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LEECHES IN THE LAKE ERIE WATERSHED: THE
IDENTIFICATION AND POTENTIAL ROLE IN DISEASE
TRANSMISSION

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Carolyn A. Schulz

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LEECHES IN THE LAKE ERIE WATERSHED: THE IDENTIFICATION AND
POTENTIAL ROLE IN DISEASE TRANSMISSION

By

Carolyn A. Schulz

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Fisheries and Wildlife

2009

ABSTRACT

LEECHES IN THE LAKE ERIE WATERSHED: THE IDENTIFICATION AND POTENTIAL ROLE IN DISEASE TRANSMISSION

By

Carolyn A. Schulz

Three species of leeches (*Actinobdella pediculata*, *Myzobdella lugubris*, and *Placobdella montifera*) were found parasitizing fish species from Lake St. Clair, Michigan, within the Lake Erie Watershed in the Great Lakes Basin (GLB). Leeches were found attached to a variety of sites on the host species. Gross and histological examination of attachment sites revealed necrotic and hemorrhagic tissue, severe swelling, expansion of muscle and dermal layers, and infiltration of macrophages, lymphocytes, and heterophils. The predominant leech in the watershed, *Myzobdella lugubris*, was further investigated for its role in disease transmission. An emerging disease in the GLB, the Viral Hemorrhagic Septicemia virus (VHSV), has been associated with several mass fish mortalities, including fish species that are susceptible to leech parasitism. Cell culture and PCR results showed that this is the first report of VHSV detected within leeches. In addition to the detection of viral pathogens, the bacterial community of *M. lugubris* was also examined. Sequences of amplified community bacterial DNA was checked for similarity to existing 16S sequences in the Ribosomal Database Project and BLAST. The phyla Bacteroidetes, Beta-proteobacteria, and Verrucomicrobia were detected in leech homogenates.

ACKNOWLEDGEMENTS

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GENERAL INTRODUCTION AND OVERVIEW

Leeches (Annelida: Hirudinea) are known to occur in a diversity of habitats, including aquatic (freshwater and marine) and terrestrial environments (Burreson 2006). They parasitize a variety of hosts, such as annelids, crabs, crayfish, fish, frogs, reptiles, aquatic birds, cows, monkeys, horses, elephants, as well as humans (Klemm 1972; Sawyer 1986; Mulcahy et al 1990; Greenblatt et al 2004; Hemmingsen et al 2005); however, this research focuses on leeches that attach to fish. Fish leeches can attach to various sites on the body of the host, including the pectoral, pelvic, dorsal, and caudal fins, the eyes, the gill chamber, the mouth cavity, and directly to the main body of the fish (Daniels and Sawyer 1975; Appy and Cone 1982; Noga et al 1990; Morrison et al 1993; Font and Tate 1994). Leeches attached to fish hosts can also cause a variety of damage, such as ulcerations, hemorrhagic or necrotic tissue, hyperplasia, thickened hypodermis, severe inflammation, and erosion of the dermis (Paperna and Zwerner 1974; Appy and Cone 1982; Noga et al 1990). In other studies, leeches have been found associated with bacteria (Kikuchi and Fukatsu 2002, 2005) and viruses (Ahne 1985; Mulcahy et al 1990; Greenblatt et al 2004). These studies demonstrate the ability of leeches to aid in the transmission of pathogenic bacteria and viruses.

Although much research has been conducted regarding the distribution and taxonomy of leeches in North America, the distribution record for the Great Lakes and its connecting waters is still incomplete, especially for the Lake Erie Watershed. The Lake Erie Watershed is of particular interest because it is a heavily utilized commercial and recreational fishery. Additionally, several mass fish mortalities associated with the

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emerging Viral Hemorrhagic Septicemia virus (VHSV) have recently occurred in the

Lake Erie Watershed. The following study was designed to better understand the role

leeches may play in disease transmission in the Lake Erie Watershed.

STUDY OBJECTIVES

The overall hypothesis of this research was that fish in the Lake Erie Watershed harbor both intermittent and semi-permanent leeches, and the leech population would most likely be dominated by a generalist leech species. It was also hypothesized that leeches in the Lake Erie Watershed could harbor microbes of potential pathogenicity to fish. To test these hypotheses, a number of tasks were performed with the following objectives:

(1) To determine the species of leeches prevalent in the Lake Erie Watershed, specifically Lake St. Clair. Since Lake St. Clair is the shallowest major lake within the Lake Erie Watershed, it is considered an optimal collection site for parasitic leeches. In specific, fish were collected to evaluate the occurrence, prevalence, intensity and abundance of leeches per each fish species of host. The prevalence and intensity of leeches at specific attachment sites on the host were also determined;

(2) To describe gross damage caused by the intermittent leeches and the histopathological damage at the attachment site of *Actinobdella pediculata* to its host, the freshwater drum (*Aplodinotus grunniens*), where *A. pediculata* spends most of its life cycle (Sawyer 1986);

(3) To determine if leeches with intermittent feeding habits, such as *Myzobdella lugubris*, harbor the emerging fish-pathogenic rhabdovirus, Viral Hemorrhagic Septicemia virus (VHSV); and

(4) To describe the bacterial pathogens that the intermittent leech *M. lugubris* harbors within its internal organs.

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CHAPTER ONE

REVIEW OF LITERATURE

Leech background information and distribution. Leeches (Annelida: Hirudinea) are

parasites that have a worldwide distribution and occur in a diversity of habitats (Burreson

2006). They live in both aquatic (freshwater and marine) and terrestrial environments

where they parasitize a variety of hosts, such as annelids, crabs, crayfish, fish, frogs,

reptiles, aquatic birds, cows, monkeys, horses, elephants, as well as humans (Klemm

1972; Sawyer 1986; Mulcahy et al 1990; Greenblatt et al 2004; Hemmingsen et al 2005).

Leeches periodically attach themselves to a host and gain their nourishment from feeding

on blood and body fluids. With the assistance of an oral and caudal sucker, leeches

attach themselves to a host and secrete an anticoagulant, which allows the blood to flow

more freely (Sawyer 1986).

Leeches can be temporary parasites, detaching from a host and reattaching to

another host, or permanent parasites, staying attached to the host for the lifetime of the

leech (Sawyer 1986). Leeches with intermittent parasitism can act as a vector for

pathogens and inflict damage in their host due to their movement and nature of feeding,

thereby facilitating the entry of opportunistic pathogens (Burreson 1982; Ahne 1985;

Kruse et al 1989; Mulcahy et al 1990; Greenblatt et al 2004; Burreson 2006; Raffel et al

2006).

By acting as prey and predator, leeches play an important role in the food chain

and can alter its dynamics. Also, due to their abundance and distribution, leeches have

been used as bioindicators for the presence of pollutants, such as polychlorinated biphenyls (PCBs) (Scrimgeour et al 1998; McCreanor et al 2008). It has also been shown that they can have similar, if not higher, levels of contaminants compared to the more often used sentinel fish species.

In a freshwater habitat, there are different physical, chemical, and biological parameters that determine if leeches will be present, such as the availability of susceptible hosts, type of substrate, depth and size of water body, water hardness, pH, and temperature, dissolved oxygen levels, turbidity and salinity (Sawyer 1986; Klemm 1991). Leeches are typically found in shallower, lentic waters with submerged vegetation; a solid substrate is preferred, as a muddy or sandy substrate will restrict their movement (Sawyer 1986; Klemm 1991). Leeches can have a wide range of tolerance for factors such as pH, water temperature, dissolved oxygen levels, and salinity; therefore these factors do not have as great of an impact on the biology of the leech as the other factors. However, the factors stated above can affect the distribution of susceptible hosts, which will in turn affect the leeches.

Leeches can exhibit several types of elementary behavior while they are searching for food, specifically relying on chemoreception to detect when a prey item is present. Once they are aware of the host, the leech will crawl or swim to attach themselves. The leech may attach itself immediately, or could explore with its oral sucker, and then may or may not attach to the host (Sawyer 1986).

Once a leech has attached itself to a host, it then must determine if it is a suitable host. Some species of leech are extremely host-specific, such as *Actinobdella pediculata* on the freshwater drum, *Aplodinotus grunniens* (Hemingway 1908; Bur 1994), and

Theromyzon tessulatum on waterfowl (Sawyer 1986), while other species are generalists

and have a wider host range, such as *Myzobdella lugubris* (Sawyer et al 1975; Miller et al

1973; Becker and Dauble 1979; Appy and Cone 1982; Font and Tate 1994).

While leeches are similar to other annelids, they differ by having and using both

anterior (oral) and posterior (caudal) suckers, which aid in attachment, feeding, and

locomotion (Klemm 1972; Sawyer 1986). Adhesion occurs when the caudal sucker

sticks to the substrate (or host) with the aid of a mucoid secretion, typically as a holdfast

structure; suction also aids in rapid locomotion and allows for flexibility (Sawyer 1986).

When a leech is feeding, the caudal sucker acts as an anchor, sometimes embedding itself

into the tissue of the host, while permitting the oral sucker to search for a suitable feeding

site (Sawyer 1986).

Leeches are classified depending upon how their mouthparts are structured, and

are either predaceous (such as those preying on invertebrates) or haematophagous

(bloodsucking). Predaceous leeches (order Pharyngobdellida) are worm-like, lack jaws

and teeth, and swallow their prey whole (Sawyer 1986). Haematophagous leeches either

have jaws with teeth to bite the host (order Gnatbobdellida), or they have a muscular

proboscis which is inserted into the host (order Rhyncobdellida) for feeding (Sawyer

1986). Also, predaceous species are nocturnal, while haematophagous are active when

hosts are active, whether it is day or night (Klemm 1972).

Some leech species stay attached to the host for the duration of their lifetime,

while others detach and reattach to other hosts (Sawyer 1986). Temporary leeches

usually leave the host soon after taking a blood meal, while semi-permanent leeches

remain on the fish and take successive blood meals, leaving only to deposit their egg eggs

on a hard substrate, such as vegetation, rocks, or even the carapace of crustaceans (Burreson 2006).

While there are several types of aquatic leeches that attach to a variety of aquatic hosts, in this review of literature, I will focus on the fish leeches. Fish leeches are in the families Glossiphonidae and Piscicolidae (Klemm 1972). They have the same general feeding and locomotion methods as stated above. Fish leeches commonly attach to various sites on the body of the host, including the pectoral, pelvic, dorsal, and caudal fins, the eyes, the interior of the gill chamber, the inside of the mouth cavity, and directly to the main body of the fish (Daniels and Sawyer 1975; Appy and Cone 1982; Noga et al 1990; Morrison et al 1993; Font and Tate 1994). Predators of piscicolid and glossiphoniid leeches consist of mainly fish, birds, aquatic insects, crayfish, and other leeches (Sawyer 1986; Young and Spelling 1986).

The distribution and taxonomy of leeches have been well documented in North America throughout the past century (Hemingway 1908; Meyer 1940; Meyer 1946; Branson 1961; White and Crisp 1973; Overstreet 1973; Miller et al 1973; Paperna and Zwerner 1974; Sawyer et al 1975; Daniels and Sawyer 1975; Becker and Dauble 1979; Smith and Taubert 1980; Schramm et al 1981; Appy and Dadswell 1981; Appy and Cone 1982; Tartar and Sheridan 1984; Muzzall et al 1987; Noga et al 1990; Woods et al 1990; Morrison et al 1993; Bur 1994; Font and Tate 1994; Klemm et al 2003; Choudhury et al 2004; Ruiz-Carus et al 2006; Wolf et al 2008). For Michigan, extensive distribution records have been published by Klemm (1972, 1977, 1991). Klemm (1972) identified 45 leech species distributed throughout the Upper and Lower peninsulas of Michigan, including two species of interest for this study, *Myzobdella lugubris* and *Placobdella*

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montifera. The third leech species of interest for this study, *Actinobdella pediculata*, was not recorded. Klemm (1972) also noted that too few specimens of fish leeches were collected to be able to make conclusions regarding their distribution.

Later, Klemm (1977) expanded the survey of leeches to include those in the entire Great Lakes (GL) region (defined as including the states of Illinois, Indiana, Michigan, Minnesota, New York, Ohio, Pennsylvania, and Wisconsin, and the Canadian province, Ontario). *Placobdella montifera* and *M. lugubris* were once again collected in Michigan and the GL region, however *A. pediculata*, while collected in the GL region, was still not found in Michigan (Klemm 1977). In his final GL comprehensive study, Klemm (1991) discussed the current status in taxonomy and distribution of leeches in the GL region, with the highest number of leech species recorded (40) in Michigan.

While the leeches of Michigan and the GL region have been extensively studied, relatively few leech species have been collected from Lake St. Clair (Appy and Cone 1982). Lake St. Clair is an ecologically and economically important part of the GL system. The economic impact due to leeches and their potential to cause damage and transmit pathogens has not been and would be difficult to quantify, yet it is still critical to review. This will be explored further later in the chapter.

Description of the leeches of interest in this study. Of the freshwater fish leeches found in Michigan, a special emphasis was given to three species of leeches; namely *Actinobdella pediculata*, *Myzobdella lugubris*, and *Placobdella montifera*. Described below is general information regarding the physical description, distribution, and life cycle for each leech. It should be noted that the annual life cycles of leeches are

extremely variable, depending on habitat, geographical location, and host biology (Burreson 2006).

Actinobdella pediculata Hemingway, 1908 (Rhynchobdellida: Glossiphonida) (Figure 1) has a flat, wide body, with a ventral oral sucker that is more or less fused with the body, and is a poor swimmer (Hoffman 1999). *Actinobdella pediculata* is not commonly found nationwide, occurring mainly in the Midwestern states, and mostly inhabiting areas where its preferred host is present (Klemm 1982). As mentioned previously, *A. pediculata* is host-specific for the freshwater drum, *Aplodinotus grunniens*, where it usually attaches itself to the isthmus below the gill chamber, or to the inside of the operculum (Sawyer 1986; Bur 1994). On one occasion, it was also reported attached to the white sucker, *Catostomus commersonii*, in Maine (DeRoth 1953), but has not been recorded attached to any other species other than the freshwater drum. While not much is known about its life history, it is believed by Sawyer (1986) to be similar to that of *A. inequiannulata*. *Actinobdella inequiannulata* is a semi-permanent parasite of freshwater fish, specifically catostomids, where it is commonly found feeding in the gill chamber of its hosts (Sawyer 1986). Juvenile *A. inequiannulata* attach themselves to their fish host where they fed for several months and then detach themselves to mature into adults for reproduction (Sawyer 1986). Due to *A. pediculata* being a semi-permanent parasite with specialized host specificity and attachment site, a similar life cycle to *A. inequiannulata* was assumed (Sawyer 1986).

Myzobdella lugubris Leidy, 1851 (Rhynchobdellida: Piscicolida) (Figure 2) has a cylindrical body, often divided into a narrow anterior and wider posterior region, with an oral sucker that is clearly set off from the body, and are described as true fish leeches, as

they are almost always found attached to fish, rarely attaching to other organisms (Hoffman 1999). *Myzobdella lugubris* has a very widespread distribution, as it is found throughout freshwater and marine waters throughout North America and has a very wide host range (Klemm 1972, 1977, 1991; Davies 1973; White and Crisp 1973; Paperna and Zwerner 1974; Daniels and Sawyer 1975; Sawyer et al 1975; Becker and Dauble 1979; Appy and Dadswell 1981; Schramm et al 1981; Appy and Cone 1982; Tartar and Sheridan 1984; Muzzall et al 1987; Woods et al 1990).

Myzobdella lugubris, also found in the marine environment, is one of the few leech species that shares its life cycle with a fish host and a crustacean host, specifically the blue crab, *Callinectes sapidus* (Daniels and Sawyer 1975). The leech is parasitic on its fish host for most of the year, and then leaves the host to deposit its eggs on the crab carapace (Daniels and Sawyer 1975). In freshwater habitats lacking *C. sapidus*, or without other suitable crustaceans available, it is assumed that stones or other hard substrates are used by *M. lugubris* in the place of the blue crab's carapace (Becker and Dauble 1979). It is of interest that *M. platensis* also utilizes a crustacean for egg deposition, the Bocourt swimming crab (*C. bocourti*) (Zara et al 2008).

Placobdella montifera Moore, 1906 (Rhynchobdellida: Glossiphonida) (Figure 3) is also a member of the Glossiphonidae family, but has different physical characteristics than *A. pediculata*: the oral sucker is distinctly expanded, forming a discoid head, and the dorsum has three prominent tuberculate ridges (Hoffman 1999). *Placobdella montifera* is distributed throughout the eastern and Midwestern United States, including Arkansas, Illinois, Indiana, Louisiana, Michigan, Minnesota, Missouri, New York, Ohio, Pennsylvania, Texas, Wisconsin, and Ontario, where it parasitizes a variety of salmonids,

sunfish, catfish, and bass (Klemm 1982, 1991; Hoffman 1999; Moser et al 2006). All *Placobdella* spp. are considered semi-permanent parasites, which are either parasitic to fish, turtles, or birds (Davies and Wilkialis 1982). Little is known about the general biology and ecology of *Placobdella* spp., primarily due to the fact that while they are widespread in range, they are also relatively rare in occurrence (Davies and Wilkialis 1982). However, *P. montifera* has been found attached to fish that prefer deeper, cooler water, which may reflect the ecology of this species (Szalai and Dick 1991). This leech has also been found in the mantle cavity of freshwater clams, but it is unknown if the leech feeds on the clam or if the leech utilizes the clam as protection (Curry 1977; Curry and Vidrine 1976).

