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**THE DIGESTIVE COMPOSITION AND PHYSIOLOGY OF WATER MITES**

by

**ADRIAN AMELIO VASQUEZ**

**DISSERTATION**

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

**DOCTOR OF PHILOSOPHY**

2017

MAJOR: PHYSIOLOGY

Approved By:

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Advisor                                                                    Date

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# DEDICATION

I dedicate this work to my beautiful wife and my eternal companion. Together we have seen what is impossible become possible!

## ACKNOWLEDGEMENTS

It has been a long journey to get to this point and it is impossible to list all the people who contributed to my story. For those that go unnamed please receive my sincerest gratitude. I thank my mentor and friend Dr. Jeffrey Ram. I was able to culminate my academic training in his lab and it has been a great blessing working with him and members of the lab. We look forward to many more years of collaboration. My committee took time out of their busy schedules to help me in achieving this milestone. The Physiology staff is second to none.

I thank my parents for their unwavering support. I thank the parents of my wife and the community which they lead. We are extremely fortunate for the support of extended family in Michigan and elsewhere. Thanks to the global community of friends that also provided support.

Specific work could not have been done without the assistance of generous collaborators. Ashley Dowling and Ray Fisher (University of Arkansas) guided us on collection and identification of water mites. Patrick Hudson and his team at USGS assisted in collecting and identification of chironomids and copepods. Dr. David Cook graciously invited me to his house where he shared his expertise. Katherine Gurdziel assisted with important bioinformatics. Gary, C.J., Ron, and Joe at the USDA lab in Beltsville, MD also provided tremendous support.

We are also grateful for the funding agencies who provided much needed assistance in completing the many different projects. These include the EPA, IMSD NIH training program, Dr. Ikuko Kato, the Physiology Department, the Wayne State University Graduate School, Office of the Vice President for Research, Stellar Links Internet Company, the Sharon Ram Award for Aquatic Research, the Belle Isle Aquarium in Detroit, and our latest donation from Dr. David Cook and an anonymous donor.

I thank God for all the opportunities I was given without which nothing would have happened.

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# CHAPTER 1 - INTRODUCTION TO WATER MITE BIODIVERSITY AND PHYSIOLOGY

Water mites (Hydrachnidiae) are colorful, biodiverse, aquatic arachnids that can be found in most freshwater habitats worldwide. Water mites belong to the Parasitengona which form part of the subphylum Chelicerata which constitute some of the most biologically diverse taxa in the phylum Arthropoda (Wohltmann 2000). Water mites can be easily found in multiple types of aquatic habitats such as ponds, rivers, lakes and even hot springs with only one family found in marine environments (Cook and Mitchell 1953, Smit and Alberti 2009). They are very conspicuous aquatic invertebrates with sizes ranging from 250 µm to well over 5 mm (Cook and Mitchell 1953). They are easy to collect and it's reported that under three hours one can typically expect to collect 600 specimens representing 13 genera in a typical Northern Michigan habitat (Cook and Mitchell 1953). Globally, it is estimated that over 6000 species have been described to date, with many more species lacking proper descriptions (Di Sabatino et al. 2008).

## Biodiversity of Water Mites in the Laurentian Great Lakes

Water mites are an underappreciated "charismatic microfauna" in freshwater environments. However, Wayne State has played a classical and critically important role in understanding the systematics of water mites (Cook 1954, 1967, 1974, 1976). My research presented in this thesis may help revive Wayne State's leadership in this area and contribute to better understanding of the biodiversity of water mites in the Nearctic especially within the Laurentian Great Lakes habitats. In the following chapters I will present biodiversity work on the water mites of Toledo Harbor (found within Lake Erie) and of Blue Heron Lagoon (a habitat on the island of Belle Isle which connects with the Detroit River near Lake St. Clair), all forming part of the connecting waterways of Lake Huron and Lake Erie. This entire habitat has been an area of intense pressure from the expansion of metropolitan Detroit and Toledo. Multiple

important uses include being a source of drinking water for metropolitan Detroit, a fishery resource, and a recreational area for thousands of American and Canadian residents (Baustian et al. 2014). This entire area has been classified as an Area of Concern (AoC) during the 1960s and has been the subject of many studies including research on chemical contamination, fisheries stock assessment, and benthic populations (Baustian et al. 2014).

Although water mites constitute a significant presence in benthic populations of invertebrates and present unique and varied forms in the ecology of their habitats, they have been mostly ignored by freshwater ecologists and have generally been reported in large groupings as “Acari,” and this may be partially due to their taxonomic complexity (Fernandez and Fossati-Gaschignard 2011). That is why studies on water mite biodiversity are important to assist future ecological aquatic studies around the Great Lakes which can enhance the health status assessment of these important freshwater habitats. In the Nearctic alone, which encompasses the Great Lakes, a conservative estimate of species number is 1500 with new genera being reported at an average of one each year (Smith et al. 2010). Not much is known about the genetic diversity of water mites in the Laurentian Great Lakes, an issue raised by a recent paper of Trebitz et al. (2015) that highlighted the lack of Laurentian Great Lakes water mite species genetic representation in public databases. Lack of knowledge on water mite biodiversity is also apparent in other regions such as the Neotropics with some locations reporting no known fauna such as Belize (Goldschmidt 2002). This work contributes to knowledge of the biodiversity of water mites in the Laurentian Great Lakes. I have worked with the leading water mite experts of North America including Dr. David Cook, Dr. Ian Smith and the team that led the 66th annual Acarology Summer Program at Ohio State University, which I attended this year. My analysis of water mite external structure using both laser confocal fluorescent microscopy and scanning electron microscopy can assist with the systematics of the biodiversity of water mites beyond the

Nearctic.

**Life History and Ecological Roles of Water Mites**

Water mites are true aquatic organisms that spend the majority of their adult life underwater although some can swim away from their benthic habitats to capture prey or as larvae when looking for a host. They undergo a complex life cycle that includes aerial parasitic larvae and predaceous deutonymphs and adults (Smith et al. 2010). Their biodiversity has been linked to that of the equally biodiverse aquatic, nematocerous Diptera such as chironomids and mosquitoes (Smith et al. 2010). In the Great Lakes water mites prey upon and parasitize chironomids (midges), some of which are known to cause allergies due to their hemoglobin (Failla et al. 2015); mosquitoes and other invertebrates of human pathological importance such as nematodes and copepods (Smith et al. 2010, Werblow et al. 2015).

It is expected that water mites, being highly diverse, would also need to partition their aquatic habitats for resources in order to persist. This is the case as they are known to be predatory on other freshwater invertebrates including cladocerans, oligochaetes, ostracods and odonate larvae (Proctor and Pritchard 1989, Martin 2004). Some genera specialize on feeding exclusively on Diptera eggs such as *Hydrachna* (Proctor and Pritchard 1989). Water mite larvae are also known to paras1989itize several groups of flying insects, including dragon flies, mosquitoes and chironomid midges (Martin 2004). They were also shown to partition the host so that many genera could be found parasitizing one host (Martin 2004).

The impact of water mites on their prey both as predator and parasite is significant. Multiple studies have demonstrated this effect on prey items such as mosquitoes, chironomids, water boatman, cladocera and copepods (Matveev et al. 1989, Ten Winkel et al. 1989, Cassano et al. 2002, Martin 2004, Esteva et al. 2006, Milne et al. 2009, Kirkhoff et al. 2013, Sanchez et al. 2015, Werblow et al. 2015). In a recent review water mites were suggested to be strong

candidates for the use of mosquito biocontrol (Atwa et al. 2017).

Water mites are also known to be prey for other aquatic organisms including fish and turtles (Marshall 1933, 1940a). I have also found water mites in the diet of round gobies (unpublished data) one of the many invasive species that have contributed to the loss of billions of dollars from the Great Lakes economy (Allan et al. 2013). Water mites may form parts of previously uninvestigated complex trophic interactions that occur in aquatic habitats such as inadvertent prey of birds that feed on parasitized dipteran (pers. comm. Ian Smith) or as parasitic larvae, such as the genus *Unionicola*, which parasitize mussels which may be food for other organisms such as raccoons (Mitchell 1955). Despite these important ecological roles in trophic interactions, water mites are typically not accurately sampled using standard techniques in biomonitoring and are therefore not well represented in collected samples (pers. comm. Dr. Ian Smith). Until more effort is exerted on the water mites of North America, population and community studies on aquatic ecosystems will not be accurately representative.

Water mites are known to inhabit diverse ecotypes and have an extensive biogeography with many groups particularly adapted to the habitat in which they are found. Some genera such as *Torrenticola* are flattened and inhabit fast moving water such as in streams and rivers (Fisher et al. 2015). Others such as *Lebertia*, which has been studied in this work, are found in both lotic and lentic habitats such as stream, ponds and lakes in colder waters such as the Great Lakes (Marshall 1912, Gerecke 2009). Some water mites are also known from subterranean waters and from hot springs that may reach temperatures up to 50 °C (Cook and Mitchell 1953). In some cases, the dietary choices of some genera of water mites found in interstitial and subterranean habitats are unknown. My research presents molecular tools that can be used to increase the knowledge in this important area and contribute to the general understanding of water mite ecology as it relates to diet composition and greater trophic interactions.

Water mites are considered a useful group of organisms for use as bioindicators for health assessments of aquatic habitats (Goldschmidt et al. 2016). Work such as presented here will encourage future researchers to include water mites in their biomonitoring assessments and will encourage better interest in mainstream ecological studies done on aquatic habitats by North American scientists. A comprehensive review by Smith et al. (2010) has outlined some reproductive and behavioral strategies of water mites. In this review the complexities of courtship behavior are mentioned and sexual dimorphism is briefly discussed (Smith et al. 2010). It is of note that water mites have a very complicated life cycle particularly when it comes to selecting a mate, choosing a site to breed, selecting a host to parasitize and determining habitat types. The scope of this work does not address some of these aspects but laboratory observations were made of clutch sizes of *Lebertia quinquemaculosa,* a species studied in this work, and an average of 30 larval water mites were observed in the clutches (Video can be seen at: http://sun.science.wayne.edu/~jram/ramlab.htm). Research on this aspect of water mite life history and ecology is still lacking.

**Physiology of Water Mites**

Water mite physiology is an area of interest because of the complex life cycle of water mites and the general lack of information on any of the major systems that they use for survival. Many questions arise when working with water mites and very few answers can be found in the literature. A brief look at the major organ systems can be obtained from the review by Smith et al. (2010) but many questions go unanswered. These questions include: How do water mites subdue their prey? Do water mites use venom as do other arachnids? What exactly are they eating in their natural habitat? What is the method of osmoregulation used by water mites? How do water mites survive the parasitic larval stage attached to aerial insects? Do water mite larvae parasitize the same species of insects whose larvae they prey upon in the water as adult predatory

water mites? This work uses molecular technology to address questions on diet composition and physiology.

The review of freshwater invertebrates by Smith et al. (2010) had only one previous and outdated citation on work done on water mite digestive physiology, which was published in 1938 (Bader 1938). More recent morphological descriptive work on the mid-gut, excretory organ and other anatomical features of two freshwater water mites and one from a marine environment revealed structural features whose functions are still not completely settled (Smit and Alberti 2009, Shatrov 2010b, Shatrov 2010a). Understanding the physiological mechanisms used by the digestive system of water mites is highly intriguing due to the unique, controversial anatomy of a blind gut (Mitchell 1970). Past work has indicated that there is no direct anatomical continuity between the mid - and hind gut (Wohltmann 2000). This dissertation includes research on defecation and electron microscopic observations of the gut in relationship to this controversy.

Water mites, just like their cousins, the terrestrial mites and ticks, feed by injecting secretions of enzymes that “pre-digest” prey tissue, allowing the water mites to feed on a liquefied diet (Di Sabatino et al. 2000, Smith et al. 2010, Chmelar et al. 2016). A study of the mouthparts of several genera of water mites demonstrates that the structural morphology of water mite mouthparts has been perfected for this type of feeding (Mitchell 1962). Although a great body of information is available for terrestrial mites and tick digestive physiology, comparable information on water mite digestive physiology is lacking. Previous work to understand water mite diets have involved laboratory feeding experiments and the use of PCR and chironomid specific primers and DNA sequencing (Martin et al. 2015). However, mites collected from the field and tested by this method have not yet been described. To my knowledge this dissertation describes the first work to implement a molecular approach to analyze water mite diet composition directly from field-collected water mites. Whether water

mites are specialists, with different species of water mites each targeting one prey species, or generalists, with a species targeting multiple prey in nature, can begin to be answered more directly using genetic and morphological methods to identify water mites and their prey.

In order to develop tailor-made or effective strategies against aquatic insects greater knowledge about the basic physiology of their predators is needed. Water mites are predatory and feed on the larvae of chironomids, some of which are called “blood worms” owing to a high concentration of an invertebrate hemoglobin (Proctor and Pritchard 1989). In ticks, water mites’ cousins, hemoglobin is considered toxic and when ingested it is digested and detoxified in the tick gut (Chmelar et al. 2016). Next generation sequencing is being used as a tool to identify tick salivary proteins that may be useful to address the many health related challenges in tick disease (Chmelar et al. 2016). Generally, when strategies are implemented to control pest arthropods, the applications are non-selective and are applied as widespread exposure affecting both unwanted and native species. The information gleaned from water mite digestive physiology has the potential of being used as a selective and targeted approach at controlling pest aquatic invertebrates.

The use of vital dyes and/or green fluorescent protein (GFP) tagged prey items has also been useful for studying digestive physiology and has led to novel discoveries including the recent revelation of alternative digestive tract pathways to remove waste in comb jelly fish (Maxmen 2016). Transmission electron microscopy has assisted in characterizing the water mite digestive system but the use of laser confocal microscopy to track vital dyes such as fluorescein diacetate (FDA) has not previously been reported. This work uses this method to characterize the digestive passage of ingested food into water mites. Water mites fed vital dyes and visualized by confocal microscopy can help to determine passage of food through the water mite digestive system and to study which water mite tissues are likely to be involved in the digestive

processes.

The digestive enzymes that water mites use in digestion have never been reported before. It is possible that the gut region of water mites contain proteases that digest hemoglobin and other proteins, esterases and lipases that digest lipids, and glycolytic enzymes that digest carbohydrates. However, without any physiological experiments this information is not available thus far. To begin to address these questions it is necessary to develop systems of laboratory water mite rearing and feeding experiments. Then the use of biochemical and molecular techniques can be applied to begin to answer the many questions on the profound lack of knowledge regarding water mite digestive physiology and the contribution water mites play in Great Lakes food web assemblages. This works provides a preliminary look into this.

The respiratory physiology of water mites presents a unique challenge since water mites have an open circulatory system and have to rely on movement of body musculature in order to get important resources such as oxygen to different parts of the body. The anatomy of trachea of water mites has been described by careful dissection and observation, revealing a complex network of disconnected trachea on the integument (Mitchell 1972). The trachea were observed as having a blind end and the other end going into the body of the mite and postulated to supply oxygen to the internal tissues (Mitchell 1972). Another study observed an anastomosed web of trachea at a point lateral to the brain in other species of water mites (Wiles 1984). Confocal microscopic work presented in this dissertation includes data that may give a new perspective on the trachea of water mites and may lead to further understanding of this important physiological system. Given the potential importance of water mites to human health and Great Lakes ecology and the great lack of knowledge about their physiology, now is an opportune time to advance our knowledge about the physiology and diets of water mites.

# CHAPTER 2 - MOLECULAR DNA BARCODES OF WATER MITES

(This chapter contains previously published material. See Appendix A.)

## Preface

This chapter is comprised of two works completed in this thesis. Section A is a study on the genetic diversity of water mites from the Toledo Harbor region where we found several genera of water mites including two genera (*Krendowskia* and *Koenikea*) for which there was no genetic molecular barcodes previously published. This paper has now been published in the International Journal of Acarology.

Section B is a study of the populations of water mites from Blue Heron Lagoon, Belle Isle Detroit where we have collected mites that were used for subsequent chapters in this thesis. Here we report three new genetic molecular barcodes for the following genera: *Albia*, *Madawaska* and *Hydrochoreutes*, which had no genetic representations in the public databases. This chapter has been prepared for submission for publication.

## Section A: New Molecular Barcodes of Water Mites (Trombidiformes: Hydrachnidiae) from the Toledo Harbor Region of Western Lake Erie, U.S.A. with First Barcodes for *Krendowskia* (Krendowskiidae) and *Koenikea* (Unionicolidae).

## Abstract

Water mites are a highly diverse group of arachnids that are morphologically difficult to analyze and for which many species have yet to be described. Furthermore, the representation of Great Lakes water mites in cytochrome oxidase 1 gene (COI) DNA barcode databases has been reported to be practically nil. To help remedy this gap in taxonomic knowledge, water mites collected in 2012 – 2013 in benthic samples from the Toledo Harbor region (Maumee River and Maumee Bay) of Western Lake Erie were identified to genus, their COI barcodes amplified and sequenced, and their relationships in a neighbor-joining tree determined. *Limnesia* yielded a clade with multiple branches, part within 1% of previous GenBank sequences and others less

than 95% similar. Barcodes of *Krendowskia* and *Koenikea* are the first for these genera in GenBank. This analysis contributes new molecular barcodes for water mites in the Laurentian Great Lakes including the first publicly available barcodes for two genera.

**Introduction**

Water mites play important roles in the freshwater environment as prey, parasite, and predator. As prey for other organisms, water mites have been found in the stomachs of various freshwater vertebrates, including trout (Marshall 1933), snapping turtles (Marshall 1940a), bayou darter (Knight and Ross 1994), queen loach (Pesic et al. 2013) and largemouth bass (Hodgson et al. 2008). Water mites feed on other animals both as parasites during their larval stages and as voracious predators as adults, particularly by preying on chironomids (Proctor and Pritchard 1989). Water mites feeding on insect larvae are capable of significantly impacting the size of their prey populations (Ten Winkel et al. 1989). Globally, there are more than 6,000 species of water mites have been identified (Di Sabatino et al. 2008), likely an underestimate of the total number of species since in North America fewer than "half the species expected to occur… are named" (Smith et al. 2010).

Despite these important roles in freshwater food webs and ecological relationships, water mite genetic information is greatly lacking in current literature. A recent review of COI barcode data for all known Great Lakes aquatic taxa highlighted the absence of DNA barcodes for Great Lakes water mite species and noted that barcode libraries were generally lacking for invertebrates that are taxonomically difficult to identify (Trebitz et al. 2015). Sequencing of DNA from a specimen can be helpful in identifying species when reference DNA sequences associated with particular taxa are present in DNA databases, such as GenBank (Benson et al. 2007) and the Barcode of Life Database (BOLD) (Ratnasingham and Hebert 2007, 2013). A frequently used sequence for identifying animal taxa is the "barcode" region of mitochondrial

cytochrome oxidase I (COI) (Hebert et al. 2003a). DNA barcode sequences can be especially useful for organisms that require complicated preparation for morphologically based identification and whose taxonomy is poorly resolved; for life stages, such as larvae, that may not have taxonomic keys; or for specimens that have been damaged making morphological characters indistinguishable. Some researchers have examined COI barcode sequences of water mites, some only to family level (Young et al. 2012), and others to genus (Telfer et al. 2015). Recently, DNA barcodes have been used to differentiate water mite species from Borneo (Pesic and Smit 2014, 2016) and Montenegro (Pesic et al. 2012). Whole mitochondrial genomes have been obtained for a few selected species (e.g., Edwards and Ernsting (2010) and Edwards et al. (2011)), and several small groups of species have been associated with COI barcodes (e.g., Martin et al. (2010), Edwards et al. (2010), Stalstedt et al. (2013), and Fisher et al. (2015)). However, in the North American Great Lakes, reference barcode databases lack numerous genera and species of water mites (Trebitz et al. 2015).

This paper examines DNA barcodes of water mites collected in benthic samples from Toledo Harbor, a port in Ohio, USA within Lake Erie. The present paper provides novel DNA barcodes of water mites referenced to morphological taxonomic identifications including the first and only molecular barcodes that are publicly available for *Krendowskia* Piersig, 1895 (Krendowskiidae) and *Koenikea* Wolcott, 1900 (Unionicolidae).

**Methods**

Benthic samples were collected with a hand-operated bottom dredge (AMS, Ben Meadows, Janesville, WI), from western Lake Erie and the Toledo Harbor region of the lower Maumee River in the spring and summer of 2012 and 2013, and from adjacent North Maumee Bay and the Ottawa River in August 2012 as described previously. Environmental variables, such as collection depth, presence of vegetation, etc. were recorded for all sites. Samples were

immediately washed on a 0.5 mm sieve, held on ice in 80% ethanol for a few hours until being washed in 90% ethanol on the 0.5 mm sieve, and then stored in 90% ethanol at 4 °C until shipment to EcoAnalysts Inc. (Moscow, ID) for sorting and identification (Failla et al., 2016).

Water mites were identified by EcoAnalysts to genus using Smith et al. (2001) and then returned to the Ram laboratory for further analysis. For two specimens of *Krendowskia* we dissected the gnathosoma in order to verify that these specimens were not *Geayia*, another genus in the Krendowskiidae.

For most specimens, COI barcode sequences were determined by the Canadian Center for DNA Barcoding (CCDB) in Guelph, Ontario. Whole mites were immersed in 30 µl of 100% ethanol in individual wells of CCDB's standard 96-well processing plate for shipping. CCDB used Folmer's universal COI primers, HCO2198 and LCO1490 for amplification and sequencing, performed quality assessment of the sequences, and then uploaded the sequences to the BOLD database for further analysis (Folmer et al. 1994). For the two specimens of *Krendowskia* that were dissected, DNA was extracted prior to dissection by lifting the dorsal plate and extracting DNA by a standard method. After morphological analysis, the exoskeletons were archived in the University of Arkansas water mite archive. The extracted DNA was amplified and sequenced using the same COI primers as CCDB as in (Failla et al. 2016).

Resultant water mite COI sequences were compared to sequences in the GenBank and BOLD databases using BLAST (Altschul et al. 1990) to identify previously sequenced organisms that matched within 3% of the specimen's COI sequence. MEGA6 software (Tamura et al. 2013) was used to construct a neighbor-joining tree using the Maximum Composite Likelihood method and to calculate pairwise differences within our sequences. Sequences reported in this paper have been uploaded to GenBank as accession numbers KX139041 – KX139059, KY111434, and KY111435.

**Results**

*Koenikea*, *Krendowskia*, and *Limnesia*, and one specimen identified only to "Acari" were identified among 21 water mites found in western Lake Erie benthic samples. *Koenikea* and *Limnesia* were found in both bay and river locations, but they did not co-occur in any individual site. Whereas the benthic samples in which *Koenikea* were recovered came from shallow bay and river sites 1 -2 m in depth, *Limnesia* specimens were found in deeper regions (2 – 3 m) of Maumee Bay, except for two *Limnesia* specimens from the Maumee River (1.5 m depth).

Five *Krendowskia* specimens were acquired in the Maumee River at depths of approximately 1.5 m. *Krendowskia* specimen 1SMB81313 4, illustrated in Figure 1, and specimen 1SMB81313 3, with a percent difference of less than 1% from each other were confirmed further by morphology. *Krendowskia* specimens were distinguished from *Arrenurus*

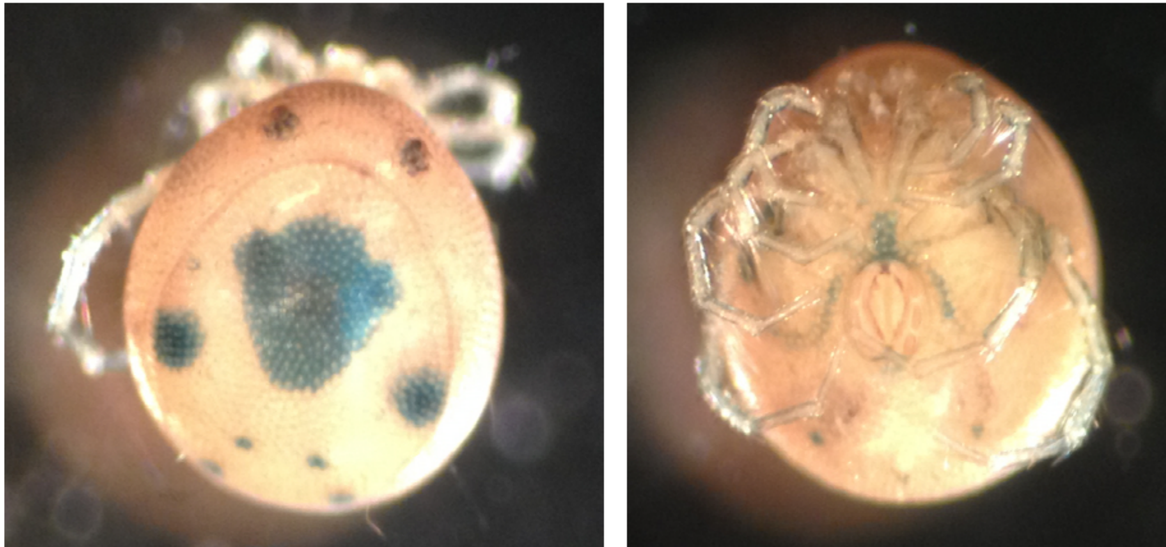


**Figure 1: Specimen of *Krendowskia*** collected from Maumee River. Left: Dorsal. Right: Ventral. The specimen is approximately 1 mm in diameter.

by the location of the coxal glandularia which were within the hind coxal plates, in contrast to *Arrenurus* in which the glandularia are between the hind coxal plates and the genital field (Smith et al. 2010, Smith and Cook 2016). The identity of both specimens of *Krendowskia* were further distinguished from *Geayia* by the shape of the gnathosome which has normal subcapitulum

dimensions in comparison to the elongated subcapitulum of *Geayia* (Smith et al. 2010, Smith and Cook 2016).

With respect to the specimens identified as *Koenikea*, the following criteria were used to identify the specimens: a dorsal and ventral shield, more than four acetabula, and six pairs of glandularia on the dorsal surface, three of which are grouped together near the postero-lateral corner of the dorsum in a triangular or crescent pattern (Smith et al. 2010, Smith and Cook 2016).

The results of BLAST analysis, shown in Table 1, revealed no previous COI barcode matching the sequences for *Koenikea* and *Krendowskia* within 3%. The most closely related sequences in existing databases are other species of water mites that are <83% similar, i.e. differing from these sequences by at least 17%. A specimen identified only as Acari had a barcode sequence nearly identical to specimens of *Koenikea* with which it groups in neighbor-joining tree analysis (Figure 2). Thus, the barcode sequence enabled us to identify the genus of a specimen that our methods could not identify.

**Table 1. EcoAnalysts' identification and closest sequence matches in GenBank and BOLD for representative specimens of water mites in this study.**

| Sample ID | EcoAnalysts' Identification | BOLD database | GenBank (closest match) % Identity |
|---|---|---|---|
| 1SMT50813 | *Limnesia* sp. | match | *Limnesia* sp., 99% |
| 3SMP73013 | *Limnesia* sp. | match | *Limnesia* sp., 99% |
| 1SMD73013 | *Limnesia* sp. | match | Limnesiidae sp., 87% |
| 1SMG50813 | *Krendowskia* sp. | no match | *Arrenurus* sp., 80% |
| 1SMN81313 | Acari | no match | *Sperchon* sp., 83% |
| 1SMW61212 | *Koenikea* sp. | no match | *Sperchon* sp., 83%. |

These specimens are marked with an asterisk in the neighbor joining tree in Figure 3.

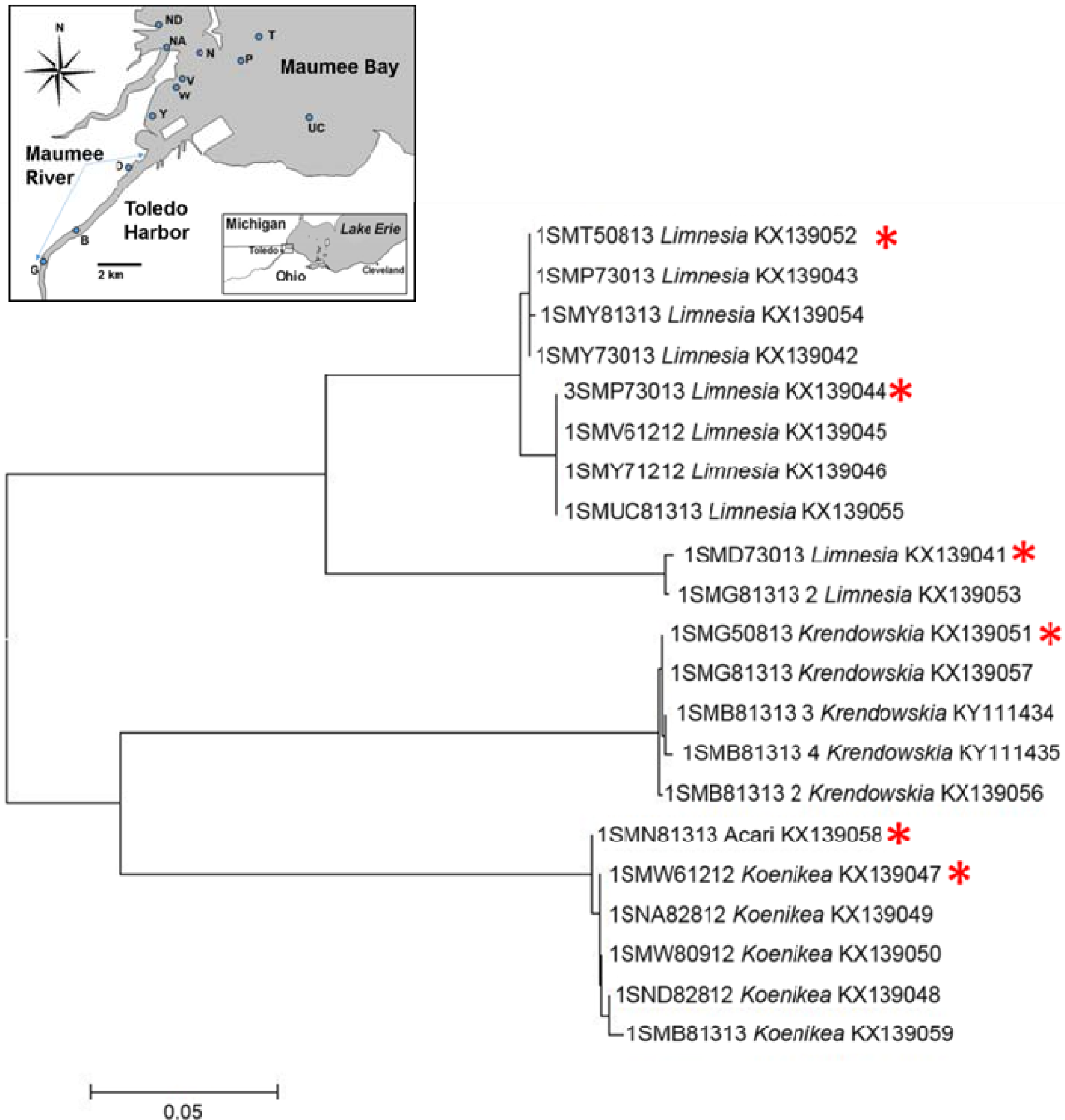



**Figure 2: Neighbor-joining tree showing sequence relationships among cytochrome oxidase I (COI) sequences at 658 nucleotide positions for 21 water mite specimens collected from Maumee River**, Maumee Bay, and North Maumee Bay. Calibration bar represents number of base substitutions per site. Terminus names (xyz##### taxon accession) represent location, (xyz = SM or SN, for sediment Maumee or sediment North Maumee respectively, z = map location letter, according to the inset map), the taxon of the sample and the GenBank accession number for that specimen. Asterisks mark sequences chosen for BLAST analysis (see Table 1).

Among the *Limnesia* specimens, those from Maumee Bay were 99% identical to previous sequences in GenBank identified as *Limnesia* (Table 1). The two specimens from the Maumee River formed a separate barcode sequences that were >99% identical to several "private" and "early release" sequences on BOLD and was 99.6% identical to one sequence from a water mite

collected along the Woodland Trail on Point Pelee (BOLD:BIN cluster AAH6535). Genbank had the most closely-related sequences identified as Limnesiidae (e.g., GenBank KM838066.1 and others), to which they were only 87% identical.

**Discussion**

More than half the COI barcode sequences observed in this study are novel and are associated with two genera, *Koenikea* and *Krendowskia*, for which no previous COI barcode with more than 97% similarity exists. These barcodes are expected to be useful for identification of damaged or immature specimens that may lack key morphological features for identifying them, as was demonstrated in this study for a specimen we could not identify but whose sequence clearly identified it as *Koenikea*.

*Krendowskia* has a Pangean distribution (Smith et al. 2010) with at least 5 known species on several continents. *Krendowskia convexa* Ribago, 1902 (Krendowskiidae) is the species most commonly found in the United States (Stang and Hetland 2015b). In Brazil, the invasion of Lake Monte Allegre by *Krendowskia* is believed to have caused restructuring of the zooplankton community (Arcifa et al. 2015). Surveys of lakes and rivers in Finland and Turkey have also noted new observations of *Krendowskia* compared to previous surveys (Hirvenoja 2000, Esen et al. 2013). While the presence of *Krendowskia* in the Great Lakes is not new (Marshall 1940b), the reports reviewed in this paragraph suggest that various species of *Krendowskia* may invade water bodies previously devoid of this genus where they may have significant effects on existing aquatic communities. By establishing baseline genetic markers of water mites in a high invasion risk area of the Great Lakes, the present study has provided data against which future invasive water mites might be compared.

*Koenikea* species also have a Pangean distribution and are known to occur in lakes (Smith et al. 2010), consistent with the locations reported here. More than twenty species have

been identified (Smit 2004, Stang and Hetland 2015a), including many in North America.

*Limnesia* is even more diverse, with more than 30 described species occurring in a wide range of habitats (Smith et al., 2010). The 13% difference in COI sequence between *Limnesia* collected here specifically in river versus bay indicates that these may represent different species of *Limnesia.* COI sequences of *Limnesia* that match the barcodes of these mites from the Maumee River have not been made public, with the nearest match being to a *Limnesia* sequence in BOLD from a water mite collected along the Woodland Trail in Pt. Pelee National Monument, which is on the Canadian side of western Lake Erie.

In future studies of water mites in the diets of fish or as parasites on insects, sequences in this paper may prove useful in identifying which genera of water mites are involved; however, in order to determine the species, a more complete COI water mite reference database is needed. Associating the observed COI barcodes with specific-species identifications is a necessary next step towards understanding the ecological roles that the various species of water mites play in the aquatic environment.

**Section B: Water Mites of Blue Heron Lagoon**

**Abstract**

Water mites are arachnids that inhabit aquatic habitats and are known for their biodiversity and impact as predators and parasites on aquatic insects. Although over 6000 species have been described, estimates are that as many as half of all North American species have yet to be described. This study uses morphological identification along with DNA molecular barcoding to improve knowledge about the genetic diversity of North American water mites.

Water mites from the Blue Heron Lagoon at Belle Isle, Detroit were collected and processed for diversity assessment. Water mite diversity in the Blue Heron lagoon is reported with up to 17 identified genera and representatives from 2 family level identifications from Blue

Heron Lagoon with the possibility of several species within each genera. Sampling distributed during two field seasons demonstrates that water mite genera such as *Arrenurus, Neumania* and *Lebertia* can be found throughout the year, and *Arrenurus* can be found in greater abundance during the summer months.

This work contributes to the DNA barcode genetic representation of water mites in public databases. This study contributes knowledge about the biodiversity of water mites in the Great Lakes region and begins to fill in gaps that may have significance in understanding their role in human health and Great Lakes ecology.

**Introduction**

Water mites are predatory arachnids that inhabit aquatic habitats and are found worldwide. Water mites are biodiverse microinvertebrates but according to leading acarologists only about half have been described in North America (Di Sabatino et al. 2008). Water mites have a predatory and parasitic stage in their life cycle and have been reported as being useful for both bioindicator species in environmental studies and as a method of biocontrol for pests such as the mosquito (Di Sabatino et al. 2000, Werblow et al. 2015, Goldschmidt et al. 2016). Over 6000 species have been described worldwide to date, however, many water mite species in North America are lacking proper descriptions (Di Sabatino et al. 2008). In addition not much is known about water mite genetic diversity in North America, an issue raised by a recent paper of Trebitz et al. (2015) that highlighted the lack of water mite genetic representation in public databases.

DNA barcodes have been shown to be useful for identification of organisms and can be accomplished with well populated databases (Hebert et al. 2003a). Reference DNA databases of sequences such as GenBank (Benson et al. 2007) and Barcode of Life Database (Ratnasingham and Hebert 2007, 2013) that have been generated from morphologically identified taxa are useful for identifying species. One such region used for molecular barcoding is the “Folmer” region of

the COI gene (Folmer et al. 1994, Hebert et al. 2003a). For instance, we used DNA barcodes to identify species of chironomid larvae that morphologically cannot be identified taxonomically to species by matching their barcode sequences to morphologically identified adult species of chironomids (Failla et al. 2016). DNA barcodes have been used to resolve the status of an invasive copepod, *Eurytemora carolleeae*, in the Great Lakes (Vasquez et al. 2016). While some groups have started to include molecular barcodes in their descriptions of water mites (Pesic and Smit 2014, Fisher et al. 2015) reference barcode databases lack numerous genera and species of water mites (Trebitz et al. 2015).

In this study, I investigated water mite populations found in Blue Heron Lagoon, an internal lagoon of Belle Isle, Detroit, MI (Figure 1, map). Blue Heron Lagoon was recently connected to the Detroit River by an EPA-funded habitat restoration project (Friends of the Detroit River 2010-2013, Blue Heron Lagoon Environmental Protection Agency's Great Lakes Restoration Initiative 2017). Belle Isle is an island in the Detroit River within the EPA designated Area of Concern (AOC) (Concern 1987). The Detroit River was declared an AOC because it is in close proximity to an urban center with overflowing sewers, industrial discharges and high levels of polychlorinated biphenyls (PCBs) and bacteria (Concern 1987). Belle Isle is managed by the Department of Natural Resources and is approximately 982 acres in size (2017). In sensitive habitats such as these it is important to continually assess the health of the ecosystem. Assessing biodiversity is one way to accomplish this, especially at the lower trophic levels that sustain the commercially important species such as fish.

The goal of this study was to use both molecular barcode data and morphological analysis to study the biodiversity of water mites in Blue Heron lagoon. As will be described in this paper, DNA barcodes reveal the presence of at least 17 genera of water mites with potentially several species represented at each genus.

## Materials and Methods

### *Water mite sampling*

Water mites were collected from several sites within and at the mouth of the Blue Heron Lagoon where it empties into the Detroit River and Lake St. Clair (see Figure 3) using methods by Fisher et al. (Fisher et al. 2015). Water mite populations were sampled at Blue Heron Lagoon, Belle Isle from July 2016 through June 2017.

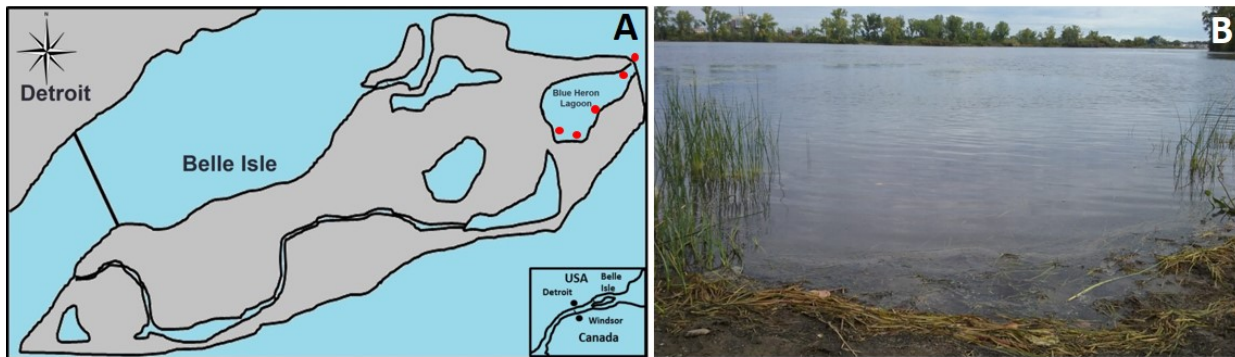



**Figure 3: Map of Belle Isle with Blue Heron Lagoon.** (A) Collection sites indicated by red dots. Belle Isle is situated between the US and Canada (inset). (B) Map of a collection site at Blue Heron Lagoon with vegetated area (lowest red dot).

