taken leeches from the field and brought them into the lab, where they deposited cocoons and those cocoons have successfully hatched. We have observed the average time it takes for these leeches to deposit cocoons, and the average time it takes for the cocoons to hatch. These new data on the leech life cycle will help in determining hatch rate, and life span of the leeches to be included into the model.

After a leech hatches from its cocoon, it can survive 9-15 days in a salinity environment of 1.1 ppt (Sawyer et al. 1975). Current research is being conducted in the Gauthier laboratory to determine exactly how long these leeches have to find a host after hatching in the Back Bay system. We have been examining hatch rates of cocoons in tubes in our laboratory, and have confirmed juvenile leeches survive for a maximum of 15 days (unpublished results).

A future logistic regression model will be run to include more data to determine if the size of the fish does have an association with leech prevalence, since the 2012-2015 data did have an association with leech prevalence, while the 2016 did not. The 2012-2015 data showed that year 2014 had a negative association with leech prevalence, but in 2016 year was not a variable. By adding more years to the 2016 data would allow for year to be a variable in the logistic regression. In 2016, high location sites had a negative association with leech prevalence, while the 2012-2015 data did not have location as a variable in the final model. This could be due to sampling sites, and sampling efforts. More data for the model would be able to determine if sampling efforts impacted the leech prevalence data. More data would also be necessary to determine the impacts of environmental and host variables on leech prevalence.

Taken together, the data presented in this thesis provide useful information to the Virginia Department of Game and Inland Fisheries (VDGIF) and enable them to make a more informed management decision regarding the stocking of largemouth bass in Back Bay.  This study also contributes to our knowledge of previously poorly understood the host-pathogen relationship and the basic biology of a locally important parasite.

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# APPENDIX A

## PRIMER SET TABLE

| Primer Set Name | Forward and Reverse Primers | 5' to 3' Sequence | Reference |
|---|---|---|---|
| 18S rDNA | 1F<br>5R | 5'-TACCTGGTTGATCCTGCCAGTAG-3'<br>5'-CTTGGCAAATGCTTTCGC-3' | (Giribet et al. 1996) |
| Universal 16S | NU16S-F<br>NU16S-R | 5'-TCCTACGGGAGGCAGCAGT-3'<br>5'-GGACTACCAGGGTATCTAATCCTGTT-3' | This work |
| LMBV | DNApolF<br>DNApolR | 5'-ATCGCCAAGAACGTGTGCTACTCT-3'<br>5'-CCGTACATGCTGTTGGCTGAAACT-3' | (Iwanowicz et al. 2013) |
| VHSV | Danish-F<br>Danish-R | 5'-AAACTCGCAGGATGTGTGCGTCC-3'<br>5'-TCTGCGATCTCAGTCAGGATGAA-3' | Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) for viral hemorrhagic septicemia virus (VHSV) (Michigan State University) |

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- Pomposini, A., Blubaugh, J., Boyce, RC., and Gauthier, D. Biology and Impacts of Leech Infestation in Largemouth Bass (*Micropterus salmoides*) in Back Bay, Virginia. Presented at 42nd Eastern Fish Health Workshop, Michigan State University, East Lansing, MI, April 2017.

- Pomposini, A., Blubaugh, J., Boyce, RC., and Gauthier, D. Biology and Impacts of Leech Infestation in Largemouth Bass (*Micropterus salmoides*) in Back Bay, Virginia. Presented at Northeast Association of Fish and Wildlife Agencies (NEAFWA), Norfolk, VA, April 2017.