Role of leeches in disease and pathogen transmission. As integral parts of the food chain, it is important to understand how leeches affect their hosts. Histological studies of leech attachment sites on fish have shown that their invasive method of attachment causes an extensive inflammatory response; displacement, erosion, and thickening of the dermis; hyperplasia and erosion of the epithelium; focal and widespread hemorrhages with clot formations; edema with mononuclear cells; and necrotic tissue (Paperna and Zwerner 1974; Appy and Cone 1982; Roubal 1986; Volonterio et al 2004). Recorded gross signs of damage caused to fish hosts have included: emaciation, bleeding ulcers, necrotic patches, inflammation, erosion of the epithelium, hemorrhage, and swelling (Paperna and Zwerner 1974; Appy and Cone 1982; Roubal 1986; Noga et al 1990). Due to the energy requirements of some leeches, it has also been shown that they are capable of stunting the growth of fish as well (Mace and Davis 1972).

Noga et al. (1990) found the leech *Myzobdella lugubris* associated with large ulcerations on the tongue and in the buccal cavity of largemouth bass, *Micropterus salmoides*, as well as small focal erosions and large, light-red ulcerations extending into the underlying musculature. Bacteria were also isolated from these lesions (*Bacillus* sp., *Pseudomonas fluorescens*, *P. putrefaciens,* and *Staphylococcus hemolyticus*), but there was not a predominant species (Noga et al 1990). This shows that not only do leeches cause damage to their hosts, but also their presence is associated with bacterial colonization.

In addition to the damage to the underlying musculature caused by leeches, they are also known as vectors for several pathogens and have been found associated with bacteria, trypanosome blood parasites, and viruses. For example, Kikuchi and Fukatsu (2005) found a *Rickettsia* spp. infection in the leeches *Torix tagoi, T. tukubana*, and *Hemiclepsis marginata. Rickettsia* sp. is a known pathogen that seriously affects many fish species (Cusack et al 2002). It has also been found that leeches which fed on infected fish contained *Aeromonas* spp. and *Pseudomonas* spp. in their digestive tracks (Snieszko and Bullock 1968). Additionally, leeches are known to disperse *A. hydrophila* from host to host (Negele 1975). The earlier studies of Dombrowski (1952) demonstrated that *Piscicola geometra* can transmit *Pseudomonas punctata* to carp. As well as potentially transmitting bacterial pathogens from fish to fish, leeches can cause bacterial infections in immuno-comprised human patients when utilizing medicinal leeches to assist with venous congestion after surgeries (Silver et al 2007; Laufer et al 2008).

In addition to bacteria, leeches are well known to transmit haematozoans among hosts. *Trypanoplasma atraria* was found in the gut of the leech *Piscicola salmositica*, as well as in the Utah chub, *Gila atraria*, redside shiner, *Richardsonius balteatus*, and speckled dace, *Rhinichthys osculus* (Cranney and Heckmann 1996). Burreson (1982) found *T. bullocki* present in the proboscis sheath of the leech *Calliobdella vivida*, which, in a laboratory experiment, then infected the hogchoker, *Trinectes maculatus*. Also, *Trypanosoma punctati* was recovered from the green snakehead, *Channa punctatus*, as well as its leech vector, *Hemiclepsis marginata* (Shanavas 1991). The leech *Johanssonia arctica* is a known vector for *Trypanosoma murmanensis*, which was found in Atlantic cod, *Gadus morhua* (Hemmingsen et al 2005).

Furthermore, leeches are vectors for viral pathogens. Greenblatt et al. (2004) reported that the fibropapilloma-associated turtle herpesvirus (FPTHV) was found within the marine turtle leech *Ozobranchus* spp. at high viral DNA loads. In the same context, the fish leech *Piscicola geometra* is known as a vector for the Spring Viraemia of Carp virus (SVCV) and was experimentally shown to transmit the pathogen mechanically from infected to non-infected carp (Ahne 1985). Also, the fish leech *P. salmositica* was found to be a vector for the Infectious Hematopoietic Necrosis virus (IHNv) in the sockeye salmon, *Oncorhynchus nerka*, where it was also suggested that the virus could be replicating within the leech itself (Mulcahy et al 1990).

Many animals that gain nourishment primarily through feeding on the blood of vertebrates possess symbiotic microorganisms, such as bacteria. Their roles are not clearly defined, but it is speculated that the symbionts can provide essential nutrients, aid in energy balance and digestion, or produce antibiotics to prevent the growth of

contaminant bacteria (Kikuchi and Fukatsu 2002; Kikuchi and Graf 2007). It is critically important to understand the structure of bacterial communities within blood-sucking leeches in order to recognize the relationship between leeches and their symbionts, as well as the potential role of leeches in transmitting bacterial pathogens.

Studies regarding leeches and their endosymbiotic bacteria have mainly pertained to the aquatic European medicinal leeches, *Hirudo* spp, and less often, the North American medicinal leech, *Macrobdella decora*. *Hirudo* spp. are of particular interest due to their ability to secrete anticoagulants, and therefore has been widely used to treat venous congestion complications following surgical procedures in humans (Sawyer 1986; Silver et al 2007).

European medicinal leeches are comprised of three species: *H. medicinalis*, *H. orientalis*, and *H. verbana* (Siddall et al 2007). It has been documented that *H. medicinalis* contains *Aeromonas veronii* Biovar sobria and *A. hydrophilia* within its digestive tract, although *A. veronii* was the dominant culturable bacteria (Graf 1999), *H. orientalis* harbors two *Aeromonas* spp., *A. veronii* and *A. jandaei* within the digestive tract (Laufer et al 2008), and similarly, *H. verbana* contains *A. veronii* and a *Rikenella*-like bacterium in the crop (Silver et al 2007). All of these bacteria are facultative pathogens. In addition, the North American medicinal leech, *M. decora*, harbors *A. jandaei* within its crop, suggesting there is a consistency among leech species in harboring motile *Aeromonas* spp., regardless of their geographical distribution (Siddall et al 2007). It has been reported that in human post-operative patients treated with *Hirudo* spp., infection with *Aeromonas* spp. can occur (Silver et al 2007; Laufer et al 2008). *Aeromonas* spp. has also been implicated in causing septicemia, wound infections, and

diarrhea in humans (Janda and Abbott 1998). In addition, *A. hydrophilia* is a common

and dangerous pathogen involved in heavy mortalities in farmed and wild fish

(Harikrishnan and Balasundaram 2005).

While there has been significant research regarding the bacterial community

structure of medicinal leeches, there is still much to learn regarding glossiphoniid and

piscicolid leeches. As of present, there is one report of endosymbiotic bacteria within the

esophageal organ of two glossiphoniid leeches, *Placobdelloides siamensis* and

Parabdella sp. (Kikuchi and Fukatsu 2002), which are parasitic on turtles and crocodiles

(Hoffman 1999). After molecular analysis, a single dominant bacterial species, which

showed an 89% similarity to *Buchnera aphidicola*, the primary endosymbiont of aphids,

was detected (Kikuchi and Fukatsu 2002). The biological function of the *B. aphidicola* is

currently unknown; however it is possible that they provide the leech host with vitamins,

as they do in their arthropod counterparts (Kikuchi and Fukatsu 2002). Piscicolid leeches

are more widespread than glossiphoniid leeches; however, their bacterial communities

are currently unknown.

The Great Lakes basin and Lake Erie Watershed background information. The

Great Lakes comprises approximately 18% of all the freshwater in the world and

provides an ecosystem for a variety of aquatic organisms, including 179 species of fish,

several species of waterfowl, and aquatic mammals (United States Environmental

Protection Agency 2006; Coon 1999). The GL are also heavily used in shipping,

recreational and commercial fishing, and recreational boating. In the Great Lakes,

Canadian, U.S., and tribal commercial and recreational fishermen compete for popular

yellow perch, walleye, lake trout, and salmonid fisheries (Brown et al 1999). In 2006, recreational fishermen spent $US 1.5 billion on their total trip and equipment expenditures for fishing on the GL, including food, lodging, guides, bait, and special equipment (United States Department of the Interior 2006). Commercial shipping vessels regularly transport iron ore, coal, and grain throughout the GL and into the Atlantic Ocean (United States Environmental Protection Agency 2006).

Within the GL system, the Lake Erie watershed contains Lake Erie (LE) and Lake St. Clair (LSC). Both lakes are greatly utilized, particularly in recreational activities. Lake Erie (Figure 4), the shallowest of the GL, is surrounded by the states Michigan, New York, Ohio, Pennsylvania, and the province of Ontario (United States Environmental Protection Agency 2006). The western portion of LE is connected to Lake Huron, via LSC and the Detroit and St. Clair rivers, and is connected to Lake Ontario by way of the Welland Canal and Niagra Falls (United States Environmental Protection Agency 2006). It also has an active commercial and recreational fishery.

Lake St. Clair is located between southeastern Michigan and southwestern Ontario and connects Lake Huron to Lake Erie via the St. Clair and Detroit Rivers (Figure 4). The lake is critical to the GL shipping industry and is also a very popular recreational lake (Michigan Department of Environmental Quality 1999). Currently, the most commonly exploited species include the muskellunge (*Esox masquinongy*), smallmouth bass (*Micropterus dolomieu*), yellow perch (*Perca flavescens*), and walleye (*Sander vitreus*) (MacLennan et al 2003; Thomas and Haas 2004). According to the Michigan Department of Environmental Quality (1999), the annual value of the sport fishery of the Michigan section of LSC was US $ 30 million and is expected to have

increased over the decade. In brief, recreation in LSC has significant socioeconomic benefits, therefore there is an increasing concern of any factor that may negatively affect the fishing industry or disturb the health of its fragile ecosystem.

Diseases of Great Lakes fish species. Over the last five decades, a number of diseases have been incriminated of devastating a number of native and introduced GL fish species (Faisal et al 2007; Faisal and Hnath 2005). Infectious agents, such as bacteria and viruses, cause many of these diseases. Among these, bacteria such as *Renibacterium salmonarium*, the causative agent of Bacterial Kidney Disease, is widespread in GL free ranging, and confined, salmonid stocks (Beyerle and Hnath 2002; Faisal and Hnath 2005). Furunculosis, which is caused by *Aeromonas salmonicida*, is another disease that also ravages salmonids in the GL basin (Cipriano et al 1996; Faisal and Hnath 2005). The bacteria *Flavobacterium* spp. is known to cause Bacterial Coldwater Disease, Bacterial Gill Disease, and Columnaris, which can cause mortalities in a wide range of fish species (Faisal and Hnath 2005). Piscirickettsia-like infections have caused raised ulcers, edema, and degenerative kidneys in muskellunge, *Esox masquinongy*, in the GL as well (Faisal and Hnath 2005).

In addition to the bacterial diseases that distress the fish populations of the GL, many viral pathogens also cause harm. The Largemouth Bass virus has caused massive mortalities among fish from several inland lakes in southern Michigan (Faisal and Hnath 2005). The Infectious Pancreatic Necrosis virus has been isolated from within hatchery-raised trout and salmon, specifically the Coho salmon, *Oncorhynchus kisutch*, which can cause up to 90% mortality rates in hatchery-raised fish, as well as melanosis and

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distended abdomens (Beyerle and Hnath 2002; OIE 2006). Garver et al. (2007) reported the presence of the Spring Viraemia of Carp virus in wild common carp in Lake Ontario, which can cause acute hemorrhages and death and is a highly contagious disease. The Lymphocystis Disease virus causes hypertrophy of connective tissue cells and is commonly found in walleye populations in the GL (Schneeberger 2000; Muzzall and Haas 1998). Recently, the lethal Viral Hemorrhagic Septicemia virus has invaded the GL and caused widespread mortalities in several fish species, including the recreationally important muskellunge (Elsayed et al 2006b).

Along with bacterial and viral pathogens, various parasites are known to negatively affect fish health and in some extreme cases, can result in death. A protozoan, such as *Myxobolus cerebralis*, the causative agent of Whirling Disease, has been found in several trout and salmon species in Michigan hatcheries (Beyerle and Hnath 2002). Larval cysts and adult parasitic worms of common cestodes, trematodes, and nematodes in the GL can be found to parasitize various organs, such as the intestine, stomach, or swim bladder, and are also found throughout the musculature of the fish (Hoffman 1999), causing erratic behavior that can result in predation, or in mortalities (Muzzall et al 2003; Torres et al 2002; Lafferty and Morris 1996).

Despite the increasing number of emerging and resurging fish diseases in the Great Lakes, very little is known about their ecology, including their mode of transmission, disease dynamics, subclinical carrier status, and pathogen reservoir. In general, pathogens can be transmitted vertically, from parent to offspring, or horizontally, from one organism to the other, either directly or indirectly via a vector (McCallum et al 2001; Begon et al 2002). Pathogens can be transmitted through the medium in which the

organism lives, such as water or air, or they can be transmitted by a vector (McCallum et al 2001). Commonly known vectors for disease include the mosquito, *Anopheles* spp., the vector for malaria, and the blacklegged tick, *Ixodes dammini*, the vector for Lyme disease (Phillips 2001; Magnarelli and Anderson 1988). Other parasites, such as *Argulus foliaceus*, are intermittent and can act as vectors for fish pathogenic viruses (Ahne 1985).

It has long been known that many organisms, such as fish, shorebirds, and mollusks, are integral in the life cycles of parasites (Roberts and Janovy 2005). For example, the tapeworm *Diphyllobothrium latum* utilizes a copepod and fish intermediate host in its life cycle to find its way to the final host, fish-eating mammals (Roberts and Janovy 2005). The bacteria *Vibrio vulnificus*, the causative agent of deadly gastroenteritis in immuno-compromised humans, employs the oyster in its dissemination (Wright et al 1996). Other recent studies on GL fish pathogens demonstrated that macroparasites, such as the sea lamprey (*Petromyzon marinus*), can harbor salmonid pathogens in their bodies, such as *Renibacterium salmonarium*, which is the causative agent of Bacterial Kidney disease, as discussed previously (Eissa et al 2006), *Flavobacterium psychrophilium* (Elsayed et al 2006b), and *Aeromonas salmonicida* (Faisal et al 2007), which are the causative agents of Coldwater disease and Furunculosis, respectively.

While it has not been shown that leeches are vectors for viral pathogens in the GL, they could become vectors for new viruses introduced to the LSC system. There are numerous ways that exotic organisms are introduced to the Great Lakes ecosystem. Mills et al. (1993) outlines several methods, including unintentional releases, ship-related introductions, deliberate releases, entry through or along canals, and movement along

railroads and highways. It is still unknown as to how a new virus, the Viral

Hemorrhagic Septicemia virus (VHSV), has arrived into the Great Lakes.

Viral Hemorrhagic Septicemia is a viral fish disease that has caused large-scale

mortalities in Europe and the North American Pacific Coast (Meyers and Winton 1995;

Skall et al 2005; Raja-Halli et al 2006). It made its first appearance in the Great Lakes in

muskellunge in LSC in 2003 (Elsayed et al 2006b). Viral Hemorrhagic Septicemia has

been implicated in several Great Lakes mass fish kills, including freshwater drum

(Lumsden et al 2007) and round gobies (Groocock et al 2007) in Lake Ontario. Fish

infected with the virus can exhibit no external signs, or can exhibit signs of petechiae or

large patches of hemorrhaging on the skin (Elsayed et al 2006b). Infected organs are

often congested with multiple hemorrhages in the kidney, liver, spleen, and intestines

with fluid-filled vesicles attached to the internal membrane of the swim bladder (Elsayed

et al 2006b).