### *Water mite identification*

Water mites were identified using a two tier method involving morphological and genetic analysis. Morphological identification used published keys, consultations with leading experts (Dr. Ashley Dowling, University of Arkansas; Dr. David Cook, retired, by personal communication), and corroboration with Ecoanalysts, a commercial taxonomy company, that I have used in several past projects including chironomids and copepods (Failla et al. 2016, Vasquez et al. 2016). Representative water mite specimens were photographed for morphological assessment with a SPOT camera mounted on a Nikon SMZ stereomicroscope. Dorsal and venter images were taken for morphology.

### *DNA molecular barcodes*

For genetic analysis, DNA from water mites was extracted using the Qiagen Easy tissue extraction protocol as in Vasquez et al. (2016). Briefly, mites were incubated in proteinase K enzyme (Qiagen, Cat. #19131) for 3 hr or overnight at 57 °C. When necessary, DNA was extracted by puncturing water mites with sharp minutien pins to allow water mite lysate to ooze out and a voucher of the exoskeleton to be retained for subsequent morphological analysis and archival storage. Sequencing and phylogenetic analysis were carried out as in Vasquez et al. (2016).

### *Bioinformatics*

After samples were sequenced bi-directionally (GENEWIZ, Plainfield, NJ), sequences were aligned and quality-checked using DNA Baser software (Heracle BioSoft SRL, Romania). Sequences were then imported into MEGA6 for sequence comparisons and construction of neighbor joining trees (Tamura et al. 2013).

## Results

### *Seasonal presence of water mites in Blue Heron Lagoon, Belle Isle Detroit*

A total of 985 water mites were accounted for during the 7 months of collection. Identifications of 15 genera and 2 family level identifications were found in the collections that were included in Figure 4, with the possibility of more than one species found in some genera for example *Arrenurus* (see Figure 4). Overall we found a total of 17 genera and 2 family level identifications with two genera that were barcoded (see Figure 4) but were collected on days that were not included in the counts. These two genera were *Hydrochoreutes* (1BHL5817AV) and *Madawaska* (BHL4717S4DY). The five most frequently observed genera were *Arrenurus* (35%), *Neumania* (31%), *Lebertia* (12%), *Hygrobates* (6%) and *Oxus* (3%). These were followed by *Limnesia*, *Mideopsis*, *Forelia* with 2% each. *Hydrachnidia* had 1% representation

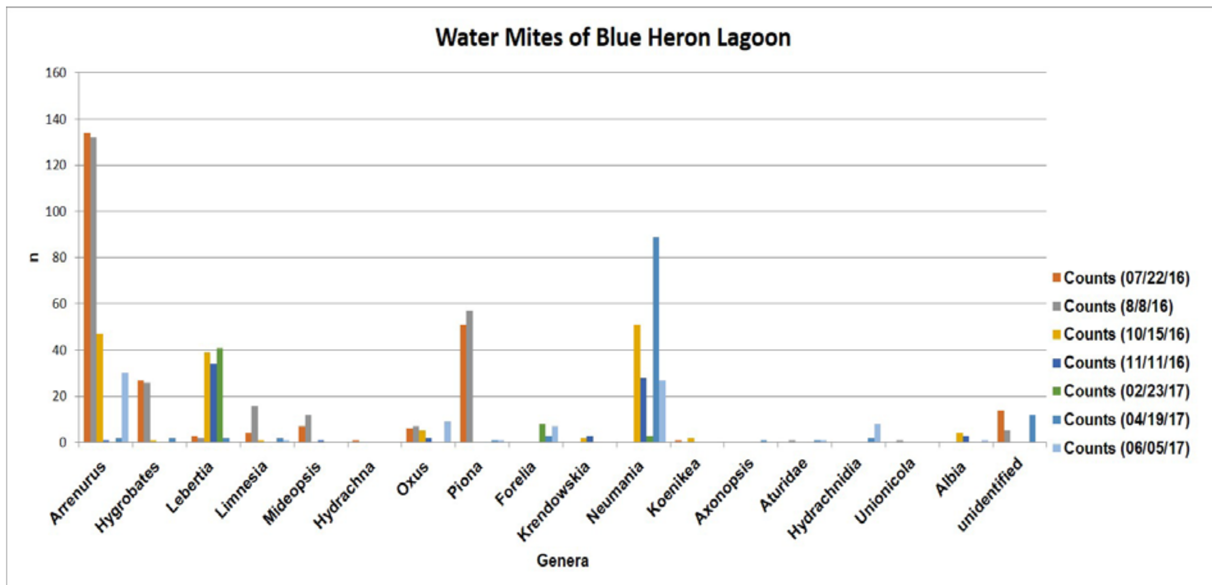



**Figure 4: Water mite populations of Blue Heron Lagoon** located on Belle Isle, Detroit, Michigan. Water mites were sampled from July 2016 to May 2017, counted and identified where possible to genera.

and the following *Hydrachna*, *Axonopsis*, *Unionicola*, *Koenikea*, *Aturidae*, *Piona*, *Krendowskia* and *Albia* were all under 1%. Unidentified mites had 3% representation in the total counts. Water mite populations fluctuated over the seasons with *Arrenurus* having high population during the months of July and August (n= 266) with *Neumania* being the second most common genus present during those months (n=108). During the fall months of October and November the populations of *Arrenurus* plummeted (n=47) while *Lebertia* populations increased (n=73). *Neumania* populations remained similar as in the summer months (n=79). During the winter most mite populations were in decline as only 52 mites were collected in total with the most common being *Lebertia* (n=41) and *Forelia* (n=8). During the spring collection (April) *Neumania* was seen to increase (n=89) but several mites were not able to be identified (unidentified specimens: n= 12) since they were immature. The early summer collection in June showed the trend for an increase in *Arrenurus* (n=30) and *Neumania* (n=89).

***Morphological diversity of the water mites of Blue Heron Lagoon***

Representative mites from all population types were photographed before molecular

analysis. The panels in Figure 5 are the phenotypic representation of the populations present in Blue Heron Lagoon. All panels are paired micrographs of the dorsal followed by the venter view

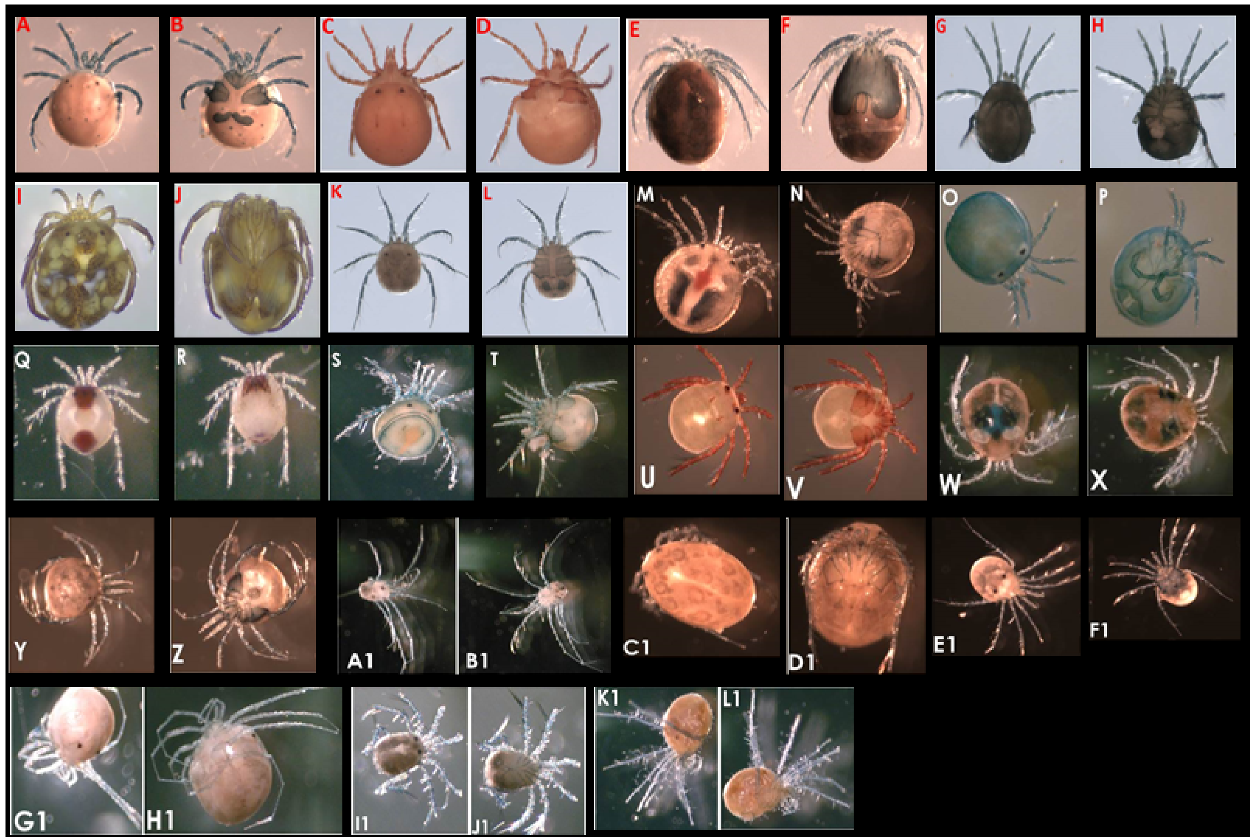



**Figure 5: The biodiversity of water mites in Blue Heron Lagoon**, Belle Isle, Detroit. Mites were photographed to represent both dorsal views and ventral views. Mite DNA was then extracted and molecular barcodes were obtained for representative mite genera. (A-B) *Arrenurus* deuteronymph. (C-D) *Hydrachna* deuteronymph. (E-F) *Oxus* (G-H) *Arrenurus* (I-J) *Lebertia quinquemaculosa*. (K-L) *Neumania* (M-N) *Mideopsis* (O-P) *Albia* (Q-R) *Axonopsis* (S-T) *Krendowskia* (U-V) *Hydrachna* (W-X) *Koenikea* (Y-Z) *Limnesia* (A1-B1) *Unionicola* (C1-D1) *Forelia* (E1-F1) *Piona* (G1-H1*)* *Hydrochoreutes* (I1-J1) *Madawaska* (K1-L1) *Hygrobates.*

of the mite (i.e. A represents the dorsal and B the venter). The panels represent micrographs of 17 of the common genera presented in this chapter. Panels A and B represent the deuteronymph form of *Arrenurus* while panels C and D are the deuteronymph form of *Hydrachna*. An adult *Arrenurus* (the most common mites in our collections) can be observed in panels G and H. Oxus represented in panels E and F belong in the same group with *Lebertia quinquemaculosa* seen in panels I and J. Panels K and L represent *Neumania* the second most abundant mite collected in

BHL. Panels M and N represent *Mideopsis* that are the "sphere" shaped water mites. Panel O and P represents *Albia,* a mite with a bluish hue and spherical in shape like *Mideopsis*. *Aturidae* (panels Q and R) are some of the smaller mites found in BHL. *Krendowskia* (panel S and T) is a mite that closely resembles *Arrenurus* but has distinct coxal plates observed in panel T. The coxa does not form a straight line across the venter but has a V shaped presentation. *Koenikea* is represented in panel W and X and can be mistaken for *Mideopsis* due to its spherical shape but its coloration is quite distinct. *Limnesia* seen in panels Y and Z is easily distinguished from other mites due to its V shaped coxa seen in Panel Z that encircles its genital field. *Unionicola* is seen in panel A1 and B1 and is very distinctive due to its spider like legs. *Forelia* is seen in panels C1 and D1, and this mite was observed in winter collections. *Piona* is represented in panels E1 and F1 and represents a large group with potentially many species. Panels G1 and H1 represent the more rarely seen mite in our collections the *Hydrochoreutes*. *Madawaska* is seen in panels I1 and J1 and has a very distinctive bend in its fourth leg. *Hygrobates*, one of the top five common mites, is also a distinctive mite with its coxa being straight in line with its genital field.

***Molecular DNA barcodes of water mites from Blue Heron Lagoon, Detroit MI***

A neighbor joining tree of molecular DNA barcoded water mites representing 16 of the 17 representative genera from our collections in Blue Heron Lagoon revealed the possibility of several species within individual branches (see Figure 5). Genera such as *Arrenurus* and *Lebertia* can be observed as having several branches that differed from each other by at least 5%. The upper branch of *Lebertia* has been identified by us as the large lake dwelling *Lebertia quinquemaculosa* and is the subject of discussion of the proceeding chapters of this work. Publication of this sequence will represent the first species level DNA molecular barcode in the database for *L. quinquemaculosa* since its description in 1928 by Ruth Marshall (Marshall 1928). *Lebertia* n sp. represents a new species of *Lebertia* whose description is discussed in Chapter 3.

Several of these barcoded water mites have no match in the genetic database GenBank (see Table 2) and may represent completely new species that are in need of descriptions. The sequences identified by the asterisk represent first time molecular DNA barcodes for the labelled genera. As of the writing of this thesis there has not been any representative molecular DNA barcodes for *Albia*, *Madawaska* and *Hydrochoreutes*.

**Table 2. Closest sequence matches in GenBank for representative water mite specimens**

| **Sample ID** | **Closest Match in GenBank % Identity** | Our identification |
|---|---|---|
| 8BHL072216 | *Lebertia* sp. 86% | *Lebertia quinquemaculosa* |
| 136BHL100916 | *Lebertia* sp. 87% | *Lebertia* sp. |
| 1BHL080816 | *Lebertia* sp. 88% | *Lebertia* n. sp. |
| 9BHL072216 | *Arrenurus* sp. 99% | *Arrenurus* sp. |
| 126BHL80816 | *Trombidiformes* sp. 99% | *Albia* sp. |
| 103BHL72216 | *Trombidiformes* sp. 83% | *Oxus* sp. |
| 11BHL070916 | *Pionidae* sp. 83% | *Neumania* sp. |
| 124BHL72216 | *Pionidae* sp. 86% | *Piona* sp. |
| 118BHL42516 | *Mideopsis* sp. 89% | *Mideopsis* sp. |
| 132BHL080816 | *Krendowskia* sp. 85% | *Krendowskia* sp. |
| 148BHL110116 | *Hydraphantes* sp. 79% | *Hygrobates* sp. |
| 150BHL80816 | *Hydraphantidae* sp. 85% | *Aturidae* sp. |
| 116BHL070916 | *Hydrachna conjecta* 80% | *Hydrachna* sp. |
| 1BHL5817AV | *Piona variabilis* 83% | *Hydrochoreutes* sp. |
| 121BHL42516 | *Limnesiidae* sp. 99% | *Limnesia* sp. |
| BHL4717S4DY | *Trombidiformes sp*. 99% | *Madawaska* sp. |

***BLAST analysis of representative water mite DNA molecular barcodes***

Representative sequences from each genus of water mites that were barcoded and listed in Table 2 showed that only four are represented by an above 97% match in GenBank (above 97% is the cutoff for a reliable species match). The other matches were mostly in the 80 percentile with the highest being 89% (*Mideopsis* sp.) and the lowest 79% (*Hygrobates* sp.). Of the 16 sequences analyzed there were 3 in which the nearest match in the GenBank database was a genus other than the one we identified; however, in all such cases the pairwise identity was less than 85%, including *Neumania* sp. mismatched with *Pionidae* (83%), family level identification *Aturidae* sp. mismatched with *Hydraphantidae* sp. (85%) and *Hydrochoreutes* sp. mismatched with *Piona variabilis* (83%). We were able to resolve 5 GenBank family level identifications

down to genus identifications (*Albia* sp., *Oxus* sp., *Hygrobates* sp., *Limnesia* sp. and *Madawaska* sp.) with the reporting of the first genus level identifications for *Albia*, *Madawaska* and *Hydrochoreutes*.

**Discussion**

In a study of the water mite populations of Blue Heron Lagoon, Detroit Michigan we found that *Arrenurus* and *Neumania* constitute by far the greatest numbers of mites found during the collection months of July 2016 to June 2017. Water mites were also seen in greatest abundance during the summer months of July and August and during the spring in April. A total of 17 genera and 2 family level identifications of water mites were found in Blue Heron Lagoon during the study period. The genera encountered are as follows: *Arrenurus*, *Hygrobates*, *Lebertia*, *Limnesia*, *Mideopsis*, *Hydrachna*, *Oxus*, *Piona*, *Forelia*, *Krendowskia*, *Neumania*, *Koenikea*, *Axonopsis*, *Unionicola*, *Albia*, *Madawaska*, and *Hydrochoreutes*. The two family level identifications that were done included *Aturidae* and *Hydrachnidia*. Water mites were selected for each genus and barcoded using the cytochrome oxidase I (COI) barcoding gene. 16 genera were successfully barcoded with an additional family level barcode. The genetic analysis involving comparisons with the public database of reference sequences (GenBank) revealed that at least 3 new genus level DNA molecular barcodes were generated as well as several new representative barcodes for the different genera studied. New barcodes for *Lebertia quinquemaculosa* and a new species of *Lebertia* n. sp. (discussed in Chapter 3) were generated.

*Arrenurus* is the most species rich genus found in North America with over 100 species described so far with the possibilities of hundreds more (Cook 1976). Although unique structures of males *Arrenurus* allow easy comparison between species the morphological differentiation amongst *Arrenurus* male and females is especially difficult which may introduce errors into the systematic taxonomy of this genera (Cook 1976). Barcode studies such as this

greatly assist the water mite taxonomy and can be used as a leverage to better manage and develop taxonomic keys. In our own study we found at least three branches of *Arrenurus* that may represent three species (see Figure. 6).

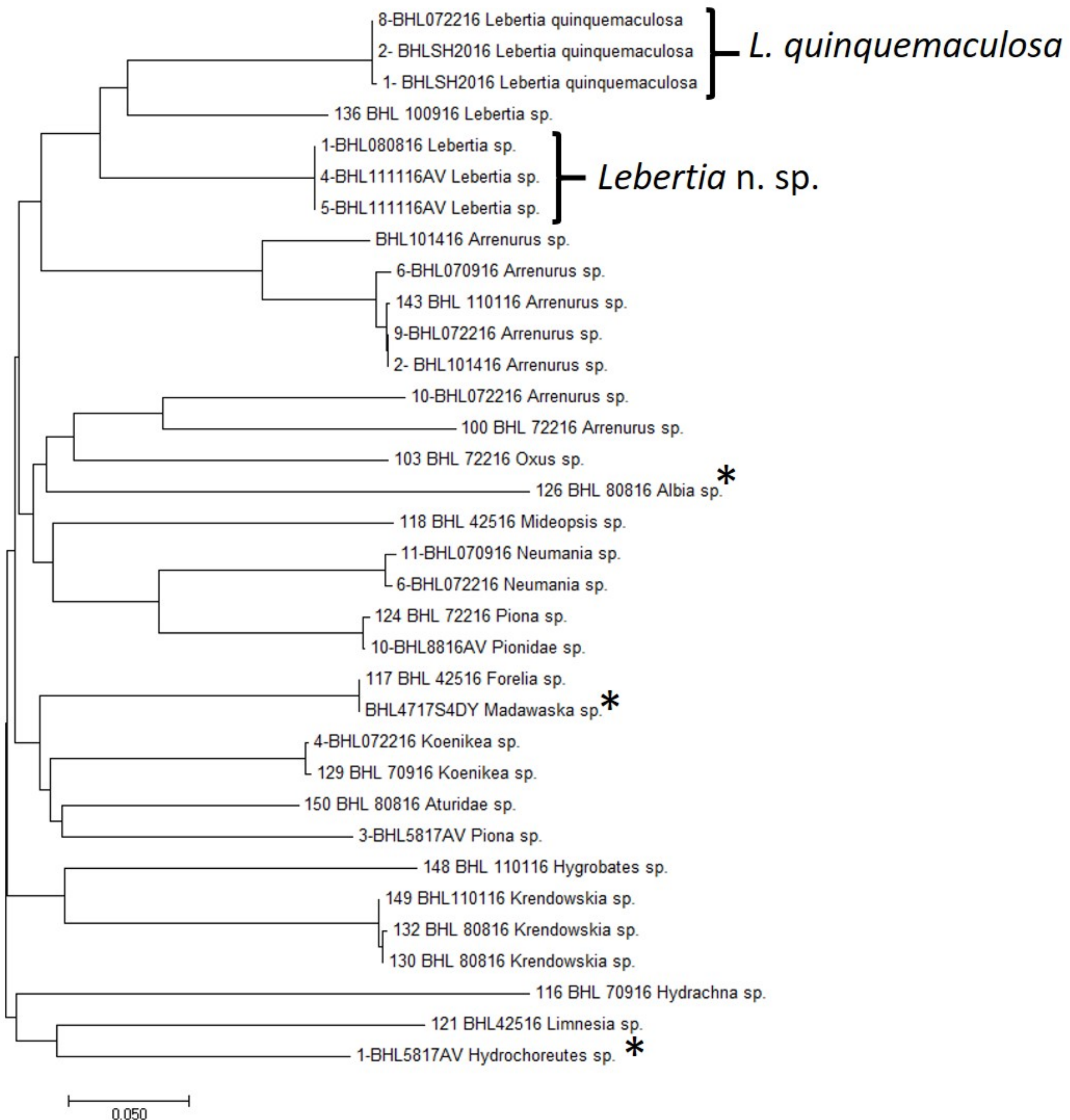



**Figure 6: Water mite genetic diversity of Blue Heron Lagoon**, Detroit MI. Neighbor joining tree showing sequence relationships among 34 water mite cytochrome oxidase I (COI) nucleotide sequences. The final dataset analyzed 600 positions and was conducted using MEGA7 (Tamura et al. 2013). Two branches of *Lebertia* sequences are identified which represent the two organisms that played a major work in this thesis. Sequences indicated by asterisk represent first ever DNA molecular barcodes for that genus in the public database.

*Lebertia* were the third most abundant genus encountered in the collection months. Our morphological analysis identified the species *Lebertia quinquemaculosa* Marshall, making this a new record for a habitat within the Great Lakes since previous descriptions and reports came from other lakes and rivers including Barton Pond in Ann Arbor, MI which the author identified in the collections of Ian Smith of the Canadian Collection during the Acarology Summer Program at Ohio State University this past summer (Marshall 1928, Young and Rhodes 1974). A new *Lebertia* species was identified from this work using both morphological and molecular methods and that has been described in Chapter 3.

The molecular barcodes of the different genera of water mites found in our sampling sites have now increased the presence of molecular barcodes for water mites from North America which were nil prior to this work (Trebitz et al. 2015). This will greatly assist in future studies on Great Lakes water mites to answer questions such as diet selection of specific groups with applications in trophic studies, conservation biology and invasive biology (Leray et al. 2013a, Leray et al. 2013b, Clare 2014, Harms-Tuohy et al. 2016). In addition, these results can be used to compare with other better-studied populations in Europe to strengthen the understanding of global water mite biodiversity and historical occurrence.

Our study characterized the genetic diversity and morphology of water mites in the Blue Heron Lagoon and is the first of this kind of in-depth study in this habitat. Blue Heron Lagoon is a nursery for Detroit River fish stock since it is connected to the Detroit River by an EPA-funded restoration project which makes it directly relevant to Great Lakes ecology (Friends of the Detroit River 2010-2013).

Earlier research around the Great Lakes has played a classical and critically important role in understanding the systematics of water mites (Cook 1954, 1967, 1974, 1976) but new work such as this is needed to continue to contribute to the sparse data set so that knowledge of

the habitat and role of water mites can be better understood. Our work verified the inadequacy of water mite genetic representation that was bemoaned in Trebitz et al. (Trebitz et al. 2015). However, we have begun to help with this issue by already publishing one chapter of this work and preparing this one for submission. Future discovery of new species using methods such as these will enhance our contributions to Great Lakes biodiversity.

# CHAPTER 3 - FOCUS ON *LEBERTIA* (ACARI: HYDRACHNIDIA: LEBERTIIDAE): DISTINCTIVE MORPHOLOGY OF *LEBERTIA QUINQUEMACULOSA* AND *LEBERTIA*

## Abstract

Water mites from Blue Heron Lagoon at Belle Isle, Detroit were collected, processed and identified. A new record of *Lebertia quinquemaculosa* Marshall and a new *Lebertia* sp. description from Blue Heron Lagoon is presented here. Light and scanning electron microscopy was used to identify the distinctive morphological characters that aid in describing the new *Lebertia* n. sp. Consultation with Dr. David Cook and review of the literature identified *L. quinquemaculosa* and led us to identify the potential new *Lebertia* species which we intend to name *Lebertia davidcooki* in honor of Dr. Cook. Some characters, comments on the biology and molecular barcodes to distinguish *Lebertia* n. sp. from *L. quinquemaculosa* are presented.

## Introduction

The *Lebertia* genus belongs to the family Lebertidae and has an extensive biogeographic region with most of this diversity found in the northern hemisphere (Di Sabatino et al. 2008). Within the family Lebertidae there are 13 known genera from North America in which the *Lebertia* genus is found (Cook 1974). There are potentially 28 species in North America, an observation that some experts think is in need of revision (personal communication: Dr. Ray Fisher) (Smith I. M. 1982). The *Lebertia* genus is considered one of the most species rich members of the family (Gulle and Boyaci 2012). A thorough revision of the *Lebertia* species of Eurasia has been completed and serves as an excellent starting point for a similar assessment of the *Lebertia* species in North America (Gerecke 2009).

In North America, the work of Marshall has been fundamental, and her work on *Lebertia quinquemaculosa* was the first description of this species (Marshall 1928). Other works described *L. porosa*, *L. distincta*, *L. artaacetabula* and *L. tyrrelli* (Marshall 1912, 1927).

However, very little descriptive work has been done on *Lebertia* since then except for Cook and Smith who studied some adults and larvae of *Lebertia* (Cook 1974, Smith I. M. 1982). The number of *Lebertia* species worldwide is thought to be 136 species but earlier estimates and reports may have included many synonyms and are in need of revision (Gerecke 2009). This is one of the reasons why work on this group is essential for having a better understanding of *Lebertia* populations in North America.

For many water mites no genus- or species–level DNA COI barcode is known, and until the present study of *Lebertia*, no reliably described species-level barcode was available for any species in this genus. A search on the Barcode of Life Database (BOLD) claimed 3 molecular barcodes of *Lebertia* to species but only one, *Lebertia inaequalis*, had a name and none of them had a reference publication attached to them. Only one of the *L. inaequalis* had a photo attached to it but it was a poor photo of the dorsum that could not be used for taxonomic purposes. The venter, showing the *Lebertia* coxal plates is what is needed for identification. In reality there are hardly any reference barcodes determined for any water mites in the Great Lakes region (Trebitz et al. 2015). In this work we contribute to making the water mites reference database of molecular barcodes more complete.

*Lebertia* biology is also inadequate but some studies have been reported that show marked circadian rhythms of activity, with some species behaving as nocturnal and others as diurnal organisms (Schmidt and Muller 1967). *Lebertia* can be collected year round (Schmidt and Muller 1967, Gerecke 2009). *Lebertia* were seen to prey on black fly larvae and are known to feed on the larvae of other dipterans (Mwango et al. 1995, Smith et al. 2010). *Lebertia* are also parasites on adult dipterans including chironomids (Martin 2004). In a study of water mite responses to changes in oxygen concentration *Lebertia quinquemaculosa* was the most sensitive of three species of mites tested (Young and Rhodes 1974).

*L. quinquemaculosa* was first described from Lake Wawasee (Turkey Lake) in northern Indiana by Marshall (Marshall 1928). Thereafter it was found in British Columbia, Canada and in Wisconsin (Marshall 1932). More recently it was reported in Hays County, Texas during summer collections (Young and Rhodes 1974). *L. quinquemaculosa* was studied in an experiment on oxygen dependence and was noted as having a negative linear relationship between number alive and the time spent in oxygen concentrations less than 0.1 mg/liter (Young and Rhodes 1974). This could be the reason why it was reported by the Environmental Protection Agency (EPA) Field and Lab method for evaluating surface waters as an organism that is intolerant to organic wastes and listed as 1 on a scale of 0 to 5 with 0 being the least tolerant (Klemm et al. 1990).

*L. quinquemaculosa* is a large lake-dwelling species that may attain lengths of up to 2 mm (Marshall 1928). It is broad and oval in form with short stout legs that have many thick bristles (Marshall 1928). The fourth coxal plate of the ventral shield is the most distinctive character as it is narrow at the posterior end and enclose a deep and broad bay in which lies the genital field (Marshall 1928). The coloration of this species has been described as distinguishable with five bright red spots seen dorsally (hence the name *quinquemaculosa*), and a large ventral spot below the coxal plates where we have seen the excretory pore (anus) being present (Marshall 1928). The coxal plates are reddish blue or purple as are the legs, with the eyes red in coloration (Marshall 1928).

*L. quinquemaculosa* is considered one of the easier species to identify due to its large size and distinctive coxal plate (personal communication, Dr. David Cook). However, the *Lebertia* population of North America is in need of revision. At the genus level *Lebertia* are easily recognized by its coxal plates (called epimera in older papers) (Marshall 1912). The coxal plates fuse into a single group that may or may not have complete fusion depending on the species, and

form a shield that covers a large area of the ventral aspect of the mite (Marshall 1912). The sexes are mostly similar and besides the ventral shield the rest of the mite is "sac like" (Marshall 1912). The feet of *Lebertia* are at times stout with many bristles as in *L. quinquemaculosa* or may have swimming setae for a different type of activity (Marshall 1912). *Lebertia* tends to inhabit colder waters, hence the discovery of several different *Lebertia* in our collections including one that may be new to science reported in this work.

The present report includes the first record of *Lebertia quinquemaculosa* Marshall from the Blue Heron Lagoon with a molecular cytochrome oxidase I (COI) barcode and the description of a new species of *Lebertia*. We propose to name this new species *L. davidcooki* in honor of Dr. David Cook, Emeritus Professor of Wayne State University. Descriptive characters that may be useful for distinguishing *L. davidcooki* are outlined here including the molecular COI barcodes.

**Material and Methods**

***Collection of Lebertia***

*Lebertia* mites were collected from Blue Heron Lagoon using the methods described previously (see Chapter 2B of this work). *Lebertia* mites were collected year round as was reported earlier. They were predominantly found in vegetated areas with *Lebertia* being found in the submerged roots and stems of nearshore terrestrial plants with aquatic vegetation earlier in the year (February to April) while they were found in deeper vegetated areas during the summer months (July to August).

***Processing and morphological identification of Lebertia***

*Lebertia* mites were processed by a modified published method where the mites are subjected to boiling water for a short period of time (20-35 seconds) while they are in a glass tube. After this they are immediately placed in cold ethanol and processed as reported (Fisher et

al. 2015). Mite specimens preserved in ethanol were examined using light microscopy, and the keys of Smith and Cook were used to distinguish *Lebertia* from other types of water mites and from each other (Smith and Cook 2016).

***Confocal laser fluorescent microscopy and scanning electron microscopy (SEM)***

In addition to examination of morphology using light microscopy we used confocal scanning microscopy and scanning electron microscopy to study the morphological features of *Lebertia* in more detail. Detailed methodological use of the confocal and scanning electron microscopy can be found in Chapter 4. Briefly, mites were studied using a confocal laser scanning microscope (Zeiss) and different filters were used to highlight different features of taxonomic value. Mites were also studied by low temperature SEM (S-4700 field emission scanning electron microscope (Hitachi High Technologies America, Inc., Dallas, TX, USA) as described previously (Bolton et al. 2014).

***DNA extraction, PCR and sequence analysis***

DNA molecular barcodes from the cytochrome oxidase 1 gene (COI) were obtained for both *L. quinquemaculosa* and *L. davidcooki* by methods previously described in Chapter 2B. Briefly, after mites were collected from the field their DNA was extracted using the Qiagen method and PCR was run using the COI molecular barcoding "Folmer" primers. PCR amplicons were then sequenced and used to construct neighbor-joining trees.

**Results and Discussion**

***Light and scanning electron microscopy reveals distinctive features of Lebertia quinquemaculosa and Lebertia davidcooki***

Photographs illustrating the major distinguishing features of *L. quinquemaculosa* and *L. davidcooki* are shown in Figures 7-9. The venter side of *Lebertia* has a fused coxa with partial fusion in between the third and fourth coxa. The fourth coxa were seen to descend and encircle

the genital field. In *L. quinquemaculosa* the distinguishing feature is that the fourth coxal plate narrows as it descends and is seen to sometime go past the genital field (see Figures 8B and 9B) (Marshall 1928). The genital field is comprised of 3 pairs of equally shaped acetabula that are covered by a genital flap (see Figures 8B and 9B). Coloration in *L. quinquemaculosa* is also distinctive (see Figure 7) but should not be used as a primary taxonomic character since many of the preparatory methods cause discoloration. *L. quinquemaculosa* is a larger mite than other *Lebertia*, at times attaining 2 mm in size (Marshall 1928). *L. quinquemaculosa* is oval in shape (see Figure 9A-B) and has stout legs with many bristles which is another descriptive character (inset, Figure 13) used to key out this species (Marshall 1928). Coloration of the legs and venter were similar in both mites.

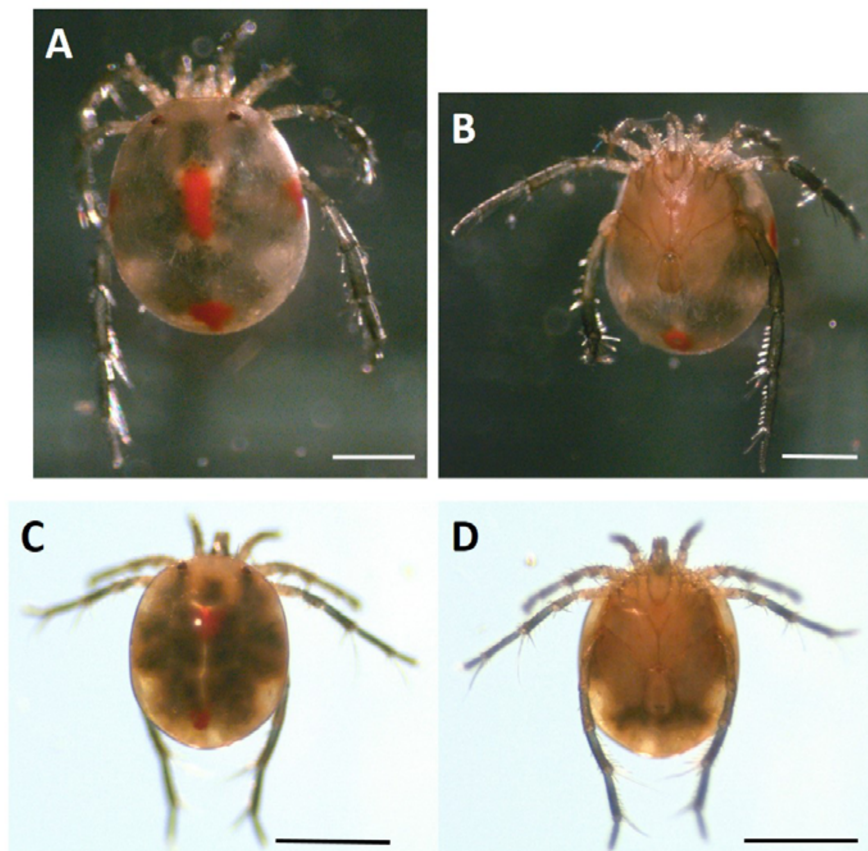



**Figure 7: Light micrographs of *L. quinquemaculosa* and *L. davidcooki*.** (A-B) Dorsal and ventral views of *L. quinquemaculosa*. (C-D) Dorsal and ventral views of *L. davidcooki*. Scale bars represent 500 µm.

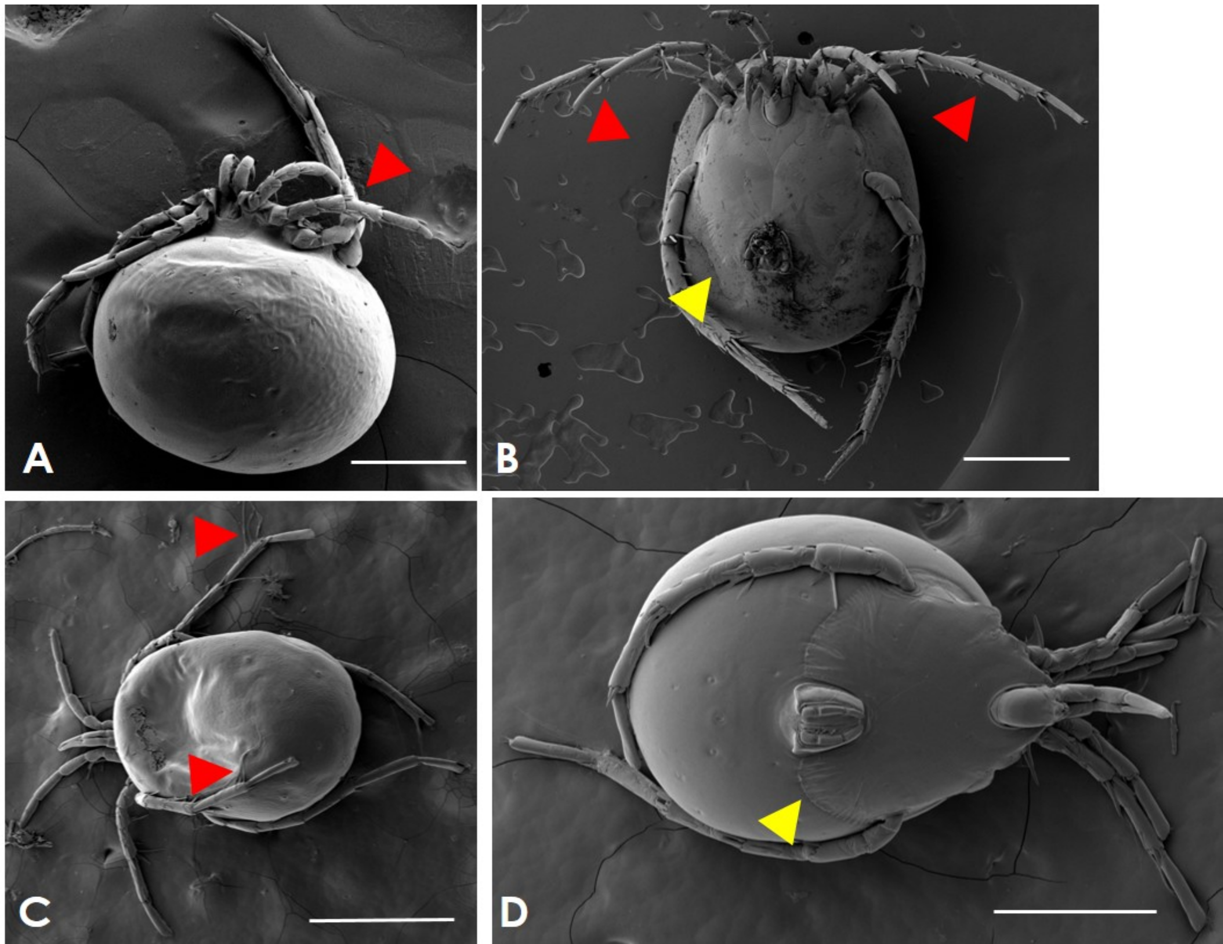



**Figure 8: SEM images of *L. quinquemaculosa* and *L. davidcooki*.** (A-B) Dorsal and ventral views of *L. quinquemaculosa* depicting described characters with red arrow showing absence of swimming setae and stout legs with bristles while yellow arrow in B shows 4$^{th}$ coxal plate narrowing as it descends past the genital field. (C-D) Dorsal and ventral views of *L. davidcooki* showing presence of swimming setae (red arrows in C) and coxal plate more circular around the genital field (yellow arrow in D). These observations were seen at least twice in SEM analysis and more than three times using light microscopy. Scale bars for A-C are 500 um, D is 400 um.