The virus has been isolated from LSC muskellunge and Lake Ontario freshwater

drum, as well as mummichog (*Fundulus heteroclitus*), three-spine stickleback

(*Gasterosteus aculeatus aculeatus*), striped bass (*Morone saxatilis*), and brown trout

(*Salmo trutta*) in eastern Canada (Elsayed et al 2006b; Gagné et al 2007; Groocock et al

2007; Lumsden et al 2007). Leeches have been found attached to muskellunge,

freshwater drum, and walleye in LSC (Schulz 2008, unpublished data). Therefore, it is

possible that leeches could transmit the VHSV from fish to fish, as previously reported

by Ahne with the SVCV (1985).
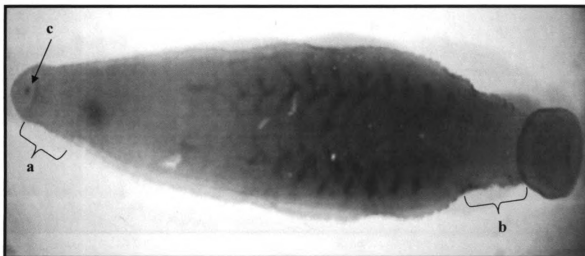
Figure 1. *Actinobdella pediculata* (Rhynchobdellida: Glossiphonida) has a flat, wide body, with a ventral oral sucker, more or less fused or continuous with the body, not distinctly expanded or set off from the body by a narrow neck (a); a caudal sucker separated from the body on a distinct pedicel with the rim of the caudal sucker thick and bulbous, without digitate processes (b); and one pair of eyes (c).
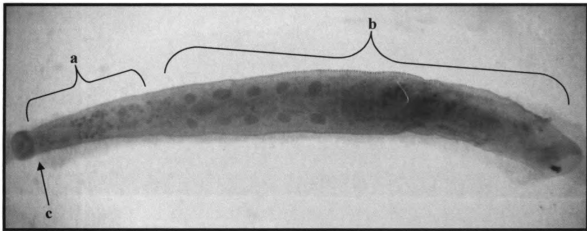
Figure 2. *Myzobdella lugubris* (Rhynchobdellida: Piscicolida) has a cylindrical body, often divided into a narrow anterior (a) and wider posterior region (b), with an oral sucker that is clearly set off from the body (c).
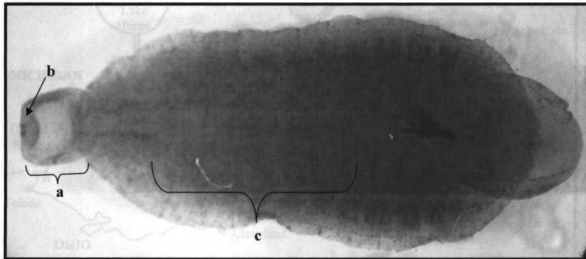
Figure 3. *Placobdella montifera* (Rhynchobdellida: Glossiphonida) has a flat body much wider than the head region; a ventral oral sucker distinctly expanded to form a discoid head, set off from the body by a narrow neck constriction (a); one pair of eyes (b); and a dorsum with 3 prominent tuberculate keels or ridges (c).
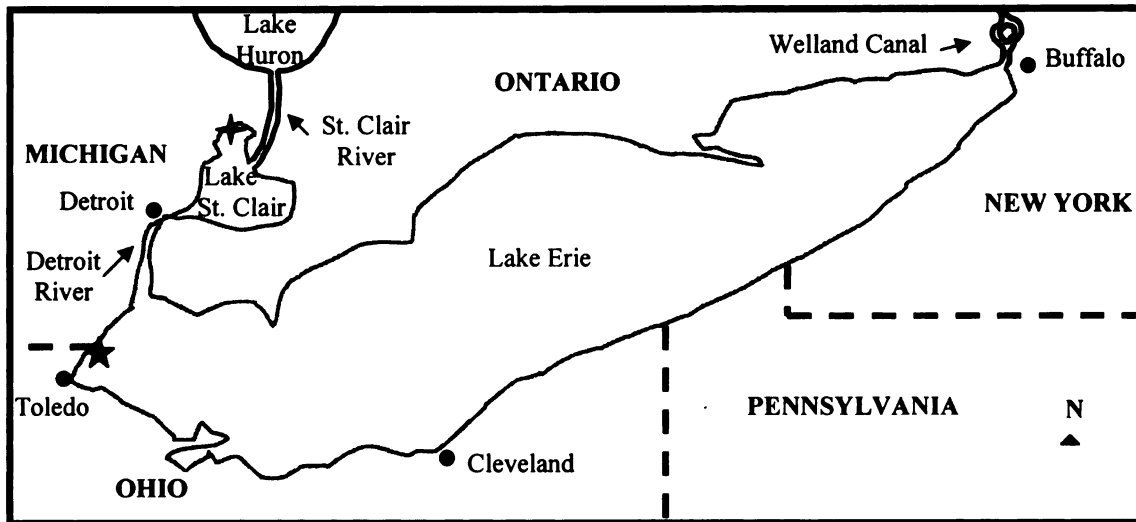
Figure 4. The Lake Erie Watershed, surrounded by the states Michigan, New York, Ohio, and Pennsylvania, and the Canadian province of Ontario, is connected in the east to Lake Ontario by the Welland canal and in the west to Lake Huron via the Detroit River, Lake St. Clair, and the St. Clair River. The four-pointed black star denotes the sampling location of the Michigan Department of Natural Resources trap nets (42°37'54.60"N, 82°45'54.60"W) in Anchor Bay, Lake St. Clair. The five-pointed black star denotes the commercial fishing trap nets (41°46'00.74"N, 83°24'58.09"W) in Lake Erie from which leeches were collected during this study.

CHAPTER TWO

LEECHES PARASITIZING FISH SPECIES IN LAKE ST. CLAIR, MICHIGAN AND

THE POTENTIAL IMPACT ON THE FISH HOSTS

ABSTRACT

Three species of leeches were found parasitizing fish species from Lake St. Clair,

Michigan, within the Lake Erie Watershed in the Great Lakes Basin. *Actinobdella*

pediculata, *Myzobdella lugubris*, and *Placobdella montifera*, identified based on

morphological keys, were attached to channel catfish, freshwater drum, northern pike,

northern shorthead redhorse sucker, quillback sucker, rock bass, smallmouth bass,

walleye, and yellow perch. This is the first report of the northern shorthead redhorse

sucker as a host for *A. pediculata*, which is highly host-specific for the freshwater drum.

The freshwater drum had the highest prevalence (36.21%), mean intensity (12.70

leeches/fish), and abundance (4.60 leeches/fish) compared to the other host species.

Leeches were found attached to the pectoral fin, pelvic fin, caudal fin, anal fin, adipose

fin, barbel cavity, body of the fish, dorsal fin, eye, gill cavity, and isthmus region. The

preferred attachment site of all leeches was the pectoral fins at 38.16%; however,

variation occurred among *A. pediculata* (gill chamber, 90.0%), *P. montifera* (body of the

fish, 60.0%), and *M. lugubris* (pectoral fins, 41.37%). Gross examination of attachment

sites revealed necrotic and hemorrhagic patches near the attachment site, severe nodular

swelling of the tissue, and a heavy infestation of leeches. Lesions associated with leech

attachment varied in size displayed hemorrhagic margins. Histological examination

revealed necrosis and expansion of muscle and dermal layers, infiltration of

macrophages, lymphocytes, and heterophils, hemorrhaging and subcutaneous edema.

INTRODUCTION

Leeches (Annelida: Hirudinea) are parasites known to cause physical damage to their hosts (Appy and Cone 1982; Roubal 1986; Kruse et al 1989; Noga et al 1990; Volonterio et al 2004). Leeches feed on blood and body fluids, thereby depriving their hosts of important nutrients (Sawyer 1986), while also transmitting trypanosomes (Lewis and Ball 1980; Kruse et al 1989) bacteria (Kikuchi and Fukatsu 2005), fungi (Raffel et al 2006), and viruses (Greenblatt et al 2004; Mulcahy et al 1990; Ahne 1985).

Leeches exhibit a marked diversity and host range as they parasitize on hosts such as annelids, crabs, crayfish, fish, frogs, reptiles, aquatic birds, cows, monkeys, horses, and elephants, as well as humans (Klemm 1972; Sawyer 1986; Mulcahy et al 1990; Greenblatt et al 2004; Hemmingsen et al 2005). Due to their abundance and distribution, leeches have also been used as bioindicators for pollution (Scrimgeour et al 1998; McCreanor et al 2008). Their ability to have similar, if not higher, levels of contaminants compared to the more often used bioindicators, such as fish, makes them a valuable resource for determining the ecological health of an ecosystem (Scrimgeour et al 1998; McCreanor et al 2008). Despite their widespread distribution in North America, little is known about leech biology and their impact on fisheries in the Great Lakes Basin (GLB).

The GLB is one of the most important waterbodies of the world and is home for 159 species of fish (Coon 1999). Lake St. Clair (LSC), which is a major lake within the Lake Erie Watershed and GLB, is a popular recreational lake that also has a diverse fish community. Lake St. Clair is the shallowest lake of the GL system, with a maximum natural depth of 6.4 m and shallow sandbars offshore of the littoral zone, which can

increase the density of macrophytes, thereby influencing the abundance of fish congregating in the shallowest portions of the lake (MacLennan et al 2003).

Leeches are typically found in shallower, lentic waters with submerged vegetation (Sawyer 1986; Klemm 1991), thus the abundance of fish in LSC is ideal for supporting leeches. Lake St. Clair is greatly utilized, particularly in recreational activities; therefore there is an increasing concern of factors that may disturb the health of its fragile ecosystem. Existing reports on leeches parasitizing fish of the GL (Klemm 1972, 1977, 1982, 1991) primarily focus on the anatomy and spatial distribution of leeches, and not their preference for host species and attachment site. The only record of leeches collected from LSC was *Myzobdella lugubris* and *Piscicola reducta* attached to logperch, *Percina caprodes*, and brown bullhead, *Ictalurus nebulosus* (Appy and Cone 1982).

Herein I report on the leeches parasitizing important LSC fish species; the preference of the leeches for different fish species and for different attachment sites on the fish; and the impact of the damage caused to the hosts.

MATERIALS AND METHODS

Study area description. Lake St. Clair is located in southeastern Michigan, and connects Lakes Huron and Erie via the St. Clair River and Detroit River (Figure 4). It is 38.6 km wide and 41.8 km long with a surface area of 1,100 km^2 (MacLennan et al 2003). As stated above, LSC is shallow compared to the GL with an average depth of 3.0 m, a maximum natural depth of 6.4 m, except for the shipping lanes, which have a maximum dredged depth of 8.0 m (MacLennan et al 2003). The fish community consists of a diverse mixture of warmwater and coolwater species (MacLennan et al 2003; Thomas and Haas 2004).

Fish and leech sampling. Fish were collected during May of 2006 and 2007 in five adjacent trap nets located in Anchor Bay, Lake St. Clair by the Michigan Department of Natural Resources (42°37'54.60"N, 82°45'54.60"W) (Figure 4). While there can be variation in the occurrence of size and number of leeches depending on the time of year, this study was conducted during May due to sampling constraints. The fish species caught in this study, as well as the specific location in Anchor Bay, are considered to be representative of the entire fish population in LSC. These locations were selected for this study due to the abundance and variety of fish species present, as well as their accessibility by the research vessel *Channel Cat* of the Mt. Clemens Fisheries Research Station, Michigan, which was made available by the Michigan Department of Natural Resources for this study. The trap nets had 1.8 m deep pots of 5.1 cm stretch mesh, 7.6 cm stretch mesh hearts and wings, and 91.4 m long leads of 10.2 cm stretch mesh.

Fish were removed from the nets at 48-72 h intervals and examined for the presence of leeches. The attachment sites were recorded for each fish and leeches were separated into vials containing lake water according to the fish number and attachment site. Fish length, weight, species, and capture location were also recorded for each fish from which leeches were collected. After collecting the leeches, the fish was released into the lake, unless needed for other investigations.

Twenty-one species of fish were examined for leeches, including the bluegill (*Lepomis macrochirus*), carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), common white sucker (*Catostomus commersonii*), freshwater drum (*Aplodinotus grunniens*), gizzard shad (*Dorosoma cepedianum*), golden redhorse sucker (*Moxostoma erythrurum*), lake sturgeon (*Acipenser fulvescens*), largemouth bass (*Micropterus salmoides*), muskellunge (*Esox masquinongy*), northern pike (*E. lucius*), northern shorthead redhorse sucker (*Moxostoma macrolepidotum*), pumpkinseed sunfish (*Lepomis gibbosus*), quillback sucker (*Carpiodes cyprinus*), rock bass (*Ambloplites rupestris*), smallmouth bass (*Micropterus dolomieu*), silver redhorse sucker (*Moxostoma anisurum*), walleye (*Sander vitreus*), white bass (*Morone chrysops*), white perch (*M. americana*), and yellow perch (*Perca flavescens*). The species and number of fish collected, as well as the number of infected fish, number of leeches, and mean weight and length for each fish species are listed in Table 1.

Data for this study is expressed as prevalence, mean intensity, and abundance. Prevalence is calculated by dividing the number of infected fish by the number of all fish examined and is reported as a percent. Mean intensity is calculated by dividing the total number of leeches in one species of fish by the number of infected fish of the same

species. Abundance is calculated by dividing the total number of leeches from one species of fish by the total number of fish examined (Bush et al 1997). The proportion of leeches is used to determine leech preference for different attachment sites, where proportion is calculated by dividing the number of leeches present at an attachment site by the total number of leeches collected in the study. Proportion is also used to calculate the leech preference for attachment sites based on the three specific leech species in this study. It is noteworthy that prevalence, mean intensity, and abundance located in Table 1 pertain only to the fish species from which the leeches were obtained.

Leech identification. Leeches are very muscular; therefore they were relaxed before preservation as recommended by Klemm (1982). Initially, two methods were compared for leech relaxation and fixation. In the first method, live leeches were placed in a Petri dish with 95% ethanol added dropwise until movement stopped. If the leech did not respond to physical stimuli, it was then fixed in 5% buffered formalin (J.T. Baker, Phillipsburg, NJ) for 24 hours, and then preserved indefinitely in 70% ethanol (Madill 1983). The second method was that described by Pritchard and Kruse (1982), which uses menthol crystals for relaxation. A Petri dish was partially filled 50% with lake water, to which menthol crystals (5-methyl-2-[1-methyl-ethyl] cyclohexanol, Sigma-Aldrich, St. Louis, MO) were added until the surface of the water was covered with the crystals. Alive leeches were then placed in the dish for 24 hours, after which the mucous was removed with paper towel, and then preserved in 70% EtOH (Pritchard and Kruse 1982). The first 100 leeches collected in this study were used in the relaxation method comparison above, with 50 leeches used for the first method and 50 leeches used for the

second method. The method of Pritchard and Kruse (1982) was found to be more effective, therefore it was used to relax the remaining leeches of the study reported here. Taxonomic identification of leeches followed primarily the dichotomous keys of Peckarsky et al (1990), while the original publications of Hoffman (1999) and Pennak (1989) were also used for validation to ensure accuracy of identification.

A representative specimen of each leech species was stained for further identification. Relaxed leeches passed through descending strengths of ethanol (70%, 50%, and 35%), a single stage of distilled water, and then stained with Mayer's hematoxylin stain (Sigma), each immersion lasting 15-30 min, depending on the size of the leech. Once the leech was stained, it was dehydrated through ascending stages of ethanol immersion (85%, 95%, and 100%), once again lasting 15-30 min depending on the size of the leech, and lastly, cleared by xylene (J.T. Baker). Cleared leeches were mounted on slides in Canada balsam (Sigma) for microscopic examination (Pritchard and Kruse 1982).

Assessment of fish damage caused by leeches. Lesions on fish caused by leech attachment were described and recorded. In the case of freshwater drum parasitized by *Actinobdella pediculata*, samples were taken from the gill musculature attachment sites and were preserved in 10% buffered formalin (J.T. Baker). The fixed samples were then embedded within paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin according to the methods detailed in Prophet (1992).

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Statistical analyses. Data on prevalence, intensity and abundance was tested for normality. If not normally distributed, the Kruskal-Wallis one way analysis of variance (ANOVA) on Ranks was performed. All pairwise multiple comparisons were performed according to Dunn's Method. In the instance where only two groups were compared, the Mann-Whitney Rank Sum Test was performed. All calculations were performed with the help of SigmaStat 3.0® (San Jose, CA). Analyses also included comparisons among the sites of leech attachment.

RESULTS

Leech identification. Along the course of this study, three leech species were found

attached to the examined fish. The leeches were identified as *Actinobdella pediculata*

Hemingway, 1908 (Rhynchobdellidae: Glossiphonidae), *Myzobdella lugubris* Leidy,

1851 (Rhynchobdellidae: Piscicolidae), and *Placobdella montifera* Moore, 1906

(Rhynchobdellidae: Glossiphonidae) with the help of taxonomic criteria outlined in

Peckarsky et al (1990) and Hoffman (1999). The proportion of the leech population in

Lake St. Clair was not equally distributed, with *M. lugubris* occurring at 92.01%,

followed by *A. pediculata* at 7.52% and *P. montifera* at 0.47% (Figure 5).

Leeches identified as *Actinobdella pediculata* had a mouth with a small pore in

the oral sucker through which a muscular proboscis was protruded; a flat body much

wider than the head region; an oral sucker ventral and more or less fused or continuous

with the body, not distinctly expanded or set off from the body by a narrow neck; a

caudal sucker separated from the body on a distinct pedicel with the rim of the caudal

sucker thick and bulbous, without digitate processes; one pair of eyes separated by less

than the diameter of one eye, without accessory eyes; a dorsum without white patches

and a white ring in neck region. These morphological criteria coincide with those

described by Peckarsky et al (1990). The leech was found parasitizing on the freshwater

drum; however, it was also found on two northern shorthead redhorse suckers.

Myzobdella lugubris had a mouth with a small pore in the oral sucker through

which a muscular proboscis was protruded; a cylindrical and narrow body often divided

into a narrow anterior region and a wider posterior region; and a concave, weakly

developed caudal sucker, which was narrower than the widest part of the body. All leeches identified as *Placobdella montifera* had a mouth with a small pore in the oral sucker through which a muscular proboscis was protruded; a flat body much wider than the head region; an oral sucker ventral and distinctly expanded to form a discoid head, set off from the body by a narrow neck constriction; one pair of eyes; a dorsum with 3 prominent tuberculate keels or ridges; and parasitic on fish. These morphological criteria also corresponded with the descriptions in Peckarsky et al (1990).