*L. davidcooki* is easily distinguished from *L. quinquemaculosa* in that its fourth coxal plate does not narrow down to encircle the genital field but it forms a circular appearance as it descends, giving the venter shield a rounded appearance when compared to *L. quinquemaculosa*. It is also a smaller mite compared to *L. quinquemaculosa* with specimens observed to about 2/3 the size of *L. quinquemaculosa*. *L. davidcooki* also has a more transparent integument and has very distinctive swimming hairs on its more slender legs when viewed under SEM analysis (see Figure 10 for detail). These swimming hairs can also be seen at high power (>50X) under a

stereomicroscope. Coloration is similar except for smaller red blotches and a very distinctive tube like structure that can be seen when viewed dorsally (Figure 14). *L. davidcooki* is seen more during the early season (February – March) and is seen intermittently throughout the summer months.

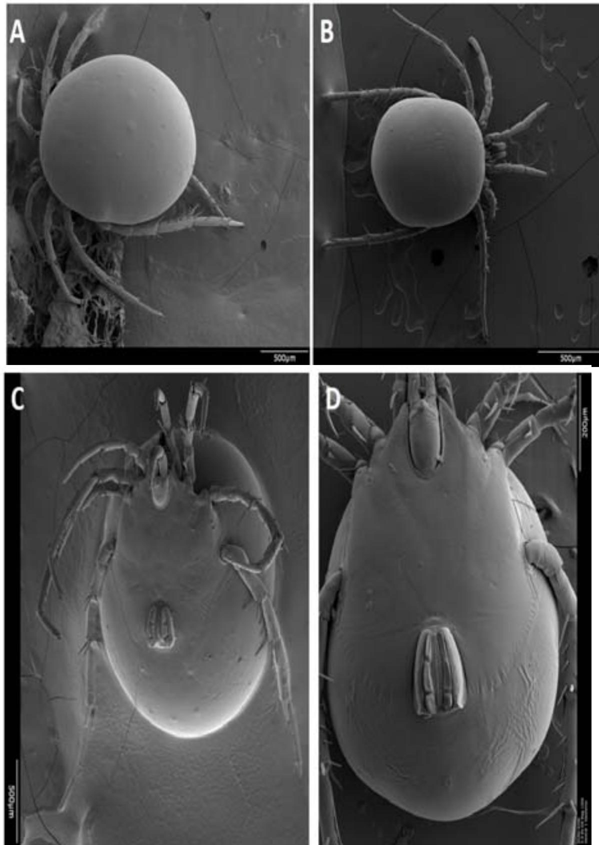



**Figure 9: SEM images of *L. quinquemaculosa* and *L. davidcooki*.** (A-B) Dorsal views of *L. quinquemaculosa* and *L. davidcooki*. (C-D) Ventral views of (C) *L. quinquemaculosa* and (D) *L. davidcooki* depicting key characters.

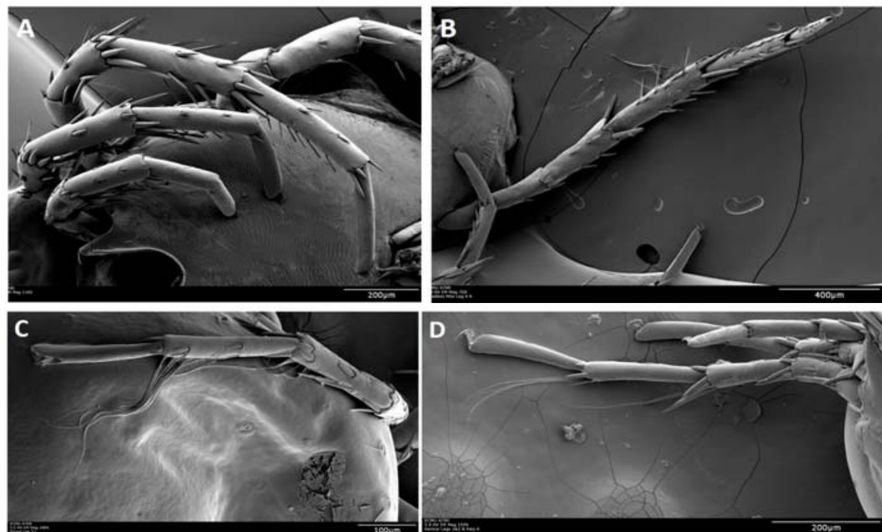



**Figure 10: SEM images of swimming hairs** of *L. quinquemaculosa* and *L. davidcooki*. (A-B) Views of *L. quinquemaculosa* appendages showing lack of swimming hairs with very few found on the fourth foot (B). (C-D) Views of *L. davidcooki* 4th leg with long swimming hairs.

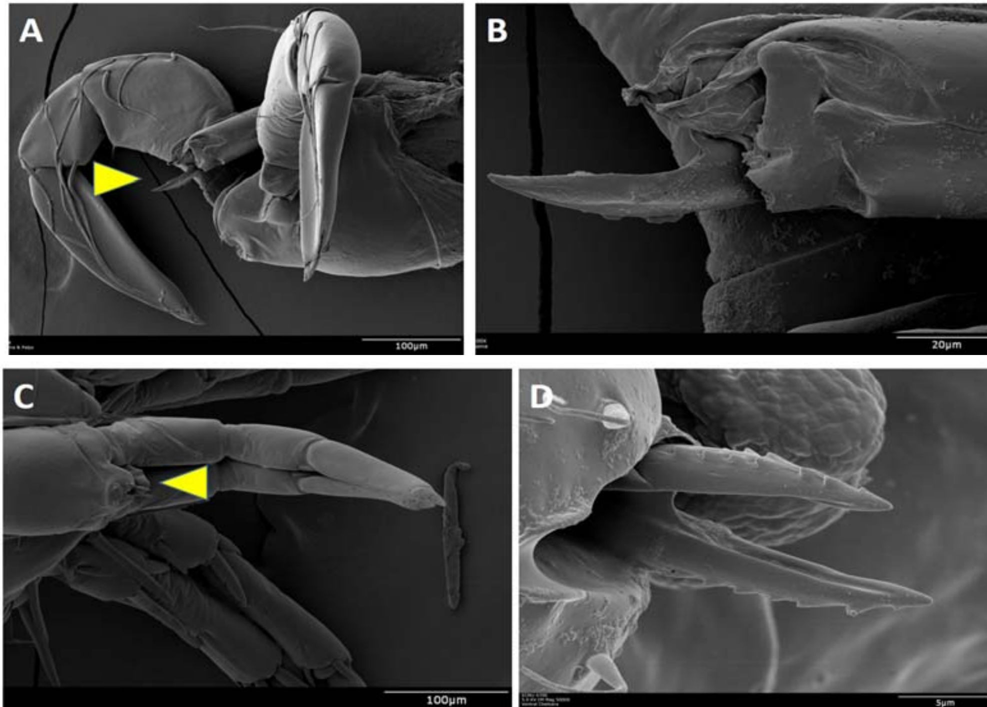



**Figure 11:SEM images of the chelicerae** of *L. quinquemaculosa* and *L. davidcooki*. (A-B) Views of *L. quinquemaculosa* chelicerae indicated by yellow arrow in (A) and showing a zoom image in (B). (C-D) Views of *L. davidcooki* chelicerae indicated by yellow arrow in (C) and showing a zoom image in (D) with distinctive jagged "harpoon" edge.

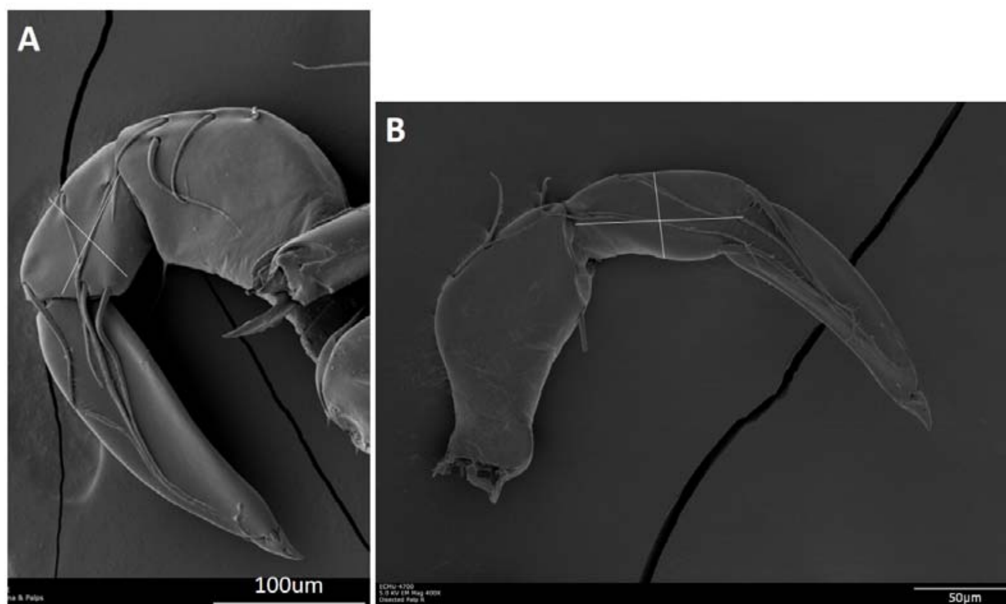



**Figure 12: SEM images of the palp** of *L. quinquemaculosa* and *L. davidcooki*. (A) Palp of *L. quinquemaculosa* with basipod indicated by white cross that might be used as taxonomic character. (B) Corresponding basipod seen in the palp of *L. davidcooki*.

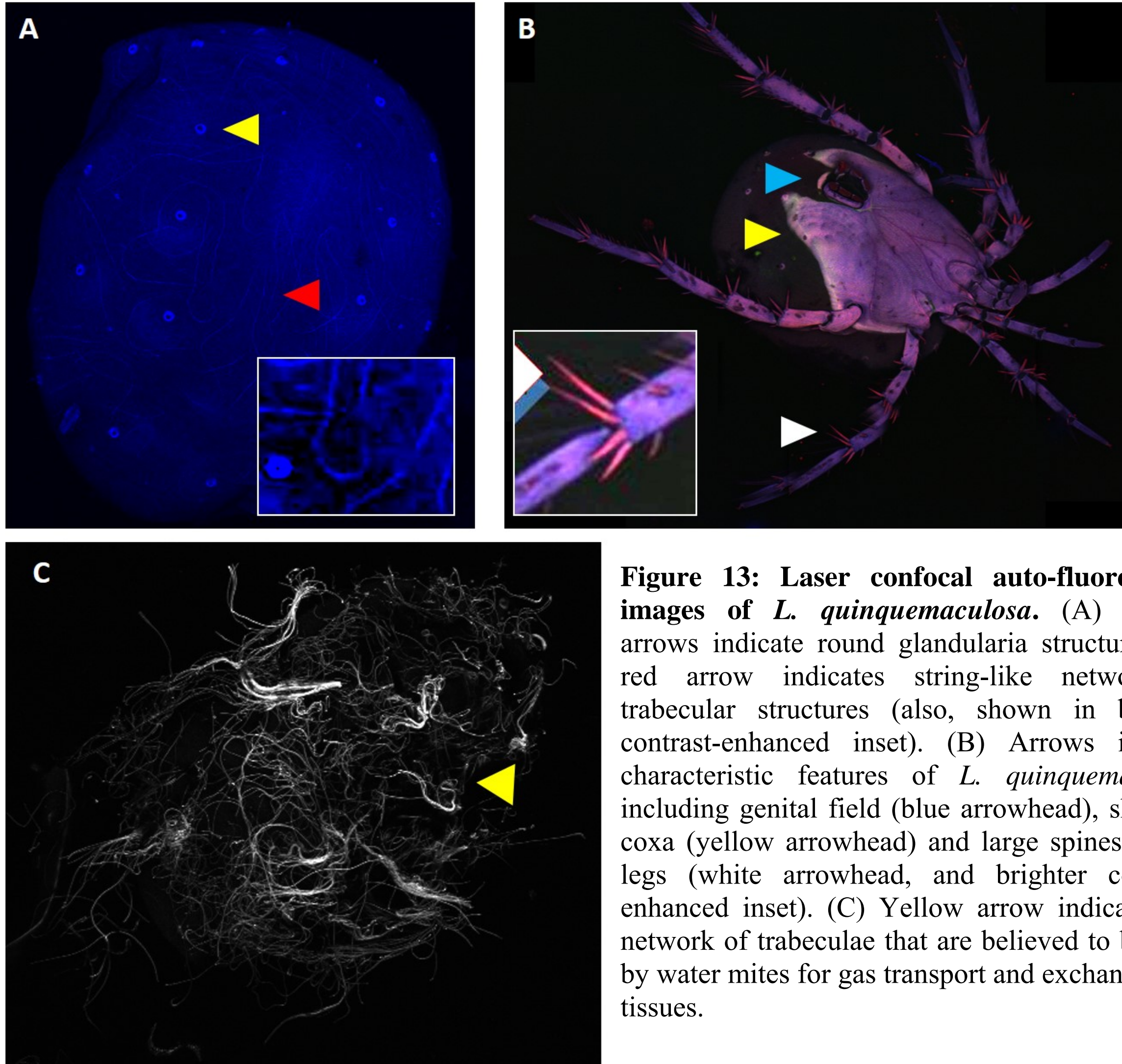



**Figure 13: Laser confocal auto-fluorescence images of *L. quinquemaculosa*.** (A) Yellow arrows indicate round glandularia structures and red arrow indicates string-like network of trabecular structures (also, shown in brighter contrast-enhanced inset). (B) Arrows indicate characteristic features of *L. quinquemaculosa* including genital field (blue arrowhead), shape of coxa (yellow arrowhead) and large spines on the legs (white arrowhead, and brighter contrast-enhanced inset). (C) Yellow arrow indicates the network of trabeculae that are believed to be used by water mites for gas transport and exchange with tissues.

Multiple specimens (>5) were examined for these features, and these were corroborated by Dr. David Cook. The analysis showed that *L. quinquemaculosa* is a larger mite than *L. davidcooki* and that their coloration is very similar. However, digestive anatomical features can more readily be seen through the integument of *L. davidcooki*. This will be more discussed in Chapter 4. Figure 11 and 12 are structures that the mite uses for feeding purposes with distinctive chelicerae for *L. davidcooki* (Figure 11C-D) that have a jagged edge when compared to *L. quinquemaculosa*. Figure 12 is the palp which the mite uses primarily for grasping prey. The palp of water mites are used to distinguish species and the basipod (highlighted by white

cross in Figure 12) could be a useful feature to distinguish *L. quinquemaculosa* and *L. davidcooki* as was used in Chapter 11B to distinguish an invasive copepod in the Great Lakes.

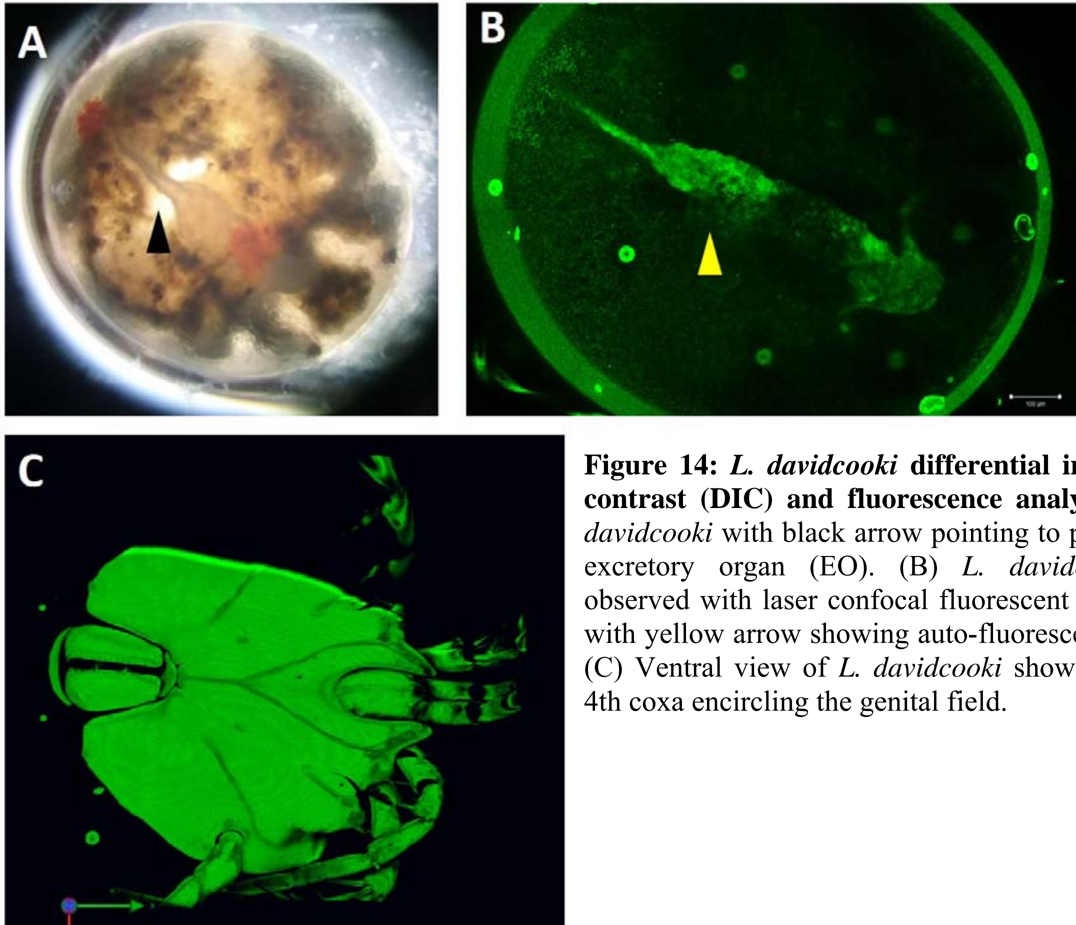



**Figure 14: *L. davidcooki* differential interference contrast (DIC) and fluorescence analysis.** (A) *L. davidcooki* with black arrow pointing to presumptive excretory organ (EO). (B) *L. davidcooki* mite observed with laser confocal fluorescent microscope with yellow arrow showing auto-fluorescence of EO. (C) Ventral view of *L. davidcooki* showing circular 4th coxa encircling the genital field.

***Confocal laser fluorescent microscopy highlights differentiating taxonomic characters of L. quinquemaculosa and L. davidcooki.***

Confocal fluorescent microscopy has been noted recently for its advantageous use in taxonomy of arthropods which may be difficult to tell apart (Valdecasas 2008, Valdecasas and Abad 2011). Here, the use of a confocal fluorescent microscope has pointed out important taxonomic features of *Lebertia* water mites (see Figure 13 and 14). This has led to the identification of *L. quinquemaculosa* by the characteristic feature of its 4$^{th}$ coxa that is indicated by the yellow arrow in Figure 13B. The large bristles on its stout legs can also be seen (magnified inset). Confocal microscopy revealed details of the trabecular network that cannot be

seen using light or scanning microscopy (see Figure. 13A and C). Whether this has any taxonomic value is not known at this point.

*L. davidcooki* was analyzed by similar methods which revealed a distinctive anatomical feature that could be seen through its more transparent integument when compared to *L. quinquemaculosa* (see Figure 14 A-B). This structure is thought to be part of the digestive system and will be further investigated in Chapter 4. This was consistently different when compared to *L. quinquemaculosa*. Confocal fluorescent micrographs of the ventral aspect of *L. davidcooki* also revealed the taxonomic character of the curved 4$^{th}$ coxa that makes the ventral shield more rounded when compared to *L. quinquemaculosa*. Table 3 summarizes the morphological characters that were used to distinguish these two species.

**Table 3. Morphological characters used to distinguish *L. quinquemaculosa* from *L. davidcooki*.**

| Character | *L.quinquemaculosa* | *L. davidcooki* |
|---|---|---|
| Body size | 1.5 to 2 mm | 1-1.5 mm |
| 4th coxa on venter | Narrow indented | rounded |
| Presence of swimming hairs | Mostly absent | Prominent |
| Chelicerae | No jagged edges | Jagged edges |
| Transparency of dorsal integument | No | Yes |

***Molecular cytochrome oxidase I (COI) barcodes of Lebertia***

*Lebertia* found at Blue Heron Lagoon were barcoded and analyzed using a neighbor joining tree (see Figure 15). The results show that there are two major population types present in BHL with the possibility of two other species. The brackets identify the two major branches that were morphologically characterized in this chapter including the new species description for *L. davidcooki*. Figure 10 shows a representative COI barcode of *L. davidcooki* (2BHL111116AV) compared to a representative COI barcode of *L. quinquemaculosa* (8BHL072216) using a 2 sequence BLAST analysis. The total length analyzed was 672 bases

and there was 567 bases that matched between the two sequences, giving 84% identity.

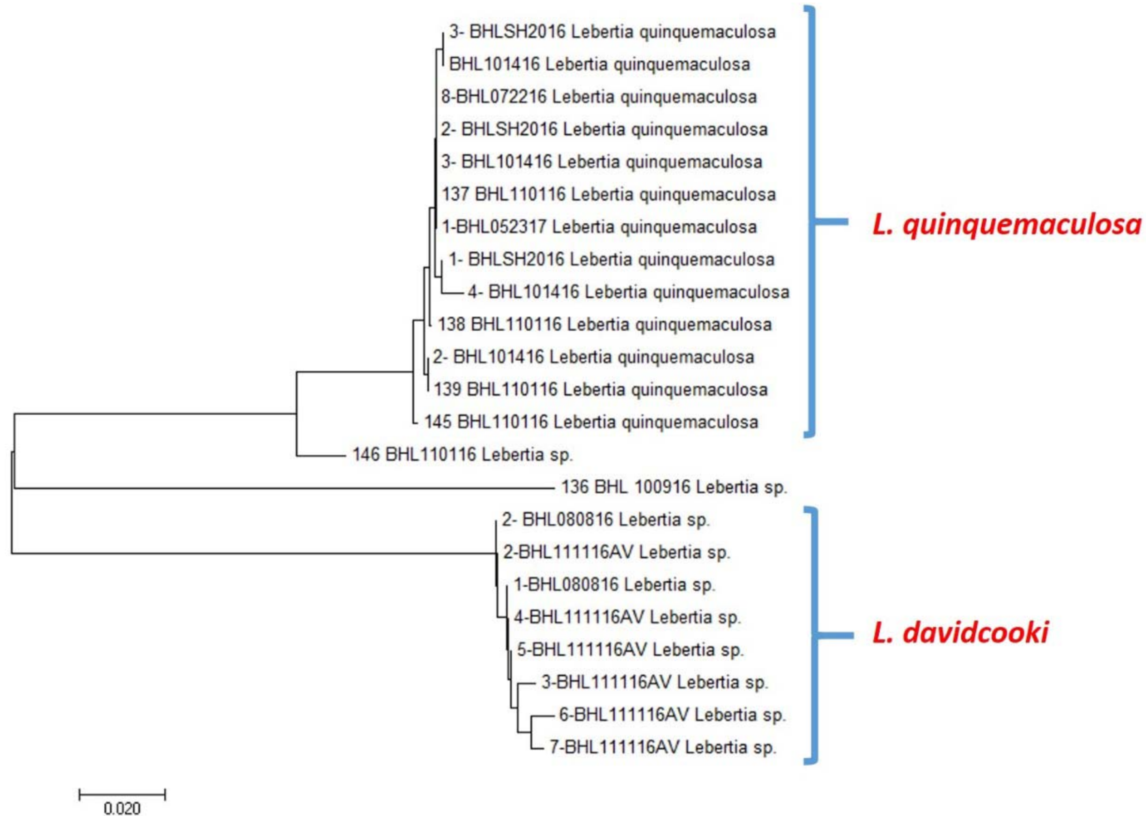



**Figure 15: *Lebertia* neighbor joining tree showing sequence relationships among COI sequences at 605 nucleotide positions for 23 *Lebertia* specimens collected from Blue Heron Lagoon**. The analysis was conducted in MEGA 6.

None of these *Lebertia* sequences were found on the public database and the average (4 sequences) percent query coverage (QC) and match hit identity (ID) for *L. quinquemaculosa* were QC:95.7% and ID:88.5% respectively and QC:97% and ID:88.5% for *L. davidcooki*, respectively. The two other potential species were also very distant from any other sequences on the GenBank database with specimen 146BHL110116 having a QC at 94% and ID at 86% while specimen 136BHL100916 was QC 97% and ID 87%, respectively. Our sequences will begin to address what Trebitz et al. (2015) bemoaned as the total absence of species identification of water mite sequences for the Great Lakes in the public databases. Species-specific primers were designed that can distinguish the two species, but are not the focus of this work and will be published elsewhere.

Query: 8BHL072216 *L. quinquemaculosa*
Subject: 2BHL111116AV *L. davidcooki*
Total length: 672 Matches: 567/672 Percent: 84%

```
Query  3    AAAATCAAAATAAATGTTGGTATAAGATAGGATCTCCTCCTCCTGCTGGGTCAAAAAATG  62
Sbjct  1    AAAATCAAAAGAGGTGTTGATATAAAATTGGATCTCCTCCTCCTGCTGGGTCAAAGAATG  60

Query  63   ATGTATTAAAATTTCGGTCAGTCAATAACATAGTGATTGCGCCAGCCAGTACTGGTAGTG  122
Sbjct  61   ATGTATTAAAATTTCGATCTGTTAGAAGTATAGTAATGGCTCCAGCTAAAACTGGAAGTG  120

Query  123  AGAGAAGTAGTAAAATAACTGTAATAAAAATTGACCATACAAATAAGGGAATTTGTTCTA  182
Sbjct  121  ATAGAAGTAATAAAATTACTGTAATAAAAATTGATCAAACAAATAAGGGGATTTGTTCTA  180

Query  183  TTTTTATTGTTTTTGGTTTTATATTAATAATTGTTGCTATGAAGTTAATTGCTCCTAAAA  242
Sbjct  181  TTTTTATAGTTTTTGGTTTTATATTAATAATTGTTGCTATGAAATTAATAGCTCCTAGAA  240

Query  243  TAGAAGAGATACCAGCTAAGTGTAAAGAGAAAATGGTTAAATCAACTGCTGGTCCGGCAT  302
Sbjct  241  TTGAGGAGATTCCTGCTAGATGGAGAGAGAAAATGGTTAAATCTACTGAAGGACCAGCGT  300

Query  303  GGGCTAAATTCCCTGATAGAGGTGGGTAAACAGTTCACCCTGTTCCAGCTCCTATGGCTG  362
Sbjct  301  GAGCTAAATTTCTTGATAAAGGAGGATAAACAGTTCATCCTGTTCCTGCTCCTATTCCTG  360

Query  363  TGAAAGAACTAGATAGTAGAAGAGTTAAAGAGGGGGGTAAAAGTCAAAATCTTATATTGT  422
Sbjct  361  TAAAGGAACTTGATAGAAGTAGAGTTAAGGATGGAGGAAGAAGTCAAAATCTTATATTAT  420

Query  423  TTATTCGTGGAAATGCTATATCTGGAGCTCTAATTATCAATGGGACTAGTCAATTTCCGA  482
Sbjct  421  TTATTCGTGGAAAAGCTATATCTGGGGCTCTGATTATTAGTGGAACTAATCAGTTTCCAA  480

Query  483  ATCCTCCAATTATTATTGGCATAACTATAAAGAAAATTATAACGAAGGCGTGAGCTGTTA  542
Sbjct  481  AACCTCCAATTATTATTGGTATTACTATGAAAAAAATTATAACGAAAGCATGAGCAGTTA  540

Query  543  CGATAGTATTATAAATTTGGTCTCTTCCTAGGAGAGTTCCTGGTTGTCCTAATTCTAAAC  602
Sbjct  541  CAATTGTATTGTAAATTTGGTCACTTCCTAGGAGTGAGCCTGGTTGTCCTAATTCAAGTC  600

Query  603  GGATTAAAGTTCTTAGTCTTGCTCCCACCATTCCTGATCAAGCCCAAAAGCGAAGTATA  662
Sbjct  601  GAATTAGGGTTCTTAATCTAGCTCCAACTATTCCGGATCATGCTCCAAAAGCAAAGTAAA  660

Query  663  GGGTTCCAATAT  674
Sbjct  661  GAGTTCCANTAT  672
```

**Figure 16: Two sequence BLAST comparing *L. quinquemaculosa* (8BHL072216) to *L. davidcooki* (2BHL111116AV).** Red circles indicate areas of mismatches while green circles show conserved regions, useful for designing primers.

## Discussion

Blue Heron Lagoon, located on an island in the Detroit River, Michigan was the site of *Lebertia* collections. Distinctive *Lebertia* specimens were identified in the collections using light microscopy and scanning electron microscopy. These tools were valuable for differentiating at least two different *Lebertia* species in BHL, including the previously described

*L. quinquemaculosa*. Molecular barcoding was carried out on the specimens, and this along with the microscopy revealed a new species that we are naming *L. davidcooki*. The discovery of this new species further illustrates the great biodiversity that is found in BHL which is located in a Michigan state park in Detroit, Michigan. Our results support the suggestion that further studies of this type throughout the Great Lakes would undoubtedly reveal many new species of water mites and expand our knowledge of native species and enable the detection of new, possibly non-native species of water mites in the Great Lakes.

# CHAPTER 4 - STRUCTURE AND FUNCTION OF FEEDING AND DIGESTION OF *LEBERTIA*

## Abstract

Water mites are aquatic arachnids with a complex life cycle that involves an airborne parasite of aquatic insects and an aquatic adult stage completely under water. This organism presents an interesting digestive physiological adaptation where it consumes a liquid diet completely under water. Here I have characterized the digestive physiology and gut structure of several closely related species of North American water mites.

Water mites from Blue Heron Lagoon at Belle Isle, Detroit were collected and processed for digestive physiological studies using light, fluorescent, scanning and electron microscopy. *Lebertia quinquemaculosa* Marshall and a newly-named *Lebertia* species (*L. davidcooki*, referred to in this chapter as *Lebertia* n. sp.) from Blue Heron Lagoon were chosen as model experimental organisms. *L. quinquemaculosa* and *Lebertia* n. sp. were studied to characterize the structures that facilitate digestive passage of ingested food. Chironomid prey that had been stained with fluorescein diacetate (FDA) were used to characterize the gut structures that water mites use to transport and process ingested food. After ingesting fluorescent chironomids, water mites were visualized using confocal fluorescent microscopy to identify and describe gut structures of the engorged water mites. Scanning electron microscopy was used to scan externally and characterize the major structures used for feeding such as the chelicerae. Transmission electron microscopy (TEM) was used to scan internally and visualize the ultrastructures of *L. quinquemaculosa* and *Lebertia* n. sp. To our knowledge this is the first time that *Lebertia* water mites have been studied this way and the first time that this combination of techniques has been applied to study water mite digestive physiology.

The excretory organ and mid gut were characterized in *Lebertia* and found to be lined

with large digestive cells. No connection between the mid gut and excretory organ was observed, consistent with previous studies in other mite species. Histochemical analysis of water mite tissues revealed the presence of lipids by Oil Red O staining. This work represents the first ever comprehensive analysis on the digestive system of *Lebertia* water mites.

**Background**

Water mites, like their cousins, the terrestrial mites and ticks, feed by injecting secretions of enzymes that then liquefies prey tissue allowing the water mites to feed on a liquefied diet (Smith et al. 2010). Although a great body of information is available for terrestrial mites and tick digestive physiology, comparable information on water mite digestive physiology is lacking. A comprehensive review by Smith et al. (2010) on freshwater invertebrates had only one previous and outdated citation on work done on water mite digestive physiology, which was published in 1938 (Bader 1938). Recent morphological descriptive works on larval water mites and marine water mites revealed several interesting structural features whose complete functionality is still in question (Smit and Alberti 2009, Shatrov 2012). Only a couple of other descriptive studies of the gut structures of freshwater water mites including; *Teutonia cometes*, from Eurasia, have been reported, with no studies of this type done on the water mites of North America (Shatrov 2010b).

As a first step in understanding the ecological role and potential use of aquatic organisms, greater knowledge about the basic physiology is needed. Since water mites are voracious predators and efficient parasites of pests that transmit disease the information that will be gleaned from this type of work has the potential of being used in development of selective and targeted approaches at controlling pest aquatic invertebrates (Werblow et al. 2015). In a following chapter I report work on which I used DNA sequencing to investigate the diet composition of water mites but the use of vital dyes or fluorescent dye-infused prey items can

also be useful for studying digestive physiology and has led to discoveries such as an alternative tract to remove waste in comb jelly fish (Maxmen 2016).

Vitals dyes have proven useful for the study of insect anatomy and for ecological studies (Hershberger 1946, Peters and Chevone 1968). Vital dyes such as Nile Blue A and Trypan Blue are especially useful because they accumulate in the gut region of insects and pose no harm to the organism being studied (Hershberger 1946, Peters and Chevone 1968). In this chapter, these dyes and also fluorescein diacetate (FDA) are used to characterize the water mite digestive tract. Fluorescein diacetate is a non-fluorescent vital substrate which, when acted upon by enzymes within living organisms, produces fluorescein, a brightly staining green fluorescent compound. Fluorescein diacetate is used here to stain the midge larvae *Chironomus riparius* (Adams et al. 2014) and other organisms that are water mite prey. Fluorescein diacetate has been determined to be a useful staining agent that has also been implemented in the Ram laboratory for use in a detection apparatus for potentially invasive organisms in ballast water (Adams et al. 2014, Akram et al. 2015). Laser confocal fluorescent microscopy is also used here to characterize the internal anatomy of the water mite digestive system. Confocal microscopy has been reported to be very useful in understanding morphological features in a wide array of arthropods including water mites (Valdecasas 2008, Haug et al. 2011, Valdecasas and Abad 2011). On water mite fixed tissues, confocal microscopy enables the visualization of the internal anatomy of the water mite digestive system by microdissection.

Previous studies on the water mite digestive system have been carried out mainly using microdissection techniques and have reported that the gut of the water mite ends in a blind sac and that there is no connection between the gut and the excretory organ which expels waste through the excretory pore (anus) (Pollock 1898). Subsequent work on Parasitengona, which water mites belong to, has suggested that loss of a sophisticated digestive system could be a

result of their efficient digestive physiology where they do not need a functional hind gut and can rely on absorptive cells to carry out all the steps of digestion including excretion (Mitchell 1970). To our knowledge there has only been a couple of other studies on the digestive structures of various species of water mites using TEM. These studies report that the mite gut is a blind sac and no connectivity to the excretory organ exists (Shatrov 2010b). We used a combination of scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to study external and internal structures of the mite that are used for feeding and digestion. This chapter reports on the analysis of *Lebertia quinquemaculosa* and *Lebertia* n. sp. feeding and digestive related structures.

**Material and Methods**

***Water mite laboratory maintenance and feeding experiments***

Mites of the genus *Lebertia* were maintained in the laboratory. Eight-well or twelve-well cell culture plates were used to house individual water mites and were checked periodically to replenish water and food if necessary. These and other species have been reported to inhabit lakes, ponds and bogs and can be found at collection sites on Belle Isle (Cook 1954). Mites were collected at Blue Heron Lagoon, Belle Isle in great numbers (see population graphs in a previous chapter) and have been maintained in our laboratory for up to three months with minimal care. These mites were acclimatized in the lab and then fed frozen or living chironomids (blood worms) and mosquito larvae. Water mites were also fed living chironomid larvae that have been exposed to FDA to assist in visualizing the gut passageway *in vivo* (see water mites vital dye tracing section).

Water mite feeding habits were studied in the lab by videography of freshly captured water mites in the presence of sampling bycatch. Sampling by-catch includes prey items from their natural environment, including copepods, chironomid larvae, ostracods and other

microinvertebrates. Water mites that are specifically fed prey items were also recorded by videography and photography (http://sun.science.wayne.edu/~jram/ramlab.htm).

***Analysis of the* Lebertia *digestive tract using vital dye: fluorescein diacetate (FDA)***

To understand water mite digestion, mites were fed with living chironomid larvae that were exposed to fluorescein diacetate (FDA) to serve as a marker of location. FDA fluorescence intensity was not a component of the analysis. An original stock of FDA was prepared by dissolving 20 mg of FDA in 1 ml dimethylsulfoxide (DMSO, Sigma Aldrich, St. Louis MO). A dilute stock was then made using 100 µl of the original stock mixed with 900 µl DMSO. An experimental stock was then made using 50 µl of the diluted stock combined with 5 ml buffer (10 mM Na-MOPS, pH 7.0). *Chironomus riparius* were placed in the solution with FDA and visualized periodically under the fluorescent microscope. After approximately 10-20 minutes the *C. riparius* was observed to be fluorescing strongly by exposure to the FDA. At this point the *C. riparius* was fed to *Lebertia* water mites that were housed in the 6 or 12 well plates. Once *Lebertia* mites were observed to start feeding on *C. riparius* they were left undisturbed for 4-6 hours. The feeding *Lebertia* were monitored periodically to ensure continued feeding. Using fluorescent microscopy the passage of the FDA was tracked to determine the passage of food through the digestive system of mites.

***Laser confocal fluorescent microscopy analyses of* Lebertia *structural features***

*Lebertia quinquemaculosa* and *Lebertia* n. sp. of mites that had been stored in 90% ethanol were both subjected to analyses using laser confocal fluorescent microscopy. Laser confocal microscopes (Zeiss, Confocal Laser Scanning Microscope) were used to study both the external and internal structures of dissected *Lebertia* mites. An inverted fluorescent microscope (Nikon, Ts2R) was also used in conjunction to visualize the structures using differential interference contrast (DIC). *L. quinquemaculosa* was also dissected and visualized under laser

confocal fluorescent microscopy.

***Scanning electron microscopy of Lebertia water mites***

The Scanning Electron Microscopy (SEM) was done with collaborators at the United States Department of Agriculture (USDA) in Beltsville, MD with my on-site and, at other times, remote assistance. Specimens were observed in the LT-SEM as described in Bolton et al. (Bolton et al. 2014). Live specimens were secured to 15 cm x 30 cm copper plates using ultra smooth, round (12 mm diameter), carbon adhesive tabs (Electron Microscopy Sciences, Inc., Hatfield, PA, USA). The specimens were frozen conductively, in a Styrofoam box, by placing the plates on the surface of a pre-cooled (-196 °C) brass bar whose lower half was submerged in liquid nitrogen ($LN_2$). After 20-30 s, the holders containing the frozen samples were transferred to a Quorum PP2000 cryo-prep chamber (Quorum Technologies, East Sussex, UK) attached to an S-4700 field emission scanning electron microscope (Hitachi High Technologies America, Inc., Dallas, TX, USA). The specimens were etched inside the cryo-transfer system to remove any surface contamination (condensed water vapor) by raising the temperature of the stage to -90° C for 10-15 min. Following etching, the temperature inside the chamber was lowered below -130°C, and the specimens were coated with a 10 nm layer of platinum using a magnetron sputter head equipped with a platinum target. The specimens were transferred to a pre-cooled (-130 °C) cryostage in the SEM for observation. An accelerating voltage of 5 kV was used to view the specimens. Images were captured using a 4pi Analysis System (Durham, NC). Individual images were re-sized and placed together to produce a single figure using Adobe® Photoshop CS 5.0.

***Toluidine Blue O staining of Lebertia structural features***

Toluidine Blue-O staining was carried out on tissues fixed in 2.5% glutaraldehyde by our collaborators at the Beltsville Laboratory. For light microscopy, semi-thin sections with a

thickness of approximately 0.25 µm were transferred to a drop of water on a glass slide, heated to 65 °C to adhere the sections onto the slide. While the slide was still warm, sections were stained with 0.25% Toluidine Blue-O, rinsed with ethanol, and sealed with Permount and a coverslip. Slides were imaged with a Zeiss Axio ZoomV16 stereo zoom microscope.

***Transmission electron microscopy of Lebertia water mites***

*Lebertia* mites were selected for transmission electron microscopy (TEM). Water mites were euthanized and preserved by immersion of a vial containing them in "barely boiling" water for one minute, followed by their transfer to freshly prepared 2.5 % glutaraldehyde with 0.1 M PBS (pH=7.4) buffer. The mites were placed in fixative for 2 hours on a rotator at room temperature or, alternatively overnight in a 4 $^{o}$C cold room. After preservation the mite was placed on a petri dish with fixative and then poked in the posterior of the mite with a minutien pin to allow easier access of fixatives to the mite interior. In some cases mites were not poked to better preserve the posterior structures of the tissue for TEM analysis. The procedures from this point were applied by collaborators at the United States Department of Agriculture (USDA) in Beltsville, MD who conducted the final fixation and sectioning of the water mites for TEM analysis. Mites were then fixed for 2 hours at room temperature in 2% paraformaldehyde, 2.5% glutaraldehyde, 0.05 M NaCacodylate, 0.005M $CaCl_2$ (pH 7.0), then refrigerated at 4°C for several days. In order to facilitate optimal diffusion of solutions into the mite, portions of the mite were cut off and legs were removed. Mites were then rinsed 6 times over the course of 1 hr with 0.05M NaCacodylate, 0.005 M $CaCl_2$ buffer and post-fixed in 1% buffered osmium tetroxide for 2 hours at room temperature. The tissue was then rinsed again 6 times in the same buffer and dehydrated in a graded ethanol series (25%, 50%, 75%, 95%, 100%). Z-6030 silane was added to the ethanol to improve the adhesion of the resin to the mite cuticle. Mites were further dehydrated in 2 exchanges of propylene oxide and infiltrated in a graded series of LX-

112 resin/propylene oxide and polymerized in 100% resin at 65°C for 48 hr. 60-90nm silver-gold sections were cut on a Reichert/AO Ultracut ultramicrotome with a Diatome diamond knife. Sections were uncompressed with chloroform vapor and mounted onto carbon/formvar-coated copper slot grids. Grids were stained with 4% uranyl acetate for 10 minutes, 3% lead citrate for 5 minutes and imaged at 80 kV with a Hitachi HT-7700 transmission electron microscope. Selection of section areas to study and photographic analysis were done with my direct involvement in the process both during a research visit to the Beltsville, MD USDA Laboratory, where the electron microscope is located and by intense interactions by phone or email.

A total of 4 specimens have so far been sectioned and examined at various levels with the TEM. In the paradigmatic specimen illustrated in this chapter, sectioning of the mite for TEM followed the schematic illustrated in the accompanying Figure 17, showing three major levels at which structures were examined.

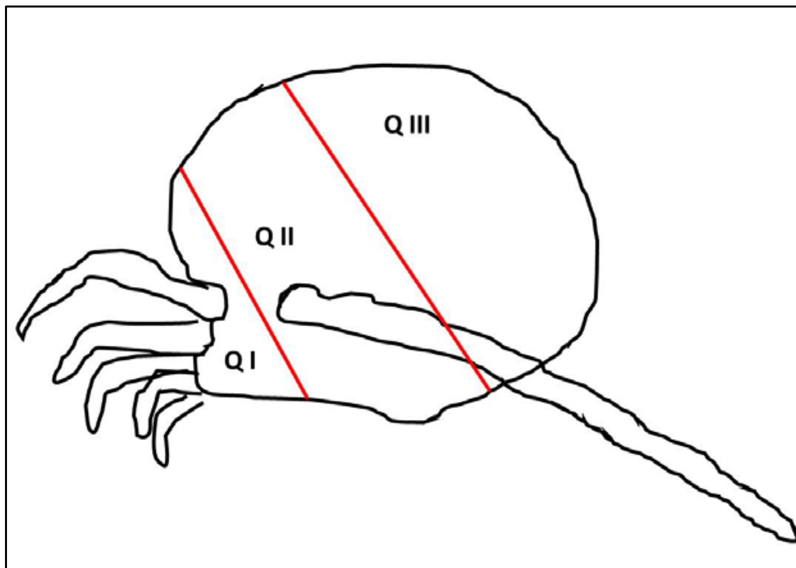



**Figure 17: *Lebertia* schematic of TEM analysis**. Sections were done in three major quadrants labelled as QI: quadrant 1, QII, quadrant 2 and QIII, quadrant 3. Red lines indicate where mites were cut for TEM analysis.

***Histochemical and structural analysis on Lebertia tissues by Oil Red O***

Histochemistry was used to identify water mite enzymes and to determine the locations of lipid storage. Mite tissues were prepared in three ways: first, by freezing mites at -80 $^{o}$C in

Tissue-Tek® O.C.T. Compound (Sakura® Finetek, VWR) and storing in -80 $^{o}$C. Water mite tissues were then sectioned using a cryostat (Thermo Scientific) and slides were frozen at -80 $^{o}$C until analysis. Water mite digestive storage cells (lipids) were localized by staining with Oil Red O which stains for lipid deposits (Bancroft and Gamble 2002, Mehlem et al. 2013). Briefly, frozen tissue sections were air-dried for 30 min at room temperature. Tissues were then fixed in ice cold 10% formalin for 5-10 minutes. Slides were then rinsed immediately in 3 changes of distilled water. Slides were allowed to dry for a few minutes. After this, slides were placed in absolute propylene glycol for 2-5 minutes to avoid carrying water into the Oil Red O (Sigma Aldrich, St. Louis). The slides were stained in pre-warmed Oil Red O solution for 8-10 min in a 60 $^{O}$C oven, placed in 85% solution of propylene glycol for 2-5 min, rinsed in 2 changes of distilled water, stained in Gill's or Mayers hematoxylin for 30 seconds, washed thoroughly with running tap water, given one final wash in distilled water, and mounted as needed.

## Results

### *Feeding and excretory behavior of water mites*

Water mites of different genera were observed feeding on prey by-catch in laboratory experiments (see Figure 18). Prey selected included ostracods, chironomid larvae, chironomid pupae and cladocerans. Further laboratory experiments were done with prey items collected from cisterns around Wayne State University Medical School. Organisms found in the cisterns included *Culex pipiens* and *Chironomus riparius* larvae (identity confirmed bother by morphological appearance and by molecular barcoding) but were not observed to have any water mites present. In the laboratory, water mites readily consumed these prey items (see Figure 19A-C). Water mites also attacked prey items not ordinarily encountered in their natural habitat, such as *Drosophila* larvae (see Figure 19D). *Lebertia* use of chelicerae (mouthparts) for this attack process was also observed (data not shown). Video-recordings of repeated experiments

illustrating these phenomena can be viewed online at the link provided above.

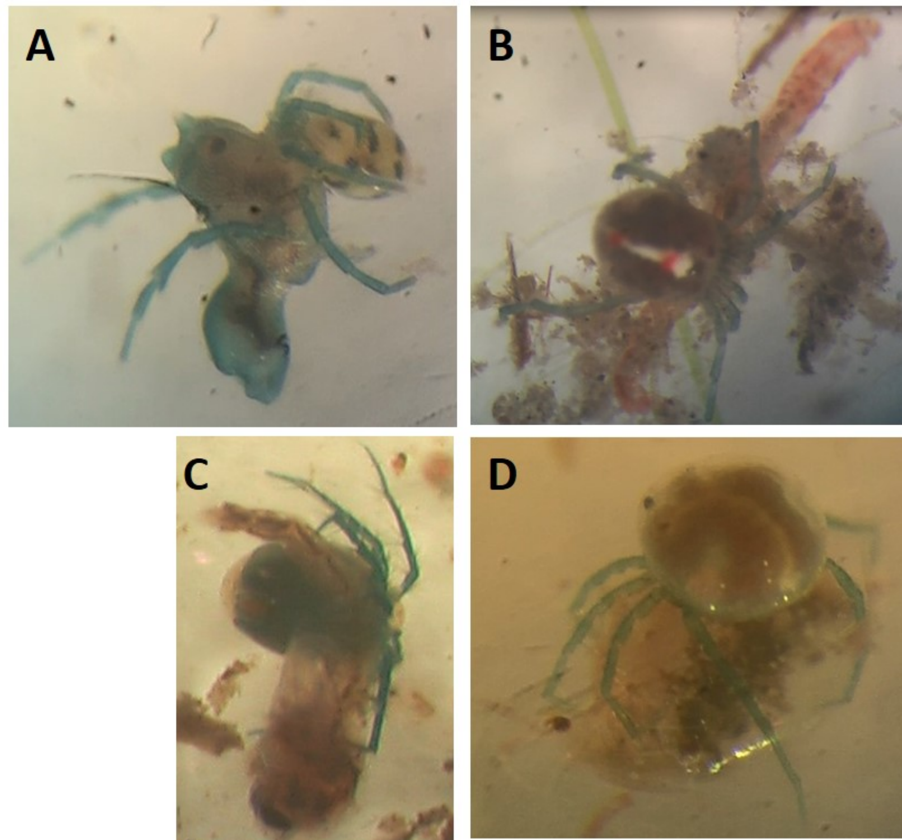
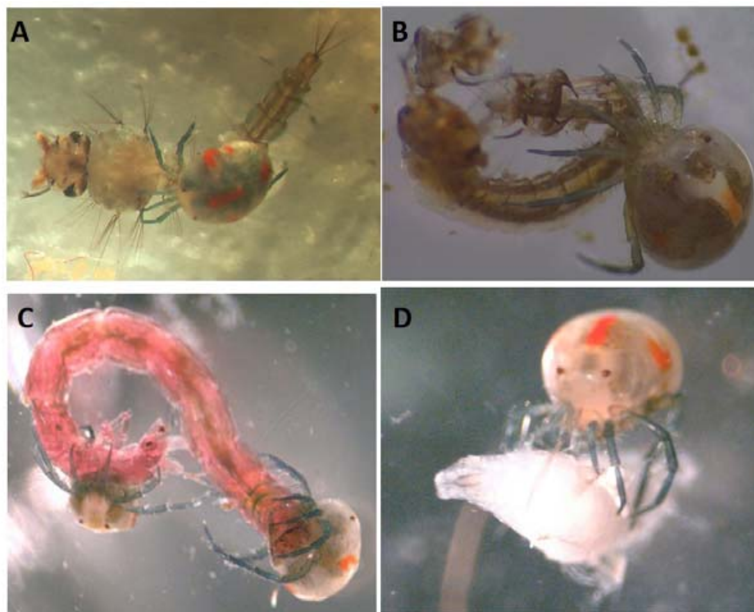



**Figure 18: Water mites preying on by-catch**. (A) *Arrenurus* feeding on an ostracod. (B) *Lebertia* feeding on chironomid larvae. (C) *Oxus* feeding on chironomid pupae. (D) *Neumania* feeding on cladoceran.

**Figure 19: *Lebertia* feeding on collected prey.** (A-B) *Lebertia* feeding on *Culex pipiens* collected from cistern. (C) *Lebertia* feeding on *Chironomus riparius* collected from cistern. (D) *Lebertia* feeding on *Drosophila* larvae.



At the other end of the mite, behavioral observations via video micrography also recorded the excretory process of water mites, including associated internal gut movements. In video recordings from which still images were captured, contractile movements of internal tissues of *Lebertia* water mites occurred prior to and during excretion of waste products from the excretory pore (Figure 20).

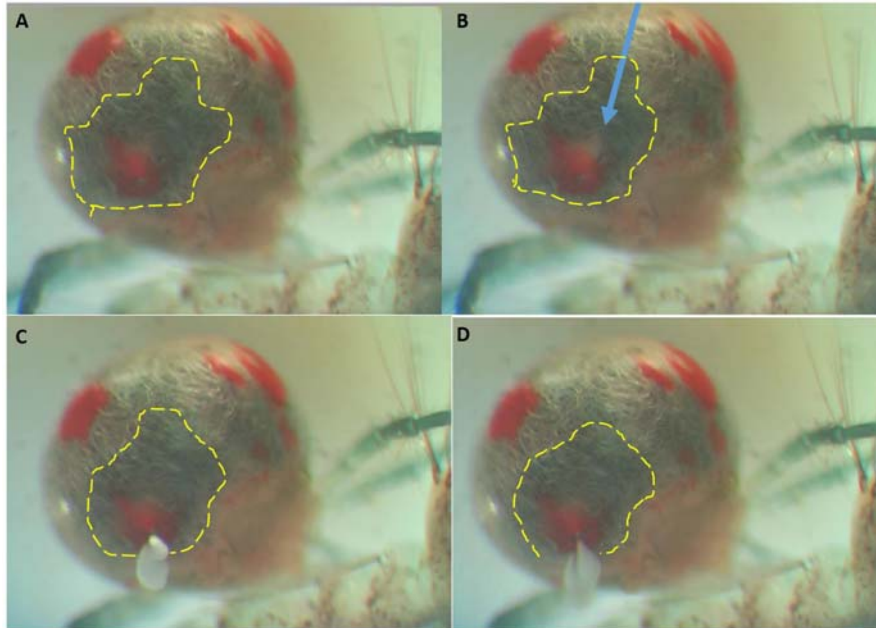



**Figure 20: *Lebertia* excretion and movement of internal structure**s. (A-D) *Lebertia* was video-recorded and movement of internal tissues was outlined (dashed yellow line) which corresponded with violent ejection of waste products (seen as milky white substance). Blue arrow indicates the excretory pore region.

***Anatomical features of the Lebertia digestive tract, from anterior to posterior***

*Overview of the feeding and digestive system of* Lebertia *mites*

Overviews of the entire organism both externally and internally illustrate several important features of the digestive tract, which will then be further examined in higher power analyses to follow. Externally, the micrographs in Figure 21 and 22 illustrate the complex of mouth parts and associated grasping structures for acquiring, holding, and piercing prey.

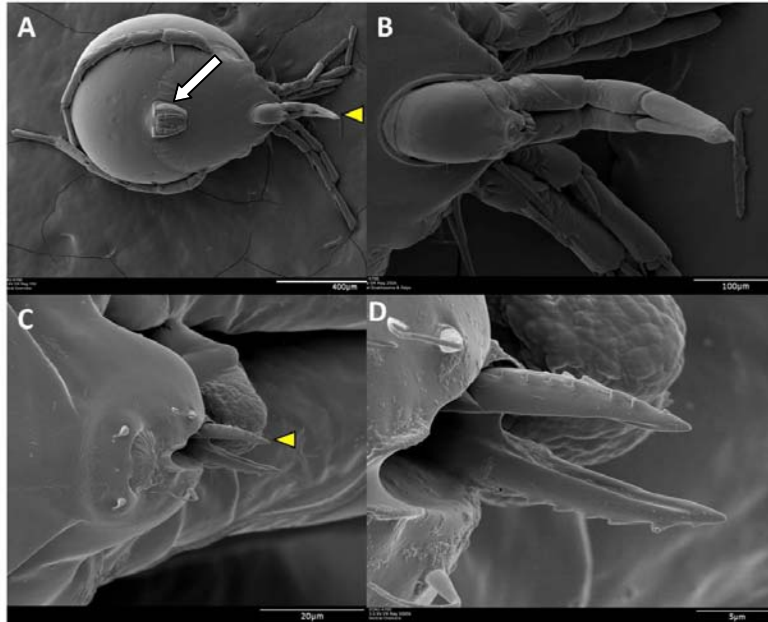



**Figure 21: SEM of *Lebertia* n. sp. mouth parts.** (A) Ventral view of *Lebertia* n. sp. showing placement of mouth parts with yellow arrow pointing to the pair of palps. White arrow points to genital field (B) Magnified view of the mouth parts with gnathosoma in view that contains chelicerae. (C) Magnified view showing gnathosome with yellow arrow pointing to chelicerae. (D) Magnified view of the chelicerae showing jagged ends.

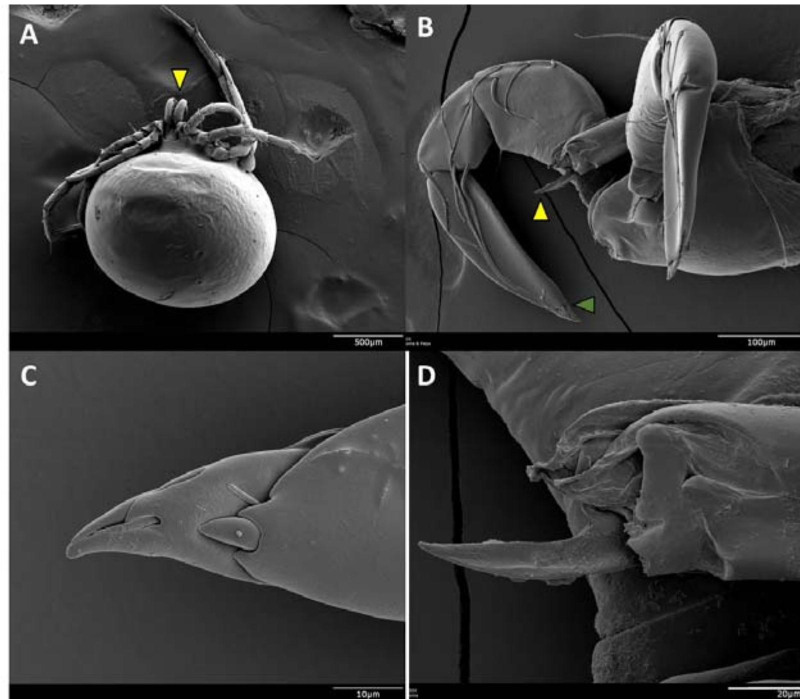



**Figure 22: SEM of *L. quinquemaculosa* mouth parts.** (A) Dorsal view of *L. quinquemaculosa* with yellow arrow pointing to mouth parts (pair of palps with chelicerae found underneath in the gnathosoma. (B) Dissected mouth parts with yellow arrow pointing to chelicerae and green arrow pointing to one of the pair of palps. (C) Magnified view of the tip of the palp. (D) Magnified view of the chelicera.

Chelicera of both species of *Lebertia* are illustrated in Figure 21C and 22D. These mouth parts are involved in the initial attack on prey and, due to their distinctive structural differences between species, are often used for differentiating mite species (see Chapter 3). The yellow

arrow indicates the chelicerae contained within the gnathosome and the green arrow indicates the palp. *Lebertia* n. sp. was also visualized using SEM and the palps are seen in Figure 21 A-B. The chelicerae can be seen in Figure 21 C-D with Figure 21D showing a magnified view of jagged, harpoon-like chelicerae. *L. quinquemaculosa* mouthparts are show in Figure 22 with the pair of palps indicated in Figure 22B.

An overview of the internal structures of *Lebertia* is provided by confocal autofluorescence microscopic pictures of dissected organisms, illustrated in Figure 23. Features to note, relevant to the digestive system include, in Figure 23A, the mouth structures on the exterior aspect of this micrograph, internal pharyngeal structures and numerous other muscle

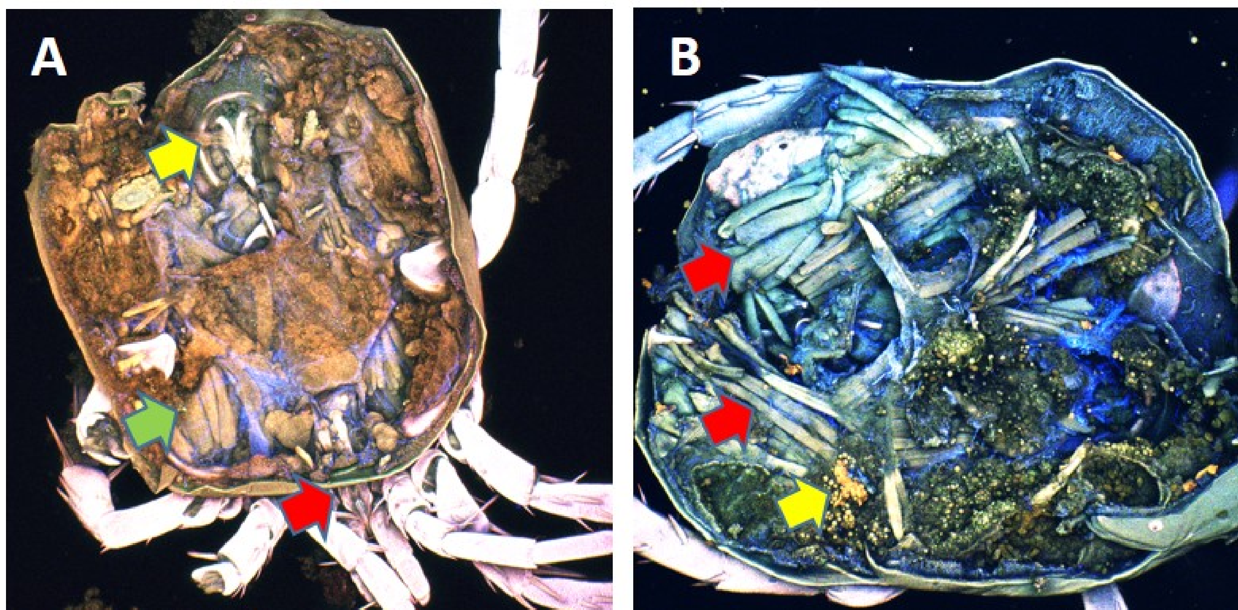



**Figure 23: Internal structures.** Dissected *Lebertia* mites reveal internal structures after fluorescent confocal microscopic analysis. (A) Dorsal view with yellow arrow pointing to the genital field, green arrow pointing to muscle bundles of legs and red arrow pointing to mouth. (C) Dorsal view with the red arrows showing distinct muscle bundles and yellow arrow showing yellow droplets. Mite orientation in A is Posterior – up and anterior – down, B posterior – left and anterior – right.

bundles that control the movement of appendages and numerous other muscle bundles that control the movement of appendages (including the palps and legs). In Figure 23B, yellow droplets that may correspond to lipids. Figure 23B also illustrates numerous other muscle

bundles, likely associated with the legs, though possibly also associated with the stomach and excretory organ which are not clearly identifiable in these images. For reference to other physiological systems, these micrographs also indicate locations of reproductive structures (genital field, identified in both Figures 21A and 23A).

***Anterior internal structures***

Figure 24A-B corresponds to TEM sections at the most anterior level (Figure 17) of a specimen of *L. quinquemaculosa*, with the black arrows in Figure 24A indicating a major group of muscles with a V-shaped orientation that presumably attaches at the pharyngeal orifice (PO). These muscles are thought to mediate a "pharyngeal pumping" movement that plays an important role in the intake of food through the pharynx. Figure 24B is a magnified image of single sarcomere within a striated muscle fiber. Figure 25 A-B shows representative sections from the first quadrant of *Lebertia* n sp. which has similar pharynx (P) morphology and the large V-oriented muscles (mus) that control the pharyngeal movements. The cuticle is represented by Cut. In Figure 25B a large secretory structure (putative salivary glands, SG) is seen in the middle of large well developed muscle groups (mus).

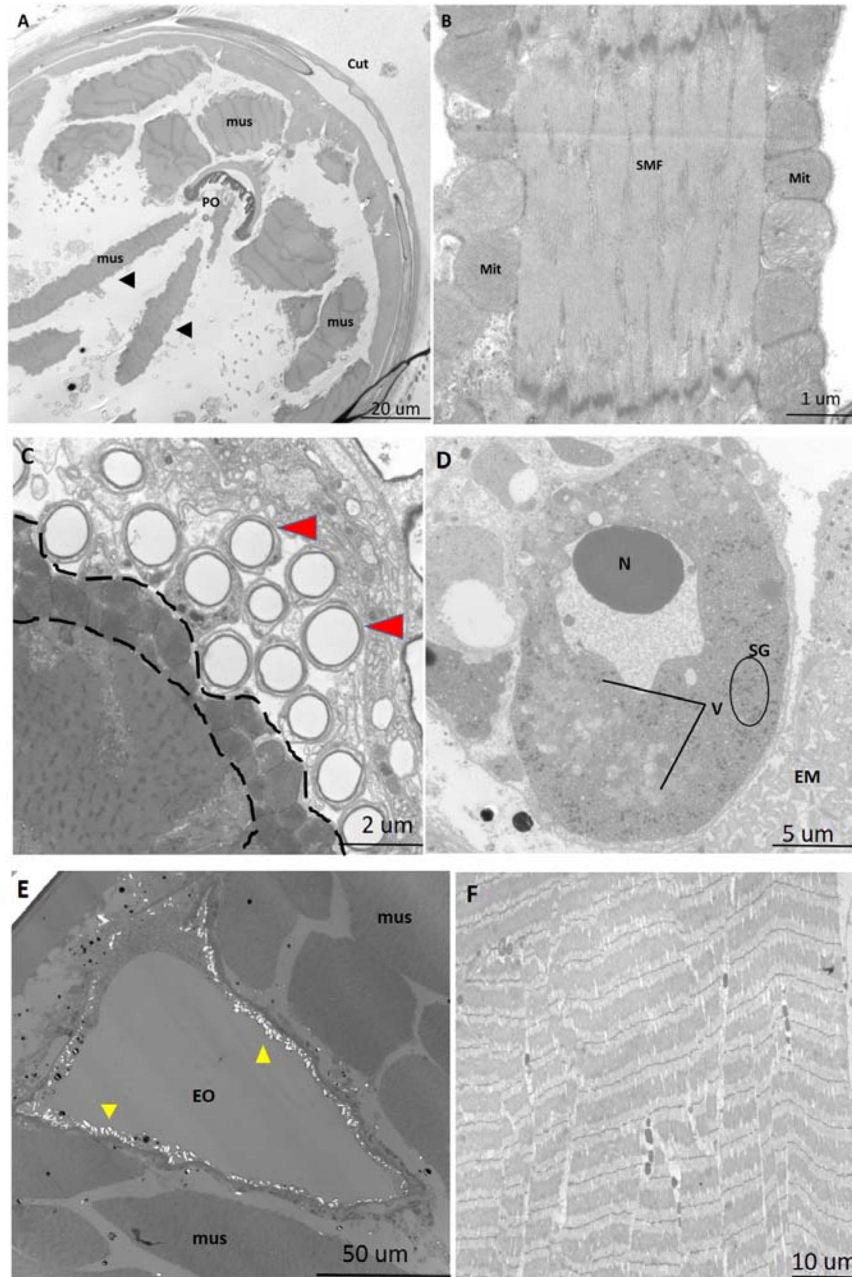



**Figure 24:** ***L. quinquemaculosa*** **TEM sections.** (A)Pharyngeal orifice (PO) can be seen with multiple striated muscles (mus) that are presumed to mediate movements of a “pharyngeal pump.” (B) A single sarcomere of a striated muscle fiber (SMF), with Z lines and large mitochondria (Mit) adjacent to the myofibrils marked. (C) Nerve tissue can be observed by red arrows adjacent to large digestive cells that are squeezed in between the open space of the mid-gut delineated by dotted lines. (D) Magnified digestive cell showing large nucleus (N), vacuoles (V), storage granules (SG) and the extracellular matrix (EM). (E) Excretory organ (EO) with the crystal waste (guanine) indicated by the yellow arrows. Musculature associated with the legs can be seen (mus). (F) Magnified muscle fiber typical of the type that assist with movement of digestive organs such as the mid gut and excretory organ.

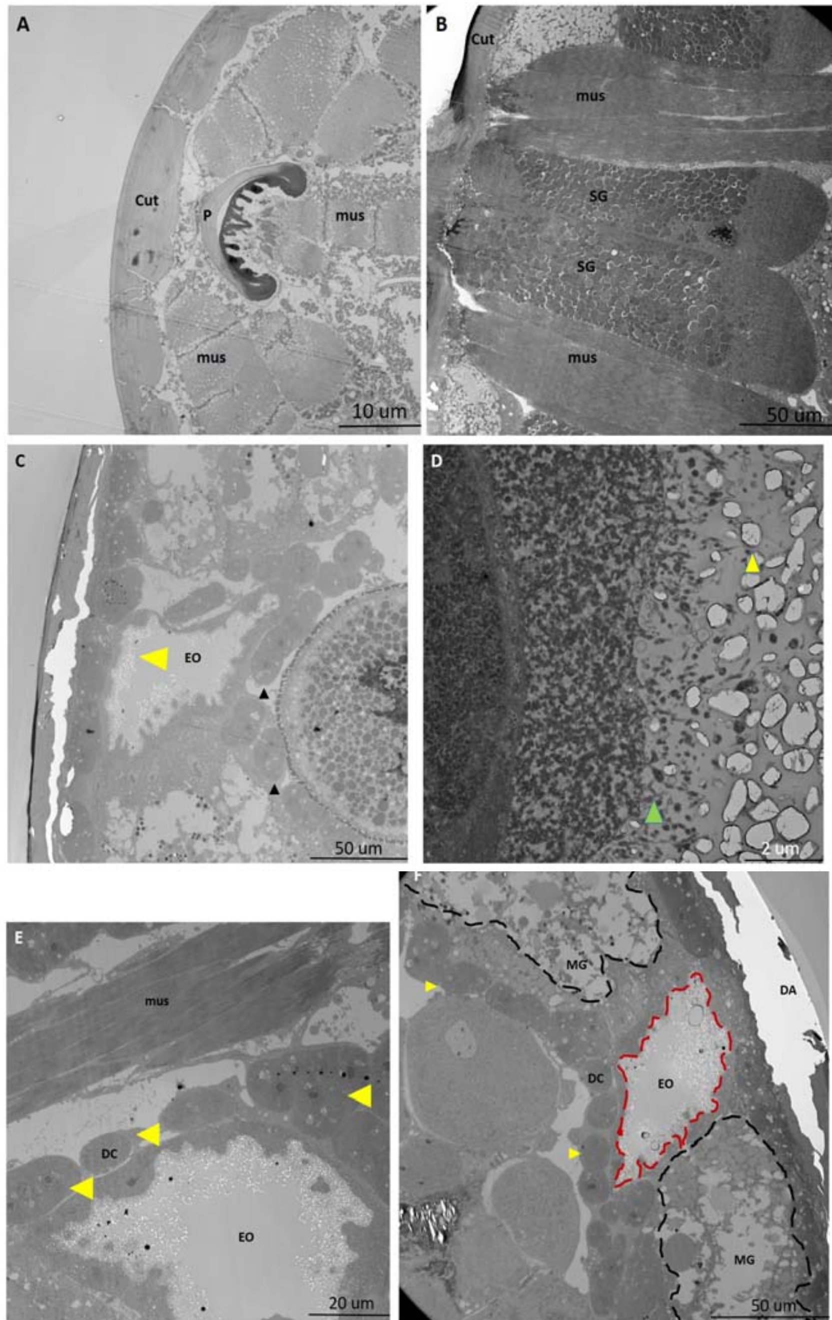



**Figure 25: *Lebertia* n. sp. TEM sections.** (A) Pharynx (P) can be seen with the characteristic large muscle groups (mus) that form part of the pharyngeal pump. Cut: cuticle. (B) Secretory granules are seen in a putative salivary gland structure situated among adjacent muscle groups (mus). (C) Excretory organ (EO) seen with yellow arrow pointing to characteristic white crystal structures within the EO. Black arrows indicate the large digestive cells. (DC) The lining of the EO is magnified with the yellow arrow pointing to the crystal like structures that are the presumed guanine crystal waste products and the green arrow pointing to the villi- like structure found at the edge of the EO. (E) The EO can be seen filled with crystals and the large digestive cells (DC) are seen lining the periphery indicated by yellow arrows. Muscle fibers (mus) are also observed. (F) The EO (red dotted line) can be seen in the middle of the two lobes of the mid gut (MG) (black dotted lines). The characteristic digestive cells (DC) are seen lining both structures indicated by yellow arrows. Dorsal aspect (DA) is labelled for context.

### *Midgut structures*

Midgut structures in the digestive system are shown first by sagittal cross sections of *Lebertia* n. sp. and *Lebertia quinquemaculosa* tissue stained with toluidine blue. The images in Figure 26A are lateral views of sagittal sections having dorsal at the top, ventral at the bottom and anterior (Ant) to the right and posterior to the left. The black dotted line represents the

midgut (MG) while the red dotted line outlines the excretory organ (EO). The EO is located within the folds of the midgut and near the dorsal aspect of the mite. The purple arrow is the salivary gland while the red arrow is an ovary. The well-developed muscle bundles that are attached to the pharynx are also seen (orange arrows) while the green arrow indicates the opening of the mouth. The synganglion can also be seen lying in between the pharynx and mid gut. When compared to *L. quinquemaculosa*, we can see similar structural features at the anterior portion of the mite (Figure 26C).

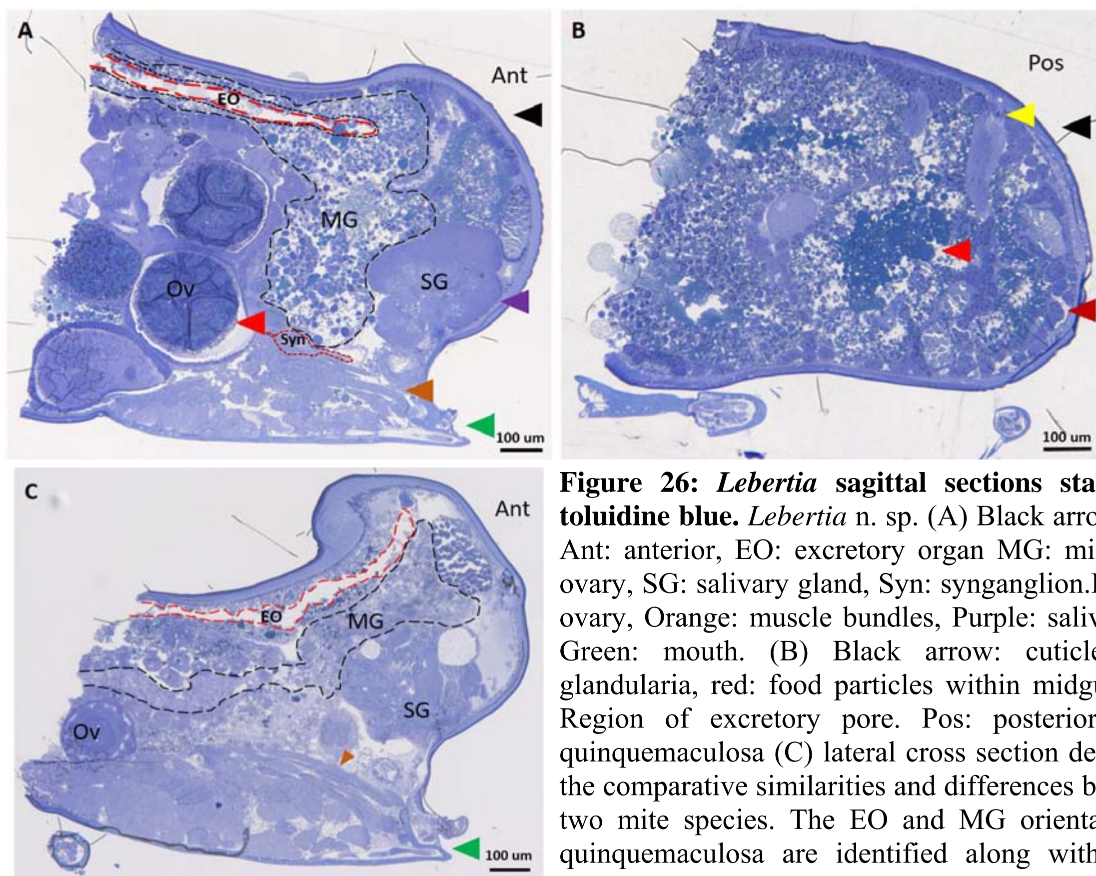



**Figure 26: *Lebertia* sagittal sections stained with toluidine blue.** *Lebertia* n. sp. (A) Black arrow: cuticle, Ant: anterior, EO: excretory organ MG: mid gut, Ov: ovary, SG: salivary gland, Syn: synganglion.Red arrow: ovary, Orange: muscle bundles, Purple: salivary gland, Green: mouth. (B) Black arrow: cuticle, yellow: glandularia, red: food particles within midgut, orange: Region of excretory pore. Pos: posterior. Lebertia quinquemaculosa (C) lateral cross section demonstrates the comparative similarities and differences between the two mite species. The EO and MG orientation in L. quinquemaculosa are identified along with the long pharyngeal muscle groups indicated by orange arrow. Green arrow points to the mouth while SG and Ov are identified. Anterior is to the right of the mite (Ant).

Several corresponding midgut structures are seen at higher power in TEM at level 2 of these specimens (Figures 24C-E and 25C-E). In Figure 24C the red arrow indicates nerve cells that could be a part of the synganglion of the mite as seen in Figure 26A. An outlined region in

Figure 24C delineated by the black dotted lines indicates the large digestive cells that line the midgut and excretory organ. Figure 24D is a magnification of one of the large digestive cells that are found throughout the mite. Vacuoles (V), secretory granules (SG) and extracellular matrix (EM) can also be seen in Figure 24D. Figures 24E-F are representative images from a level 3 section, showing in Figure 24E, the excretory organ (EO) with the white crystal structures found dispersed in the periphery (yellow arrows). Multiple large muscles are observed surrounding the EO in this image. Figure 24F shows an example of the many striated muscle fibers, possibly corresponding to some of the many muscle bundles illustrated in Figure 23B. Figure 24C-E shows the excretory organ (EO) in another specimen, with characteristic crystal structures found throughout. Large digestive cells are indicated with black arrows (Figure 24C). Figure 24D is a magnified region of the EO where the villi-like extensions on the periphery and white crystal structures are observed within the EO. Figure 24E-F are representative images of level 3 sections and the EO can be seen with large muscle groups (mus) adjacent to it. Large digestive cells (DC) are seen clearly lining the EO. Figure 24F shows the organization of the mid gut (MG) and the EO. The red dotted line outlines the EO and the black dotted line outlines the MG. Large digestive cells (DC) are located on the periphery of both the EO and the MG.

An alternative method of viewing the midgut region is possible in *Lebertia* n. sp. whose dorsal integument is sufficiently translucent to observe midgut structures directly with DIC and confocal autofluorescence microscopy without the need for dissection or sectioning (Figure 27). These *Lebertia* mites were more translucent on the dorsal aspect than *L. quinquemaculosa*, enabling the anatomical structures of the presumptive excretory organ (EO) to be visualized directly. This structure is also clearly seen in the auto-fluorescence microscopy image in Figure 27C. All three images show a clear delineation of the EO including a tube extending towards the excretory region of the posterior aspect of the mite.