Leech prevalence, intensity, and abundance. Of the 2,117 fish examined, 165 individual fish were found with leeches attached, with a prevalence of 7.79% of examined fish being infected with leeches. The leeches were attached to nine of the 21 fish species examined (Table 1). The infected fish species were channel catfish, freshwater drum, northern pike, northern shorthead redhorse sucker, quillback sucker, rock bass, smallmouth bass, walleye, and yellow perch. The prevalence of leeches, however, varied greatly among fish species (P<0.001). The freshwater drum exhibited the highest prevalence (36.21%) of attached leeches, followed by the channel catfish (24.44%), yellow perch (13.89%), and walleye (12.24%, Table 1).

Statistical analysis indicated that, while there were no significant differences between the freshwater drum and channel catfish in terms of leech prevalence, both species had a higher prevalence than all other fish species examined (P≤0.05). No significant differences were observed among the Northern pike, quillback sucker, rock bass, smallmouth bass, and Northern shorthead redhorse in leech prevalence, which ranged between 2.68-4.84%. *Myzobdella lugubris* preferred the freshwater drum as a

host, with a prevalence of 32.76% (Table 3); however, this was not significantly different from the channel catfish or yellow perch. There were significant differences in *M. lugubris* prevalence between freshwater drum and walleye, quillback sucker, rock bass, northern pike, and smallmouth bass ($P \leq 0.023$). Additionally, the channel catfish was statistically different than the northern pike, rock bass, and smallmouth bass as a host for *M. lugubris* ($P \leq 0.017$).

A total of 1,064 leeches were collected from 165 fish, with a mean intensity of 6.45 leeches/infected fish. The freshwater drum accounted for the highest mean intensity (12.70 leeches/fish) and was significantly different than the other fish species ($P < 0.05$). The mean intensity of the walleye (5.50 leeches/fish, Table 1) and channel catfish (2.85 leeches/fish, Table 1) were also statistically significant compared to other species ($P < 0.05$). Differences among the northern pike, quillback sucker, smallmouth bass, and northern shorthead redhorse sucker were not statistically significant. The freshwater drum had the highest mean intensity of *Actinobdella pediculata* (2.26 leeches/fish, Table 2) compared to the northern shorthead redhorse sucker (1.50 leeches/fish, Table 2), which was significantly different ($P = 0.011$). However, the host preference of *Placobdella montifera* was not statistically significant among its host species (Table 4). The freshwater drum also had the highest mean intensity of *M. lugubris* (12.65 leeches/fish, Table 3) and was statistically different than all other fish species ($P \leq 0.023$), with the exception of the channel catfish.

The overall abundance of leeches was 0.50 leeches/examined fish, regardless if it was parasitized by leeches or not. The freshwater drum had the highest abundance of leeches (4.60 leeches/fish) and was statistically different than all other fish species

(P≤0.021). The abundance of leeches attached to the channel catfish (0.70 leeches/fish) differed significantly from the northern pike, quillback sucker, rock bass, smallmouth bass, and northern shorthead redhorse sucker (0.03-0.05 leeches/fish, Table 1, P≤0.022). As a host for *A. pediculata*, the freshwater drum had a higher abundance (0.44 leeches/fish) than the northern shorthead redhorse sucker (0.03 leeches/fish) and was statistically significant as well (P=0.011). However, the difference in abundance of leeches among the host species for *P. montifera* did not differ significantly (Table 4). The freshwater drum had the highest abundance of *M. lugubris* (4.14 leeches/fish, Table 3) and was statistically different than the other host species (0.03-0.67 leeches/fish, Table 3, P≤0.024), with the exception of the channel catfish (0.70 leeches/fish, Table 3). The channel catfish did differ significantly from the northern pike, rock bass, and smallmouth bass (0.03-0.04 leeches, Table 3, P≤0.015) as a host for *M. lugubris*.

Leech site of attachment. Leeches were attached at various external sites of the host (Table 5). The proportion of leeches showed that the pectoral fins were the most common attachment sites for leeches (38.16%), followed by the pelvic (19.92%), caudal (12.88%), and anal fins (10.43%, Table 5). Additionally, the attachment of leeches to the pectoral fins were statistically different than all other attachments sites (P≤0.043). The proportion of leeches attached to the pelvic fins (P≤0.004), anal fin (P≤0.004), and caudal fin (P≤0.002) all differed significantly from the leeches attached to the less common attachment sites, such as the barbel, body, dorsal fin, eye, gill chamber, and isthmus (Table 5).

The leech *Myzobdella lugubris* was the most abundant leech found in this study; therefore the specific attachment sites of *M. lugubris* were further scrutinized. The pectoral fins had the highest proportion of *Myzobdella lugubris* attached (41.37%, Table 5) and were statistically different than all other attachment sites ($P \leq 0.004$), with the exception of the caudal fin. The pelvic fins accounted for 21.45% of the proportion of *M. lugubris* and differed significantly from leeches attached to the barbel, body, dorsal fin, eye, gills, and isthmus ($P \leq 0.004$, Table 5). The proportion of *M. lugubris* attached to the caudal fin (13.79%, Table 5) also differed significantly from the aforementioned sites, including the anal fin ($P \leq 0.001$). In addition, the anal fin (11.24%, Table 5) was statistically different than the eye, gills, and isthmus ($P \leq 0.004$).

Actinobdella pediculata was predominantly attached to the gill chamber (90.0%), and to a much lesser extent, it was attached to the trunk of the fish (3.75%), and pelvic (2.5%), caudal (2.5%), and anal (1.25%) fins (Table 5). On the other hand, *P. montifera* was primarily found on the trunk of the fish (60.0%) and to a lesser degree on the isthmus and pectoral fins (20.0%, Table 5).

Gross and histopathological damage. Attachment of *Myzobdella lugubris* and *Placobdella montifera* to the fins and musculature of the fish were similar, therefore *M. lugubris* was used as a representative leech. The size of *M. lugubris* varied from host to host, however there was also variation of size at individual attachment sites (Figure 6a). Attachment of *M. lugubris* was also associated with pathological changes in the form of necrotic and hemorrhagic patches on fin tissue (Figure 6b). The invasive attachment of the caudal sucker caused hemorrhages surrounding the attachment site (Figure 6c).

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Additionally, while the intensity of leeches attached to a particular site varied from host to host, there were a few instances that exhibited a severe infestation (Figure 6d).

Actinobdella pediculata is a semi-permanent leech, therefore it attaches as a young leech to the gill chamber of its host, remains for the duration of its lifetime, and then detaches itself to deposit its eggs (Sawyer 1986). The caudal sucker of the juvenile leech was embedded into the opercular tissue as a holdfast structure to allow the oral sucker to search for suitable feeding sites; however, the feeding behavior of *A. pediculata* resulted in hemorrhagic areas surrounding the leech (Figure 7a). The attachment of *A. pediculata* resulted in severe nodular swelling of the tissue; however, there was no hemorrhage associated with it (Figure 7b).

As *A. pediculata* grew in size, it was more commonly found attached to the musculature in the gill chamber, as opposed to the opercular tissue (Figure 8a). In addition to juvenile *A. pediculata*, medium-sized leeches were also found associated with hemorrhagic areas surrounding the attachment site (Figure 8b). Larger specimens of *A. pediculata* were frequently attached to the gill chamber in groups of one to three leeches (Figure 9a); however, in a few instances, if *A. pediculata* was extremely large, only one specimen was found (Figure 9b). Additionally, as the size of the leech continues to grow, it can prevent the operculum from closing entirely, making it difficult for the host to breathe.

Once *A. pediculata* was removed from the gill chamber, lesions at the site of attachment remained. The lesions varied in size, mostly dependent upon the size of *A. pediculata*, and frequently had hemorrhagic margins (Figure 10a). The larger, more

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severe sites of attachment displayed a relatively large, circular, one to two cm in diameter, raised lesion with a hemorrhagic margin and central depression (Figure 10b).

In hematoxylin and eosin stained sections of the attachment sites, severe focal muscle necrosis, massive expansion of the subcutis by macrophages and lymphocytes, and infiltrates of macrophages, lymphocytes, and heterophils were observed. Healthy muscle tissue is usually more developed and lighter in color with more defined nuclei (Figure 11a), whereas necrotic muscle tissue is shrunken and darker in color and has less pronounced nuclei (Figure 11b). It was also found that the necrotic areas were surrounded by lymphocytes and macrophages, which are part of the inflammatory response to close off and isolate the dying tissue and cells (Figure 11c). Less common findings at the attachment sites were hemorrhaging and subcutaneous edema. Edema can cause marked expansion to occur, as seen in the dermis layer of a leech attachment site from a freshwater drum (Figure 11d).

DISCUSSION

Leech identification. The findings of this study demonstrated that the diversity of the leech community in Lake St. Clair is very low, as it was composed of only three species. As stated previously, there can be variation in the occurrence of size and number of leeches depending on the time of year; however, this study was conducted during May due to sampling constraints. One species of leech in particular, *Myzobdella lugubris*, was quite dominant (92.1%) throughout this study (Figure 5). *Myzobdella lugubris* has a very general distribution throughout freshwater and marine waters in North America and has a widespread host range as well (Klemm 1972; Davies 1973; White and Crisp 1973; Paperna and Zwerner 1974; Daniels and Sawyer 1975; Sawyer et al 1975; Klemm 1977; Becker and Dauble 1979; Appy and Dadswell 1981; Schramm et al 1981; Appy and Cone 1982; Tartar and Sheridan 1984; Muzzall et al 1987; Woods et al 1990; Klemm 1991). Due to its ability to exist in a wide range of environments, it was not unexpected for *M. lugubris* to be so abundant in this study.

Actinobdella pediculata is commonly found in the Midwestern states, mostly inhabiting areas where its preferred host, the freshwater drum, is present (Klemm 1982). It is known for having a very high host specificity for the freshwater drum (Hemingway 1908; Branson 1961; Sawyer 1972; Klemm 1982; Sawyer 1986; Bur 1994; Wolf et al 2008); although it has also been found free-living, under a rock (Bere 1931), and on one occasion, attached to a white sucker, *Catostomus commersonii* (DeRoth 1953). Therefore, the northern shorthead redhorse sucker (n=2) is a new host record for *A. pediculata*. The northern shorthead redhorse sucker has similar habitat requirements and

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physical characteristics to the freshwater drum (Trautman 1981) and could explain the

attachment of *A. pediculata.*

Actinobdella pediculata (Hemingway, 1908) (*Placobdella pediculata*, Hemingway, 1908)

Prevalence, mean intensity, and abundance (Table 2)

Site of infection: Anal fin, body of fish posterior to anal fin

Location: Lake St. Clair, Michigan, U.S.A.

New Host: Northern shorthead redhorse sucker (*Moxostoma macrolepidotum*)

Other reported hosts: Freshwater drum (*Aplodinotus grunniens*, Hemingway 1908;

Branson 1961; Sawyer 1972; Klemm 1982; Sawyer 1986; Bur 1994; Wolf et al 2008),

white sucker (*Catostomus commersonii*, DeRoth 1953)

Other reported localities: Lake Ontario, Lake Erie, Lake St. Clair, Mississippi River

(Illinois), Kentucky Lake (Kentucky), North America (Illinois, Iowa, Maine, Minnesota,

Missouri, Oklahoma, Wisconsin), Lake Texoma (Oklahoma), Lake Pepin (Minnesota)

Specimen deposited: USNPC, in submittal process

Placobdella montifera* was found attached to only a few individual fish and was

the least dominant leech species in this study (0.47%, Figure 5). Other studies have

indicated that *P. montifera* is rare in occurrence despite its generalist nature. It is found

throughout the eastern and Midwestern U.S., where it parasitizes a variety of salmonids,

sunfish, catfish, and bass (Harms 1960; Klemm 1982, 1991; Hoffman 1999; Moser et al

2006). Little is known about the biology and ecology of the *Placobdella* spp.; however,

P. montifera has been found attached to fish that prefer deeper, cooler water (Szalai and

Dick 1991). In this study, *P. montifera* was found attached to the freshwater drum, northern pike, northern shorthead redhorse suckers, and walleye; indicating that they might not be restricted to only deep, cool water fish species as hosts.

Leech prevalence, intensity, and abundance. The generalist *M. lugubris* was very dominant in LSC fish. Despite the wide host range and high abundance, the leech was not found on a number on species. For example, the rock bass was the most commonly examined fish, yet it accounted for one of the lowest prevalence of attached *M. lugubris*. The freshwater drum was the most common host species for leeches in this study. The feeding habits of freshwater drum consist of searching through sediment and vegetation for aquatic invertebrates and mollusks (Moyle and Cech 2003), which is also suitable habitat for leeches (Sawyer 1986). It is possible that, due to their habitat and feeding behavior, bottom-feeding fish are more susceptible to leech attachment, but more research is needed to support this.

Regarding leech prevalence, the northern pike, quillback sucker, and northern shorthead redhorse sucker were not significantly different compared to the freshwater drum. While the quillback sucker and northern shorthead redhorse sucker are similar in feeding behavior and habitat requirements to the freshwater drum, fewer specimens of northern pike, quillback sucker, and northern shorthead redhorse sucker were examined for leeches during the study, and could explain the low prevalence of each one.

The freshwater drum had the highest prevalence of *Myzobdella lugubris* and it differed significantly compared to the walleye, quillback sucker, rock bass, northern pike, and smallmouth bass. While *M. lugubris* is a commonly occurring leech (Davies 1973;

White and Crisp 1973; Paperna and Zwerner 1974; Daniels and Sawyer 1975; Sawyer et al 1975; Becker and Dauble 1979; Tartar and Sheridan 1984; Woods et al 1990; Klemm 1991), the freshwater drum has shown in this study that it is a preferred host for leech attachment, regardless of leech species. Therefore, it is not unexpected for the freshwater drum to have a high prevalence of *M. lugubris*. In addition to the freshwater drum, the channel catfish was also preferred as a host for *M. lugubris*, compared to the northern pike, rock bass, and smallmouth bass. As stated previously, the channel catfish also exists in suitable habitats for leeches, and it is not unexpected to have a high prevalence for leech attachment.

Leech site of attachment. The distribution of leeches differed greatly by attachment sites. The most common attachment sites were the pectoral fins, which were significantly different than all other attachment sites. The pectoral fins are a suitable site for leech reproduction, simple adhesion, and feeding (Sawyer 1986). They are also an area of least water resistance, particularly the interior portion, and could provide protection and prevent the leech from dislodging from the fish. The pectoral fins are also located near the gills and heart, which is a site of increased blood flow, thereby possibly making it an appealing site for feeding. Additionally, the pelvic fins, anal fin, and caudal fin were significantly different than the less common attachment sites, the barbel, body of the fish, dorsal fin, eye, gills, and isthmus region. The isthmus region is an exposed surface where leeches could easily be dislodged by the host (Rupp and Meyer 1954). The barbel, body of the fish, dorsal fin and eye are also more exposed than the other sites, making it possible for leeches to be easily dislodged from these sites as well.

The gills had a low distribution of leeches attached (7.52%, Table 5), which is most likely because the gills are the preferred attachment site for *Actinobdella pediculata*, and not the other two leech species (Sawyer 1986; Bur 1994). Therefore, while its distribution was low among all leech species, it was the most common site of attachment for *A. pediculata* (Table 5).

Due to its common occurrence in North America and this study, the attachment sites of *M. lugubris* were further investigated. The pectoral fins had the highest distribution of *M. lugubris* and were significantly different than all other attachment sites, with the exception of the caudal fin. As previously described, the pectoral fins are a suitable site for reproduction, simple adhesion, and feeding and could provide protection and prevent the leech from dislodging.

Gross and histopathological damage. Observation of gross and histopathological damage caused to the fish hosts at attachment sites were similar to earlier findings (Paperna and Zwerner 1974; Appy and Cone 1982; Roubal 1986; Noga et al 1990; Woods et al 1990). *Myzobdella lugubris* was found attached to the freshwater drum in high intensities (Table 1, Figure 6a). Previous studies have documented severe infestations of *M. lugubris* attached to the white bass, *Morone saxatilis* (Woods et al 1990) and *Cystobranchus virginicus* attached to the white catfish, *Ictalurus catus* (Paperna and Zwerner 1974). In addition to these findings, circular, necrotic areas were noted surrounding attached leech specimens (Figure 6b). Due to the consistency in size and shape of the circular areas, it is most likely that these were previous attachment sites. Inflammation and swelling were also associated with leech attachment (Figure 6b, 6d).

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The location of attachment for *Actinobdella pediculata* seemed to vary based on the size of the leech. Juvenile *A. pediculata* were mainly found attached to the inside of the operculum, embedded in tissue (Figure 7), where as the medium and larger adult *A. pediculata* were mostly found attached to the musculature in the gill chamber (Figures 10 and 11). Juvenile *A. inequiannulata*, a species with a similar life history to *A. pediculata*, are known to initially infest the gills, and then move on to the inside of the operculum (Sawyer 1986). Swollen, nodular lesions were also noted in this study (Figure 7b), along with hemorrhagic feeding sites (Figure 7a and 8b, Figure 10). Large, reddened lesions, swelling, and hemorrhagic areas have also been associated with the attachment of leeches in other studies (Appy and Cone 1982; Roubal 1986; Noga et al 1990). Lesions may allow the introduction of pathogenic microorganisms into the fish, especially where the dermal layer is missing (Appy and Cone 1982); however, more research is needed to support this.

The histopathological results reported here are also consistent with previous records of damage caused to fish, such as an extensive inflammatory response, focal and widespread hemorrhages, necrotic muscle tissue, and edema, which led to expansion (Figure 10; Paperna and Zwerner 1974; Appy and Cone 1982; Roubal 1986; Volonterio et al 2004). Sections from the freshwater drum revealed necrotic muscle tissue, which was surrounded by lymphocytes and macrophages (Figure 11b and c). The yellowfin bream, *Acanthopagrus australis*, showed an extensive inflammatory response in the upper palate, associated with the attachment of the marine fish leech, *Austrobdella bilobata* (Roubal 1986). While the histopathological findings do not show that the

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damage caused by the leeches is fatal, there is the potential for secondary pathogens to gain entry to the fish host (Burreson 2006).

It is evident that three specific leech species are parasitizing fish species in the Lake Erie Watershed, specifically Lake St. Clair, Michigan. While they were found on a variety of hosts, the leeches showed a high prevalence for the freshwater drum as a suitable host. The leeches also showed a high prevalence for attaching themselves to the fins of the fish in general, specifically the pectoral fins. In addition, gross and histopathological damage were noted, however the implications of long-term damage are still unknown and require further research.

Table 1. Prevalence, intensity, and abundance of leeches parasitizing fish caught from Lake St. Clair, Lake Erie Watershed, Michigan. (N/R = Not Recorded)

[a]The freshwater drum had the highest prevalence, mean intensity, and abundance, despite not having the greatest number of fish collected, such as the rock bass[b].

Fish species	Fish Weight (mean ± SD) (g)	Fish Length (mean ± SD) (cm)	# of Collected Fish	# of Fish with Leeches	Prevalence (%)	# of leeches	Mean Intensity	Abundance
Freshwater drum	1700 ± 430	46.6 ± 11.8	174	63	36.21[a]	800	12.70[a]	4.60[a]
Channel catfish	2640 ± 1430	46.9 ± 10.6	135	33	24.44	94	2.85	0.70
Yellow perch	200 ± 40	47.0 ± 9.0	36	5	13.89	13	2.60	0.36
Walleye	850 ± 550	50.7 ± 7.9	147	18	12.24	99	5.50	0.67
Northern pike	2450 ± 700	39.6 ± 11.1	62	3	4.84	3	1.00	0.05
Quillback sucker	1700 ± 920	48.9 ± 4.6	24	1	4.17	1	1.00	0.04
Rock bass	250 ± 90	48.4 ± 13.7	954[b]	31	3.25	42	1.35	0.04
Smallmouth bass	710 ± 170	45.9 ± 15.5	262	8	3.05	8	1.00	0.03
Northern shorthead redhorse	810 ± 220	40.9 ± 15.7	112	3	2.68	4	1.33	0.04
Bluegill	100 ± 70	15.2 ± 3.6	2	0	0	0	0	0
Carp	N/R	N/R	20	0	0	0	0	0
Common white sucker	970 ± 490	45.9 ± 5.3	16	0	0	0	0	0
Gizzard shad	100	20.3	1	0	0	0	0	0
Golden redhorse sucker	1000 ± 140	43.1 ± 3.6	2	0	0	0	0	0
Lake sturgeon	23600	156.2	1	0	0	0	0	0
Largemouth bass	N/R	36.2 ± 7.6	3	0	0	0	0	0
Muskellunge	8830 ± 3370	105.0 ± 11.0	25	0	0	0	0	0
Pumpkinseed	100 ± 50	15.9 ± 1.5	18	0	0	0	0	0
Silver redhorse sucker	2320 ± 1040	59.5 ± 5.2	56	0	0	0	0	0
White bass	620 ± 330	31.8 ± 1.5	4	0	0	0	0	0
White perch	170 ± 120	20.4 ± 2.7	63	0	0	0	0	0
TOTAL			2117	165	7.79	1064	6.45	0.50
MEAN ± SD							1.40 ± 2.93	0.31 ± 1.00

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Table 2. Prevalence, intensity, and abundance for *Actinobdella pediculata* attached to fish collected from Lake St. Clair, Lake Erie Watershed, Michigan.

Fish species	# of Fish Collected	# of Fish with *A. pediculata*	# of *A. pediculata*	Prevalence (%)	Mean Intensity	Abundance
Freshwater drum	174	34	77	19.54[a]	2.26[a]	0.44[a]
Northern shorthead redhorse	112	2	3	1.79	1.50	0.03
TOTAL	286	36	80	12.59	2.22	0.28
MEAN ± SD					1.88 ± 0.54	0.23 ± 0.29

[a]The prevalence, mean intensity, and abundance of leeches attached to the freshwater drum differed significantly (P=0.011) from the northern shorthead redhorse sucker.

Table 3. Prevalence, intensity, and abundance for *Myzobdella lugubris* attached to fish collected from Lake St. Clair, Lake Erie Watershed, Michigan.

Fish species	# of Fish Collected	# of Fish with *M. lugubris*	# of *M. lugubris*	Prevalence (%)	Mean Intensity	Abundance
Freshwater drum	174	57	721	32.76[a]	12.65[a]	4.14[a]
Channel catfish	135	33	94	24.44	2.85	0.70
Yellow perch	36	5	13	13.89	2.60	0.36
Walleye	147	17	98	11.56	5.76	0.67
Quillback sucker	24	1	1	4.17	1.00	0.04
Rock bass	954	31	42	3.25	1.35	0.04
Northern pike	62	2	2	3.23	1.00	0.03
Smallmouth bass	262	8	8	3.05	1.00	0.03
TOTAL	1794	154	979	8.58	6.36	0.55
MEAN ± SD					3.53 ± 4.03	0.75 ± 1.40

[a]**The freshwater drum had the highest prevalence, mean intensity, and abundance of leeches attached to it and was significantly different (P<0.005) than all other species.**

Table 4. Prevalence, intensity, and abundance for *Placobdella montifera* attached to fish collected from Lake St. Clair, Lake Erie Watershed, Michigan.

Fish species	# of Fish Collected	# of Fish with *P. montifera*	# of *P. montifera*	Prevalence (%)	Mean Intensity	Abundance
Northern pike	62	1	1	1.61	1.00	0.02
Freshwater drum	174	2	2	1.15	1.00	0.01
Northern shorthead redhorse	112	1	1	0.89	1.00	0.01
Walleye	147	1	1	0.68	1.00	0.01
TOTAL	495	5	5	1.01	1.00	0.01
MEAN ± SD					1.00 ± 0.00	0.01 ± 0.004

Table 5. Proportion of leeches attached to a specific external attachment site based on the total number of leeches collected in this study, as well as the individual number of leeches collected from *Actinobdella pediculata*, *Myzobdella lugubris*, and *Placobdella montifera*.

Attachment site	Total	A. pediculata	M. lugubris	P. montifera
Pectoral fins	38.16%[a]	0%	41.37%[c]	20.00%
Pelvic fins	19.92%	2.50%	21.45%	0%
Caudal fin	12.88%	2.50%	13.79%	0%
Anal fin	10.43%	1.25%	11.24%	0%
Gills	7.52%	90.00%[b]	0.72%	0%
Isthmus	3.76%	0%	3.98%	20.00%
Dorsal fin	3.48%	0%	3.78%	0%
Barbel	1.79%	0%	1.94%	0%
Body	1.79%	3.75%	1.43%	60.00%[d]
Eye	0.19%	0%	0.20%	0%
Adipose fin	0.09%	0%	0.10%	0%

[a]Based on the total number of leeches, as well as [c]*M. lugubris*, the pectoral fins had the most leeches attached, whereas [b]*A. pediculata* mostly attached to the gill chamber and [d]*P. montifera* was mostly attached to the body of the fish.
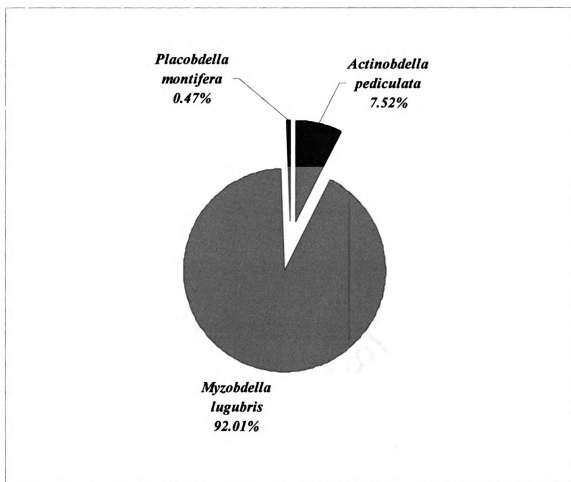
Figure 5. Proportion of each leech species (*Actinobdella pediculata*, *Myzobdella lugubris*, and *Placobdella montifera*) identified in the study, expressed as a percent.
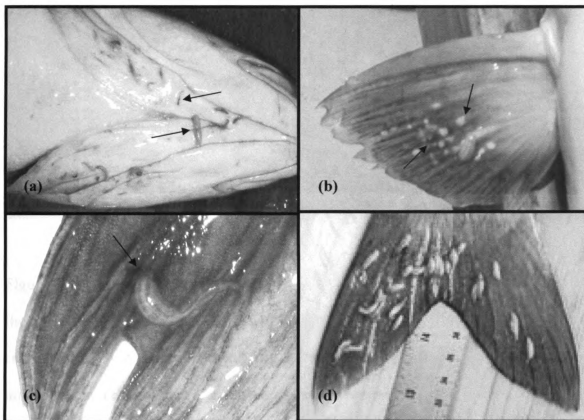
Figure 6. Gross attachment of *Myzobdella lugubris* attached to various locations on hosts: (a) the isthmus of a freshwater drum demonstrating the variation in size of *M. lugubris* (arrows); (b) *M. lugubris* attached to the pectoral fin of a channel catfish, surrounded by necrotic and hemorrhagic patches (arrows), which are former feeding sites; (c) the caudal sucker of *M. lugubris* (arrows) embedded into the anal fin of a northern pike, surrounded by a hemorrhagic ring; and (d) the caudal fin of channel catfish demonstrating the a heavy infection of *M. lugubris*.
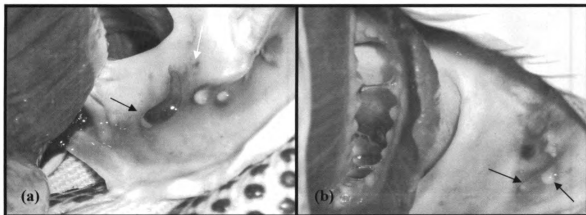
Figure 7. A juvenile specimen of *Actinobdella pediculata* attached to the interior of the operculum of two freshwater drum, (a) the caudal sucker of *A. pediculata* is embedded into the opercular tissue (black arrow), while the oral sucker left behind multiple hemorrhagic sites of previous feeding (white arrow); and (b) the attachment sites left behind by juvenile *A. pediculata* caused severe swelling (arrows), but had no hemorrhage associated with it.
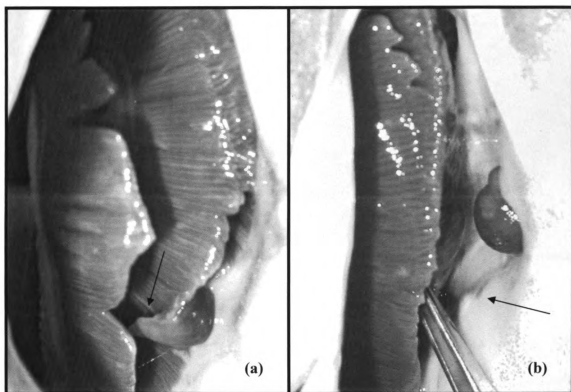
Figure 8. As *Actinobdella pediculata* increases in size, the location of attachment changes in the gill chamber of the freshwater drum, (a) the caudal sucker of *A. pediculata* embedded into the musculature of the gill chamber, while the oral sucker (arrow) searched for a suitable feeding site; and (b) the hemorrhagic feeding sites of *A. pediculata* (arrow).
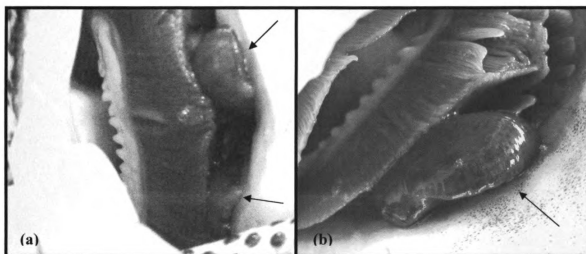
Figure 9. Adult *Actinobdella pediculata* were attached to the gill chamber of freshwater drum in groups of 1-3 leeches, (a) two adult *A. pediculata* attached to the gill chamber (arrows); and (b) whereas, if *A. pediculata* was large enough, only one was attached to the gill chamber (arrow).
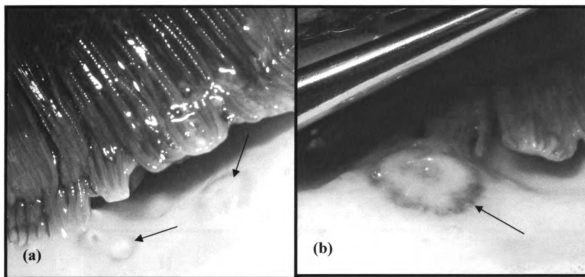
Figure 10. The gill chamber of the freshwater drum, where *Actinobdella pediculata* was attached and resulted in lesions, (a) the lesions varied in size, dependent upon the size of *A. pediculata*, and were frequently surrounded by a hemorrhagic ring (arrows); and (b) the attachment site of larger specimens of *A. pediculata* resulted in more severe lesions, with a hemorrhagic margin and central depression where the caudal sucker was attached.
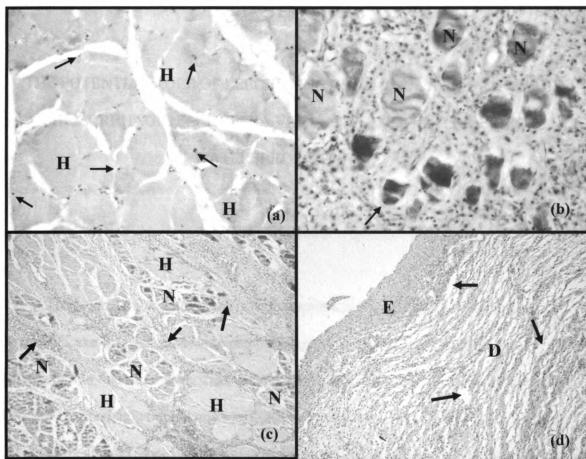
Figure 11. Histopathological damage caused by *Actinobdella pediculata* to the freshwater drum. Figure (a) demonstrates healthy (H) muscle tissue with prominent nuclei (arrows) (x200, H&E), whereas (b) shows necrotic (N) muscle tissue with less pronounced nuclei (arrow) (x200, H&E); (c) demonstrates healthy and necrotic muscle tissue, being surrounded by lymphocytes and macrophages (arrows), in order to engulf the dying tissue and cells (x100, H&E); and (d) shows epidermis (E) and dermis (D) where massive expansion (arrows) has occurred due to edema (x100, H&E).

CHAPTER THREE

THE POTENTIAL ROLE OF LEECHES IN THE TRANSMISSION OF VIRAL HEMORRHAGIC SEPTICEMIA VIRUS (VHSV) IN THE LAKE ERIE WATERSHED, MICHIGAN

ABSTRACT

The leech *Myzobdella lugubris* is widespread in the Lake Erie Watershed, especially Lake St. Clair, its role in pathogen transmission, is however, not fully understood. In this same watershed, several widespread fish mortalities associated with the Viral Hemorrhagic Septicemia virus (VHSV) were recorded. VHSV is an emerging disease in the Great Lakes Basin that is deadly to the fish population, yet little is known about its mode of transmission. To assess the potential role of *M. lugubris* in VHSV transmission, leeches were collected from Lake St. Clair and Lake Erie and pooled into samples of five. Cell culture and polymerase chain reaction (PCR) were used for virus isolation. Results showed that 57 of the 91 pooled leech samples were positive by cell culture for VHSV and 66 of the 91 pooled leech samples were positive by PCR for the VHSV. Two representative samples were sequenced for further genetic confirmation and genotype classification. VHSV detected within *M. lugubris* was homologous to the Great Lakes strain of VHSV, Type IV. This is the first record of the VHSV being detected from within a leech, specifically *M. lugubris*, and demonstrates the potential of *M. lugubris* being involved in VHSV transmission.