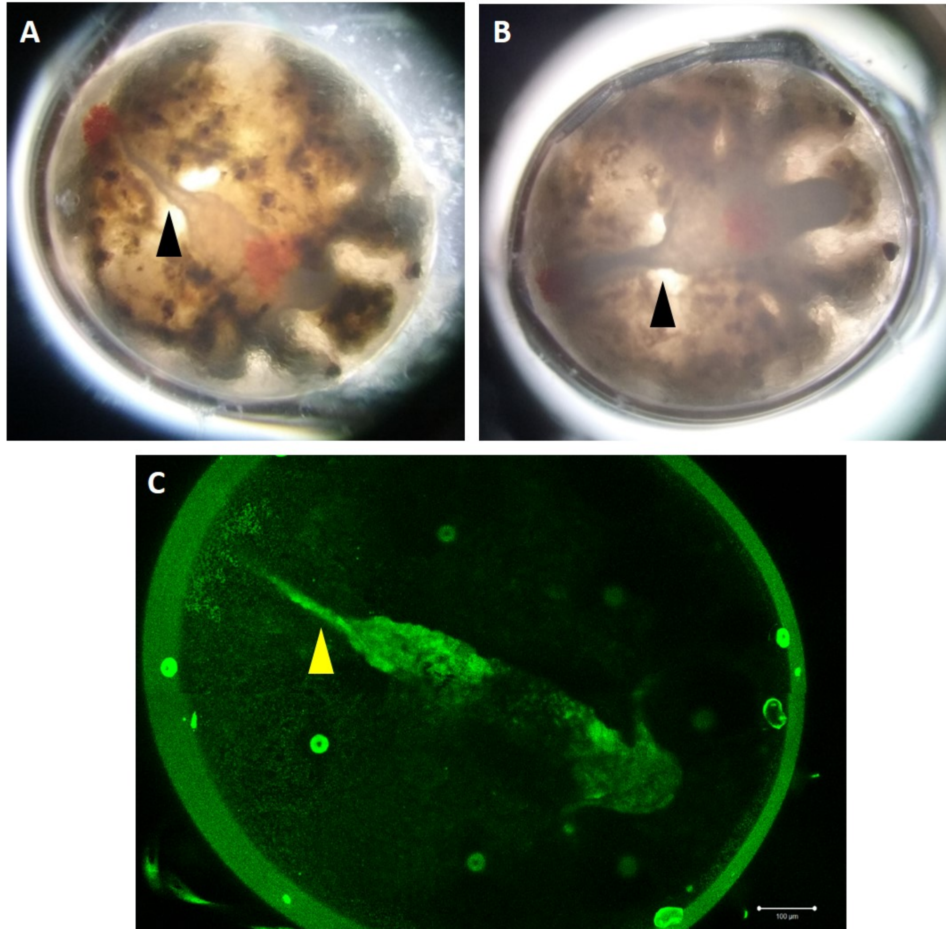


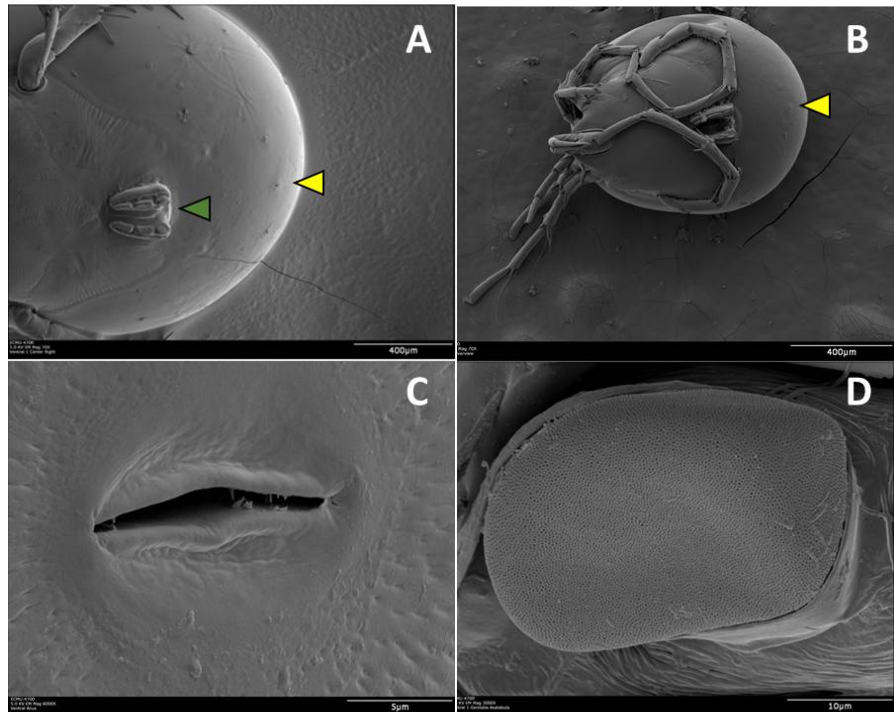

**Figure 27: *Lebertia* mite differential interference contrast (DIC) and confocal autofluorescence analysis.** (A-B) *Lebertia* mites with black arrows pointing to presumptive excretory organ (EO). (C) *Lebertia* mite observed with laser confocal fluorescent microscope with yellow arrow showing auto-fluorescence of EO.

### *Anal pore region*

In the toluidine-stained sections, Figure 26B shows the posterior region of a specimen, oriented with dorsal at the top and posterior (Pos) to the right. The red arrow highlights densely stained structures that likely correspond to particles in the EO shown in Figure 24E in the process of digestion. The lower orange arrow is the region near the excretory pore (anus) of the mite. In SEM images in Figure 28 A and B the placement of the excretory pore (yellow arrows) is compared in both *L. quinquemaculosa* and *Lebertia* n. sp. The anal pore is a "puckered lips" appearing structure on the midline posterior ventral surface. For reference to other easily recognized structures, Figure 28A shows the location of the genital field (green arrow points)

where 3 pairs of acetabula can be clearly seen opposite each other with the genital opening in the center. A magnified view of one of the acetabula can be seen in Figure 28D where porosity of the acetabula can be observed. Correspondingly, Figure 28B shows the position of the anal pore in relation to the coxal plates and legs on the ventral surface. The location of the anal pore in these structural studies clearly correspond to the observed excretion site illustrated in videographic observations in Figure 3.

**Figure 28: SEM of *Lebertia* genital field and excretory pore (anus).** (A) Ventral view of *Lebertia quinquemaculosa* with green arrow indicating genital field containing 3 pairs of acetabula and yellow arrow showing placement of excretory pore. (B) Ventral view of *Lebertia* n. sp. with yellow arrow pointing to excretory pore. (C) Magnified view of excretory pore of *Lebertia* n. sp. (D) Magnified view of acetabula from *Lebertia* showing porous structure.




***Functional studies of ingestion, in relation to the digestive structures***

*L. quinquemaculosa* imaged before (Figure 29A) and after (Figure 29B) feeding with FDA labelled *Chironomus riparius* larvae shows regions of enhanced fluorescence after feeding compared to the general lack of autofluorescence prior to feeding. The increased fluorescence intensity can be seen in the regions where the presumptive mid gut and excretory organ are located. *Lebertia* n. sp. was also imaged before (Figure 30A) and after (Figure 30B) feeding. Yellow arrows highlighted the areas that had an increase in fluorescence intensity in the

presumptive region of the excretory organ with a tube leading to the excretory pore. All figures are representative illustrations of at least three experiments.

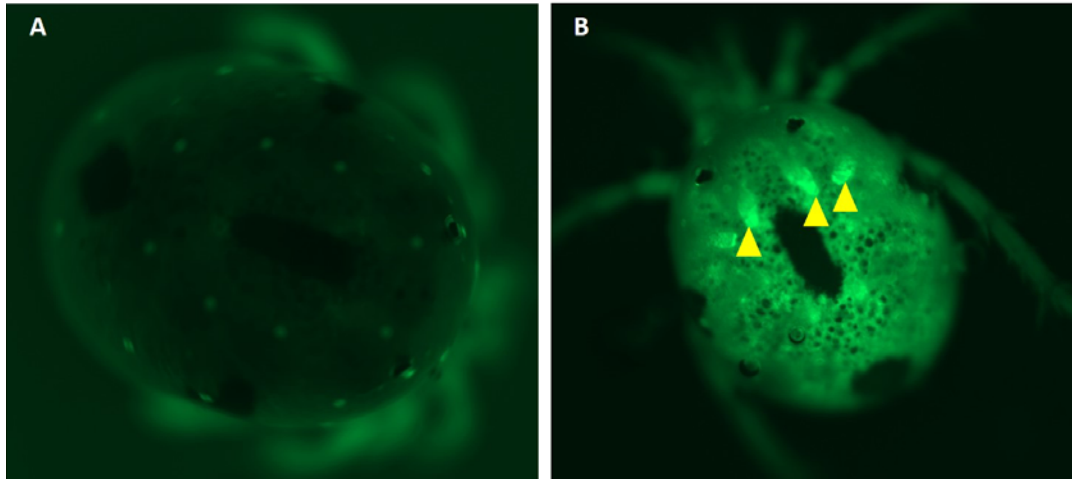



**Figure 29: Fluorescence of *L. quinquemaculosa* before and during a feeding experiment.** (A) Unfed mite. (B) Mite fed with *C. riparius* that had previously been exposed to FDA. Yellow arrows indicate region of lobed mid gut with enhanced fluorescence resulting from ingesting FDA-exposed *C. riparius*.

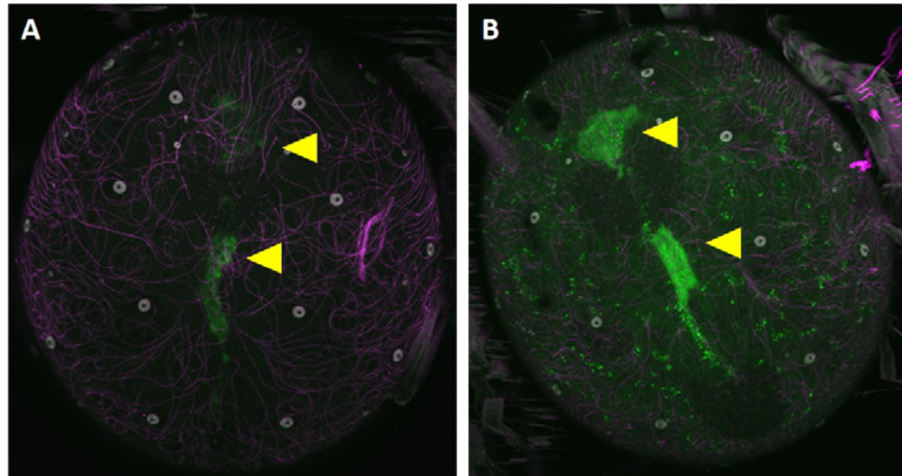



**Figure 30: *Lebertia* mite feeding experiment.** (A) Unfed mite. (B) Mite fed with *C. riparius* exposed to FDA. Yellow arrows indicate regions where fluorescence is enhanced after feeding with FDA exposed *C. riparius*. The anterior of the mite is near the top of the images.

***Oil Red O histochemical detection of lipids***

Histochemical analysis of water mite tissues using Oil Red O is shown in Figure 31. Oil Red O staining is indicated in Figure 31B and D with blue arrows pointing to positive staining. The purple arrow in D points to the cuticle.

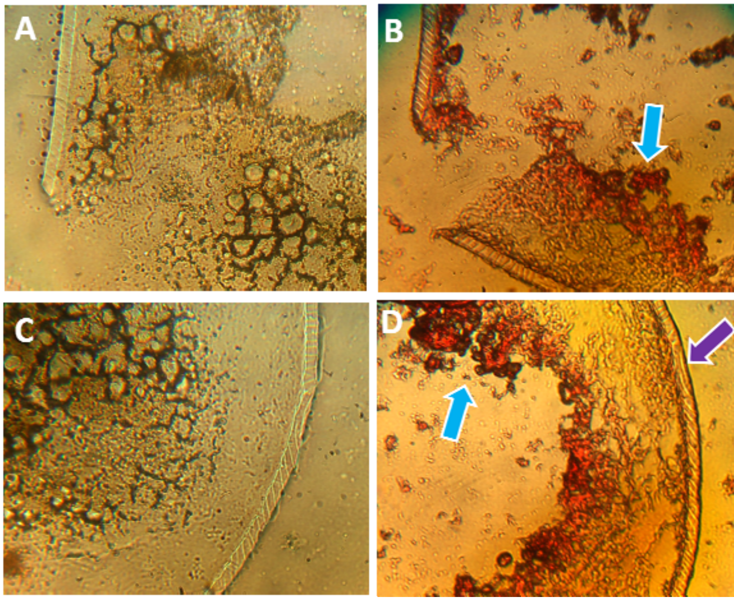



**Figure 31: *L. quinquemacolosa* Oil Red O staining.** Water mite tissue sectioned from whole mounts was stained with Oil Red O (ORO) and Mayer's hematoxylin. (A, C) Unstained tissue. (B, D) ORO stain. ORO stains lipids and is shown by blue arrows. Purple arrow shows water mite exoskeleton with no staining.

## Discussion

Analysis of behavior, structure and experimental data on water mites in this study reveal details about the behavior and adaptations that water mites use for the liquefied diet that they ingest. Water mites, aquatic arachnids that are members of Parasitengona (terrestrial and water mites), are predators with a diverse choice of prey. The two species of water mites on which the present chapter focused, *L. quinquemaculosa* and *Lebertia* n. sp., while similar in many features, also have distinctive differences in size and transparency that facilitate different types of studies. This chapter shows that the water mite genus, *Lebertia* can predate different types of prey. A variety of microscopic methods, ranging from scanning and transmission electron microscopy to confocal fluorescence microscopy, were used to reveal structural and anatomical features of *Lebertia* that are used to capture and process the organisms that they prey upon. Among these structural features are a large array of striated muscle fibers that likely play important roles in the movements of both the external and internal components of the mite's feeding and digestive system. This study is the first analysis of water mite digestive tract using FDA-exposed *Chironomus riparius* larvae as prey. We speculate that subsequent to digestion, water mites may

store energy in the form of lipids, which is supported by observations in this study (see Figure 31).

Among the distinctive features of *L. quinquemaculosa* and *Lebertia* n.sp. that affected our observations is the degree of pigmentation. *L. quinqumaculosa* is heavily pigmented dorsally in contrast to the comparatively translucent integument of *Lebertia* n. sp. Thus, the transparent integument of *Lebertia* n. sp. enabled the visualization of presumptive digestive structures (see Figure 27). In *Lebertia* n. sp. feeding experiments, there was a distinct increase in fluorescence at two regions in the presumptive gut structures seen with differential interference contrast (DIC) microscopy (see Figure 30).

The palps and mouth parts of the two species of water mites also have distinctive features, revealed by scanning electron microscopy (SEM) in Figures 21 and 22, that mediate feeding behavior. The palps are primarily used for grasping prey items, and work in coordination with the chelicerae found in the gnathosome that are used as the cutting “tool” of the mite to penetrate prey tissue. It is through here that the salivary glands (e.g, Figure 21B) secrete enzymes that liquefy the prey tissue. The liquefied tissue is then pumped into the mite midgut through the pharynx. In *Lebertia* n sp., (Figure 22), the palps can be seen again with the chelicerae in between, however, in this species the chelicerae are very distinctive with jagged edges that might be responsible for this smaller mite being able to hold on to larger prey as it is thrashing about (observed in video micrographs).

Microscopic and behavioral studies on *Lebertia quinquemaculosa* and *Lebertia* n. sp. suggest that striated muscles have important roles in the digestion of these organisms. An analysis of dissected *Lebertia* mites, using confocal fluorescence microscopy, revealed a large network of well-developed muscle bundles throughout the mite body (see Figure 23 A-B). Transmission electron microscopy (TEM) analysis of these structures revealed V-shaped muscle

bundles that most likely play a critical role in moving the pharyngeal pump which is one of the major structures used for feeding. Since water mites feed by liquefying their food outside of their body they need an efficient apparatus that can pump in the liquid into their midgut. Magnified views of these muscle groups in Figure 24B demonstrate how advanced and well developed the striated muscle fibers are. Some muscle bundles that likely play key roles in moving the palps during feeding and protrusion of the chelicerae are indicated by red arrows in Figure 23A. Muscles that are associated with the pharynx likely enable these structures to function basically as a pump to ingest the liquid diet once external chemical digestion has occurred.

These muscle bundles could also be used to squeeze internal tissues to assist in movement of nutrients and hemolymph. Parasitengona do not have a circulatory system and have multiple muscle bundles that may be capable of moving material inside the mite (Mitchell 1970). Some of these internal gut movements are highlighted in Figure 20, in which a yellow dotted line outlines the internal gut which gradually contracts until the mite expels waste from its excretory pore. These gut structures could be moving by the use of well-developed muscle fibers seen throughout the mite body. Figure 27 illustrates the last part of the digestive tract; the excretory pore [EP] (anus). Figure 27A-B shows the placement of the EP between two glandularia near the posterior of the mite. A zoom of the EP shows no clear structure at the very entrance of the EP and expulsion of waste must be dependent on the movement of the mid gut by muscle bundles as discussed.

The size of the fibers and the sarcomeres are larger in *Lebertia* water mites than in many other organisms. Whereas in vertebrate striated muscle the thick filaments are approximately 1.5 μm long (Franzini-Armstrong 1970), in these mite muscle fibers the thick filaments seem to be about 4 μm in length. The relaxed sarcomere is nearly 8 μm long. Other invertebrates also seem

to have longer sarcomeres. For example, in the horseshoe crab (an arachnid) the thick filaments can be 4 µm or a bit more in length (Dewey et al. 1977). On closer inspection (see Figure 24B), the muscle bundles can be seen lined with large mitochondria consistent with high contractile activity. Another distinctive feature of the *Lebertia* striated muscle seems to be the unevenness of the Z-lines which has been reported in other studies using frog skeletal muscle that has been stretched during active periods then rapidly fixed for TEM analysis (Edman 2012). This is still an area of active research, and water mites may be able to provide an excellent comparative physiological model.

These results revealed that food is able to travel quickly through the water mite digestive system, and this was consistent with observations of mites defecating right after feeding in laboratory experiments (see on-line video supplement). It has been postulated that the digestion of these types of organisms is so efficient that it does not need a hind gut and has lost some of these anatomical features present in higher animals (Mitchell 1970).

In lateral views of the anterior sagittal sections of *Lebertia* (see Figure 26) the midgut (MG) structure was indicated by dotted black lines. Sitting in the same plane one could see the excretory organ (EO) indicated by the red dotted line. The EO lies within the folds of the MG but no connection between the two structures has been seen despite decades of investigation. Our work confirms that both structures are proximate to each other, a thin translucent layer separates the two structures, and no visible direct connection is present. Figure 24D is a magnification of one of the large digestive cells showing several vacuoles (V) and possible storage granules (SG). These large digestive cells (DC) were found throughout the mite TEM sections primarily near the midgut and the excretory organ (see Figure 24 and 25). Perhaps these cells might play major roles in trafficking and excretion of waste products in water mites. Besides the midgut and EO there has been no evidence of other structures used for transporting

digestive products. Recent investigations on midgut ultrastructure also identified these large DC and attempted to characterize their villi like peripheral structures (Shatrov 2010b). Our work showed that the EO also contains villi like structures at the periphery (see Figure 25D).

Another structure of interest is the synganglion that is found beside the esophagus (Shatrov 2010a). In Figure 24C nerve structures are observed, indicated by the red arrows, and they can be seen around the circumference of the putative mid gut structure which itself is lined with large digestive cells indicated by the region enclosed by black dotted lines. What role the synganglion plays in digestion in water mites can only be speculated at this point. It might be a means of communication between the other parts of the mite internal anatomy.

When dissected, water mites were observed to release several large globular lipid-like spheres (personal observation, and personal communication from Dr. Ian Smith). To begin to study whether these structures were storage features we applied the technique of Oil Red O staining on the cryo-sectioned internal tissues of *Lebertia* that could be sites for storage of lipids. Cuticular structure on the periphery did not stain with Oil Red O which is expected of the non-reactive cuticle made primarily of chitin in the exoskeletons of arthropods (see Figure 31B-D). However, when compared to unstained tissues there was significant staining on internal tissues suggesting that water mites may have a substantial storage capacity of lipids which may contribute to their ability of surviving without feeding for up to five months (personal observation unpublished results). A study on the biomass of water mites reported that lipids comprise the third highest percentage of the total dry biomass out of mean levels of proteins, lipid, carbohydrate, chitin and ash (Kabbe and Meyer 1991). This preliminary histochemical analysis of cryo-sectioned water mite tissues is a first good step to begin to characterize the lipids present in water mites.

My research on this water mite model can be adapted as a rapidly accessible aquatic

invertebrate that can be studied easily with limited resources. Due to its important role in freshwater aquatic ecosystems already discussed in earlier chapters, this work can contribute to better understanding the health of important habitats such as the Laurentian Great Lakes. Feeding experiments, where multiple prey items were made available to mites (see Figure 18B) demonstrated that *Lebertia* mites had a diverse appetite for prey. *Lebertia* mites that were kept captive and starved would even feed on other prey items including mosquito larvae and *Drosophila* larvae (not usually found in its habitat) (see Figure 19D). The topic of what *Lebertia* actually eat in the natural environment is the subject of a subsequent chapter.

We have already suggested multiple practical applications of water mites such as biocontrol for mosquitoes such as those currently plaguing the Americas with Zika virus and causing global panic (Werblow et al. 2015, Imperato 2016) and as bioindicator organisms (Goldschmidt et al. 2016). Given the lack of knowledge on the structure and function of feeding and digestion of water mites and the importance that water mites play in the Great Lakes ecosystem our work contributes the first of this kind of in-depth study of any North American water mite and has now opened the door to many future possibilities and contributions to Great Lakes aquatic invertebrate physiology and ecology.

# CHAPTER 5 - MORPHOLOGICAL IDENTIFICATION AND COI BARCODES OF ADULT FLIES HELP DETERMINE SPECIES IDENTITIES OF CHIRONOMID LARVAE (DIPTERA, CHIRONOMIDAE)

(This chapter contains previously published material. See Appendix B and C.)

## Abstract

Establishing reliable methods for the identification of benthic chironomid communities is important due to their significant contribution to biomass, ecology and the aquatic food web. Immature larval specimens are more difficult to identify to species level by traditional morphological methods than their fully developed adult counterparts, and few keys are available to identify the larval species. In order to develop molecular criteria to identify species of chironomid larvae, larval and adult chironomids from Western Lake Erie were subjected to both molecular and morphological taxonomic analysis. Mitochondrial cytochrome c oxidase I (COI) barcode sequences of 33 adults that were identified to species level by morphological methods were grouped with COI sequences of 189 larvae in a neighbor-joining taxon-ID tree. Most of these larvae could be identified only to genus level by morphological taxonomy (only 22 of the 189 sequenced larvae could be identified to species level). The taxon-ID tree of larval sequences had 45 operational taxonomic units (OTUs, defined as clusters with >97% identity or individual sequences differing from nearest neighbors by >3%; supported by analysis of all larval pairwise differences), of which 7 could be identified to species or "species group" level by larval morphology. Reference sequences from the NCBI and BOLD databases assigned six larval OTUs with presumptive species level identifications and confirmed one previously assigned species level identification. Sequences from morphologically identified adults in the present study grouped with and further classified the identity of 13 larval OTUs. The use of morphological identification and subsequent DNA barcoding of adult chironomids proved to be beneficial in revealing possible species level identifications of larval specimens. Our sequence

data from this study also contributes to currently inadequate public databases relevant to the Great Lakes region, while the neighbor-joining analysis reported here describes the application and confirmation of a useful tool that can accelerate identification and bioassesment of chironomid communities.

**Introduction**

Chironomids represent a dominant group of benthic macro-invertebrate populations and have been observed as one of the principal groups of aquatic organisms both in terms of number and distribution in sampling studies (Carr and Hiltunen 1965). Chironomids are ecologically important due to their contribution to the food web, nutrient cycling, and pollutant accumulation; however, the adaptive ability of chironomids and ease of both natural and anthropogenic-mediated transport warrant a concern for their potential role as invasive pests, especially in recently disturbed environments (Failla et al. 2015). The terrestrial adult stage is short-lived and often characterized by swarms of mating adults, sometimes presenting a great nuisance to humans in environments where emergences occur (Ali et al. 1985, Tabaru et al. 1987, Iwakuma 1992, Hirabayashi and Okino 1998). The ecological roles of chironomids have made sampling and subsequent species-level identification an important and useful biological tool for monitoring lake health (Langdon et al. 2006).

The identification of chironomid larvae to species level represents a challenging task for taxonomists. Identifying characteristics are more difficult to distinguish among the immature features of larval specimens, as compared to mature adults (Oliver 1971). Adults possess more developed and specific features and thus are more amenable to establishing species level identifications (Ekrem et al. 2007). Larval keys usually identify chironomids only to genus level (Ram et al. 2014), and few chironomid keys exist that enable identification to species level. Identification of larvae to species level is usually accomplished by rearing larvae and collecting

the pupae or adults (Inoue et al. 2008). The fully developed pupae or adults are then morphologically identified in order to assign species level identifications for the corresponding larvae.

DNA barcoding has been instrumental in facilitating identification of cryptic larval chironomid species (Sharley et al. 2004, Pfenninger et al. 2007, Carew et al. 2011). Studies combining the use of adult and larval DNA sequences have aided the species level identification of larvae within specific genera such as *Cricotopus* (Sinclair and Gresens 2008), *Cladopelma* (Carew et al. 2005), *Procladius* (Carew et al. 2011) and *Corynoneura* (Silva and Wiedenbrug 2014). However, these techniques have not been applied to describing the composition of diverse communities of chironomid larvae in the Great Lakes.

The present study specifically addresses the assessment of a benthic community from the standpoint of aquatic species monitoring and identification with its application in the Western Lake Erie region. Studies of larval and adult mitochondrial cytochrome c oxidase I (COI) genes have shown that DNA barcodes are an excellent tool provided that a comprehensive DNA barcode library at the species level is available. Such DNA libraries should contain a set of sequences that have been obtained from diverse larvae or adults that have been morphologically identified by expert taxonomists (Ekrem et al. 2007).

Although the public databases are abundant, only a small number of sequences are useful in the Great Lakes region. Expansion of the reference databases in regions where chironomids are of interest would make species level identifications from molecular analysis more accessible and consistent. The present study uses newly determined sequences of taxonomically identifiable adult specimens to enrich the chironomid sequence database for Western Lake Erie and to improve identification of the diverse larval community in this region.

## Materials and Methods

### *Collection of larvae*

Benthic organisms were collected from the Maumee Bay area of Western Lake Erie and the Toledo Harbor region of the Maumee River in the spring and summer of 2012, at 14 sites illustrated in Figure 32. On May 2, May 30, June 12, July 12, August 9 and August 28, 2012 a total of 128 benthic samples were collected from 14 different sites.

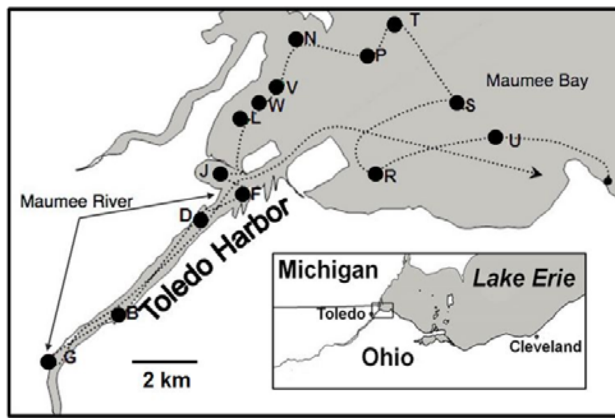



**Figure 32: Map of 14 benthic collection sites near Toledo Harbor** in 2012. Benthic communities of the Maumee Bay and Maumee River of the Western Lake Erie region (inset) were sampled at the lettered locations.

Benthic grab samples were obtained via a hand-operated bottom dredge (AMS, Ben Meadows, Janesville, WI), as previously described by (Ram et al. 2014). Samples were collected from most sites on each date, washed on a 0.5 mm sieve to remove fine sediments while retaining organisms, and stored temporarily on ice in the field in 80% ethanol obtained by adding a four-fold volume of 100% ethanol (Fisher Science, Pittsburgh, PA). Upon delivery to the lab, samples were washed again on a 0.5 mm sieve and stored in 90% ethanol at 4°C until shipment to EcoAnalysts, Inc (Moscow, ID) for sorting and morphological identification.

### *Morphological taxonomy of larvae*

EcoAnalysts performed morphological identification of all organisms in each sample, sorting different taxa into separate vials. These animals included 2410 chironomid larvae that were mostly classified only to genus level by morphological features. Only a small proportion could be identified to species, as described in results.

***DNA sequencing of larvae***

Sorted, identified chironomid larvae were returned to the laboratory for molecular analysis. All specimens of rare taxa (those identified in each collection by Ecoanalysts fewer than 5 times) were sequenced. Among more common taxa, at least 5 specimens of each taxon randomly chosen from among the available specimens were sequenced from each collection. Due to particular interest in *Cricotopus* spp., known agricultural pests, all *Cricotopus* larvae that were collected were sequenced. The result of this selection, designed to obtain multiple representative sequences, whenever possible, from every taxon identified by Ecoanalysts, was that a total of 189 larvae out of the 2410 larvae collected were chosen for sequencing.

The full body, anterior aspect, and posterior aspect of chironomid larvae chosen for sequencing were photographed, and then sterile dissecting methods were used to obtain a small piece of tissue from the mid-portion of each selected specimen. Each dissected piece was placed in 30 μL of 100% ethanol in individual wells of a 96-well micro-plate and sent to the Canadian Center for DNA Barcoding (CCDB; Biodiversity Institute of Ontario, University of Guelph, Ontario, Canada) for sequencing of up to 658 base pairs of the mitochondrial COI gene using forward and reverse primers HCO2198 and LCO1490 (Folmer et al. 1994, Hebert et al. 2003a). The anterior and posterior ends of each larva were retained as voucher specimens.

***Collection of adults***

Adults were collected from the Maumee Bay region, including from the hull and sides of the boat while the benthic collections already described were in process. Briefly, the flies were trapped via a hand-held vacuum cleaner and subsequently emptied into a series of 50 ml collection tubes containing isopropyl alcohol (pilot studies indicated that DNA was as readily obtained from specimens preserved in isopropyl as in ethyl alcohol). Adult flies were then sorted, and each individual was placed in its own vial containing 85% ethanol. Thirty-nine

undamaged adults of diverse macroscopic characteristics were chosen for morphological and molecular analysis.

***DNA sequencing of adult chironomids***

For the first set of 20 specimens, two legs were detached from each adult chironomid and preserved in 90% ethanol for DNA analysis. DNA isolation was performed according to a Qiagen DNA spin-column protocol (https://www.qiagen.com/us/resources/resourcedetail?id=21b4511b-4aaa-470a-a141-191ed91c54be&lang=en). Isolated DNA was amplified by PCR using COI forward and reverse primers, HCO2198 and LCO1490 (Folmer et al. 1994, Hebert et al. 2003a). The PCR product was purified and diluted with sterile PCR water to a concentration appropriate for sequencing. Genewiz (South Plainfield, N.J.) sequenced the purified COI product in the forward and reverse directions. A consensus sequence was determined using DNAbaser software (DNA Baser Sequence Assembler v4.x 2014, Heracle BioSoft SRL, www.DnaBaser.com), evaluating chromatograms and aligning sequences from both directions. For the remaining 19 adults, two legs were removed from each fly and placed in a microplate that was sent to CCDB for COI sequencing, based on the protocol described above for larvae. Altogether, 37 of the 39 specimens belonged to the Chironomidae. The remaining two specimens, a culicid (*Anopheles* species) and a chaoborid (*Chaborus punctipennis*) were excluded from the analysis.

***Morphological identification of adults***

Morphological identification of the 39 specimens (minus the two legs used for molecular taxonomy) was completed at the Great Lakes Science Center (Ann Arbor, MI). The tissues were digested, and the remnants were mounted on slides to identify key morphological features. Initially, a specimen's size, color and shape were noted and then a pair of legs, wings and one antenna were mounted on a glass slide while the rest of the body was cleared of muscle tissue

and then mounted. Keys by Townes (1945) and Cranston et al. (1989) were used to key adult specimens to genus. Species within a genus were identified using Townes (1945), Dendy and Sublette (1959), Roback (1971), (Epler 1988), Heyn (1992), Saether (2009), and Saether (2011). When available, at least two specimens of each species were mounted and identified; additional specimens were studied for confirmation based on size, color and shape and returned to their labeled vials pending any possible need to mount and reconfirm based on DNA analysis.

***Initial database search***

All of our larvae and adult sequences were initially screened with the BOLD species level identification engine (http://www.boldsystems.org/index.php/IDS_OpenIdEngine) to determine if there were matches that differed by <3% in the BOLD database. If no match was found, the sequence was then subjected to a Genbank BLAST search to confirm that no match existed in either database. If the result from BOLD included published results that also existed in the Genbank database, no further search was done. If a sequence had a database match differing by <3%, representative sequences from the matches were used as reference sequences in subsequent analysis.

***Neighbor-joining analysis of larvae, adults and reference sequences***

Neighbor-joining analysis was performed using MEGA software (Tamura et al. 2011). Pairwise differences of all larval sequences were calculated and analyzed graphically to determine natural groupings of sequences to define operational taxonomic units (OTU) of sequences. COI barcode relationships were determined by constructing neighbor-joining trees and calculating pairwise differences using a maximum composite likelihood algorithm (Tamura et al. 2011).

In addition to the reference sequences chosen from the results of BOLD and Genbank searches, a database of useful species-level reference sequences was developed by downloading

all sequences that registered as "chironomidae" in the NCBI database and selecting quality sequences from among them. After aligning the potential reference sequences using Clustal W in MEGA, sequences that were too short or of poor quality (e.g., with multiple "N"s, less than seventy-five percent contiguous, etc.) were removed from the analysis.

COI barcode relationships of larval sequences were inferred from these quality reference sequences, comparing 619 nucleotide positions. Since the analysis of average pairwise differences supported defining OTUs as clusters having no more than 3% pairwise differences within the OTU (see below), reference sequences that differed from the larval OTUs by more than 3% were removed and redundant reference sequences were eliminated. Subsequent neighbor-joining analysis defined OTUs as clusters having >97% identity. Any genetic grouping that is described in this paper as being the same species or OTU adhered to this standard. Sequences obtained from chironomid adults were then added to the analysis, creating a final tree with sequences from larval specimens, taxonomically identified adult specimens, and quality reference chironomid sequences from NCBI and BOLD.

Genus and species names, their authorities and years, and their family and subfamily identities were verified by reference to http://zipcodezoo.com/Key/ and through use of the NCBI taxonomy browser (http://www.ncbi.nlm.nih.gov/taxonomy/).

**Results and Conclusions**

The numbers of larval and adult specimens that were classified by morphological criteria are summarized in Table 4, along with a subset of specimens for which COI barcodes were sequenced. Altogether, 2410 larvae were identified morphologically by EcoAnalysts, revealing 23 genera, among which only 6 genera had specimens that could be identified to species level. Of the 2410 specimens, 189 were selected for sequencing, including 22 (11.6%) specimens that had been identified by EcoAnalysts to species (Table 4). Out of 39 adult specimens subjected to

morphological and molecular analysis, 37 were adult males, along with two other insect specimens. Of the 37 adult chironomid specimens 33 (89%) were identified to species level, comprising the 15 different species listed in Table 4. COI barcode sequences were obtained from all 39 adult specimens. All of these newly identified sequences have been submitted to NCBI for inclusion in the Genbank database as accession numbers KP954634-KP954653 (adults), KR085203 – KR085223 (adults), and KR085224 – KR085412 (larvae).

**Table 4. Species Level morphological identification of adult and larval chironomids.**

| Item | Adults | Larvae |
|---|---|---|
| Total number of specimens examined by taxonomists | 39 (39 sequenced) | 2410 (189 sequenced) |
| Total number of specimens (# of chironomids) identified to genus level | 39 (37 chironomids) | 2410 (2410 chironomids) |
| Total number of chironomid specimens (species) identified to species or species group level; *list of species* | 33 (15)<br>*Ablabesmyia annulata* (Say, 1823)<br>*Axarus festivus* (Say, 1823)<br>*Chironomus crassicaudatus* (Malloch, 1915)<br>*Chironomus decorus* (Johannsen, 1905)<br>*Cladopelma viridulum* (Linnaeus, 1767)<br>*Coelotanypus scapularis* (Loew, 1866)<br>*Cryptochironomus fulvus* (Johannsen, 1905)<br>*Cryptochironomus digitatus* (Malloch, 1915)<br>*Dicrotendipes lucifer* (complex) (Johannsen, 1907)<br>*Glyptotendipes senilis* (Johannsen, 1937)<br>*Glyptotendipes meridionalis* (Dendy & Sublette, 1959)<br>*Procladius bellus* (Loew, 1866)<br>*Procladius denticulatus* (Sublette, 1964)<br>*Stictochironomus devinctus* (Say, 1829)<br>*Tanypus stellatus* (Coquillett, 1902) | 73 (6); 22 were sequenced<br>*Dicrotendipes simpsoni* (Epler, 1987)<br>*Dicrotendipes modestus* (Say, 1823)<br>*Polypedilum halterale* (Coquillett, 1901)<br>*Polypedilum scalaenum* (Schrank, 1803)<br>*Cricotopus bicinctus* (Meigen, 1818)<br>*Ablabesmyia annulata* (Say, 1823) |
| Percentage of chironomid specimens identified to species level | 89% (33/37) | 3% (11.6% of sequenced) |

***Pairwise differences of larval sequences***

A histogram of pairwise differences of 189 larval sequences illustrated in Figure 33A shows structure that helped define OTUs for this study. Graphic analysis reveals 3 major peaks: (1) pairwise differences of <3% that we used in subsequent analysis to define OTUs; (2) a second peak between 3% and 6% pairwise differences. The pairs within this peak include four different genera (*Cryptochironomus, Procladius, Microchironomus,* and *Dicrotendipes*) for which the members of each pair had the same genus but whose species taxa had been designated

only as “sp.” by EcoAnalysts; and (3) pairwise differences that were greater than 11%, peaking at ~20% as the most frequent pairwise difference. Pairs within this peak included both intragenus (e.g., *Chironomus,* 16%; *Coelotanypus,* 14%; *Cryptochironomus,* 14%; and *Polypedilum* 11%) and intergenus (e.g., *Glyptotendipes* and *Procladius*, 20%) differences.

***Reference sequences***

Out of over 2000 species level “chironomidae” COI sequences downloaded from NCBI and BOLD, deletion of short or poor quality sequences left 1447 as reference database sequences for COI barcode analysis. Of these 1447 sequences, 11 non-redundant sequences differed by less than 3% from one or more larval and adult sequences and were used as reference sequences in constructing neighbor joining taxon-ID trees. Altogether, 250 larval, adult and reference database sequences were compared at 619 nucleotide positions. The histogram of pairwise differences retained its three-peak structure with the inclusion of the adult and reference database sequences (Figure 33B).

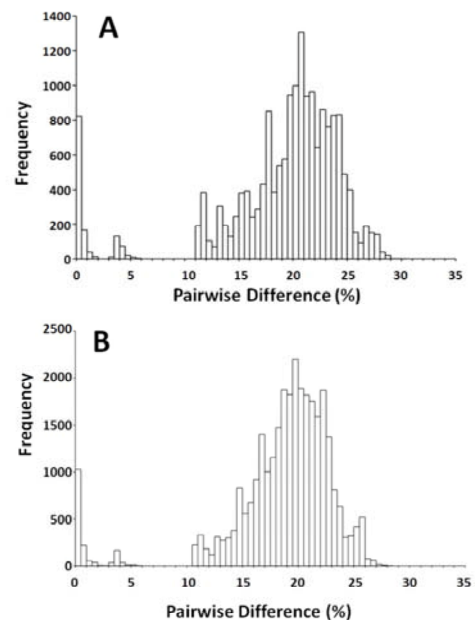



**Figure 33: Histograms of pairwise distance values** of (A) larval sequences and (B) all sequences (larvae, reference database, and adults) combined.

***Neighbor-joining analysis larvae, adults and reference sequences***

A neighbor-joining taxon-ID tree of all larval sequences contained 45 OTUs (Figure 34). Seven of these (15.6%) were supported by reference sequences from NCBI, as shown in detail (Figure 35). OTUs in Figure 35 were identified as follows: *Chironomus entis/plumosus* (OTU 2), *C. quinnitukqut/decorus* (OTU 13), *Micropsectra insignilobus* (OTU 22), *Paratanytarsus natvigi* (OTU 23), *Paratanytarsus grimmii* (OTU 33), *Cricotopus sylvestris* (OTU 35) and

*Cricotopus bicinctus* (OTU 36). OTUs 2, 23, 33, 35 and 36 were identified reliably based on close relationships to reference sequences from NCBI. However, OTU 13 grouped with both an adult and a reference sequence, the identification of which differed from each other and will be discussed below. For OTU 22, the genus of the reference sequences from NCBI differed from the genus of the larva morphologically identified by EcoAnalysts, a discrepancy that will also be discussed below. In OTU 2, two reference sequences enabled identification of both larvae and adults that had previously only been identified to genus level.