INTRODUCTION

The Viral Hemorrhagic Septicemia virus (VHSV) is a recent invader to the Great Lakes Basin (GLB) and has been associated with mortalities in a number of fish species, such as mummichog (*Fundulus heteroclitus*), three-spine stickleback (*Gasterosteus aculeatus aculeatus*), striped bass (*Morone saxatilis*), brown trout (*Salmo trutta*), round goby (*Neogobius melanostomus*), freshwater drum (*Aplodinotus grunniens*), and muskellunge (*Esox masquinongy*) (Elsayed et al 2006; Gagné et al 2007; Groocock et al 2007; Lumsden et al 2007). It has spread to four of the Great Lakes (not including Lake Superior), as well as several inland lakes in the states surrounding the GL. Viral hemorrhagic septicemia has been responsible for fish mortalities since 2003 in the GLB (Elsayed et al 2006b). It has also been associated with large-scale mortalities in Europe and the North American Pacific Coast (Meyers and Winton 1995; Raja-Halli et al 2006; Skall et al 2005).

While there are four known genotypes of VHSV (Types I-IV), the GL strain is a new sub-lineage, Type IVb, and little is known about its biology and epidemiology (Elsayed et al 2006b). Kim and Faisal (in press) have shown that the virus has a wide host range infecting many freshwater species, although some species (e.g., muskellunge and largemouth bass, *Micropterus salmoides*) are more susceptible than others. Despite its relative rapid spread within the GLB, little is known about the modes of its transmission.

Leeches (Annelida: Hirudinea) have a widespread distribution in the GLB (Klemm 1972, 1977, 1991; Muzzall et al 1987; Klemm et al 2003). In a previous study

(Schulz Chapter 2), leeches were found to parasitize nine out of the 21 fish species examined from Lake St. Clair, with the highest prevalence found on the freshwater drum, channel catfish (*Ictalurus punctatus*), rock bass (*Ambloplites rupestris*), and walleye (*Sander vitreus*). The leech population in Lake St. Clair was found to be dominated by *Myzobdella lugubris*, an intermittent, haematophagous feeder that is capable of causing hemorrhagic ulcerations to underlying musculature, as well as allowing opportunistic pathogens to gain entry (Noga et al 1990; Faisal in preparation; Schulz Chapter 2). In the study conducted on LSC, *M. lugubris* was especially abundant on the freshwater drum (Chapter 2), a species that has experienced extensive VHSV-related mortalities in Lake Ontario (Lumsden et al 2007). This raises the concern that leeches may be playing a role in its transmission.

There are other leech species that are known to contribute to the transmission of fish pathogenic viruses. For example, the marine turtle leech *Ozobranchus* sp. has been incriminated as a mechanical vector for fibropapilloma-associated turtle herpesvirus (FPTHV), based on molecular diagnostic assays (Greenblatt et al 2004). The fish leech *Piscicola salmositica* is a mechanical, and possibly biological, vector for the Infectious Hematopoietic Necrosis virus in sockeye salmon, *Oncorhynchus nerka* (Mulcahy et al 1990). In the same context, the fish leech *P. geometra* has been shown to be a mechanical vector for the Spring Viraemia of Carp virus (Ahne 1985).

In addition to being an intermittent feeder, *M. lugubris* is a good candidate leech to act as a vector because of its widespread distribution in North America, its wide host range, and it has been shown to heavily infest its host (Klemm 1972; Davies 1973; Paperna and Zwerner 1974; Daniels and Sawyer 1975; Sawyer et al 1975; Klemm 1977;

Appy and Dadswell 1981; Appy and Cone 1982; Muzzall et al 1987; Woods et al 1990;

Klemm 1991). Therefore, *M. lugubris* is an ideal leech species to determine if leeches

can contribute to the spread of VHSV.

MATERIALS AND METHODS

Study area description. Leeches were collected from the Lake Erie Watershed, which encompasses two major lakes in the GLB, Lake Erie (LE) and Lake St. Clair (Figure 4). These locations were chosen because of the VHSV-associated fish mortalities that have occurred in both lakes and the abundance of suitable leech hosts present. Lake Erie is the smallest and shallowest of all the GL, and is located near the southeastern portion of Michigan. It is 92 km wide and 388 km long with a surface area of 25,700 km^2. Lake Erie has an average depth of 19 m, but can be as deep as 64 m in some areas (USEPA 2006). Although it has declined over the past several years in Michigan, LE supports a commercial fishery for a wide variety of fish, including, but not limited to, the channel catfish, freshwater drum, bigmouth buffalo (*Ictiobus cyprinellus*), and white perch (*Morone americana*) (Baldwin et al 2002). Lake Erie is the warmest of the GL and also very biologically active, making it a suitable environment for leeches (USEPA 2006).

Lake St. Clair is also located in southeastern Michigan, and connects Lakes Huron and Erie via the St. Clair and Detroit River. It is 38.6 km wide and 41.8 km long with a surface area of 1,100 km^2 (MacLennan et al 2003). Lake St. Clair is shallow compared to the GL with an average depth of 3.0 m and a maximum natural depth of 6.4 m (MacLennan et al 2003). The fish community consists of a diverse mixture of warmwater and coolwater species (MacLennan et al 2003; Thomas and Haas 2004). In a previous study, LSC was determined to have an abundant population of leeches present (Schulz Chapter 2).

Leech collection. Leeches were collected on five separate dates, within the months of May and June 2008, from trapnets in Anchor Bay, LSC (42°37'54.60"N, 82°45'54.60"W) and the western basin of LE (41°46'00.74"N, 83°24'58.09"W) (Figure 4). While there can be variation in the occurrence of size and number of leeches depending on the time of year, this study was conducted during May and June due to sampling constraints. Fish species inspected for leeches include bluegill (*Lepomis macrochirus*), carp (*Cyprinus carpio*), channel catfish, common white sucker (*Catostomus commersonii*), freshwater drum, gizzard shad (*Dorosoma cepedianum*), golden redhorse sucker (*Moxostoma erythrurum*), lake sturgeon (*Acipenser fulvescens*), largemouth bass, muskellunge, northern pike (*Esox lucius*), northern shorthead redhorse sucker (*Moxostoma macrolepidotum*), pumpkinseed sunfish (*Lepomis gibbosus*), quillback sucker (*Carpiodes cyprinus*), rock bass, smallmouth bass (*Micropterus dolomieu*), silver redhorse sucker (*Moxostoma anisurum*), walleye, white bass (*Morone chrysops*), white perch (*M. americana*), and yellow perch (*Perca flavescens*). Fish species that had leeches attached include channel catfish, freshwater drum, rock bass, yellow perch, and walleye caught in trapnets. Due to the intermittent feeding nature of *Myzobdella lugubris*, samples collected during this study were not separated according to fish species or location.

Fish were removed from the nets after 24-48 hrs and inspected visually for attached leeches. Suspected *M. lugubris* specimens were detached from the hosts and deposited into large, one liter bottles of lake water. Overall, 456 leeches were removed and divided in 91 pools of ~five leeches. Leeches remained alive until returned to the laboratory, where they were identified as *M. lugubris* based on the morphological key of Peckarsky et al (1990).

Sample processing. All leech samples were briefly immersed into 100% ethanol (EtOH) (Thermo Fisher Scientific, Pittsburgh, PA) to eliminate any superficial bacteria that might have been present. Bacteria-free leech samples were then sectioned into smaller pieces with sterile scissors and scalpels in sterile Petri dishes. The purpose of sectioning the leech samples was to maximize extraction of leech viscera due to the thick cuticle of the leech, which cannot be easily homogenized. The sectioned tissue of pooled samples was placed into labeled whirlpak bags.

Leech samples were homogenized using a Biomaster Stomacher (Wolf Laboratories Ltd, Pocklington, York, UK) at the high speed setting for 2 min. Homogenates were diluted with Earle's salt-based minimal essential medium (MEM, Invitrogen, Carlsbad, CA) supplemented with 12 mM tris buffer (Sigma-Aldrich, St. Louis, MO), Penicillin (300 μg mL^{-1}), Streptomycin (300 μg mL^{-1}) (Invitrogen), and Amphotericin B (750 μg mL^{-1}, Invitrogen) to produce 1:4 dilution (w/v) of original tissues. Homogenized leech contents were removed with a sterile transfer pipette and dispensed into a sterile 15 ml centrifuge tube (Denville Scientific, Inc., Metuchen, NJ). Samples were then centrifuged at 5300 rpm for 20 min in the IEC Multi RF Centrifuge (Thermo Scientific).

Cell culture. Virus isolation was performed according to the standard protocols published by the American Fisheries Society Blue Book (2007). The *Epithelioma papulosum cyprinii* (EPC) (Fijan et al 1983) cell line was used for virus isolation. EPC was maintained and sub-cultured in 150 cm^2 tissue culture flasks (Corning, Lowell, MA) at 25°C using a growth formulation of Earle's salt-based Minimal Essential Media

(MEM) (Invitrogen) supplemented with 29.2 mg mL^{-1} L-glutamine (Invitrogen), Penicillin (100 IU mL^{-1}) and Streptomycin (0.1 mg mL^{-1}) (Invitrogen), 10% fetal bovine serum (Hyclone, Logan, UT), and sodium bicarbonate (7.5% w/v; Sigma-Aldrich).

After centrifugation, the supernatants were removed and inoculated into individual wells of a 96-well plate containing EPC cells grown with MEM (5% fetal bovine serum). Plates were incubated at 15°C for 7 days, and were observed for the formation of cytopathic effects (CPE) periodically. Second and third blind passages were performed and assessed for the presence of the VHSV.

Reverse-transcriptase polymerase chain reaction (RT-PCR) procedure. Total RNA was extracted from inoculated cells from all samples, regardless if CPE was noticed or not, using a QIAamp® Viral RNA Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Reverse transcription was accomplished by a two-step protocol using the Affinity Script Multiple Temperature Reverse Transcriptase RT-PCR™ (Stratagene, La Jolla, CA) following the manufacturer's instruction. The primer set used in this assay was recommended by the Office of International Epizootics (OIE 2006) for detection of a 811 base pair sequence of the VHSV nucleocapsid (N) gene: 5'-GGG GAC CCC AGA CTG T-3' (forward primer) and 5'-TCT CTG TCA CCT TGA TCC-3' (reverse primer). Polymerase chain reaction (PCR) was achieved by adding 2.5 μl of complimentary DNA, 1.5 μl of each primer, 12.5 μl of 2X GoTaq Green Mastermix (Promega, Madison, WI), and nuclease free water (Promega), to create a final volume of 25 μl, into each reaction tube. The reverse transcriptase was inactivated by subjecting the mixture to a 94°C for 2 min, and 30 cycles of PCR (denaturation for 30 s at 94°C,

annealing for 30 s at 52°C, and polymerization at 68°C for 1 min) in a Mastercycler

Personal Thermal Cycler (Eppendorf, Hamburg, Germany). The polymerization was

finalized by maintaining the mixture for a period of 7 min at 68°C. The product was

visualized by gel electrophoresis in 1.5% agarose gels.

Sequence analysis. Two representative VHSV-positive samples were chosen for further

confirmation of the VHSV by sequencing. The PCR products were purified with the

Promega Wizard® SV Gel and PCR Clean-up System. The concentration of the PCR

product was determined with the Quant-iT™ dsDNA BR Assay Kit (Invitrogen) and the

Qubit Fluorometer. The concentration of the PCR product was used to develop the 30 ng

DNA sample required for cycle sequencing. The final reaction for sequencing consisted

of 30 pM VHSV forward (or reverse) primer, 30 ng of viral DNA. Samples were

submitted to the MSU Research Technology Support Facility. After quality trimmed, the

two sequences were aligned by BL2SEQ (Tatusova and Madden 1999). The aligned

contig was used for multiple alignments performed by ClustalW (Thompson et al 1994)

with seven other VHSV genotypes, namely type I, Ia, Ib, II, III, Iva and IVb. The

phylogenetic analysis of the VHSV leech strain with 19 nucleoprotein encoding genes

from other species of rhabdovirus was done by using bootstrap test of phylogeny in

MEGA 4 (Tamura et al 2007). The Neighbor-Joining algorithm was chosen to create the

phylogenetic dendrogram containing 1000 bootstrap samplings. The VHSV leech

sequence was submitted to GenBank nucleotides database (Accession no. pending).

RESULTS

Virus isolation. Thirteen out of the 91 samples of leech homogenates caused CPE on EPC cell lines. Cytopathic effect was in the form of focal areas of rounded, refractile cells that progressed to full lysis of the cell monolayer within two to four days post-inoculation (Figure 12). Since tissue homogenates can be toxic to cultured cells, a second passage was performed. Results indicated that nine out of the original 13 positive samples from the first passage caused CPE, while samples #19, 52, 57, and 73 did not (Table 6). Moreover, pooled samples #51, 56, 77, and 87, which were negative in the first passage, caused CPE in the second passage (Table 6). A third passage was then performed during which the original 13 positive samples, three of the four second passage positive samples, and an additional 41 homogenate samples exhibited CPE (Table 6).

RT-PCR results. This assay was performed on third passage EPC cells, which had been incubated for 14 days. The primers 5'-GGG GAC CCC AGA CTG T-3' (forward) and 5'-TCT CTG TCA CCT TGA TCC-3' (reverse) produced an approximately 811 base pair product, which is characteristic of VHSV (Figure 13). A positive PCR result for the VHSV was obtained in 66 out of 91 samples (Table 6). Results from 80 out of the 91 samples were consistently positive or negative with both the third passage of cell culture and PCR (Table 6). However, 11 out of the 91 samples were not consistent with cell culture and PCR testing (Table 6).

Sequence analysis. Sequencing of the VHSV leech sample produced a 780 base pair

sequence that was identical to Great Lakes strain of VHSV IVb (Accession No.

DQ427105). Multiple alignment of the VHSV leech sequence was a 100% match to

VHSV Type IVb (Figure 14). The phylogenetic analysis indicated that within the

rhabdovirus family, the VHSV leech strain was grouped into a big clade with the seven

known strains of VHSVs (Figure 15). It showed a fairly short distance between the

VHSV leech strain and the VHSV strain type IVb. The closest clade to VHSV contained

a strain of rabies virus and two bat lyssus virus from Europe and Australia, respectively

(Figure 15). However, the BLASTn results suggested that the VHSV as a independent

species was much different than the other species of rhabdovirus in nucleoprotein genes.

DISCUSSION

Virus isolation. This is the first time that VHSV (of any genotype) has been isolated from leeches, or other invertebrates. *Myzobdella lugubris* is an intermittent, generalist species; therefore the detection of VHSV within *M. lugubris* poses a threat to VHSV-susceptible host species. In addition to residing in the same family, *P. geometra* and *M. lugubris* share similar distribution and host specificity (Meyer 1940; Davies 1973; Klemm 1972, 1991; Kvach and Skróa 2007; Öktener et al 2007). Also, similar to VHSV, the Spring Viraemia of Carp virus is an acute hemorrhagic and contagious disease among fish and is also a rhabdovirus (Garver et al 2007). As VHSV has been associated with mass fish mortalities in the GLB, the potential for *M. lugubris* to play a role in VHSV transmission is eminent.

While quantification was not performed in this study, the titer levels of the virus were most likely very low due to the lack of CPE in most samples until the third passage. This is common in many viruses; therefore, typically two blind passages are required for virus detection.

RT-PCR results and sequence analysis. Polymerase chain reaction is widely considered to be more sensitive and accurate than cell culture (Devold et al 2000; van Elden et al 2002; Knüsel et al 2007); therefore PCR was used as a preliminary confirmation of the VHSV. Due to the sensitivity of PCR, the ten samples that were cell culture negative, but PCR positive, were considered to be positive for the VHSV. It is most likely that the concentration of the virus was so low that it went undetected in cell

culture, but was detected by PCR. Also due to the sensitivity of PCR, the one sample that was cell culture positive and PCR negative was considered to be negative for VHSV. There is the potential that a virus other than the VHSV was present or that there might have been intracellular bacteria causing the CPE to occur. After initial detection of VHSV via PCR, the USDA APHIS National Veterinary Services Laboratory in Ames, Iowa also confirmed representative samples to be VHSV positive.

This is the first study documenting the presence of VHSV within *Myzobdella lugubris*. Due to the intermittent feeding behavior of *M. lugubris* and the still unknown mode of transmission of VHSV, it is imperative to determine if *M. lugubris* is a vector for VHSV or not. Additionally, because the VHSV leech genotype is the same that has been associated with mass mortalities of muskellunge and freshwater drum, there is an increased chance that *M. lugubris* might be spreading the virus. To fully determine if *M. lugubris* is a vector for VHSV, experimental infections and transmissions of the virus need to be conducted.

Table 6. Pooled leech sample results for the first (P1), second (P2), and third (P3)

cell culture passages, as well as PCR (~five leeches/pool).

[a]Samples which initially tested negative via cell culture, but were PCR-positive for

VHSV, and [b]samples which initially tested positive via cell culture, but were PCR-

negative for VHSV.

Sample #	P1	P2	P3	PCR	Sample #	P1	P2	P3	PCR
1	-	-	-	-	47	-	-	+	+
2	+	+	+	+	48	+	+	+	+
3	-	-	+	+	49	-	-	+	+
4[a]	-	-	-	+	50	-	-	-	-
5	-	-	+	+	51	-	+	-	-
6	-	-	+	+	52	+	-	+	+
7	-	-	+	+	53	-	-	-	-
8	-	-	+	+	54	-	-	-	-
9[a]	-	-	-	+	55	-	-	-	-
10	-	-	-	-	56	-	+	+	+
11	-	-	+	+	57	+	-	+	+
12	-	-	+	+	58	-	-	-	-
13	+	+	+	+	59	-	-	+	+
14	-	-	+	+	60	-	-	-	-
15	-	-	+	+	61[a]	-	-	-	+
16	-	-	+	+	62	-	-	-	-
17	-	-	+	+	63	-	-	+	+
18	-	-	+	+	64	-	-	+	+
19	+	-	+	+	65[b]	-	-	+	-
20	-	-	+	+	66	-	-	+	+
21	-	-	+	+	67	+	+	+	+
22	-	-	+	+	68[a]	-	-	-	+
23	-	-	+	+	69	-	-	+	+
24	+	+	+	+	70[a]	-	-	-	+
25	-	-	-	-	71	-	-	+	+
26	-	-	+	+	72[a]	-	-	-	+
27	-	-	+	+	73	+	-	+	+
28	-	-	+	+	74	-	-	-	-
29[a]	-	-	-	+	75	-	-	+	+
30	-	-	+	+	76	-	-	-	-
31[a]	-	-	-	+	77	-	+	+	+
32	-	-	+	+	78[a]	-	-	-	+
33	-	-	-	-	79	-	-	+	+
34	-	-	+	+	80	-	-	+	+
35	-	-	-	-	81	-	-	-	-
36	-	-	-	-	82	-	-	-	-
37	+	+	+	+	83	-	-	+	+
38[a]	-	-	-	+	84	-	-	+	+
39	+	+	+	+	85	-	-	+	+
40	-	-	-	-	86	-	-	+	+
41	+	+	+	+	87	-	+	+	+
42	-	-	+	+	88	-	-	-	-
43	-	-	-	-	89	-	-	-	-
44	-	-	+	+	90	-	-	+	+
45	+	+	+	+	91	-	-	-	-
46	-	-	-	-					
+	8	7	32	37	+	5	6	25	29
-	38	39	14	9	-	40	39	20	16
Total	46	46	46	46	Total	45	45	45	45

Figure 12. The comparison of (a) the cell monolayer of normal EPC growth in 96-well plates, and (b) the cell monolayer exhibiting cytopathic effect in the form of focal areas of rounded, refractile cells.
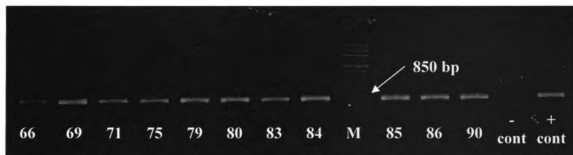
Figure 13. Agarose gel showing the bands from RT-PCR, used for the detection of VHSV (811 base pair). Pooled leech samples (#66, 69, 71, 75, 79, 80, 83-86, and 90) are representative VHSV-positive samples. The marker (M) used was 1.0 kb plus (Invitrogen).
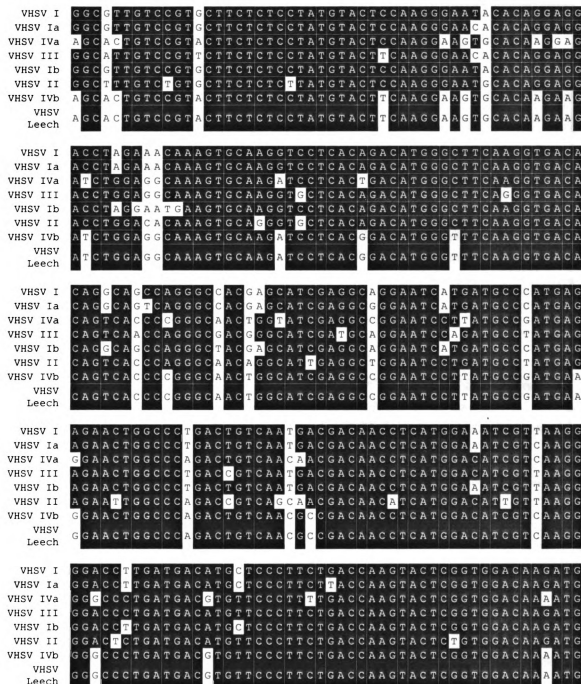
Figure 14. Multiple alignments of VHSV sequences, showing 100% match of the VHSV Leech

sequence to the DQ427105 VHSV IVb sequence, as well as homology to other VHSV genotypes,

VHSV I (Accession No. Z93412), VHSV Ia (Accession No. AJ233396), VHSV Ib (Accession No.

AY356632), VHSV II (Accession No. AY356743), VHSV III (Accession No. AY356720), VHSV IVa

(Accession No. AJ130926), VHSV IVb (Accession No. DQ427105).
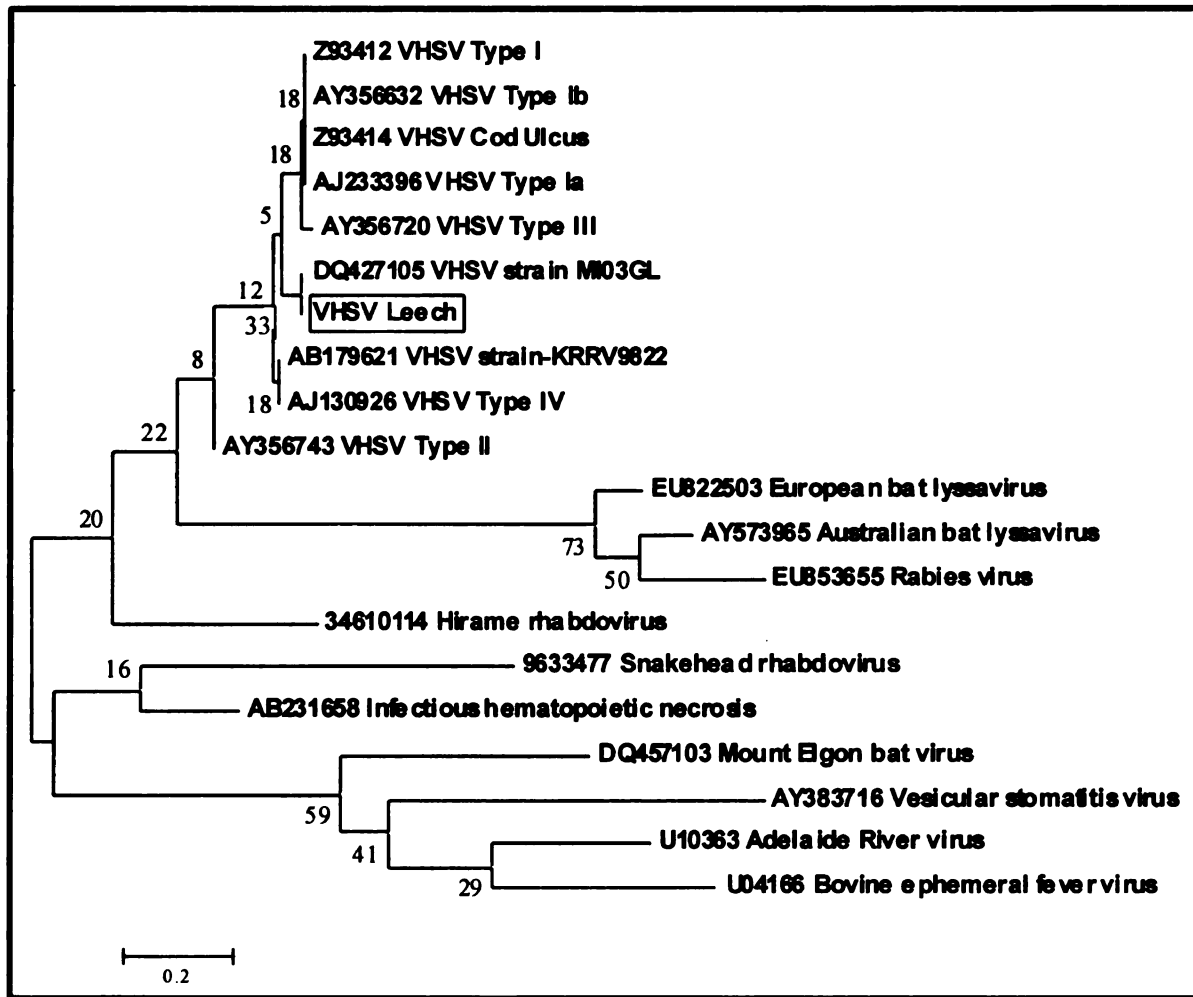
Figure 15. Phylogenetic distance tree generated by neighbor-joining analysis of 20 complete nucleoprotein gene open-reading frame sequences representing the Rhabdoviridae group, with special interest going to the VHSV genotypes. Bootstrap confidence values are shown at branch nodes. Notice the VHSV leech is homologous to the MI03GL strain (type IVb).

CHAPTER FOUR

BACTERIAL COMMUNITY ASSOCIATED WITH THE LEECH *MYZOBDELLA LUGUBRIS* FROM THE LAKE ERIE WATERSHED, MICHIGAN

ABSTRACT

Leeches are widespread in the Great Lakes basin, yet their potential to harbor disease-causing agents has not been investigated. The purpose of this study was to identify the bacterial community of the commonly occurring leech, *Myzobdella lugubris*, within the Lake Erie Watershed. Leech samples were collected from the pectoral fins of channel catfish and freshwater drum from Lake Erie in commercial trap nets and pooled into two samples based on host attachment. Bacteria from within the viscera of the leech were identified by sequencing the 16S rRNA (rDNA) gene of amplified community bacterial DNA extracted from pooled leech homogenate samples. One-hundred thirteen 16S gene sequences were checked for similarity to existing 16S sequences contained in two public databases: the Ribosomal Database Project and BLAST. A total of five genera belonging to three bacterial subdivisions were detected in leech homogenates. Bacteria belonging to the phyla Bacteroidetes, Beta-proteobacteria, and Verrucomicrobia were present in the leech samples. Samples also contained sequences from bacteria that are currently unclassified. The majority of bacteria found within leeches attached to channel catfish consisted of a number of sequences that could not be classified beyond the Domain Bacteria (63.64%). However, many of these sequences were homologous

(<45%) to the phyla Bacteroidetes. Beta-proteobacteria was the most abundant phyla within leeches attached to freshwater drum (86.21%), while Verrucomicrobia was the least abundant (1.72%). One of the five genera detected in the leech homogenates, *Flavobacterium psychrophilum*, is the causative agent of Bacterial Cold Water Disease. While the frequency of genera varies, bacteria associated with the two samples in this study are comparatively similar. This study represents the first report of microbial communities within the leech *Myzobdella lugubris* in the Lake Erie Watershed.

INTRODUCTION

Leeches (Annelida: Hirudinea) have been shown to be widespread throughout North America, and recently, Lake St. Clair and the Lake Erie Watershed (Klemm 1972, 1977, 1991; Muzzall et al 1987; Klemm et al 2003; Schulz Chapter 2). In a previous study in the Lake Erie Watershed, leeches were attached to nine out of 21 fish species examined, with a prevalence of 7.79% (Schulz Chapter 2). In addition, 1,064 leeches were found parasitizing 165 individual fish, with a mean intensity of 6.45 leeches/fish and an abundance of 0.50 leeches/fish (Schulz Chapter 2). *Myzobdella lugubris*, an intermittent, haematophagous feeder, was found to dominate the leech population.

Leeches can harbor potentially harmful bacteria and transmit it from host to host (Graf 1999; Kikuchi and Fukatsu 2005; Silver et al 2007; Laufer et al 2008). While there have been many studies regarding bacteria within leeches, no studies have been conducted concerning the bacterial communities within leeches from the Great Lakes Basin, specifically the Lake Erie Watershed.

The European medicinal leech, *Hirudo* spp., is of particular interest due its use in aiding venous congestion complications that occur following surgical procedures in humans and its unique relationship with *Aeromonas* spp. (Sawyer 1986; Silver et al 2007). It has been documented that *Hirudo* spp. can contain multiple *Aeromonas* spp., such as *A. veronii* Biovar sobria, *A. hydrophilia*, and *A. jandaei* (Graf 1999; Silver et al 2007; Laufer et al 2008). Infections have been known to occur in human post-operative patients, for which *Hirudo* spp. have been used for bloodletting (Silver et al 2007; Laufer et al 2008).

Pathogenic bacteria have also been found within the digestive organs of leeches. Kikuchi and Fukatsu (2005) found a *Rickettsia* spp. infection in the leeches *Torix tagoi*, *T. tukubana*, and *Hemiclepsis marginata*. *Rickettsia* is a known pathogen that seriously affects many fish species (Cusack et al 2002). Also, leeches that fed on infected fish contained pathogenic strains of *Aeromonas* spp. and *Pseudomonas* spp. in their digestive tracks (Snieszko and Bullock 1968). Additionally, Dombrowski (1952) demonstrated that *Piscicola geometra* transmits *Pseudomonas punctata* to carp. *Pseudomonas* species, such as *P. putida*, have been implicated as the cause of fish mortalities in aquaculture (Altinok et al 2006). Leeches are also known to disperse *A. hydrophila* from host to host (Negele 1975). *Aeromonas* spp. cause Furunculosis and mortalities in farmed and wild fish (Harikrishnan and Balasundaram 2005), as well as septicemia and diarrhea in humans (Janda and Abbott 1998).

The purpose of this study was to identify the bacterial community of *Myzobdella lugubris* within the Lake Erie Watershed. Due to its ability to provide a more detailed description of the bacterial communities within the leeches, the molecular method of 16S rRNA (rDNA) gene sequencing was preferred over traditional culture methods.

MATERIAL AND METHODS

Study area description. Leeches were collected from the western portion of Lake Erie, where *Myzobdella lugubris* and fish hosts were abundant. Lake Erie is the smallest and shallowest of all the Great Lakes (GL), and is located near the southeastern portion of Michigan (Figure 4). It is 92 km wide and 388 km long with a surface area of 25,700 km^2. Lake Erie has an average depth of 19 m, but can be as deep as 64 m in some areas (USEPA 2006). Although it has declined over the past several years in Michigan, Lake Erie supports a commercial fishery for a wide variety of fish, including the channel catfish (*Ictalurus punctatus*), freshwater drum (*Aplodinotus grunniens*), bigmouth buffalo (*Ictiobus cyprinellus*), and white perch (*Morone americana*), to name a few (Baldwin et al 2002). Lake Erie is the warmest of the GL and also very biologically active, making it a suitable environment for leeches (USEPA 2006).

Leech collection. Leech samples were collected during October of 2008 from Lake Erie in commercial trap nets (41°46'00.74"N, 83°24'58.09"W) (Figure 4). While there can be variation in the occurrence of size and number of leeches depending on the time of year, this study was conducted during October due to sampling constraints. In addition to the abundance of fish hosts and leeches, this specific location was chosen due to the accessibility by commercial fishermen. The trap nets had 1.8 m deep pots of 5.1 cm stretch mesh, 7.6 cm stretch mesh hearts and wings, and 91.4 m long leads of 10.2 cm stretch mesh.

Fish were removed from the nets after 48 hrs and examined for the presence of leeches. The attachment sites were recorded for each fish and leeches were separated into whirlpaks containing lake water according to the fish number and attachment site. *Myzobdella lugubris* heavily parasitized the channel catfish and freshwater drum in Lake St. Clair (Lake Erie Watershed) and were therefore chosen as hosts of interest for this study and examined for leeches. Leeches remained alive until returned to the laboratory, where they were identified as *Myzobdella lugubris* based on the morphological key of Peckarsky et al (1990).

Leech preparation. Leeches ranged in size from 7.5 mm to 20 mm. Leeches were dipped in 100% ethanol baths prior to dissection, in order to remove any contaminants and limit the presence of external bacteria. Leech viscera were aseptically removed, with the assistance of a dissecting microscope, and placed in a microcentrifuge tube containing 80% ethanol (Thermo Fisher Scientific, Pittsburgh, PA). Due to the small size of the leeches, intestinal contents were pooled into two samples and are referred to hereafter as leech guts. Eight leeches were collected from the pectoral fins of channel catfish and four leeches were collected from the pectoral fins of freshwater drum.

Sequence analysis. Genomic bacterial community DNA was harvested from the leech gut homogenates (30-50 mg) using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA) following the manufacturer's protocol. The PCR amplification of the bacterial 16S gene were performed using the universal eubacterial primer set 27f-1387r (27f: 5'-AGAGTTTGATC(AC)TGGCTCAG-3' and 1387r (5'-GGG

CGG WGT GTA CAA GGC-3') (Marchesi et al 1998). The PCR mixtures

(25µl/reaction) contained 20 pmol 27F and 1387R primers, 22mM Tris-HCL (pH 8.4),

55mM KCL, 1.65 mM MgCl$_2$, 220 µM dNTP's, 0.55 units recombinant *Taq* DNA

Polymerase, and 50 ng template DNA (all reagents from Invitrogen Life Technologies,

Carlsbad, CA, USA unless otherwise stated). The PCR amplification was carried out for

30 cycles of 94°C for 4 min., 56°C for 30 sec. and 72°C for 1.5 min. and final 7 min.

incubation at 72°C (modified after Sambrook and Russell 2001).