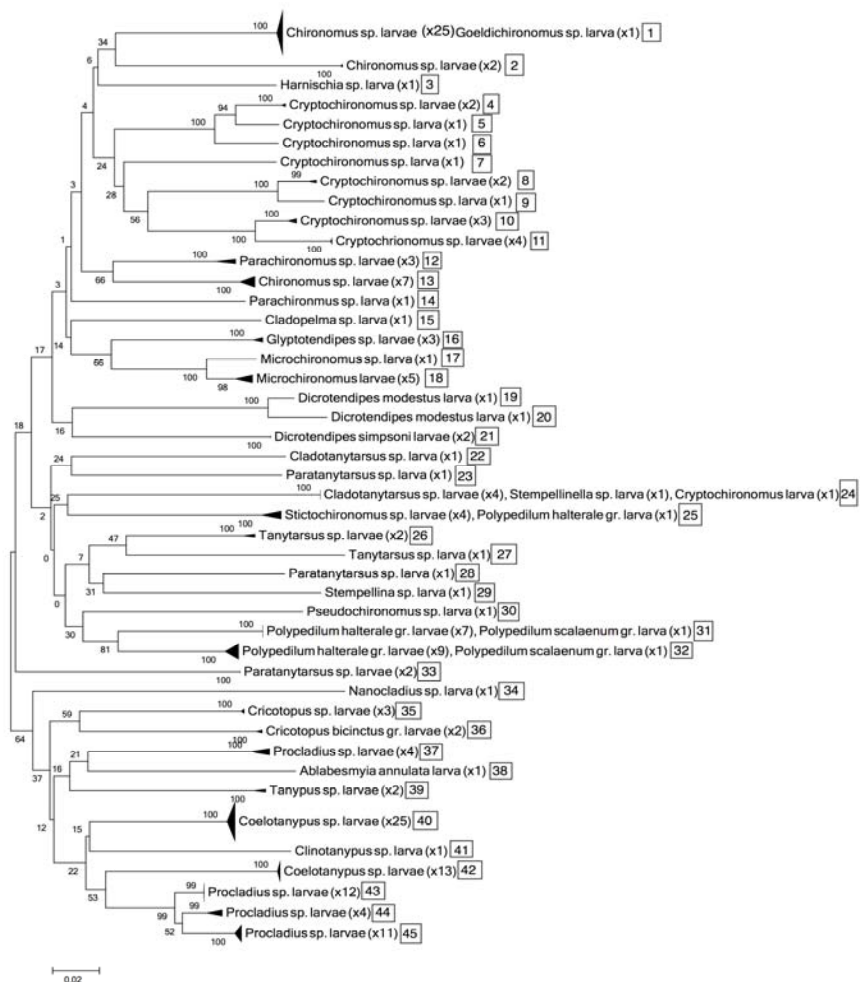



**Figure 34: Condensed neighbor–joining tree of chironomid COI barcodes** with maximum composite likelihood algorithm depicting 45 distinct larval operational taxonomic units (OTUs) based on mitochondrial COI DNA sequences. Values at nodes correspond to a bootstrap 1000 test. Numbers in parentheses indicate the number of sequences within each branch. Numbers in blocks to the right of each branch correspond to the OTU numbers referred to throughout the rest of the paper. The analysis is based on 619 nucleotide positions in 189 larval sequences. The identifications are according to the highest taxonomic level achievable by EcoAnalysts. The scale represents fractional difference of sequence according to the length of the branch. For condensed branches, triangle height represents the number of sequences in the OTU, while width indicates the distance value corresponding to the lowest branch point.

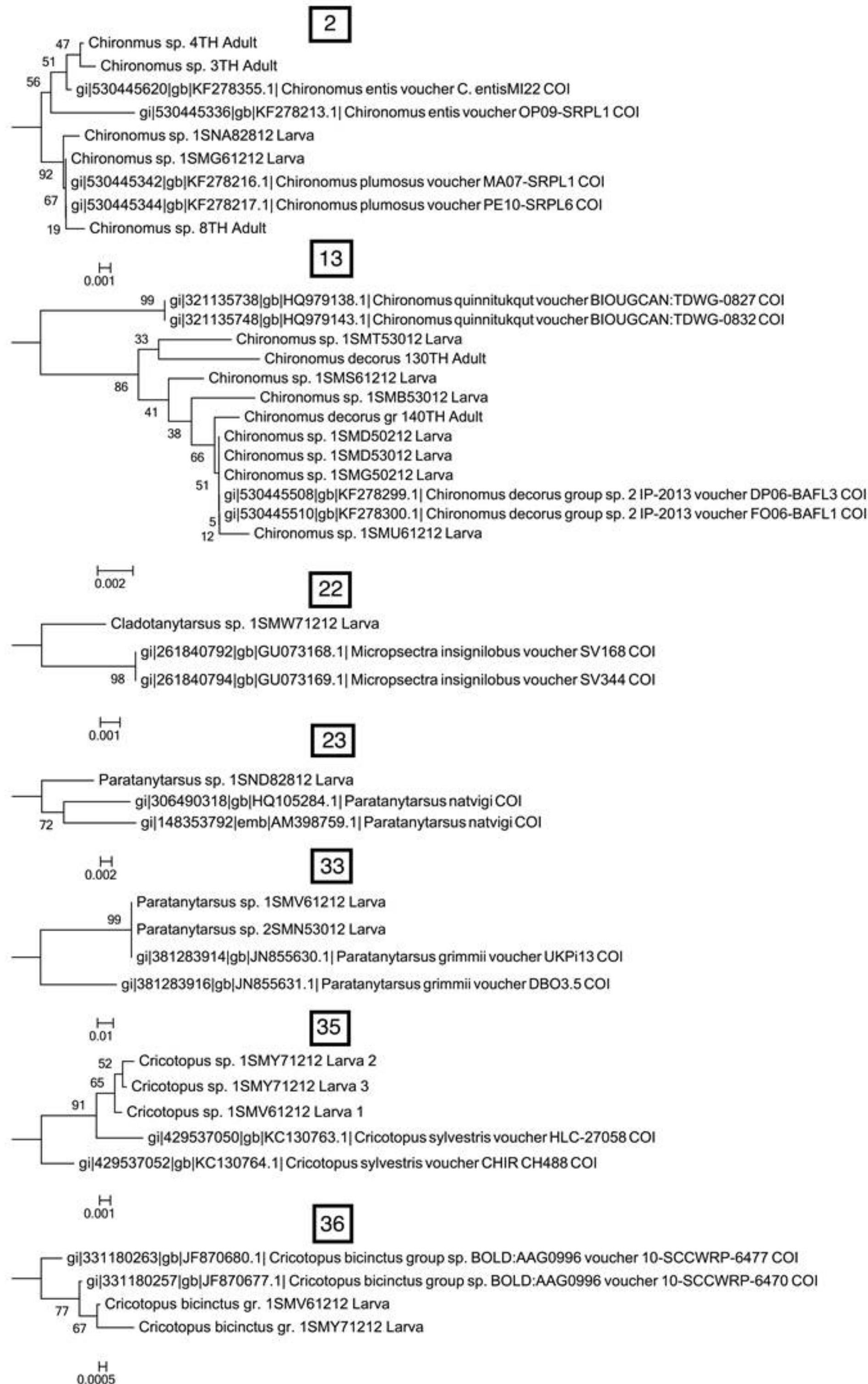



**Figure 35: NCBI reference sequences identified seven larval OTUs to species leve**l. Each subtree has its own scale in the bottom left corner. Numbers in boxes indicate the clade number that corresponds to the larval tree (Figure 3). For OTU 33, only the UKpi13 reference sequence of *Paratanytarsus grimmii* was within a 3% distance. The DBO3.5 reference sequence is included to indicate the scale.

Figure 36 shows 13 larval OTUs out of 45 (29%) that could be compared with sequences of identified adult chironomids, from which they differed by less than 3%. OTUs 4, 11, 15, 16, 37, 38, 39, 40, and 45 were identified reliably (*Cryptochironmus digitatus*, *C. fulvus*, *Cladopelma viridulum*, *Glyptotendipes meridionalis*, *Procladius bellus*, *Ablabesmyia annulata*, *Tanypus stellatus*, *Coelotanypus scapularis*, *Procladius denticulatus*, respectively). OTUs 1, 13, 21 and 25 (*Chironomus crassicaudatus*, *C. decorus*, *Dicrotendipes lucifer*, *Stictochironmus devinctus*, respectively) contain discrepancies when compared to adult and reference sequences. Of the 15 different chironomid species identified as adults (Table 4), only *Axarus festivus* failed to have a corresponding sequence among the larvae. In addition, a *Glyptotendipes senilis* adult was taxonomically identified but not included in the phylogeny due to poor sequence quality and length. While 19 out of the 45 OTUs (42%) are identified, 26 out of the 45 still have no reference sequences or identified adults from which to assign the larvae with putative species identifications.

Based on COI sequence relationships, OTU 22 (Figure 35) shows a specimen classified by EcoAnalysts as *Cladotanytarsus* sp. larva that is most likely to actually be *Micropsectra insignilobus*. In OTU 22, the claimed *Cladotanytarsus* larva falls within a large *Micropsectra* reference sequence clade, being most closely related to *Micropsectra insignilobus* as indicated by Figure 37. Furthermore, the sequence of this specimen differs from 4 other larvae also identified by EcoAnalysts as *Cladotanytarsus* sp. by a pairwise difference of ~20%. Contamination of this sample during sequencing by *Microspectra* DNA from another specimen is highly unlikely because no other *Micropsectra* larvae were collected or handled during the present study.

**Figure 36: Larval OTUs identified by adult sequences.** Each subtree has its own scale in the bottom left corner. Numbers in boxes indicate the clade number that corresponds to the larval tree (Figure 3).

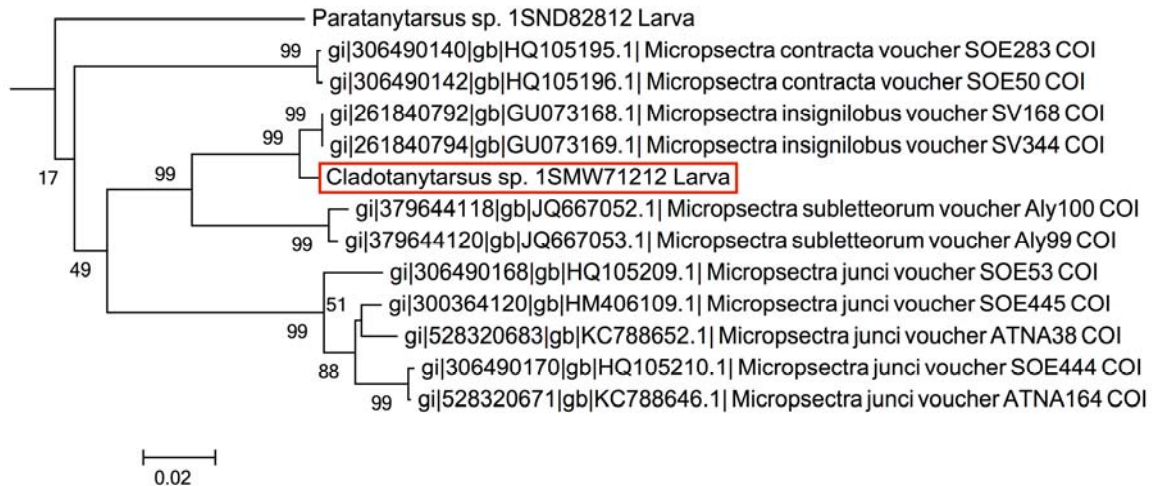



**Figure 37: A subtree extracted from the neighbor-joining taxon-ID tree** constructed with Genbank reference and larval sequences before redundant sequences were removed. The relationship between the Genbank reference sequences and the sequenced larva in OTU 22 that had been classified by EcoAnalysts as *Cladotanytarsus* sp. suggests that this specimen was most likely misclassified during morphological analysis, as its position within a large *Micropsectra* clade is evident.

## Discussion

This study improves the reference databases of COI barcodes for chironomid larval identification by conducting a quality review of existing database sequences of chironomid COI barcodes and determining additional sequences from newly collected morphologically identifiable adult chironomids. This study also provides support, through its analysis of pairwise differences in COI barcodes, for using 97% identity as a natural amount of within-species variation defining chironomid OTUs. Sequences provide specific, reliably generated data for classifying specimens. Nevertheless, as will be discussed, the need for referencing the sequences to potentially ambiguous morphological identifications and to databases that may vary in sequence quality and taxonomic reliability means that ambiguities, inconsistencies, and errors may still occur, and care must be taken in using sequence data for identification.

### *Improvement of chironomid reference databases*

A search for high quality reference sequences in Genbank to identify 45 OTUs of chironomid larvae specimens from Maumee River and the Maumee Bay area of Western Lake

Erie revealed that only 15.5% of sequenced larvae OTUs had corresponding reference sequences in Genbank. In order to achieve even that degree of identification this study reviewed over 2000 chironomid sequences in Genbank to assure selection of only high quality sequences of sufficient length and adequate annotation. This study adds 33 new reference sequences to the public database, based on careful taxonomic identification and COI barcoding of adult specimens. Addition of the sequences from taxonomically identified adult specimens to these databases aided in identification of OTUs 1, 4, 11, 15, 16, 21, 25, 37, 38, 39, 40 and 45, which would not have been possible prior to this study. Nevertheless, more work needs to be done, as 26 of the 45 OTUs have yet to be identified in the phylogeny. In addition, we have yet to collect larvae with sequences corresponding to the adult specimens of *Axarus festivus*. Absence of these corresponding larvae in our dataset could indicate that *Axarus festivus* larvae occupy different or harder substrate habitats than those we sampled. In any case, addition of *Axarus festivus* to the Genbank database will make future identifications of *Axarus* larvae possible.

***Relationship of taxa to pairwise differences***

Data in Figure 33 show gaps in the distribution at around 3% difference and between 6% and 10%. A threshold of 3% for species differentiation has been used for various animal groups (Hebert et al. 2003a, Hebert et al. 2003b, Sinclair and Gresens 2008). In some cases, different species appear within the same COI cluster (e.g., OTU 24 and 25). Although these specimens remain within the confines of the 3% threshold designated by molecular morphology, the taxonomic designations do not agree. Evolutionary processes such as hybridization or rapid speciation where divergent mutations have not yet accumulated may explain this phenomenon (Michailova and Fischer 1986, Ekrem et al. 2007, Proviz 2008, Abbott et al. 2013). The second peak in pairwise differences, between 3 and 6%, suggests that some chironomid groups may have a greater within species variation. The question also arises: could the specimens in the

region between the two larger histogram peaks represent instances of incipient speciation? In most cases, in our data pairwise differences greater than 11% clearly represent different species and usually different genera. However, cases where apparently the same species has pairwise differences this large might also represent cryptic species (Anderson et al. 2013), revealed by large intraspecific pairwise differences. *Polypedilum halterale*, represented by OTU 31 and 32, which differ by 11%, is one such example.

***Ambiguities, inconsistencies, and possible errors***

Despite the care with which larvae and adults were sequenced and the adult specimens were identified, the results shown here exhibit several ambiguities, inconsistencies, and possible database or identification errors. Examples of ambiguities in identifying larvae are OTU 13 and 21 (Figures 35 and 36). In OTU 13, a reference sequence and a sequenced adult were classified as different species within the genus *Chironomus*. For some *Chironomus* species, the COI barcode and morphological identification alone may be inadequate for establishing species level identifications (Ekrem et al. 2010, Proulx et al. 2013). For example, *C. quinnitukqut* is a part of the *C. decorus* group, and species within this group are often separated based on karyotype analysis (Martin et al. 2011). In OTU 21, *Dicrotendipes simpsoni* represents a species within the *Dicrotendipes lucifer* complex. Similarly, difficulty in assigning species level identifications to chironomids within the genus *Cricotopus* exists, specifically within the *Cricotopus sylvestris* species group (Gresens et al. 2012). Assignment of species level identifications within these types of difficult genera will be ambiguous unless additional markers are utilized, techniques such as karyotyping are incorporated, or morphological keys are improved.

Ambiguity is also present in OTU 2 (Figure 35) and OTU 24 (Figure 34). For OTU 2, identification as *Chironomus entis* is evident, yet examination of a karyotype could place it as a closely related species, such as *Chironomus borokensis* Kerkis, Filippova, Shobanov, Gunderina

& Kiknadze, 1988 (Proviz and Bazova 2013). Both of these taxa belong to the *C. plumosus* group, which contains sibling species differentiated by karyotype (Kiknadze et al. 2000, Golygina and Kiknadze 2012). For OTU 24, six larval specimens with identical sequences were classified as *Cladotanytasus* sp. (4 specimens), *Stempellinella* sp. (1 specimen), and *Cryptochironomus* sp. (1 specimen). Since this OTU is fully within a clade in which identification of most other specimens are in the tribe Tanytarsini, and *Cryptochironomus* is not classified as a member of this tribe, the *Cryptochironomus* identification is likely to be in error. Whether the correct designation of OTU 24 is *Cladotanytarsus* or *Stempellinella* remains ambiguous.

In some cases, the assignment of a larval taxon by qualified taxonomists (e.g., those who work for EcoAnalysts) even at the genus level is inconsistent with identifications derived from molecular data. In the present study, the morphological identification of some larval specimens in OTUs 1, 13, 21, 22, and 25 did not agree with the identification determined from Genbank reference or adult chironomid COI sequences. Morphological identification may be compromised by condition and maturity of specimens, preservation, and inadequate reference materials available to taxonomists. It is also possible that after identification a specimen might get cross-contaminated by DNA from another specimen or a specimen or vial might be mislabeled, leading to such inconsistencies. Avoiding errors is essential to use this process effectively. To as great an extent possible, we consulted expert taxonomists and sterilized dissecting equipment with ethanol and flame between specimens-to-minimize the risk of sample-to-sample cross-contamination prior to subsequent barcoding.

Since mistakes in reference databases could also lead to ambiguities or incorrect identification, we consider here several instances in which reference databases may have errors. For example, in Figure 35, OTU 33 has two *Paratanytarsus* sp. larval sequences grouping with a

100% identical *P. grimmii* reference sequence; however, another *P. grimmii* reference sequence (label: DBO3.5) is greater than 3% divergent and is suspected to be incorrectly labeled as *P. grimmii*. taxon-ID trees of quality reference sequences constructed without the inclusion of adult sequences (Figures 38 and 39) reveals that the highly divergent "*Paratanytarsus grimmii*" sequence is the only one out of 23 very closely related sequences with this designation, leading us to suspect that this identification is likely incorrect (Figure 38). Instead, the correct identity of this reference sequence is more likely to be *P. kathleenae*, in agreement with the other 22 specimens in this clade (Figure 39).

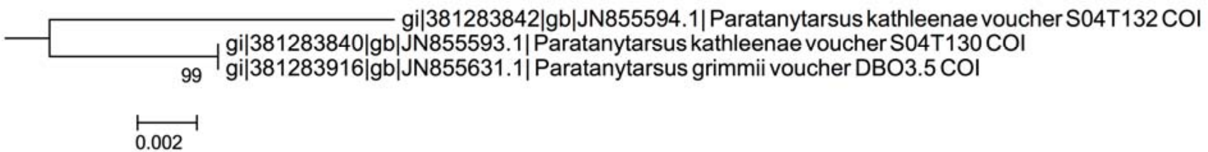



**Figure 38: The neighbor-joining taxon-ID tree with reference sequences only,** which was constructed as an intermediate step in obtaining the final tree.

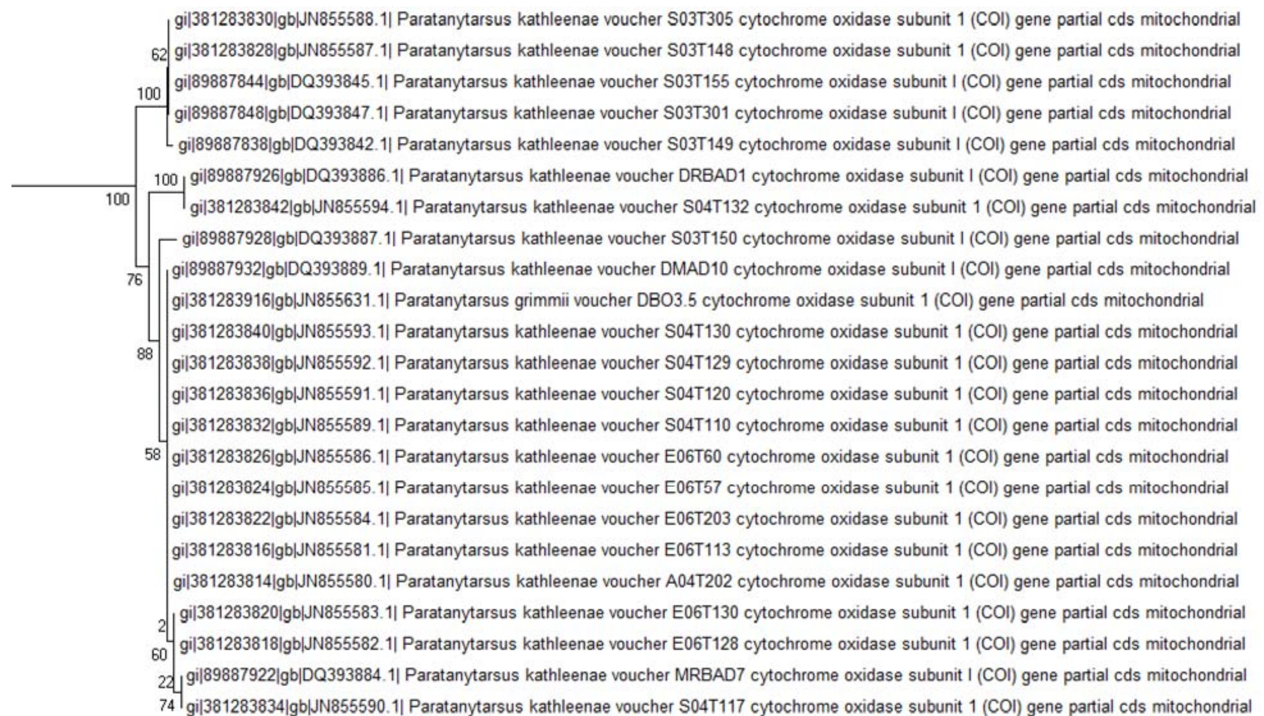



**Figure 39: *Paratanytarsus* sequence relationships** for a part of the uncut reference sequence tree.

Despite the existence of such ambiguities, inconsistencies, and possible errors, the clades of the taxon-ID tree of larvae in Figure 34 mostly show excellent congruence with previous morphological taxonomic classification to the family, subfamily, or tribe levels. Thus, the clades of OTUs 1 to 21 and 30 to 33 contain genera that are all classified as subfamily Chironominae in the tribe Chironomini; genera of OTUs 37 to 45 are all members of the subfamily Tanypodinae; and genera of OTUs 34 to 36 are all members of the subfamily Orthocladiinae. Only OTUs 22 to 29 represent an exception to this general congruence of molecular clades with known subfamilies and tribes: while the majority of these genera are classified as subfamily Chironominae of the tribe Tanytarsini, exceptions are one specimen in OTU 24 and 5 specimens of OTU 25, whose genera are classified as members of the tribe Chironomini. The mix of tribes within this clade may simply indicate the difficulty of determining larval morphology or it may reflect identification errors.

***Significance of identifying chironomid larvae***

Species level identification of chironomid larvae is useful due to the importance of larvae in aquatic food webs (Oliver 1971, Armitage et al. 1995) and the negative impacts of some species as pests (Ali 1996, Broza et al. 2003) and potential invaders (Failla et al. 2015). For example, OTU 33 (Figure 35) confirms the presence of *Paratanytarsus grimmii,* a parthenogenic nuisance species known for colonizing water treatment ponds and their associated equipment, such as granular activated carbon absorbers (Langton et al. 1988, Olsen et al. 2009). Also, OTUs 35 and 36 identify the presence of two species of *Cricotopus*, that are both known to be colonizers and detrimental pests of rice fields in California (Clement et al. 1977).

In addition to identifying known nuisance species, this method has the potential to identify the presence of new species that are either previously undetected or invasive. For example, OTU 22 indicates the presence of *Micropsectra insignilobus*, a species associated with

waters of low organic content that has a very limited record of detection in the Great Lakes, but whose distribution is well described in Northern Europe (Saether 1979, Ilyashuk and Ilyashuk 2001). To our knowledge, no previous records exist of this species in Lake Erie, although new species of the *Micropsectra* genus, such as *M. subletteorum*, a sister species of *M. insignilobus*, have recently been described in Minnesota via similar use of molecular and morphological methods (Anderson et al. 2013).

**Future Research Needs**

Despite the possibility of ambiguities, inconsistencies, and reference database errors, we recommend the molecular barcode methods used in this study to identify chironomid larvae in future studies. Disagreements in molecular identifications exist, suggesting that developing a more comprehensive library of diverse genetic markers and employing additional identification techniques, such as karyotyping, may resolve some issues. Because some OTUs are only identified by one reference sequence, confirming their identity with barcodes of replicate reference specimens would be beneficial. In addition, improving the quality of existing databases is needed. To some degree, errors were avoided by sorting out sequences that had low quality scores or many ambiguous bases (N's, for example). COI is able to provide presumptive species-level identifications in many cases and in general is considered accurate and reliable (Silva et al. 2013, White et al. 2013). Nevertheless, COI does not provide as great a resolution as CAD or *Cyt b* genes (Ekrem et al. 2010, Carew et al. 2011). The use and establishment of other DNA markers in the future could contribute significantly to the reference data and improve the field of DNA-based taxonomic identification.

The larvae and adults in this study were collected from just one small portion of Lake Erie and the Maumee River. For studies throughout the Great Lakes, reference databases ought to be established for specimens elsewhere in the region since reference sequences are likely to be

region-specific. While we have expanded the number of reference sequences available for this relatively little studied portion of Western Lake Erie, if this study were performed in Scandinavia, a heavily studied region, the number of reference sequences that would match sequences of environmentally collected midge sequences would likely be higher. Establishing barcodes and analyzing the phylogeny is important in regions where chironomids have not been heavily studied in this manner (Bergsten et al. 2012).

In order to improve the results obtained from the methods of chironomid identification used in this study, a more comprehensive collection and identification of adult flies within the Western Lake Erie region needs to be done. For future studies, obtaining a larger sample size and a more diverse assortment of adult flies from the region in question may increase the number of identifiable larval OTUs. Establishing quality reference sequences that are supported by professional taxonomists is integral to utilizing this process. As more quality sequences are submitted to public databases species identifications based on molecular taxonomy will be more accessible.

Because chironomid larvae contribute significant biomass and diversity to aquatic ecology, it is important to have reliable methods of species level identification. The use of barcodes from adult midges collected from Western Lake Erie helped to resolve the species level identity of several larval clades collected in benthic samples. The present study validated the use of adults for further resolution of larval species identification. Our study enhances existing work in regions where chironomid populations are prominent and allows species level identifications to be more reliable, accurate and accessible. Establishing a comprehensive reference database of multiple DNA barcodes using reliable, cross-referenced adults identified by expert taxonomists, as was done in this study could potentially resolve problems of species level taxonomy of larvae in the family Chironomidae.

**Section A: Chironomid Biodiversity Beyond Lake Erie**

Biodiversity studies on adult chironomids expanded beyond the Lake Erie region with new collections from 2014 through 2017. We collected new adult chironomids and identified them as already described in this chapter. After morphological identification chironomids were barcoded and sequence analysis was conducted as previously described. These sequences and their identifications were combined with the previously developed chironomid database described in Chapter 5A. Because many sequences in that combined database were represented multiple times (e.g., 25 nearly identical sequences for *Chironomus* sp. [branch #1 in Figure 34), and only one representative well-identified sequence was needed as reference for a particular sequence, the tree was "pruned" to have only one or two sequences per branch. These branches were "curated" in that decisions were made as to which sample sequence out of many to keep, usually choosing the sequence with the more specific taxon identification (species level, for example, preferred over a sequence identified only to the genus level). In addition, sequences were reviewed for the total length of the sequence, since some sequences may have been truncated due to lower quality at their 5' or 3' end. Following this pruning and curation, sequences were uploaded to MEGA6, aligned, and the curated, pruned tree in Figure 40 was produced. The tree has 98 sequences, with 67 identifications to species level, and 6 to genus level. One mosquito sequence (*Anopholes* sp.) has also been included in the tree.

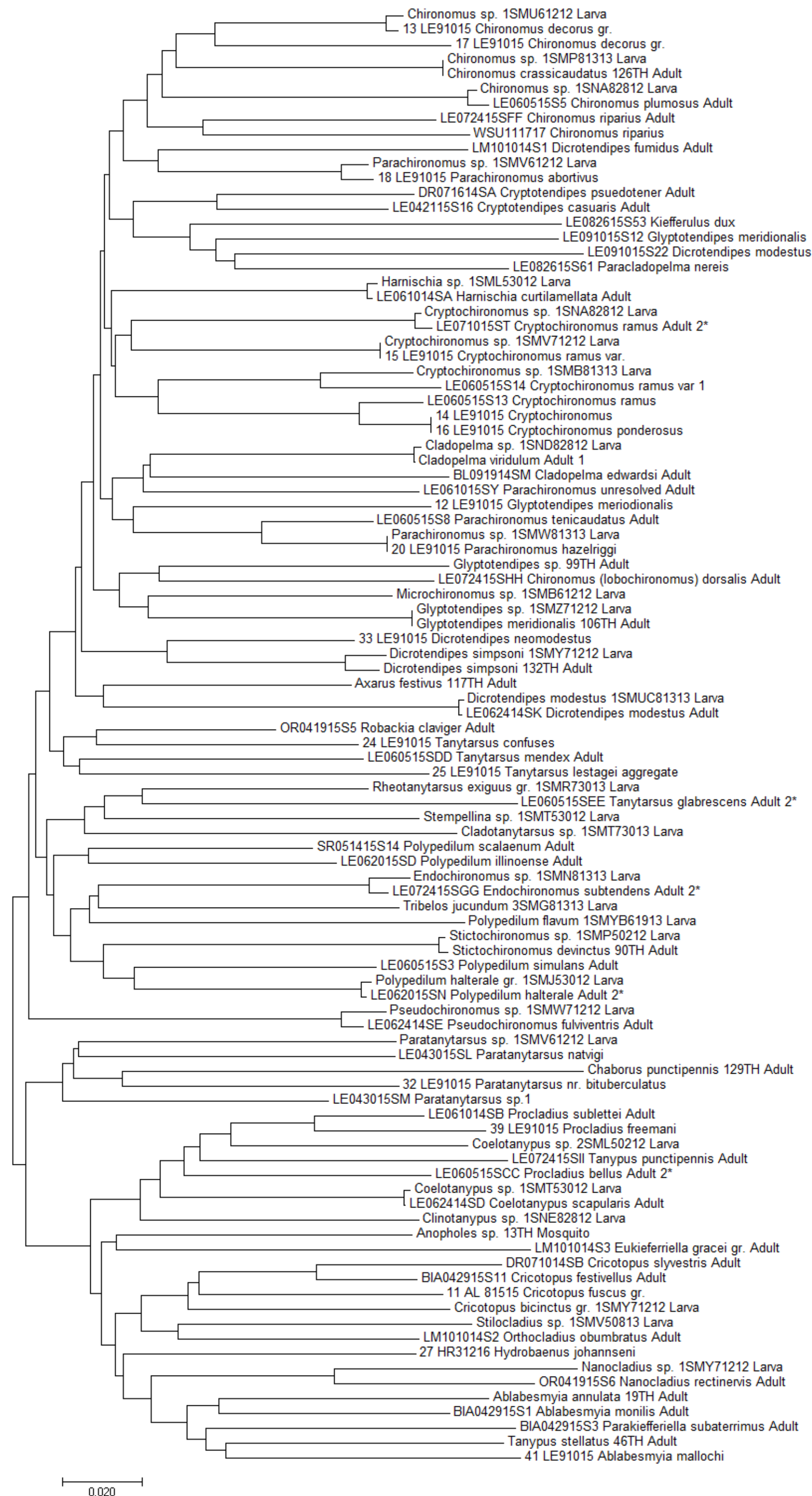



**Figure 40: Chironomid curated pruned reference tree.** The tree was computed using the number of differences method (base differences per sequence). The analysis involved 98 nucleotide sequences and there was a total of 539 positions in the final dataset. MEGA6 was used to carry out this analysis.

**Section B - *Eurytemora carolleeae* in the Laurentian Great Lakes Revealed by Phylogenetic and Morphological Analysis.**

**Abstract**

In the Laurentian Great Lakes, specimens of *Eurytemora* have been reported as *E. affinis* since its invasion in the late 1950s. During an intensive collection of aquatic invertebrates for morphological and molecular identification in Western Lake Erie in 2012-2013, several specimens of *Eurytemora* were collected. Analysis of these specimens identified them as the recently described species *E. carolleeae* Alekseev and Souissi 2011. This result led us to assess *E. carolleeae's* identifying features, geographic distribution and historical presence in the Laurentian Great Lakes in view of its recent description in 2011. Cytochrome oxidase I (COI) DNA sequences of *Eurytemora* specimens were identified as closer (2 - 4% different) to recently described *E. carolleeae* than to most *Eurytemora affinis* sequences (14% different). *Eurytemora* from other areas of the Great Lakes and from North American rivers as far west as South Dakota (Missouri River) and east to Delaware (Christina River) also keyed to *E. carolleeae*. Morphological analysis of archival specimens from 1962 and from all the Great Lakes were identified as *E. carolleeae*. Additionally, *Eurytemora* drawings in previous publications from studies in the Holarctic region were reassessed to determine if these specimens were *E. carolleeae*. Additional morphological characters that may distinguish the North American *E. carolleeae* from other taxa are also described. We conclude that *E. carolleeae* is the correct name for the species of *Eurytemora* that has inhabited the Great Lakes since its invasion, as established by both morphological and COI sequence comparisons to reference keys and sequence databases in present and archival specimens.

**Introduction**

In the Laurentian Great Lakes, introductions of non-native copepods have occurred over

several decades (Engel 1962, Horvath et al. 2001, Hudson and Bowen 2002). However, some publications that list copepods have either mischaracterized their native distribution (Mills et al. 1993, Drake and Lodge 2007a, b) and/or the taxonomy of the species (Reid and Hudson 2008). The introduction of the estuarine copepod *Eurytemora* to the Great Lakes was noticed quickly since it is easily distinguished from native calanoid copepods by its long caudal ramus, long pointed metasomal wings, and relatively shorter antennae. *Eurytemora* was likely introduced to the Great Lakes due to the construction and opening of the St. Lawrence Seaway since introductions of many non-native freshwater tolerant marine taxa coincided with the opening of the Seaway or followed shortly thereafter (Mills et al. 1993). *Eurytemora* sp. was first recorded in Lake Ontario at the genus level in 1958 (Anderson and Clayton 1959) and thereafter reported as *Eurytemora affinis* Poppe 1880 in all the Great Lakes (Mills et al. 1993). However, the recent description (Alekseev and Souissi 2011) of *Eurytemora carolleeae* Alekseev & Souissi, 2011 raised questions about the appropriate identification of the *Eurytemora* populations in the Great Lakes, which the present study seeks to answer.

Significant work has been completed in studying the life history, mechanisms of invasion and biogeography of *Eurytemora* taxa in North America (Lee 1999, Lee and Frost 2002, Winkler et al. 2008, Dodson et al. 2010, Favier and Winkler 2014, Posavi et al. 2014, Cabrol et al. 2015). *Eurytemora*, typically identified as *E. affinis*, has been known to play an important role as a dominant grazer in marine, estuarine, and freshwater systems and is considered to be a cosmopolitan species due to its broad biogeographic range encompassing subtropical to subarctic areas (Lee 2000, Suarez-Morales et al. 2008). Historically, this coastal-estuarine copepod was considered to be a marine species (Croskery 1978). Nevertheless, surveys within freshwater systems in North America and Mexico have identified *Eurytemora* clades far from the coastline (Lee and Frost 2002, Suarez-Morales et al. 2008). Evolutionary and physiological

osmoregulatory adaptations may have enabled *Eurytemora* taxa to invade freshwater environments from its typical saline habitats (Lee 1999, Johnson et al. 2014, Posavi et al. 2014).

*E. affinis* has a geographic range that spans the northern hemisphere and habitat types that range from hypersaline salt marshes to fresh water suggesting a cryptic species complex (Dodson et al. 2010). Sequences of the mitochondrial cytochrome oxidase I (COI) gene have been shown to be very useful for distinguishing calanoid and harpacticoid copepods including cryptic and sibling species in biogeographic studies (Laakmann et al. 2013, Miracle et al. 2013, Peterson et al. 2013, Gutierrez-Aguirre et al. 2014). Previous genetic analyses of the COI gene in *Eurytemora* populations described specimens from the Great Lakes as belonging to an Atlantic clade of *E. affinis* (Lee and Frost 2002, Winkler et al. 2008). Phylogenetic analysis of North American *Eurytemora* collected from several marine and freshwater sites, including specimens from Lake Michigan and the Detroit River, revealed several distinct clades but did not distinguish any differences in the morphological characters of the specimens associated with the different clades using keys available at that time (Lee and Frost 2002). Recently, Alekseev and Souissi (2011) identified *E. carolleeae* as a previously undescribed sibling species to *E. affinis* native to the North American Atlantic coast, with distinct characters to enable its morphological identification. *E. carolleeae* was also reported to be a potentially new invasive copepod in the Baltic Sea and European Atlantic coast estuaries first based on COI sequence data and then through taxonomic identification (Alekseev et al. 2009, Sukhikh et al. 2013). *E. carolleeae* observations in North America were from the Chesapeake Bay and the St. Lawrence estuary with the possibility of distributions in the inland waters of the Great Lakes to Mexico (Alekseev and Souissi 2011). COI sequence data was used to corroborate the morphological identification of the *E. carolleeae* invasion of the Baltic Sea and European Atlantic coast estuaries (Sukhikh et al. 2013). These recent analyses indicated more than one species of *Eurytemora* contributed to the

Great Lakes invasion, which led us to re-examine the classification of *Eurytemora* specimens collected in the Great Lakes.

In order to determine which *Eurytemora* species or clade had invaded the Great Lakes, this present study used morphological and COI molecular barcoding methods to identify the *Eurytemora* taxa. Morphological analysis was carried out for archival specimens from the Great Lakes dating back to 1962, and we reviewed drawings and photographs in past literature. Additionally, this paper describes our analysis of samples collected in 2012-2014 from the Great Lakes and from rivers as far west as South Dakota and east to Delaware to determine the possible distribution and morphological variation associated with this species complex.

**Methods**

***Sampling***

Specimens of *Eurytemora* came from various locations in the Great Lakes including western Lake Erie, Detroit River, Lake St. Clair, Lake Huron, Lake Michigan (including from Muskegon Lake, an estuarine lake of Lake Michigan (Weinke et al. 2014)), and two river systems, the Christina River in Delaware and the Missouri River (Lewis and Clark Lake) in South Dakota (Figure 41). Plankton samples from western Lake Erie were collected using a hand-thrown Wisconsin plankton tow net with a 80 µm mesh (Wildco, USA) during the summer of 2012 and 2013 at sites in and near Toledo Harbor, Ohio USA. Fourteen sites were repeatedly sampled over the 2012 and 2013 summer months beginning in May and ending in August (see Electronic Supplementary Material S1). Samples were split and preserved in 80% ethanol for molecular analysis and in Lugol's solution for morphological analysis. The sample in Lugol's solution was shipped to EcoAnalysts (Moscow, ID) for taxonomic analysis. Sampling during 2014 was limited to spot locations using either a Wisconsin net near shore or in shoreline aquatic vegetation using a bucket and multiple grabs, filtered with an 80 µm sieve and stored in 91%

isopropyl alcohol.

**Figure 41: Map depicting zooplankton collection sites** within the Laurentian Great Lakes system and North American rivers. Circles represent samples collected during 2012, 2013 and 2014. Triangles represent sites where archival samples were collected (see Table 1). Inset in map is of Toledo Harbor and western Lake Erie where *Eurytemora carolleeae* was first found in this study.

***Taxonomy***

For identifying specimens of *Eurytemora* we used several characters to separate *E. carolleeae* from its congener *E. affinis* including a large outside dent on the mandible and setal segmentation on the caudal rami and swimming legs, which we documented in some of our specimens (see Table 6). However, for routine separation we chose to use the wing-like outgrowths on the genital double-somite (Figure 42a) and a small spine near the distal seta insertion point in P5 (Figure 42b) in females, and the naked caudal rami (Figure 42c) and cylindrical shape (length/width (L/W) ratio >1.3) of the second segment of the exopod (also

known as the basipod) on the left P5 (Figure 42d) in the male to identify specimens of *E. carolleeae* as described in Alekseev and Souissi (2011). These characters were either easily seen under a dissecting microscope or when the P5 was placed under a coverslip on a slide and viewed under a compound microscope at higher magnification. In addition, Great Lakes specimens from the US Geological Survey Great Lake Science Center collections of alcohol preserved plankton samples and specimens archived on microscope slides were examined (Table 5) using the same characters. A similar analysis was applied, when possible, to drawings and photographs in descriptions of *Eurytemora* in previous publications from studies in the Holarctic region (see list in Table 6).

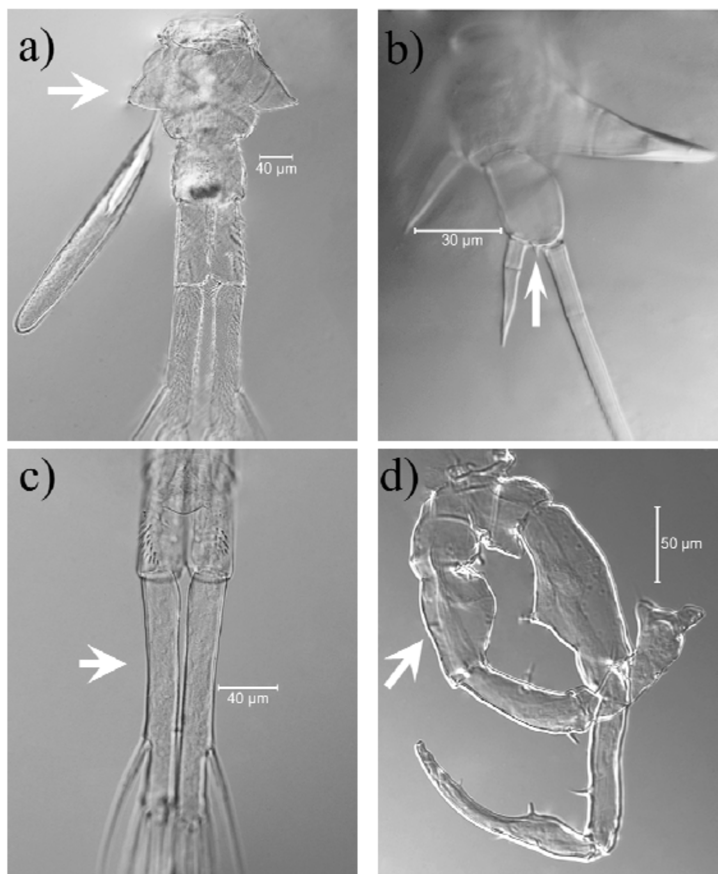



**Figure 42: Micrographs of morphological characters used to key *Eurytemora carolleeae*** Alekseev and Souissi, (2011). (a) Female, genital somite (arrow); ( b) Female fifth leg showing the small spine (arrow) near the distal seta insertion point; (c) Male spineless caudal ramus (arrow); (d) Male fifth leg indicating basipod (arrow).

**Table 5. Historical biogeography of *Eurytemora carolleeae* using archived specimens and archived slides from the Laurentian Great Lakes.**

| Lake | Location | Latitude (°)/ Longitude (°) | Depth (m) | Date | Sex |
|---|---|---|---|---|---|
| Superior | Bibon Lake wetland# | 46.784/-91.398 | 1 | Aug. 5, 1993 | F |
| Michigan | Milwaukee Harbor* | NR | NR | Aug. 27, 1969 | M |
| | Little Bay de Noc* | NR | NR | Aug. 31, 1970 | MΘ |
| | Green Bay* | NR | NR | May, 1969 | F |
| Huron | Saginaw Bay* | NR | 5 | July 10, 1974 | M |
| | Saginaw Bay# | 43.598 -83.664 | 1 | Aug. 17, 1997 | M |
| | Saginaw Bay# | 43.817/-83.919 | 1 | July 28, 1994 | M |
| St. Clair | Clinton R. cutoff* | 42.562/-82.847 | NR | July 10, 1973 | M,MΘ |
| Erie | Sandusky Bay* | 41.500/ -82.702 | NR | May 26, 1967 | F,M,M |
| Ontario | Fair Haven, NY# | 43.428 -76.722 | 55 | Nov. 2, 1992 | M |

Source and sex of archived specimens from the Great Lakes region are provided. Specimens on archived slides (*) were collected by John Gannon. Specimens preserved in alcohol (#) were collected by Patrick Hudson. NR means not recorded. Θ means only caudal ramus and not basipod was available for morphology. Multiple specimens for a particular collection are indicated by more than one sex designation separated by commas.

To further characterize *E. carolleeae* morphologically, the presence/absence and placement of setae on the fifth leg of female and male specimens were analyzed. To supplement this, drawings of the female and male fifth legs of *Eurytemora* in research papers listed in Table 6 were reviewed for setae presence and placement. Dr. Eduardo Suarez-Morales from El Colegio de la Frontera Sur (ECOSUR), Chetumal, Mexico assisted us by further confirmation of his observations of setae placement and contributed additional morphometrics of the female and male fifth legs of his specimens reported in Suarez-Morales et al. (2008) and evaluated morphological differences. *Eurytemora* specimens analyzed by Dr. Suarez-Morales are deposited in the collection of Zooplankton of ECOSUR under Colina Lake ECO-CHZ-03662, and Balmorhea Lake ECO-CHZ-03440, 03441. Comparisons to the specimens in this study were used to investigate a basis for possible diagnostic characters to further separate the *E. affinis* complex.

**Table 6. Summary of illustrations in referenced literature analyzed for presence of *Eurytemora carolleeae* in previously described specimens.**

| Reference (Geographic source & date) | Figure in Reference | Structures | Abbreviation |
|---|---|---|---|
| Alekseev & Souissi (2011) Chesapeake Bay, USA (2008) | 6, 9 | ♂P5, caudal ramus; ♀P5, genital somite | A |
| Busch & Brenning (1992) North Sea and Baltic Estuaries (1988) | 2, d, e, f, g, m, | ♂P5; ♀P5, genital somite | B |
| Davis (1943) Chesapeake, Bay (1943) | Plate 9: 7 | ♂P5 | D |
| Gurney (1931) England (prior to 1931) | 305 | ♂P5 | G |
| Katona (1971) Oyster Pond, MA (1969) | 88, 89 | ♂P5 | $K_{88}$, $K_{89}$ |
| Lee & Frost (2002) Germany (prior to 1896) | 2, redrawn from Pl. XI, Fig 10 of Schmeil (1896) | ♂P5 | L |
| Suarez-Morales et. al (2008) Presa Falcon, Mexico (2000-2001) | 2, 3, 4 | ♂P5; ♀P5, genital somite | $S_3$, $S_4$ |
| Wilson & Yeatman (1959) Lake Providence, Louisiana (prior to 1959) | 29.15 | ♂P5 | W |

"Abbreviation" is the single letter with or without subscript used in an illustration (Figure 45) in the Results to identify the reference source.

### *DNA extraction*

Individual ethanol-preserved specimens were lysed in ATL lysis buffer (cat. no. 19076, Qiagen, Hilden, Germany), with Proteinase K (cat. no. 19133, Qiagen, Hilden, Germany), followed by DNA purification with the DNAeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and Qiagen Spin Columns according to standard protocols (https://www.qiagen.com/us/resources/resourcedetail?id=6b09dfb8-6319-464d-996c-79e8c7045a50&lang=en). Elution with Low TE (Invitrogen, Carlsbad, CA) used a small volume (28 µl) since the resultant purified DNA is from a single microscopic organism.

***Polymerase chain reaction (PCR)***

Purified DNA was amplified by PCR using COI forward primer HCO2198 and reverse primer LCO1490 prepared as stock solutions of 10 pmol/µl (Folmer et al. 1994). DNA was added to PCR reactions at a quantity of 1.5 µl per 25 µl reaction. PCR master mix contained 0.5 µl of each forward and reverse primer stock solutions, 12.5 µl of SSO Advanced Universal SYBR Green Supermix (BioRad, Irvine, CA), and 10 µl sterile water. Reactions were run on an iCyclerQ Realtime thermocycler (BioRad, Irvine, CA), initiated by heating to 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 51 °C for 30 s, 72 °C for 1 min, and then a final extension of 72 °C for 7 min followed by a hold at 15 °C until further processing within 3 hours. PCR products were visualized on 1% agarose gels with SYBR Safe DNA Gel Stain (Invitrogen, Grand Island, NY), and images were documented with a DarkReader (Clare Chemical, Dolores, CO). A 100 bp eXact gene DNA ladder (Fisher Scientific, NH) was run alongside the products for size calibration. After purifying PCR amplicons using QIAquick PCR purification columns (Limburg, NL), DNA quality and concentration was measured by spectrophotometry (Tecan US Inc. Infinite F200, Morrisville, NC), and the amplicons were sequenced by Sanger DNA Sequencing services of GeneWiz (South Plainfield, NJ).

***Sequence and phylogenetic analysis***

Samples were sequenced bi-directionally and analyzed with DNA Baser software (Heracle BioSoft SRL, Romania) and Mutation Surveyor (Softgenetics, State College, PA) to visualize the chromatogram files to determine quality and accuracy of the sequences. Sequences that were trimmed by the DNA Baser software Trimming Engine to less than 626 bases in length (trimming removed regions where >35% of bases in 16 base windows had quality scores <22) were not used. The remaining sequences were >625 base pairs long and had average quality scores >40. Forward and reverse complement chromatograms were then compared manually to

ascertain agreement of base identities bi-directionally and the high quality consensus sequence of each sample was used in subsequent analysis. Sequences were then compared to the existing NCBI GenBank and Barcode of Life Data Systems (BOLD) databases (as of January 5, 2015) focusing particularly on comparisons to sequences uploaded by the laboratory that had identified morphological features that distinguished *E. carolleeae* from other *Eurytemora* species (Sukhikh et al. 2013). MEGA6 was used for phylogenetic analysis, including constructing neighbor-joining trees (Tamura et al. 2013) and, in the supplement, calculations of pairwise differences of nucleotides between sequences.

**Results**

***Toledo Harbor specimens***

A neighbor-joining tree examining the relationship of COI sequences from eight Toledo Harbor specimens to reference sequences in the NCBI and BOLD databases revealed that the Toledo Harbor sequences were on a different branch than almost all of the sequences annotated as *E. affinis* (Figure 43). Branch lengths of the tree and calculations of average pairwise difference (see Electronic Supplementary Material S2, Figure S2) indicated that the Toledo Harbor COI sequences differed from *E. affinis* by an average of 14%. The group of sequences in the NCBI database with the closest similarity to the Toledo Harbor sequences were barcodes of specimens annotated by Sukhikh et al. (2013) as *E. carolleeae*, from which the Toledo Harbor specimens differed by 2 - 4%. The outgroup, *Eurytemora lacustris*, differed from the Toledo Harbor specimens by an average of 18%. Seven of the eight Toledo Harbor specimens as a distinct group within an *E. carolleeae* clade, different from most sequences reported as *E. affinis*. Only one Toledo Harbor specimen (THP2J53012S5) has a sequence that is in the same branch as the type specimens for *E. carolleeae*.

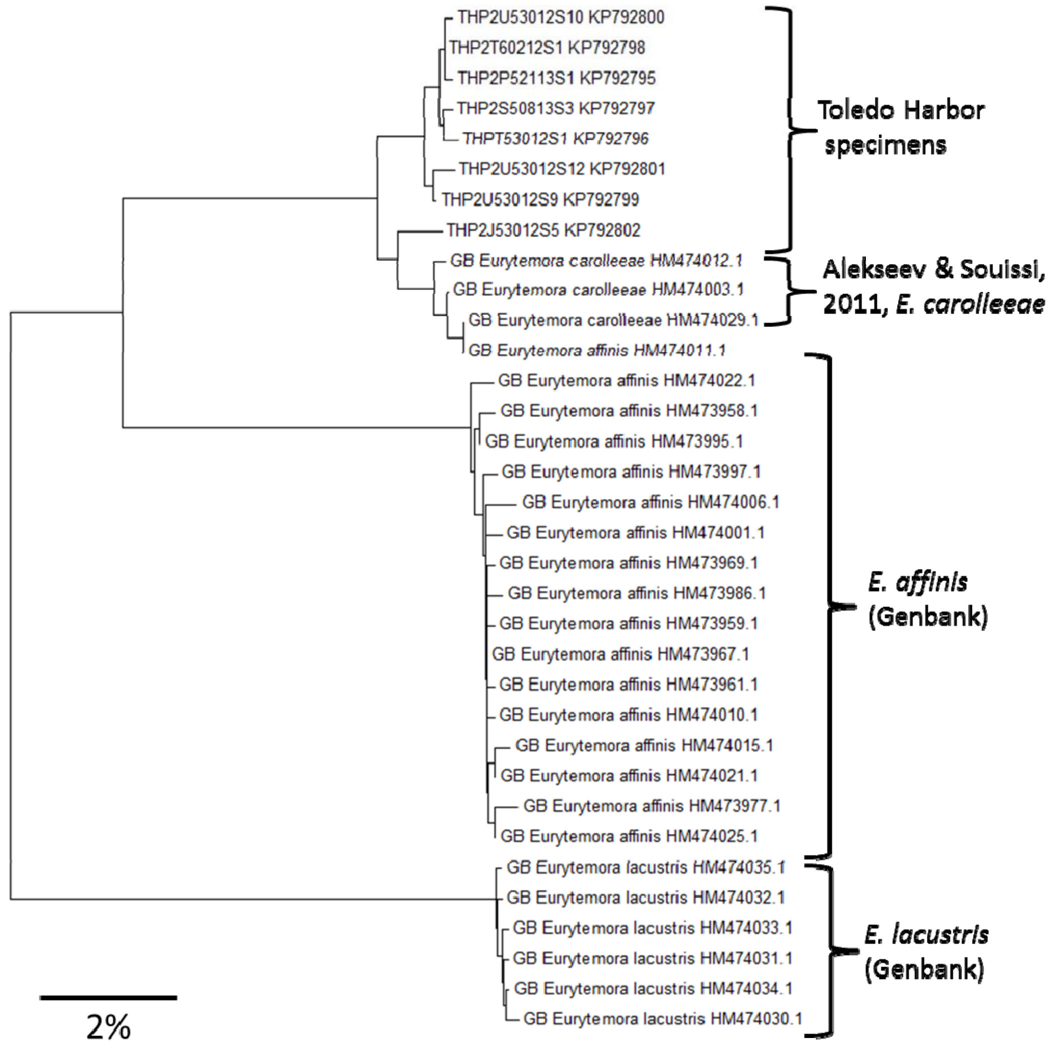



**Figure 43: Neighbor-joining tree based on number of base differences per cytochrome oxidase I (COI) sequence for eight specimens from Toledo Harbor** (GenBank accession numbers KP792795-KP792802) compared to *Eurytemora carolleeae, Eurytemora affinis* and *Eurytemora lacustris* sequences from the GenBank database. Sequences were selected from sequences submitted by Victor Alekseev. The analysis of 34 sequences is expressed as percent of 631 nucleotide positions.

***Identification of Eurytemora spp. beyond Toledo Harbor***

To determine whether the sequences found in Toledo Harbor *Eurytemora* are present over a broad geographic range we investigated samples from other Great Lakes and elsewhere. Collection sites ranged from the Great Lakes basin (Lake Huron, Lake Michigan, Lake St. Clair and the Detroit River) to Delaware (Christina River) and South Dakota (Missouri River) (Figure 41).

None of the sequences obtained from more than 25 specimens from these locations were closely related to *E. affinis* or *E. lacustris*, from which they all differed by at least 13% (Figure 44 and Electronic Supplementary Material S2, Figure S3). In fact, most of the new sequences in Figure 44 were nearly identical to sequences of previous Toledo Harbor specimens (< 2%

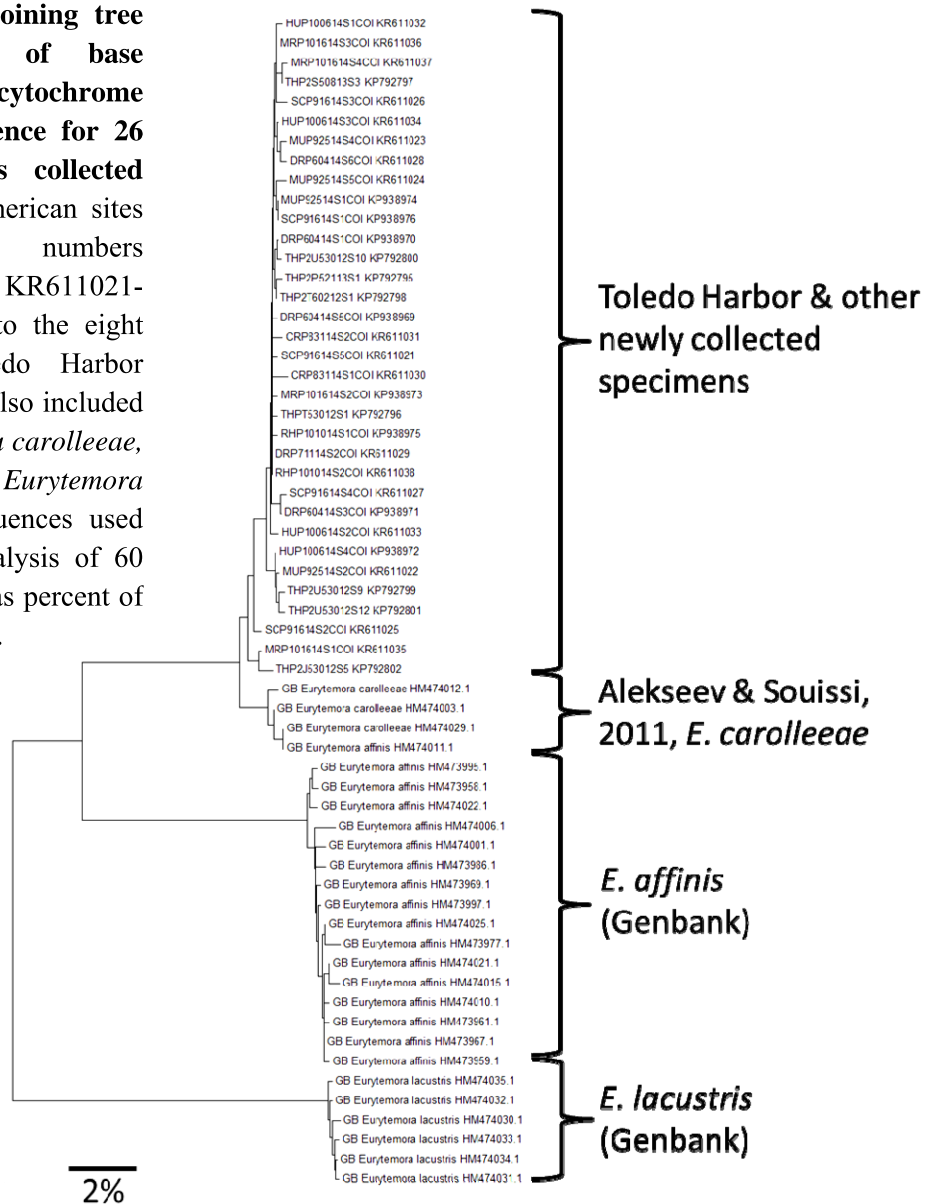



**Figure 44: Neighbor-joining tree based on number of base differences per cytochrome oxidase I (COI) sequence for 26 *Eurytemora* specimens collected** from various North American sites (GenBank accession numbers KP938969-KP938976, KR611021-KR611038) compared to the eight specimens from Toledo Harbor analyzed in Figure 3. Also included are the same *Eurytemora carolleeae, Eurytemora affinis* and *Eurytemora lacustris* GenBank sequences used in Figure 43. The analysis of 60 sequences is expressed as percent of 626 nucleotide positions.

difference). The added North American sequences are seen distributed within the branch that includes the Toledo Harbor specimens (Figure 44). All of the added North American sequences

differed from the *E. carolleeae* sequences of Alekseev and Souissi (2011) by 1.5 – 3% (See Electronic Supplementary Material S2, Figure S4).

***Morphological considerations***

Routine review of specimens collected for this study using descriptive morphological features (Figure 42) clearly identified Great Lakes specimens as *E. carolleeae*. For example, as summarized in Figure 45, the cylindrical shape of the basipod (arrow in Figure 42d) of the left P5 leg in males was longer than its width, (in agreement with the L/W ratios of *E. carolleeae* reported by Alekseev and Souissi 2011). The L/W ratio of this segment in 9 mature male North American specimens collected in this study averaged 1.51 $\pm$ 0.045 (mean $\pm$ sem, Figure 45a). A similar analysis for 8 archived male specimens (Table 5) from each of the five Great Lakes and Lake St. Clair averaged 1.47 $\pm$ 0.064 (mean $\pm$ sem, Figure 5b). Examination of drawings in previous research papers listed in Table 6 indicated that L/W ratios of the male P5 basipod segment fell into two distinct clusters, one with averages less than 1.0 and another cluster with L/W ratios >1.3. The summary drawing of the P5 basipod segment of the type material for *E. carolleeae* from Chesapeake Bay in Alekseev and Souissi (2011) had a L/W ratio of 1.44.

Assessment of multiple morphological features in the drawings of male and female *Eurytemora* spp. in papers by Gurney (1931), Busch and Brenning (1992), and Lee and Frost (2002) verified the identification as *E. affinis*. The basipod L/W ratios of the male P5 were < 1.0 in all of these publications (Figure 45c). The specimens of Davis (1943), Wilson (1959), and Suarez-Morales et al. (2008) are clearly most similar to *E. carolleeae*, with male basipod L/W ratios > 1.3 (Figure 45c). Katona (1971) drew several specimens and may have been illustrating more than one *Eurytemora* species as the male P5 L/W ratio was 1.0 for Figure 89 and 1.3 for Figure 85. Male and female North American specimens and archived specimens from the US

Geological Survey Great Lake Science Center collections (Table 6) had characters that key them more closely to *E. carolleeae* than to *E. affinis*.

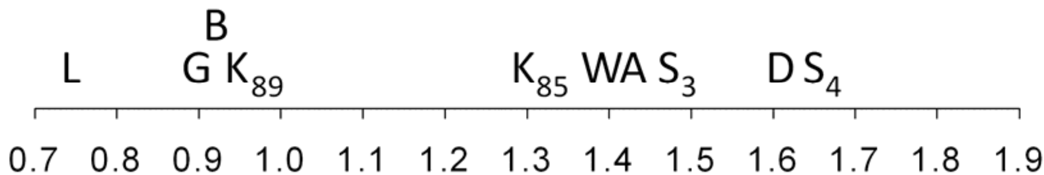



**Figure 45: Length/width ratios for left basipod in the male fifth leg** in (a) North American specimens collected for this study from sites shown by circles shown in Figure 41, (b) archived specimens from sites shown by triangles in figure 1 and listed in table 1, (c) drawings in the scientific literature. In (c) the abbreviations listed in table 6 are positioned corresponding to their L/W ratios.

***Setae size and locations***

Drawings representing the typical placement and sizes of setae on the male and female fifth legs (P5) of specimens from the Great Lakes collected for this study are illustrated in Figure 46a, b. This setation pattern was then compared to relatively complete and detailed drawings of the male P5 in the literature (Table 6) that were identified (see previous paragraph) as either *E. affinis* or *E. carolleeae*.

The right P5 in males (Figure 46a) has a robust basipod (segment A) with a strong inside lateral spine (RA2) plus two strong spines in the middle of the segment centered on each side of

the segment (RA1, RA3). The next segment B, or first exopodal, has a strong inside lateral spine (RB3) about 0.7 distance from the base, two setae about midway on the segment (RB1, RB2) and one seta at the distal end of the segment (RB4). The last segment C on our specimens have a seta on the inner surface (RC1) where the segment starts constricting, another one just above and a little further out on the outer surface (RC2), and a peg-like seta on the middle inner surface at the scythe-like end of the segment (RC3). The representative right leg drawn in Figure 46a is most similar to drawings by Lee and Frost (2002), Busch and Brenning (1992), and Katona (1971) except for the presence of a single setae between RB3 and RB4 seen in Lee and Frost (2002) and Katona (1971).

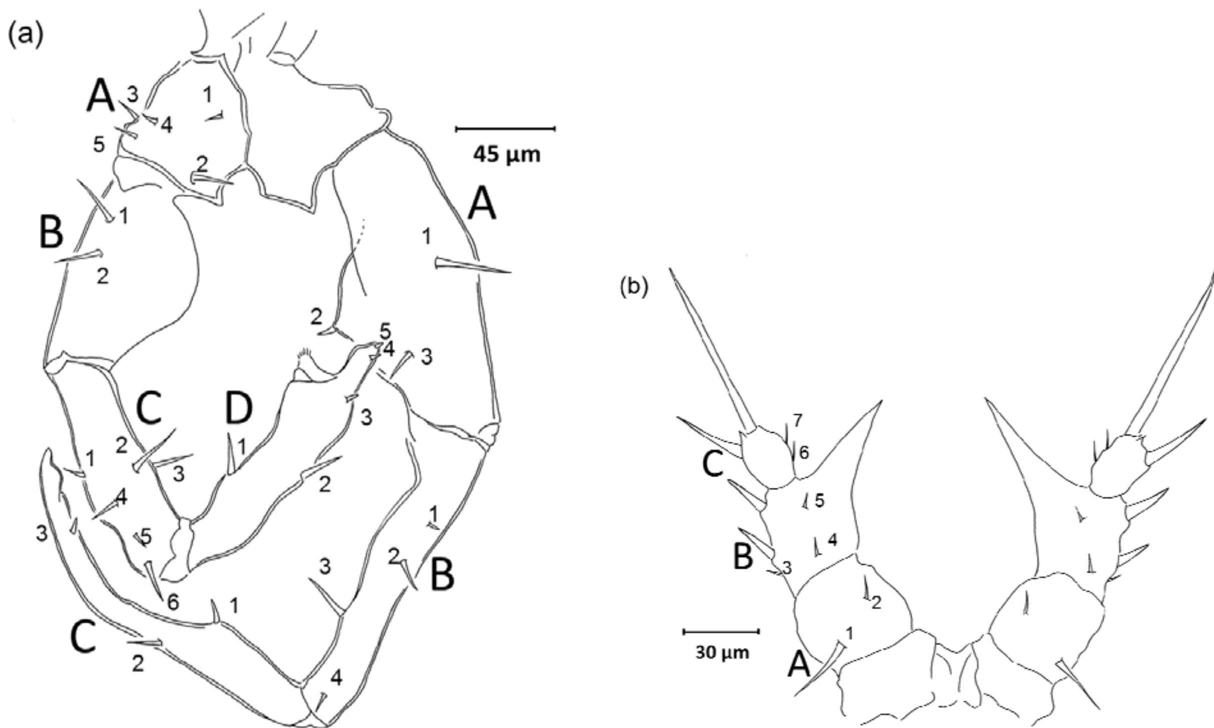



**Figure 46: Drawings of *Eurytemora carolleeae* fifth legs in** (a) **male and** (b) **female**. The segments are lettered from distal to proximal; setae within a pod are numbered from distal to proximal. In the text particular setae are referred to by the designation Side-Podite-Seta # (SPS#). For example RA2 refers to the strong inside lateral spine on the right segment A.

For the left P5 in males (Figure 46a) the setal patterns of the coxal segment (labeled segment A), the basipod (B) and the two exopodal segments (C and D) were examined. Comparisons could not be made with Busch and Brenning (1992) because they did not include the entire left leg. The coxal segment in Figure 46a has two to three setae on the outer surface (LA3, LA4, and LA5), one seta on the midline of the segment (LA1) and one seta on the distal inner surface (LA2). The basipod of our specimens have two setae on the outer edge (LB1 and LB2). Segment C (first exopodal segment) in Figure 46a has two setae midway on the inner surface of the segment (LC2, LC3), a single seta opposite the two on the outer surface (LC1), and setae on the distal end of the segment (LC4, LC5, LC6). The last segment (segment D, the second exopodal) in Figure 46a has a very strong seta midway on the inner edge (LD1) and a seta on the outer edge about 0.6 distance from the base (LD2) and other features (see below). Segments A and C on the left leg are quite variable, as similarly illustrated by Busch and Brenning (1992). The setal pattern of the left leg (Figure 46) is identical to those illustrated by Katona (1971).

In the left male exopod, the end of the second segment (Segment D) resembles a "dragon's head" at high power (Figure 47), but only two of the seven references listed in Table 5 illustrate the complexity present in this structure. A row of short setae covers the inner portion of the bifurcated end of the second exopodal segment and at some positions several spicules come out of small protuberances. The outer surface of the segment has three setae placed as if one were viewing an eye (LD3) and a pair of nostrils (LD4, LD5). Wilson (1959) only showed the row of short setae at the end of the segment but did not show setae LD3-LD5, and Davis (1943) and Katona (1971) both display the row of short setae at the end of the segment but setae LD3-LD5 are not clearly represented. Examination of archived specimens from Dr. Suarez-Morales (personal communication, Dr. Eduardo Suarez-Morales, ECOSUR, 2016) described in Suarez-

Morales et al. (2008) identified the presence of setae LD4 and LD5; Dr. Suarez Morales also verified (personal communication, Dr. Eduardo Suarez-Morales, ECOSUR, 2016) that the dragon's head structure of segment D in his Balmorhea Lake specimens has a longer "neck" and more bulbous ending illustrated in Figure 3c of Suarez-Morales et al. (2008), distinctively different from Figure 47 of this paper.

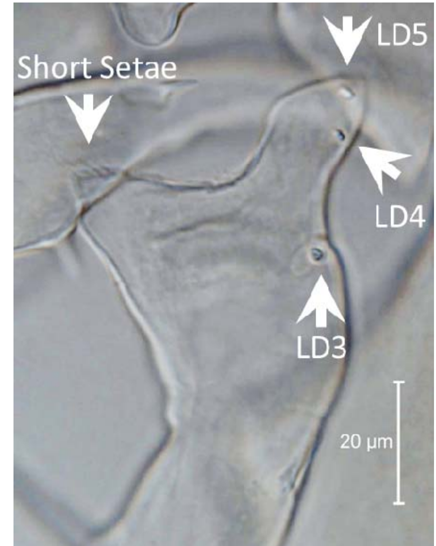



**Figure 47: "Dragon's head" structures at the end of the left endopod of male fifth leg.**

On the female P5 (Figure 46b), seta 1 on segment A (designated seta A1) is a strong seta that occurs on the anterior to lateral face of the basipod plus a finer seta occurs in the middle portion of the anterior face (A2). Most authors have not described the finer setae illustrated in the present study. Only the stronger seta (A1) appears in Suarez-Morales et al. (2008), as further confirmed by review of Dr. Suarez-Morales' archived *Eurytemora* from Lake Balmorhea (personal communication, Dr. Eduardo Suarez-Morales, ECOSUR, 2016). In contrast, both setae are illustrated in Katona (1971). In Figure 46b, the first exopod (segment B) have two fine setae on the central part of the anterior face (B4, B5) which are also drawn or photographed in Suarez-Morales et al. (2008) and Katona (1971). The strong seta 3 on the lateral edge of segment B (B3) also appears to have its counterpart in the lateral position in Figure 45c and on the posterior face in Figure 2f of Suarez-Morales et al. (2008). This seta is absent in the Katona (1971) drawing

which appears to be a drawing of the anterior view. The drawing by Katona (1971) of the last exopodal segment has three setae. Our specimens have two (C6, C7), but these setae are absent in Suarez-Morales et al. (2008), who further confirmed the fewer number of setae in general on his archived specimens than have been found in this study of Great Lakes *Eurytemora*.

**Discussion**

***E. carolleeae as a distinct species in the Eurytemora complex.***

Previous studies on the morphological and genetic divergence of members of the *Eurytemora* complex from Alaska, Europe and North America demonstrated great diversity and suggested that there were undiscovered clades (Lee 2000, Winkler et al. 2008, Dodson et al. 2010, Winkler et al. 2011). Mitochondrial DNA analysis performed during the past fifteen years suggested that two clades inhabit the St. Lawrence estuary region, an invasive Atlantic clade and a non-invasive North Atlantic clade (Lee 1999, Winkler et al. 2008, Favier and Winkler 2014, Cabrol et al. 2015). Attempts to mate individuals from two populations with each other did not produce viable reproductive adult offspring (Lee 2000). Alekseev and Souissi (2011) identified the existence of a new species, *E. carolleeae*, and deduced that the Atlantic clade previously referred to as *E. affinis* might be considered the separate species of *E.carolleeae*. Alekseev and Souissi (2011) suggested that a reassessment of the population found in the Great Lakes be done since previous surveys reporting *E. affinis* might actually have been observing *E. carolleeae*. Indeed, the initial reports from EcoAnalysts we received counted all specimens as *E. affinis*, based on the standard taxonomic keys used by many plankton specialists. Nevertheless, the distinct morphological features described by Alekseev and Souissi (2011) and COI sequences differing from most *E. affinis* specimens by a much larger margin than 3% support the identification of *E. carolleeae* as a distinct sibling species within the *Eurytemora* complex.

Small (1.5% - 3%) but consistent differences in the sequences of the Great Lakes

specimens in the present study compared to the European sequences by Sukhikh et al. (2013) might indicate further incipient speciation events. To determine if speciation is occurring additional genes should be examined. Such speciation events could occur as a result of geographic separation of populations and subsequent selection or founder effects.

The reanalysis of specimens from the US Geological Survey Great Lake Science Center collection of alcohol preserved plankton samples and microscopic slide collection spanning from 1962 to present identified only *E. carolleeae*. These records from all 5 Great Lakes and Lake St. Clair suggest that historically, *E. carolleeae* was the species of record, and that *E. affinis* was probably never present in the Great Lakes. The drawings on plate 26 of *E. affinis* in Balcer et al. (1984) which are clearly *E. carolleeae*, are further historical evidence. Thus, analysis in the present study of archived, and freshly collected specimens, and literature drawings from Lake Erie, Lake Michigan, Lake Huron, Lake Superior, Lake Ontario, Lake St. Clair and the Detroit River revealed only *E. carolleeae*.

Now that *E. carolleeae* is considered a distinct species, several previous papers and annotated sequences should be updated to reflect our current understanding of *Eurytemora* diversity and to assist with proper reporting and identification of current populations. Especially relevant to invasion biology are two sequences (Accessions GQ924685 and GQ924686) by Briski et al. (2011) that were uploaded to GenBank in 2009 as *E. affinis* but are 97% and 99% identical to the *E. carolleeae* sequences in GenBank. These two specimens were sequenced from diapaused copepod eggs obtained from sediments in ship ballast tanks of transoceanic vessels that had arrived at a Canadian port (Sept-Iles, QC). In additional neighbor-joining tree analysis (See Electronic Supplementary Material S3) with these shorter sequences (545 and 549 bases, respectively), one of the sequences of Briski et al. (2011) grouped closer to the Sukhikh et al. (2013) sequences than to the Toledo Harbor specimens in this study; the other sequence was

closer to the sequences of Toledo Harbor specimens and was 99% identical to specimens collected for the present study from the Detroit River, Lake Huron, and Muskegon Lake. Aside from suggesting that the annotations of GQ924685 and GQ924686 should be updated to reflect their likely re-identification as *E. carolleeae*, the presence of these specimens in ballast tank sediment suggests the possibility that *E. carolleeae* might invade ports in ship's ballast sediments as suggested by Sukhikh et al. (2013).

***Morphological features***

Historical comparisons of *Eurytemora* taxa have used various widths, lengths and shapes of various body parts with some parameters involving presence/absence. These comparisons worked well for separating most of the species within the genus as a whole (Dodson et al. 2010) but did not differentiate several subtypes within the *E. affinis* complex (Busch and Brenning 1992, Lee and Frost 2002, Dodson et al. 2010). However, Alekseev and Souissi (2011) revealed features that morphologically differentiated the *E. carolleeae* subtype from *E. affinis.*

In the present study, the most useful morphological measurement in comparisons to previous *Eurytemora* descriptions was the L/W ratio of the basipod in the left fifth leg of males. Quantitative analysis of this trait was described for *E. affinis* and *E. carolleeae* by Alekseev and colleagues (Alekseev and Souissi 2011, Sukhikh et al. 2013). While the average values for this trait differ significantly (1.43 $\pm$ 0.13 [mean $\pm$ SD] in *E. carolleeae* v. 0.96 $\pm$ 0.05 in *E. affinis* in Alekseev and Souissi (2011)), this measure is not absolute. For example, Figure 5B of Sukhikh et al. (2013), who studied populations from a broader geographic area, illustrated similar averages to Alekseev and Souissi (2011) for the two species but values for about 12% of the specimens crossed over the value of 1.3 that we have used to differentiate the species. Thus, the L/W ratio of the basipod in the left fifth leg of males represents a useful key feature, but for definitive identification, additional features or DNA sequences are needed.

***Setal and exopodal patterns***

In an effort to provide a more complete description of the diagnostic fifth legs of males and females and to potentially identify additional key features; this study investigated the pattern of setae on the fifth legs in detail. The illustration by Alekseev and Souissi (2011) of the setal pattern on the male P5 of *E. carolleeae* was limited to a few major seta. In reviewing the drawings from past literature, the precision and accuracy of the various artists or the quality of the specimen, the preparation, or the type of microscope used are difficult to assess. For example, Davis (1943) did not illustrate any of the setae on the female P5 and it appears that a number of setae on the male P5 were likely omitted. Busch and Brenning (1992) suggested that the number and positions of setae on the male $5^{th}$ leg were variable. When we compared the setal patterns of our male specimens with the figures of the authors in Table 6 only the drawings of Katona (1971) matched ours. Since we suspect that he was dealing with two species, this may mean that the male $5^{th}$ leg setal patterns of *E. affinis* and *E. carolleeae* are virtually identical. The drawings from Schmeil (1896) as illustrated in Lee and Frost (2002), which definitely illustrate *E. affinis*, also match ours except for the absence of seta LA1 and an additional seta on segment B on the right side.

In contrast, the drawings of Suarez-Morales et al. (2008) properly characterize the material they studied and they suggest that their specimens may represent the Gulf subclade of *Eurytemora affinis* of Lee and Frost (2002). Interestingly, Segment D (exopod 2) on the left side of the male $5^{th}$ leg of Suarez-Morales et al. (2008) lacks a few setae (even after review of Suarez-Morales' archived specimens); however, the marked differences in shape of the "dragon's head" ending of Segment D (personal communication, Dr. Suarez-Morales, ECOSUR, 2016) may especially be of some diagnostic value since it is a gross difference in shape and not just setae that may be difficult to see or easily damaged in preparation. Whether the presence or absence

or position of any of these setae or the differences in the dragon's head structure has any taxonomic value may come from further detailed morphological studies of the P5 of various clades of the *Eurytemora* complex accompanied by molecular data. This is especially true since the differences in the structure and ornamentation of the male fifth leg is evident in copepods coming from transitional environments such as the Temoridae and Pseudodiaptomidae family as well as from continental waters as in the case of Diaptomidae (personal communication, Dr. Suarez-Morales, ECOSUR, 2016).

An important question is whether the presence of these structures may have any selective role or effect on fitness. As previously pointed out by Lee and Frost (2002), copepods have no image-forming vision. Therefore the reproductive behavior of copepods is unlikely to use fine details of physical structures such as spines and setae, as visual cues. Copepods instead use pheromone trails and olfactory senses to mediate sexual tracking and mating (Yen et al. 2011, Seuront 2013). Nevertheless, at short range (i.e., once the male tracks down the female) spines could help as a tactile signal in mate recognition (Holynska 2000). Future detailed comparisons of reproductive or other behaviors in *E. carolleeae* and *E. affinis* might help determine their adaptive role, if any, and their utility for taxonomy.

**Conclusions**

Although morphological features that distinguish *E. carolleeae* from *E. affinis* were described more than four years ago, the identification of *E. carolleeae*, rather than *E. affinis*, in the Great Lakes was facilitated by the use of genetic barcoding. The previous observations from Chesapeake Bay and the St. Lawrence estuary apparently did not catch the attention of taxonomists in the Great Lakes, and *E. carolleeae* was not integrated into the commonly used keys for the Great Lakes, such as Dodson et al. (2010), which leads only to *E. affinis*. As a result of the present study, which combined sequence analysis of contemporary specimens of

*Eurytemora,* all of which were most similar to sequences of *E. carolleeae*, with morphological analysis of contemporary and historical Great Lakes specimens dating as far back as 1962, we conclude that *Eurytemora carolleeae* is the correct name for the species of *Eurytemora* that invaded the Great Lakes.

As in the present study, the observation of novel COI sequences may alert analysts to examine specimens more closely. Phylogenetic research on the many species of copepods in the Great Lakes should be encouraged in view of their abundance, diversity, and important roles in most freshwater systems, so that routine sequence identification of copepods could be used to determine if rare or non-native species are present.