The expected size of PCR products was 1.36 kb. The PCR products were used to

construct 16S gene clone libraries using a TOPO TA Cloning Kit® (with pCR®2.1-

TOPO® vector and One Shot® TOP10 Chemically Competent *E. coli*, Invitrogen)

following the manufacturer's protocol. All clones were cultured on Luria-Bertani agar

plates (Fisher Scientific Inc., Pittsburgh, PA) containing 50 µg/ml Kanamycin, as

directed by the protocol supplied in the TOPO TA Cloning Kit®. Clones were screened

for positive transformation with PCR using the primer set M13 forward (5'-GTT TTC

CCA GTC ACG AC-3') and M13 reverse (5'-CAG GAA ACA GCT ATG ACC-3').

A total of 190 screened clones were submitted to the Michigan State University

Genomic Technology Support Facility (GTSF) for cell preparation and sequencing on a

3730 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). Sequences were

aligned and classified using the Ribosomal Database Project II (RDP II) Release 9.47

produced by Wang et al (2007). The RDP-II provides aligned and annotated rRNA

sequences and uses a naive Bayesian classifier to assign sequences to the RDP

Taxonomy. It provides taxonomic assignments from domain to genus, with confidence

estimates for each assignment. Fifty-five clones from the channel catfish leech sample

and 58 clones from the freshwater drum leech sample were successfully aligned. The RDP "Seq Match" was used to identify bacteria, while the RDP "Classifier" (95% confidence interval) was used to compare relative abundance of sequences. Multiple sequences were rechecked with the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information of the National Institute of Health.

RESULTS

Sequence analysis revealed the presence of four bacterial groups within the leech

viscera (Beta-proteobacteria, Bacteroidetes, Verrucomicrobia, and RDP-unclassified

Bacteria), although the frequency at which each group was found in the two samples

differed (Figure 16). With the exception of Verrucomicrobia, which only occurred in the

freshwater drum leech sample, all phyla were detected both leech samples. In the

channel catfish leech sample, RDP-unclassified Bacteria had the highest occurrence at

63.64% (Figure 16). While these were determined to be unclassified Bacteria, the RDP

library produced homologous match scores (<45%) to the Sphingobacteriales order

(Bacteroidetes). Interestingly, the group with the least amount of clones present in the

freshwater drum leech sample was RDP-classified as Bacteroidetes (16.36%). In the

channel catfish leech sample, Beta-proteobacteria were the most abundant bacterial group

detected (86.21%, Figure 16). The least abundant bacterial group in the channel catfish

leech sample was the Verrucomicrobia (1.72%, Figure 16).

A number of sequences within the Bacteroidetes phylum showed high homology

to *Flavobacterium* spp. (*F. johnsoniae* and *F. psychrophilium*) (Bacteroidetes) according

to RDP and BLAST (Table 7). In the freshwater drum leech sample, six of the nine

Bacteroidetes sequences were similar to *Flavobacterium* sp. (AAXX01000001.1l), with

the highest match at 94% (BLAST) and three of the nine sequences were similar to *F.*

psychrophilium (NC_009613.1l), with the highest match at 98% (BLAST, Table 7). In

the channel catfish leech sample, the only two sequences homologous to the phylum

Bacteroidetes were similar to *Flavobacterium* sp. (AAXX01000001.1l) with the match at

95% and *F. johnsoniae* (NC_009441.1l) with the match at 92% (Table 7).

The class beta-proteobacteria was the most abundant group of bacteria detected,

with RDP and BLAST searches producing matches for the genera *Chromobacterium*,

Thiobacillus, and *Vogesella*. In the freshwater drum leech sample, three of the 11 beta-

proteobacteria sequences closely resembled *T. denitrificans* (NC_007404.1l), with the

highest match at 92% (BLAST); however RDP matched the same three samples as

unclassified members of the Neisseriaceae family of bacteria (Table 7). The remaining

eight sequences of beta-proteobacteria were similar to *Vogesella* sp., with the highest

match at 100% (RDP, Table 7). In the channel catfish leech sample, 48 of the 50 beta-

proteobacteria sequences were also closely related to *Vogesella* sp., with the highest

match at 100% (RDP). The last two sequences from the channel catfish leech sample in

the Beta-proteobacteria were determined to be unclassified members of the Neisseriaceae

family of bacteria according to RDP, but according to BLAST, they were similar to

Chromobacterium violaceum, with the highest match at 96% (Table 7).

One sequence from the freshwater drum leech sample was determined to belong

to the phylum Verrucomicrobia. This sequence showed high homology to

Verrucomicrobium genera incertae sedis, with a match of 86% (RDP, Table 7).

DISCUSSION

This is the first report addressing the microbial community of *Myzobdella lugubris*. Results indicated that there are multiple bacterial groups within the viscera of *M. lugubris*. Bacterial communities have been documented in other species of leech, either as intestinal symbionts or as an infection within the leech. *Aeromonas* spp. such as *A. jandaei* and *A. veronii* have a symbiotic relationship with the European medicinal leeches *Hirudinea orientalis*, *H. verbana*, and *H. medicinalis* (Graf 1999; Kikuchi and Graf 2007; Laufer et al 2008). Infectious bacteria, such as *Rickettsia* sp., have been found within *Torix* spp. and *Hemiclepsis* spp. (Kikuchi and Fukatsu 2005).

With the exception of Verrucomicrobia, the bacterial communities within the freshwater drum sample and the channel catfish sample consisted of mostly similar phyla. Although the group that had the highest occurrence in the channel catfish leech sample was unclassified bacteria, they were similar to the order Sphingobacteriales (Bacteroidetes). The order Sphingobacteriales includes several species, such as *Sphingobacterium composti*, *S. antarcticus*, and *S. anhuiense*, which are bacteria commonly found in forest soil, composts, and aquatic environments (Shivaji et al 1992; Yoo et al 2007; León-Galván et al 2008; Wei et al 2008). However, a bacterium from Sphingobacteriales was recently isolated from diseased fish (Loch and Faisal, personal communication). It is not unexpected since *M. lugubris* feeds exclusively on blood and tissue fluids; however, it is unknown as to how bacteria from Sphingobacteriales found its way to the interior of *M. lugubris*.

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Bacteria from the Bacteroidetes phylum were found to be the least abundant

group in the channel catfish leech sample. A number of these sequences were similar to

Flavobacterium spp., including *F. psychrophilum*. *Flavobacterium psychrophilum* is the

causative agent of Bacterial Cold-Water Disease and Rainbow Trout Fry Syndrome,

which are known to cause high mortalities in salmonids and can increase their

susceptibility to other diseases (Nematollahi et al 2003). This finding underscores the

potential role of *M. lugubris* in *Flavobacterium* spp. transmission among susceptible fish.

The most abundant group present in the freshwater drum sample was unclassified

members of Neisseriaceae (beta-proteobacteria). Most of the sequences were similar to a

Vogesella sp. (85-100%, RDP), of which there are only two known species, *V. indigofera*

and *V. perlucida* (Grimes et al 1997; Chou et al 2008). *Vogesella indigofera* and *V.*

perlucida have both been found in groundwater (Grimes et al 1997; Gu and Cheung

2001; Whiteley et al 2001; Chou et al 2008). However, *V. indigofera* has been found

within the pyloric cecae of wild fish which had leeches, specifically *Piscicola geometra*,

attached to them (Goldschmidt-Clermont et al 2008). The bacterial community from

within *P. geometra* was not investigated; therefore, it is unknown if the leeches contained

V. indigofera or not (Goldschmidt-Clermont et al 2008). While the sequences in this

study were similar to a *Vogesella* sp., further research is needed to determine the potential

symbiotic, or pathogenic, relationship there might be between *M. lugubris* and *Vogesella*

sp.

The least abundant bacteria group in the freshwater drum sample was

Verrucomicrobia, which only had one sequence similar to a *Verrucomicrobium* species.

Verrucomicrobium spp. are found in soil and aquatic environments and are significant for

understanding the evolution of bacteria (Paissé et al 2008; Lee et al 2009). Verrucomicrobia bacteria have also been detected in toxic cyanobacterial blooms, although their role in the toxic bloom is unknown (Pope and Patel 2008). Bacteria from the Verrucomicrobia phylum have recently been found to oxidize methane, which enables the bacteria to reduce methane emissions to the atmosphere (Dunfield et al 2007). This is also the first report of a *Verrucomicrobium*-like species detected within the leech viscera, however the phylum Verrucomicrobia is still relatively new and it is unknown as to what kind of relationship the bacteria and leeches might have.

There is little variation in bacterial community composition in *Myzobdella lugubris* collected from channel catfish and freshwater drum. As expected, most of the bacteria detected from within the leeches were environmental bacteria typically found in soil and the aquatic environment; however, a further investigation is warranted to determine if there is a pathogenic relationship between *M. lugubris* and *Flavobacterium psychrophilum*.

This is the first study ever conducted on bacterial communities in leeches that are parasitic on fish. These types of studies are needed to better understand the role played by leeches in shaping the bacterial community structure of an ecosystem and in disease transmission. In general, the *M. lugubris* bacterial community seemed to be of extreme low diversity, especially when compared to other invertebrates collected from the same environment, such as the zebra mussel, *Dreissena polymorpha* (Winters 2008). This may be attributed to the haematophagous nature of leech feeding.

Table 7. The presence of bacteria from the Bacteroidetes phyla (*Flavobacterium* sp., *F. johnsoniae*, and *F. psychrophilum*), Beta-proteobacteria phyla (*Chromobacterium violaceum*, *Thiobacillus denitrificans*, and *Vogesella* sp.), and Verrucomicrobia phyla (*Verrucomicrobium genera incertae sedis*) detected within viscera from leeches collected to the channel catfish and freshwater drum.

Genus species	Channel catfish leech samples	Freshwater drum leech samples
Chromobacterium violaceum		x
Flavobacterium johnsoniae		x
Flavobacterium psychrophilum	x	
Flavobacterium sp.[a]	x	x
Thiobacillus denitrificans	x	
Verrucomicrobium genera incertae sedis		x
Vogesella sp.[a]	x	x

[a]Only *Flavobacterium* sp. and *Vogesella* sp. were present in both the channel catfish and freshwater drum samples.
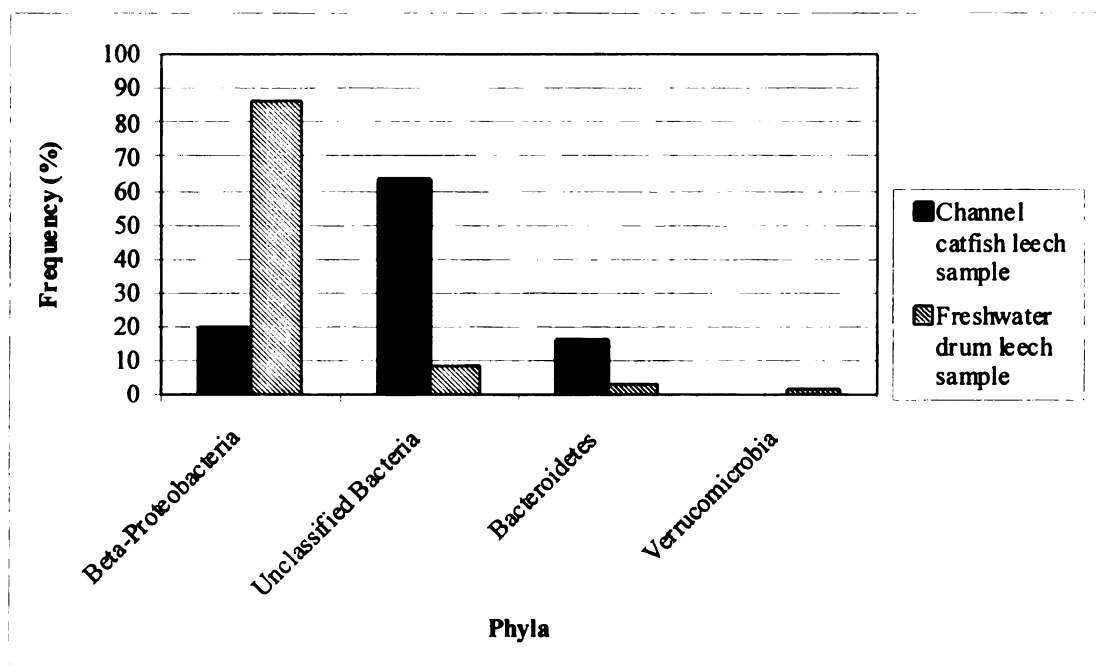
Figure 16. The occurrence of bacteria phyla (expressed as frequency) within the two leech samples; based on the RDP Classifier, using a 95% confidence threshold.

CHAPTER 5

CONCLUSIONS AND FUTURE RESEARCH

Although there have been studies regarding the distribution and ecology of leeches in the Great Lakes, there is still a lack of knowledge pertaining to leeches of the Lake Erie Watershed. This study has shown that leeches have the potential to cause damage to fish hosts as well as possibly spread disease, which may have negative effects on the fish population in the Lake Erie Watershed. Three species of leech, *Actinobdella pediculata*, *Myzobdella lugubris*, and *Placobdella montifera*, were found attached to fish hosts.

The leech *A. pediculata* was thought to be highly host-specific to the freshwater drum. This study is the first report of *A. pediculata* attached to two northern shorthead redhorse suckers. The *A. pediculata* that were attached to the northern shorthead redhorse sucker were smaller in size and were found attached to the anal fin and the musculature just posterior to the anal fin. As an adult, *A. pediculata* detaches from its host to deposit its eggs; then as a juvenile, it searches for its preferred host, attaches anywhere on the body, and then gravitates towards its preferred attachment site, the gill chamber. Due to the size and attachment site of *A. pediculata* on the northern shorthead redhorse sucker, the leeches were most likely juveniles that had not gravitated to the gill chamber yet. It is unknown if larger *A. pediculata* will attach to the northern shorthead redhorse sucker in the gill chamber, or only juveniles.

Leeches have an annual life cycle and different life stages can be found during different periods. This study was conducted over only one month; therefore, it would be of interest to sample for leeches intermittently throughout the year, specifically during winter, spring, summer, and fall months. It would also be helpful to conduct this study over at least two years, in order to better compare prevalence, intensity, and abundance data and statistical analyses.

In addition to determining the leech species present in the portion of the Lake Erie Watershed that was sampled, one species in particular, *Myzobdella lugubris*, was further examined in Chapters 3 and 4. *Myzobdella lugubris* is a wide-ranging leech in regards to distribution in North America, as well as preferred host species. There are few studies which have shown leeches to act as vectors for viral diseases. While this study was not able to confirm that *M. lugubris* is a vector for VHSV, it is the first report of VHSV being detected within *M. lugubris*. Due to the still unknown mode of transmission, the implication of *M. lugubris* acting as a vector for VHSV could have severe effects on the fish population. Further research is required in order to determine if *M. lugubris* is a vector for VHSV or not. Experimental infections of susceptible host species, particularly the freshwater drum, should be conducted with *M. lugubris*. Freshwater drum in replicate tanks would be injected with the VHSV, and would then be exposed to VHSV-free leeches over a period of time. Leeches, now infected with VHSV, would be detached from infected hosts, and reattached to VHSV-free fish hosts. After a period of time, both leeches and fish hosts would be tested for VHSV, which will determine whether leeches can transmit the disease.

It would also be of value to determine if they are mechanical vectors, passing the virus from one host to another, or biological vectors, having the virus replicate within the leech while the virus is transmitted. While *A. pediculata* and *P. montifera* are semi-permanent hosts, it would also be of interest to discover if they too harbor VHSV. Since they are not intermittent feeders, the presence of VHSV from within both leeches would imply that leeches can acquire the virus through the water and not just through blood feeding.

Myzobdella lugubris was found to not only contain the VHSV, but different groups of bacteria as well. The bacterial communities within leeches have been studied extensively regarding medicinal leeches, but this is the first report of bacterial communities within leeches that are parasitic on fish, specifically *M. lugubris*. While the bacterial community of *M. lugubris* was not very diverse, the pathogenic *Flavobacterium psychrophilum* was detected, which could have implications regarding the spread of *F. psychrophilum* in the fish population. Due to the size of leeches collected for this study, the entire viscera was removed and examined for bacteria. It would be beneficial to focus on specific internal organs of the leeches, because the different locations of the bacteria within the leech could have different implications as to their purpose.

Overall, it would be valuable to compare the data in all three studies on both Lake St. Clair and Lake Erie. Due to sampling constraints (i.e., suitable access to sites), the Chapter 2 study was conducted on only Lake St. Clair, the Chapter 3 study was conducted on both lakes, and the Chapter 4 study was conducted on only Lake Erie. Since both of these lakes are similar to each other in fish distribution, and they share waterways, it would be of interest to determine how the leech distribution and the

presence of bacterial communities compare. The three studies mentioned above have implications regarding the health of the fish population of the Lake Erie Watershed, and therefore should be continued in order to determine the role leeches have in the spreading of diseases in the Great Lakes.

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