# CHAPTER 6 - DNA ANALYSIS OF GUT CONTENTS OF WATER MITES

## Abstract

Water mites are aquatic arachnids with great biodiversity. They are found worldwide in most aquatic habitats except Antarctica. They are known for their impact both as predators and parasites on aquatic arthropods. However, only laboratory experiments on observations of their predatory behavior has been done. Water mite's predatory roles in aquatic systems have been consistently underappreciated possibly due to difficult taxonomy and because they ingest a liquefied diet, and therefore, analysis of what they eat using microscopy is not possible.

Water mites from Blue Heron Lagoon at Belle Isle, Detroit were collected and processed for assessment of molecular gut contents. Water mites of the *Lebertia* genus were chosen for molecular gut content analysis because they were found throughout the collecting season and were observed to feed on Diptera, particularly chironomids, another biodiverse aquatic arthropod. Molecular gut contents were assessed using primers targeting both the COI gene that has been used for molecular barcoding, and the 18S region of ribosomal DNA. The 18S primers targeted Chironomid taxa while "Arthropod-specific" primers that were used were shown first not to amplify water mite DNA and subsequently used to elucidate dipteran and other prey that might be consumed without interference with water mite DNA amplification. While sequences obtained by Sanger Sequencing likely showed the predominant organism that had been consumed recently, Next Generation Sequencing yielded sequences of a range of prey consumed by the mite.

The results of our next generation sequencing identified *Lebertia* as a generalist and opportunist. A diverse set of sequences were identified from each water mite, and identified, in many cases to species level, by comparisons to reference sequences in GenBank and sequences in new species-level databases that we have developed for chironomids. *L. davidcooki* and *L.*

*quinquemaculosa* have diverse diets that include chironomids, ostracods, and oligochaetes. To our knowledge, this work represents the first ever digestive composition experiments using next generation sequencing done on any water mite, and the first to demonstrate that oligochaetes may be part of water mite diets.

**Background**

Water mite adults are known to be predatory on freshwater invertebrates including cladocerans, copepods, ostracods and dipteran larvae (Proctor and Pritchard 1989, Martin 2004). Water mite larvae are also known to parasitize several groups of flying insects including dragon flies, mosquitoes and chironomid midges (Martin 2004, Kirkhoff et al. 2013). The impact of water mites on their prey in one study demonstrated the decline of chironomid prey up to 50% with water mites being the primary predator (Ten Winkel et al. 1989). Another study reports that water mites may potentially limit the invasion of an introduced species by parasitizing the adult forms (Sanchez et al. 2015). Water mites are clearly critical in their environment as possible apex predators; however, their life cycle and morphological complexity has made the study of water mites difficult.

As adults, water mites prey on several insect larvae and their eggs including mosquitoes and chironomids (Smith et al. 2010). Water mites are excellent predators and may have a significant impact on controlling organisms that transmit pathogenic organisms that cause human disease. In fact, water mites have been reported as being underappreciated as predators although they feed on and parasitize mosquitoes, chironomids and nematodes all of which either transmit pathogenic organisms or are pestiferous to humans (Proctor and Pritchard 1989, Failla et al. 2015). Therefore, given the potential importance of water mites to human health and Great Lakes ecology there is a need to fill in these knowledge gaps about water mites.

We have done extensive work on one of the categories of water mite prey items,

chironomids (Failla et al. 2016). Our work identified many chironomid larvae and adults that inhabit the Great Lakes region and may be part of water mite diets (Failla et al. 2016). In the Great Lakes some genera of water mites feed on chironomid larvae, some of which are known to cause allergies due to their invertebrate hemoglobin (Failla et al. 2015); water mites are voracious predators that feed on and parasitize many other prey items but lack of research on these abundant and diverse predators has been an ongoing concern (Proctor and Pritchard 1989, Smith et al. 2010, Werblow et al. 2015).

Recent work to understand water mite diets have involved laboratory feeding experiments and the use of PCR and chironomid specific primers and DNA sequencing (Martin et al. 2015). However, mites collected from the field and tested by this method have not yet been described. Water mites macerate their prey items using their chelicera, likely secrete enzymes to digest prey tissues prior to ingestion, and then ingest their liquefied food similar to other arachnids like ticks. The use of molecular tools might be the only way to determine what water mites are actually feeding on in their natural habitat. Diet studies in spiders, which have similar feeding habits as water mites, have shown that use of advanced technology in DNA sequencing has greatly facilitated the study of these types of trophic interactions (Hamback et al. 2016). The analysis of the diet composition of species that are aquatic is particularly difficult especially when there might not be any morphologically intact prey items in the gut in order to apply traditional gut dissection and morphological analysis of gut contents. Molecular analysis provides a suitable alternative (Boyer et al. 2013). If the organism is a generalist the molecular gut contents will give complex amplicons after PCR which would result in difficulty determining the prey items unless high throughput sequencing is implemented (Boyer et al. 2013).

DNA sequencing technology can be applied to answer questions such as diet selection with applications in trophic studies, conservation biology and invasive biology (Leray et al.

2013a, Leray et al. 2013b, Clare 2014, Harms-Tuohy et al. 2016). Here we used two pairs of primers; one that amplifies only chironomids using the 18S gene and the other that amplifies the COI barcode region of Arthropod organisms and is known not to amplify arachnid DNA (Martin et al. 2015, Hamback et al. 2016).

This study chose to focus on *Lebertia* because, as shown in previous studies (previous chapter 2B) in our local environment it is (a) a large, commonly occurring water mite in the Blue Heron Lagoon, a readily accessible water body in a nearby state park; (b) several species of *Lebertia* are present in Blue Heron Lagoon spanning every month of the year that the collecting site has been ice-free (February – November), (c) a highly detailed review, analysis, descriptions, and renaming of European species of *Lebertia* has recently been published by Gerecke (Gerecke 2009) to which North American species of *Lebertia* might usefully be compared, and (d) the diet of *Lebertia* had been said to consist of dipterans, especially chironomids, for which we have an extensive molecular database (Failla et al. 2016) but these reports (Proctor and Pritchard 1989) were based on laboratory feeding of *Lebertia* and not studies of *Lebertia* in natural settings. Laboratory experiments are not the best way to assess what a mite predator may be feeding on since the conditions are different such as the prey item may not be in its anti-predatory mode or prey items not usually encountered by the mite such as planktonic organisms in a benthic setting might give false indications (Walter and Proctor 2013). While the present study includes some initial laboratory-based feeding experiments to confirm that the DNA of ingested organisms (e.g., chironomids and mosquitoes) is retained in its water mite predator in a form that can be detected and sequenced, the focus of the present study is to identify what species of organisms is/are in the gut of *Lebertia* specimens freshly collected from their natural environment.

## Materials and Methods

### *Water mite sampling*

Water mites were collected from Blue Heron Lagoon (Figure 48) using methods by Fisher et al. (2015).

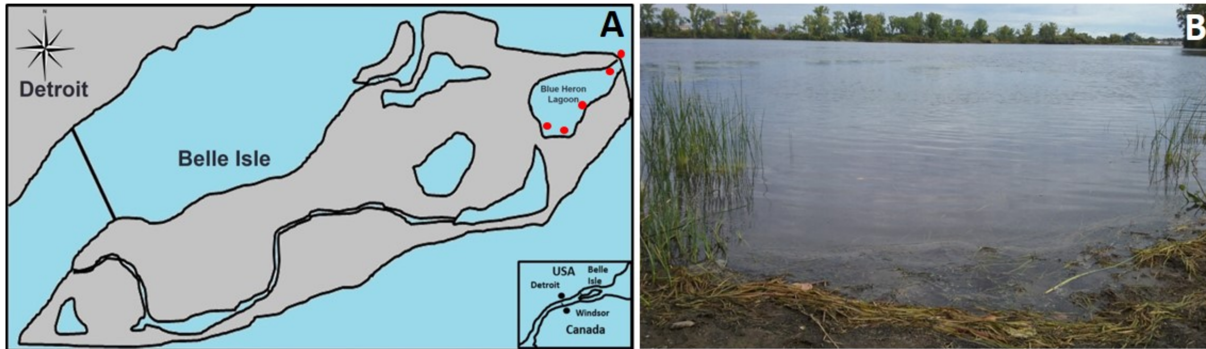



**Figure 48: Map of Belle Isle with Blue Heron Lagoon and collection sites** indicated by red dots. (B) Representative Blue Heron Lagoon habitat.

### *Water mite identification*

Water mites were identified using a two tier method involving morphological and genetic analysis as described previously in Chapter 2.

### *Water mite feeding experiments*

Mites were collected along with by-catch. By-catch included other aquatic arthropods seen in the collecting trays along with the mites. Sampling by-catch included prey items from their natural environment, such as copepods, chironomid larvae, ostracods and other microinvertebrates. Mites were then observed under a stereomicroscope and observations of predation were video recorded or photos were taken. Additionally, mites of the genus *Lebertia* were collected and kept in 6-well cell culture plates and checked periodically to replenish water if necessary. These mites were not fed for 1 to 4 months. At this point they were fed frozen or live chironomids (blood worms) or live mosquito larvae. For DNA analysis, the satiated water mite was separated from its prey, preserved in ethanol, washed in ethanol to remove any

externally contaminating DNA, and then processed for PCR and sequencing as in the following section.

***Identification of putative water mite prey with molecular analysis of gut contents***

Water mites were sampled from the field and immediately stored in ethanol for diet analysis. This allowed us to assess what non-mite microinvertebrate DNA (presumed to be in the gut) is associated with freshly collected water mites. Mites were sorted according to genus (*Lebertia* was our study model) and isolated from the rest of the sample. Each mite underwent a washing step with ethanol to avoid cross-contamination with other organisms. Any mite that was observed as damaged or pierced due to the sorting method was not selected for this analysis. Whole mite DNA was extracted by puncturing water mites with sharp minuten pins to allow water mite lysate to ooze out and a voucher of the exoskeleton to be retained for future morphological analysis. The punctured mites were then incubated in proteinase K enzyme overnight at 57 $^{\circ}$C and extraction was completed the following day. DNA extraction was carried out using the Qiagen Easy tissue extraction protocol as described in Vasquez et al. (2016). PCR experiments with primers to amplify water mite diet contents were done using primers from Table 7. Care was taken to maintain the contamination integrity of each experiment, and appropriate controls were run during each experiment which included negative controls: PCR grade water with no DNA template, non-arthropod DNA from fish tissue and water mite only DNA (taken from dissected legs of water mites). Positive controls run during each experiment included oligochaete DNA (that was seen to be amplified in previous experiments), chironomid DNA and mosquito DNA.

***Molecular analysis of water mite diet: primer selection and design***

Primers used in this study are shown in Table 7, together with their annealing temperature conditions. "Folmer" primers were used as general COI amplification as they are

known to amplify the COI gene from a wide range of organisms, including water mites (as seen in Chapter 2), and many water mite potential prey items (e.g., chironomids, as seen in chapter 5). Chironomid-specific primers that target 18S nuclear genes were selected from previously published studies that used the primers to amplify chironomid prey DNA in laboratory fed *Hygrobates* water mites, whose DNA was not amplified by the primers (Martin et al. 2015). So-called "Arthropod-specific COI primers" (mLep), which were said not to amplify arachnids but would amplify dipterans (Hajibabaei et al. 2006, Rougerie et al. 2011, Hamback et al. 2016) were also tested. The choice of these primers was on the basis that *Lebertia* has been reported to prey on chironomids, and we have extensive databases of chironomid sequences from the Great Lakes. These primers were then modified, by adding an Illumina adapter (TAG), for next generation sequencing. All primers used in this study are listed in Table 7.

**Table 7. Primers used in this study.**

| Name of Primers | Primer Sequence | Annealing Temperature | Reference |
|---|---|---|---|
| Folmer HCOI<br>Folmer LCOI | 5'TAAACTTCAGGGTGACCAAAAAATCA3'<br>5'GGTCAACAAATCATAAAGATATTGG3' | 51°C | (Folmer et al. 1994) |
| mLep<br>Folmer LCOI | 5'CCTGTTCCAGCTCCATTTTC3'<br>5'GGTCAACAAATCATAAAGATATTGG3' | 50 °C | (Hajibabaei et al. 2006) |
| mLep+TAG<br>Folmer LCOI+TAG | 5'TACGGTAGCAGAGACTTGGTCTCCT GTTCCAGCTCCATTTTC3'<br>5'ACACTGACGACATGGTTCTACA GGTCAACAAATCATAAAGATATTGG3' | 50 °C | This work |
| 18SF<br>18SR | 5'GAACTAGTTAACTATGTT3'<br>5'TATTCCATGCAAAAATATTCA3' | 51 °C | (Martin et al. 2015) |

***Molecular analysis of water mite diet: polymerase chain reaction (PCR)***

PCR was carried out on DNA extracted from the freshly collected water mites. PCR reaction protocols were designed based on the primer sets used. Reactions were run on an iCyclerQ Realtime thermocycler (BioRad, Irvine, CA) with the following protocol used for both 18S and mLep primers. Reaction initiated by heating to 95 °C for 4 mins, followed by 40 cycles of 95 °C for 30 secs (melt), 50 °C (mLep) or 51 °C (18S) for 30 secs (annealing), 72 °C for 1.5

mins (extension), and then a final extension of 72 °C for 10 mins followed by a hold at 25 °C until further processing within 3 h.

***Sequence analysis and comparisons***

PCR products were purified and sequenced bi-directionally by GENEWIZ (Plainfiedl, NJ). Sequences were analyzed initially with DNA Baser software (Heracle BioSoft SRL, Romania), to determine sequence quality and accuracy, align forward and reverse sequences, and produce a quality consensus sequence of the COI barcode. Parameters used to determine sequence quality by DNA Baser software are detailed in Vasquez et al. (2016). MEGA6 (Tamura et al. 2013) was used for comparisons of various mite sequences, including alignments, pair-wise comparisons, and constructing neighbor joining trees.

***Next generation sequencing of water mite diets***

The Illumina MiSeq v2 was used to analyze the diet composition of water mites collected from Blue Heron Lagoon. Samples were prepared by using mLep, and Folmer primers with Fluidigm CS1 or CS2 oligomers fused to their 5' ends (primers listed in Table 7). The amplicons were loaded in a 96 well plate and shipped to the Michigan State University RTSF Genomics Core for next generation sequencing on an Illumina MiSeq platform. Amplicons were processed to remove dNTPs, primer dimers, and other small side-products (less than 100 bp in size), using the Agencourt AMPure XP system (Beckman). PCR with sample-indexed primers targeting the CS1/CS2 oligos was performed to add dual-indexed, Illumina compatible adapters at the ends of the PCR products. The sample-indexed PCR products were batch-normalized using Invitrogen SequalPrep DNA normalization plates and the recovered products pooled. The pool was quality controlled and quantified using a combination of Qubit dsDNA HS, Caliper LabChipGX HS DNA and Kapa Illumina Library Quantification qPCR assays. It was loaded on an Illumina MiSeq v2 standard flow cell and sequenced in a 2x250 bp paired-end format using a v2 500

cycle reagent cartridge. Primers complementary to the Fluidigm CS1/CS2 oligonucleotides were added to appropriate wells of the reagent cartridge to act as primers for the forward, reverse and index sequencing reads. Base calling was done by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.19.0.

***Bioinformatics***

FastQ files were unzipped and demultiplexed using Perl software, clustered using SEED (Bao et al. 2011), and all SEEDS (basically, clusters that differ less than 1% from their SEED sequence) were BLASTed against the GenBank database to identify the closest identity in that database. Bioinformatics assistance and advice was provided by Wayne State University's Applied Genomics Technology Center http://agtc.med.wayne.edu/). MEGA6 (Tamura et al. 2013) was used for comparisons of various sequences, including alignments, pair-wise comparisons, comparisons to the RamLab chironomid sequence database, and construction of neighbor joining trees.

***Chironomid prey identification using a curated reference database of Great Lakes chironomids***

We have collected and identified larvae and adult chironomids from the Great Lakes and used the adults to identify the more taxonomically difficult to ID larvae. This work is presented in Chapter 5A, and the reference data used in this chapter represents both unpublished and published work (Failla et al. 2016). A total of 67 sequences of species level and 4 sequences of genus level chironomid taxa, illustrated in a neighbor-joining tree presented in the results section of this chapter were used to identify prey chironomid sequences that the public database was unable to identify. Further descriptions on sampling and molecular methods used to construct this tree are in Chapter 5A.

## Results

### *Laboratory observations of feeding behavior by Lebertia*

Water mites of different genera were observed feeding on prey by-catch including ostracods, chironomid larvae, chironomid pupae and cladocerans (see Chapter 4). Further laboratory experiments were done by feeding *Lebertia* prey items such as *Culex pipiens* larvae and chironomid larvae (see Figure 49).

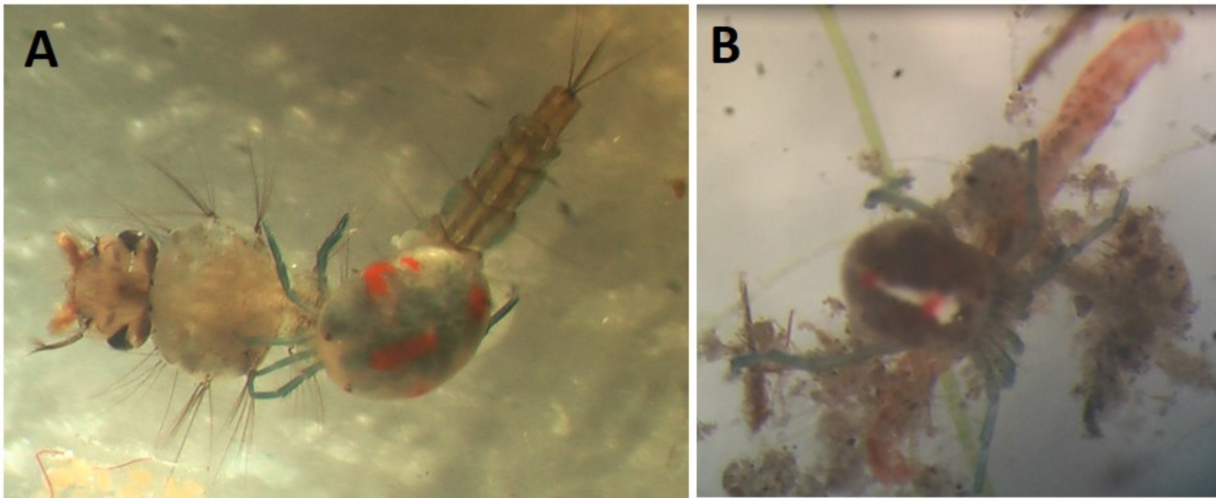



**Figure 49: Represent-ative micrographs of *Lebertia* feeding on collected prey.** (A) *Lebertia* feeding on *Culex pipiens* collected from cistern. (B) *Lebertia* feeding on chironomid.

### *Amplification of water mite extracts of laboratory fed water mite with mLep "arthropod-specific" primers and 18S chironomid-specific primers*

*Lebertia* water mites that were observed feeding in laboratory experiments were analyzed to test the proof of principle that molecular gut contents would match what the mite was observed feeding on. The results in Table 8 show that this method reliably yielded DNA sequences that matched the item that the mite had been preying upon, as was previously reported using 18S primers for laboratory-tested water mites (*Hygrobates*) feeding on chironomid prey (Martin et al. 2015). In negative control experiments, DNA was extracted from legs of water mites (i.e., not including the gut region), shown to amplify as expected with Folmer primers, yielding the expected water mite sequences, and the same DNA subjected to amplification by

mLep primers, which yielded no PCR product. Tests of mLep primers with fish DNA were also negative.

**Table 8. Prey DNA identified in mites after laboratory feeding.**

| **Predator** | **Prey** | **Match of PCR product (mLep) on GenBank. Query and ID%** |
|---|---|---|
| *Lebertia* | *C. pipiens* | *C. pipiens* Q: 100% & ID: 99% |
| *Lebertia* | *C. pipiens* | *C. pipiens* Q: 100% & ID: 99% |
| *Lebertia* | *C. pipiens* | *C. pipiens* Q: 98% & ID: 99% |
| *Lebertia* | Chironomid | *Chironomidae* sp. Q:100 % & ID: 97% |
| *Lebertia* | Chironomid | *Cricotopus* sp. Q: 99% & ID: 97% |

***Amplification of water mite extracts of field collected water mites with 18S chironomid-specific primers***

Sequences of DNA obtained from water mites that had been freshly caught in Blue Heron Lagoon, immediately preserved, and amplified with 18S chironomid primers indicated that *Lebertia* and other water mite species were feeding on at least three species of chironomids. Table 9 illustrates the diversity of chironomid DNA associated with water mites. The data show that the closest matches to known chironomid sequences were associated with *Lebertia* mites, with DNA associated with *L. quinquemaculosa* having excellent chironomid sequence matches (>97%) to *Dicrotendipes*, *Polypedilum prasiogaster*, and *Tanypus* and a distant match for one specimen to *Sublettea*. Among other wáter mite species that were tested (*Arrenurus*, *Neumania*, *Mideopsis*, and *L. davidcooki* ), matches to known chironomid sequences were 88% to 92% identical to known mite sequences, indicating possible identification only at family or higher level. Sometimes the query coverage was low (<50%), possibly indicating sequencing “noise” due to a mixture of other PCR products in the PCR amplicons.

**Table 9. BLAST results of DNA amplified by 18S chironomid primers from DNA molecular gut contents from water mites**

| Water mite identification (sample ID) | Amplicon name of closest match in GenBank: query coverage, BLAST ID %, Accession Number |
|---|---|
| *Arrenurus* sp. deuteronymph (6-BHL070916) | *Imparipecten pictipes*<br>Query 63%, ID 90%, HQ440608.1 |
| *Mideopsis* sp. (4-BHL072216) | *Cladotanytarsus* sp.<br>Query 94%, ID 90%, HQ440574.1 |
| *Neumania* sp. (11-BHL070916) | *Cladotanytarsus* sp.<br>Query 49%, ID 92%, HQ440574.1 |
| *Lebertia quinquemaculosa*(8-BHL072216) | *Sublettea* sp.<br>Query 43%, ID 87%, HQ440684.1 |
| *Lebertia* sp.(2-BHL072216) | *Chironomus tepperi*<br>Query 94%, ID 88%, KC177280.1 |
| *Lebertia quinquemaculosa*(3-BHL101416) | *Tanypus* sp.<br>Query 34%, ID 98%, FJ570805.1 |
| *Lebertia quinquemaculosa* (4-BHL101416) | *Polypedilum prasiogaster*<br>Query 33%, ID 99%, GU356735.1 |
| *Lebertia quinquemaculosa* (BHL101416) | *Dicrotendipes* sp.<br>Query 95%, ID 97%, HQ440587.1 |
| *Lebertia quinquemaculosa* (138 BHL 110116) | *Dicrotendipes* sp.<br>Query 99%, ID 98%, HQ440587.1 |

***Amplification of water mite extracts with mLep "Dipteran" primers***

Sequences obtained from using mLep primers and Sanger sequencing to determine what mites were preying on in the field are summarized in Table 10. In all experiments negative controls and positive controls were used. Negative controls included a water only sample which did not have any amplification. DNA that should not be amplified by the mLep primers, including fish DNA and mite leg DNA, also yielded negative results. Positive controls of DNA from chironomids or mosquitoes, that mLep would reliably amplify, produced the expected PCR products.

**Table 10. Non-mite DNA amplified by mLep primers-DNA extracted from water mites.**

| Water mite identification | Amplicon closest match in GenBank: BLAST ID %+Accession # |
|---|---|
| *Lebertia* sp. (8-BHL022317) | *Chironomidae* sp. Query:95% ID 87% KP045212.1 |
| *Lebertia* sp. (6-BHL022317) | *Paratanytarsus* sp. Query: 93% ID 100% KM988017.1 |
| *Lebertia* sp. (2-BHL022317) | *Paratanytarsus* sp. Query: 75% ID 99% KR276527.1 |
| *Neumania* sp. (113-BHL072216) | *Chironomus riparius* Query: 94% ID 99% KR657116.1 |
| *Lebertia quinquemaculosa* (138-BHL110116) | *Slavina appendiculata* Query: 85% ID 88% GQ355375.1 |
| *Lebertia quinquemaculosa* (145-BHL110116) | *Nais elinquis* Query: 88% ID 87% GQ355369.1 |
| *Lebertia quinquemaculosa* (8-BHL072216) | *Diaphasoma* sp. Query: 42% ID 99% LC060041.1 |
| *Neumania* sp. (11-BHL070916) | *Macrocyclops* sp. Query: 36% ID 99% KM611739.1 |

The data shows close matches of *Lebertia* gut DNA with chironomids. For example associated with *Lebertia* (6-BHL022317) is *Paratanytarsus* sp., with a percent match in identity of 100% with a good query cover. Another *Lebertia* collected during the same period (2-BHL022317) also had a high match to *Paratanytarsus* sp. at 99%. *Neumania* (113-BHL072216) gut DNA included *Chironomus riparius* with a 99% match. Two *Lebertia quinquemaculosa* (138-BHL110116 and (145-BHL110116) had DNA from oligochaetes, but the percent match was <90%, at 88% and 87%, respectively. The last three mites had high percent matches for *L. quinquemaculosa* feeding on a *Diaphasoma* but a very low query cover at 42%. The same was the case for *Neumania* (11-BHL070916) with a high match to a *Macrocyclops* (copepod) at 99% but very low query coverage at 36%. This type of analysis led us to conclude that the preferred way to get a full perspective on water mite diet composition would be next generation sequencing.

***Next generation sequencing of water mite molecular gut contents***

*Lebertia* water mites collected from Blue Heron Lagoon were chosen for an in depth analysis of their diet composition by Illumina MiSeq next generation sequencing. A total of 26 water mite specimens were processed by using the mLep primers and the resulting amplicons underwent next generation sequencing with the results of a few representative samples presented here in Figures 50 through 54. Table 11 summarizes the main prey items with 97% or higher match identities with sequences that were longer than 200 bases in length. In addition, because some specimens had many “best matches” to oligochaetes but never with sequence identities above 90%, Table 11 also reports which oligochaete sequences showed up most frequently with matches in the 80 – 90% sequence identity range.

**Table 11. Predominant taxa in *Lebertia* mLep amplicons.**

| Mite ID | Chironomids (>97%) | Oligochaetes & related phyla (83% - 90%) |
|---|---|---|
| *L. quinquemaculosa*<br>(NG1BHL110116) | *Chironomidae sp.*<br>*Cricotopus sp.*<br>*Chironominae sp.*<br>*Orthocladiinae sp.*<br>*Dicrotendipes tritomus*<br>*Paratanytarsus sp.* | *Progizzardus varadiamensis*<br>*Chaetogaster limnaei*<br>*Amynthas phaselus*<br>*Pheretima camiguinensis* |
| *L. quinquemaculosa*<br>(NG2BHL110116) | *Chironomidae sp.*<br>*Cricotopus sp.*<br>*Dicrotendipes tritomus*<br>*Paratanytarsus* sp.<br>*Chironmus riparius* | *Vejdovskyella sp.*<br>*Slavina appendiculata*<br>*A. phaselus*<br>*Nais communis* |
| *L. quinquemaculosa*<br>(NG3BHL110116) | *Chironomidae sp.*<br>*Chironominae sp.*<br>*Cricotopus sp.*<br>*D. tritomus*<br>*Paratanytarsus sp.* | *Chaetogaster diastrophus*<br>*Vejdovskyella sp.*<br>*S. appendiculata*<br>*N. communis*<br>*Henlea ventriculosa* |
| *L. quinquemaculosa*<br>(NG8BHL101516) | | *Nais bretscheri*<br>*Nais elinguis*<br>*N. communis*<br>*Vejdovskyella sp.*<br>*S. appendiculata*<br>*Amynthas papulosos*<br>*P. varadiamensis* |
| *L. quinquemaculosa*<br>(NG9BHL101516) | *Cricotopus sp.* | *S. appendiculata*<br>*Vejdovskyella sp.*<br>*P. varadiamensis*<br>*Rhyacodrilus falciformis*<br>*P. camiguinensis* |
| *L. davidcooki* (1BHL101516) | *Chironominae sp.*<br>*Paratanytarsus sp.*<br>*C. riparius* | |
| *L. davidcooki* (2BHL101516) | *Chironominae sp.*<br>*Paratanytarsus sp.*<br>*C. riparius*<br>*Tanyponinae sp.* | |
| *L. davidcooki* (2BHL111116) | *Cricotopus sp.*<br>*D. tritomus*<br>*Chironominae sp.*<br>*Chironomidae sp.*<br>*Paratanytarsus sp.* | *C. diastrophus*<br>*Amphichaeta raptisae*<br>*Vejdovskyella sp.*<br>*S. appendiculata*<br>*P. varadiamensis*<br>*Amynthas sp.*<br>*Rhyacodilus sp.*<br>*Nais christinae* |
| *L. davidcooki* (147BHL110116) | *Cricotopus sp.*<br>*D. tritomus*<br>*Chironominae sp.*<br>*Orthocladiinae sp.*<br>*Chironomidae sp.*<br>*Paratanytarsus sp.*<br>*C. riparius*<br>*Psectrocladius sp.*<br>*Cricotopus trifasciatus*<br>*Dicrotendipes sp.* | *Amynthas taiwumontis*<br>*Amynthas sp.*<br>*C. diastrophus* |
| *L. davidcooki* (123BHL40916) | *C. trifasciatus*<br>*C. riparius*<br>*Chironomidae sp.* | |

***Chironomid DNA in Lebertia***

Figure 50 shows the diet profile of a representative *Lebertia* n. sp. water mite (123BHL40916) that was feeding primarily on chironomids. All branches are primarily

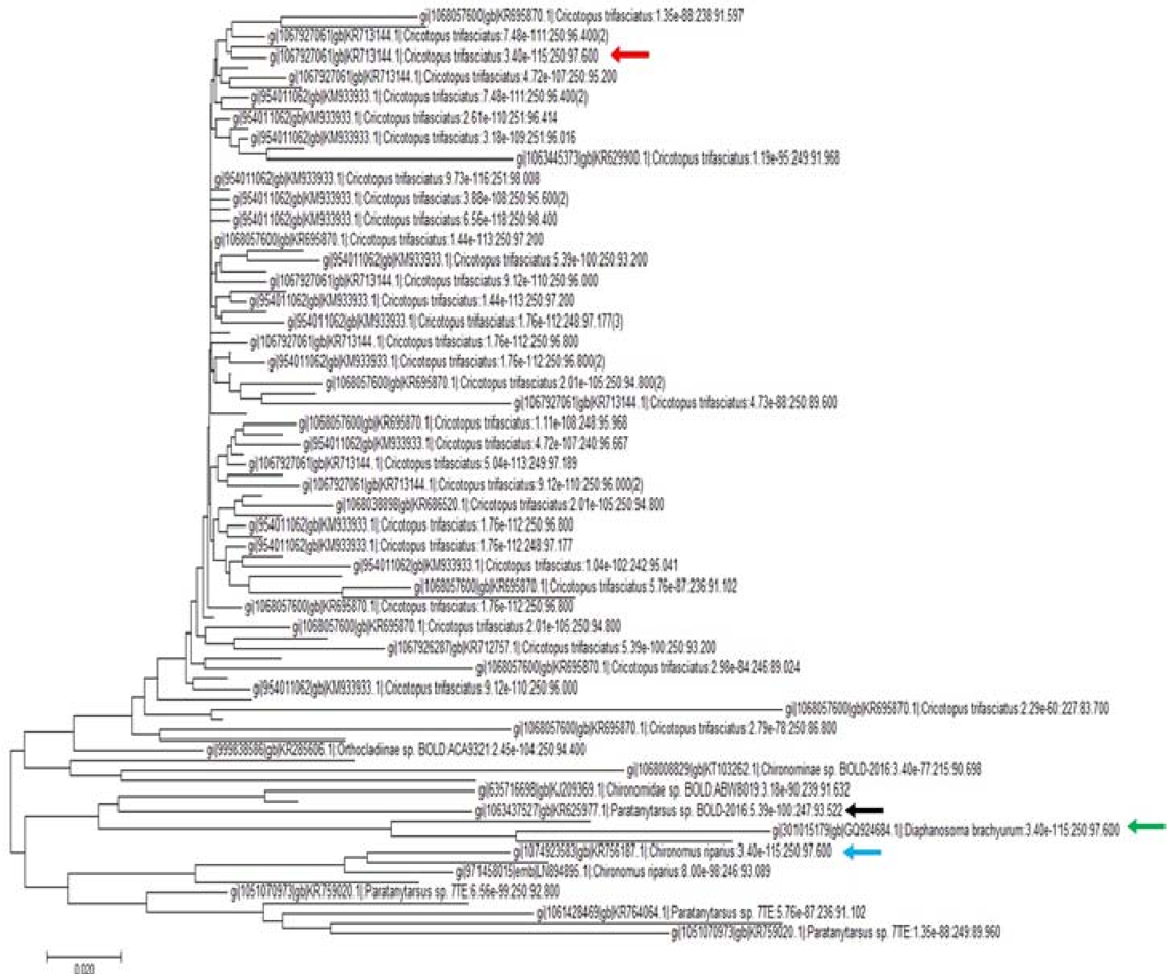


**Figure 50: Condensed neighbor joining tree of DNA sequences amplified by mLep-TAG/Folmer LCOI-TAG primers, from *Lebertia* (123BHL40917).** The tree was computed using the number of differences method (base differences per sequence). The analysis involved 96 nucleotide sequences resulting from a prior SEED clustering of the Illumina MiSeq sequencing from this sample. MEGA6 was used to implement ClustalW alignment and after truncation to the shortest sequence in the dataset, a total of 206 positions in the final dataset MEGA6 constructed this neighbor-joining tree. The format of the branch names for this tree is: gi|Genbank identifier|gb|GenBank accession ID|:name of sequence (usually genus or genus species):GenBank E value:length of sequence:percent identity. Arrows highlight the names on several different branches, usually with high percentage matches, which are discussed in the text.

represented by chironomids and when the sequences are filtered to determine which sequences have match identities to GenBank above 97% match ID, we observed 17 *Cricotopus trifasciatus*,

1 *Diaphanosoma brachyurum*, 1 *Chironomus riparius*, and 1 *Chironomidae* sequences. Above 95% but lower than 97% identity were represented by 32 *C. trifasciatus*. Many other sequence identities to chironmids also lie in the range of 90 – 95% sequence identity to GenBank reference sequences, e.g. *Paratanytarsus* sp.

***Worm and chironomid DNA in Lebertia***

In Figure 51 a representative example of the full diet composition of a single *L. quinquemaculosa* water mite (NG9BHL101516) can be seen. In the compressed tree a separation of branches is noted with the upper branch reflecting mostly oligochaete matches albeit no higher than 90% match. The most frequent matches to oligochaetes are *Slavina appendiculata*, up to 87.3% identical; *Vejdovskyella* sp., up to 86.4% identical; and *Progizzardus varadimensis*, up to 86.2% identical. The lower branch reflects chironomids. Analysis of the sequences with greater than 97% identity to GenBank reveals one genus that predominates, *Cricotopus* sp., with 31 sequences represented. All other sequences above 95% are also represented by *Cricotopus* sp. Review of all sequence matches in the BLAST output reveals several other chironomid matches in the 90 – 95% identity range, including *Tanytarsus* sp.

***Predominantly worm DNA in Lebertia***

Figure 52 is a condensed tree from a *L. quinquemaculosa* (NG8BHL101516) that shows associated non-mite DNA primarily comprised of matches to oligochaetes, albeit the highest match not exceeding 90%. No chironomids with sequence matches >95% were present. The oligochaete matches had a similar set of genera to the mite in Figure 51 but several additional oligochaete genera appeared more frequently in this mite, including various species of *Nais* with match identities as high as 88%; *Megascolecidae* sp., up to 85.1%; and Rhyacodrillus, up to 85.9%.

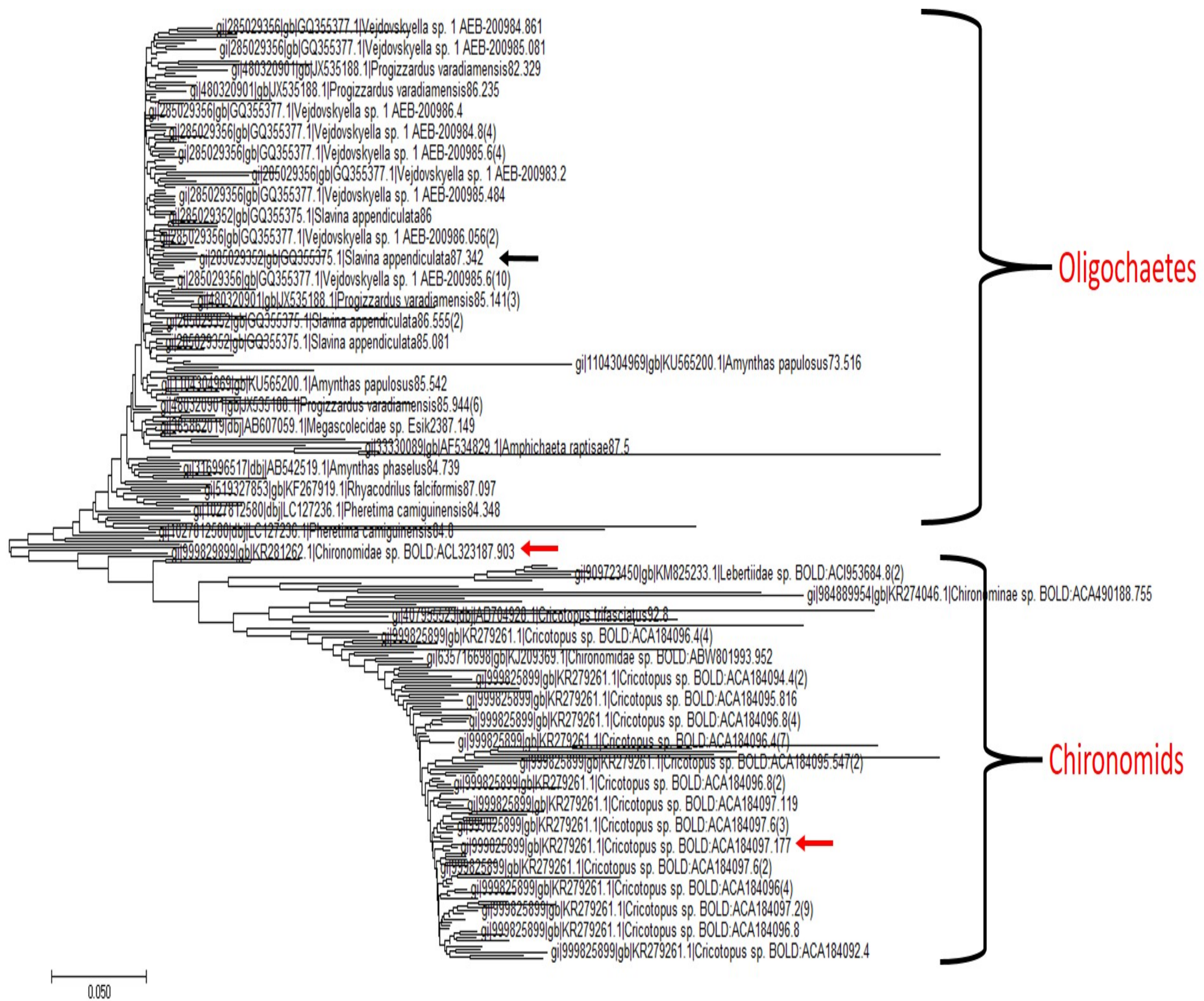



**Figure 51: Compressed neighbor joining tree of DNA sequences amplified by mLep-TAG/Folmer LCOI-TAG primers, from *Lebertia quinquemaculosa* (NG9BHL101516)**. Two major groups of sequences are identified by parenthesis. The tree was computed using the number of differences method (base differences per sequence). The analysis involved 311 nucleotide sequences and there were a total of 189 positions in the final dataset. The branch names have the following format: gi|GenBank identifier|gb|GenBank accession number|name of sequence, percent identity (up to 5 digits with decimal point).

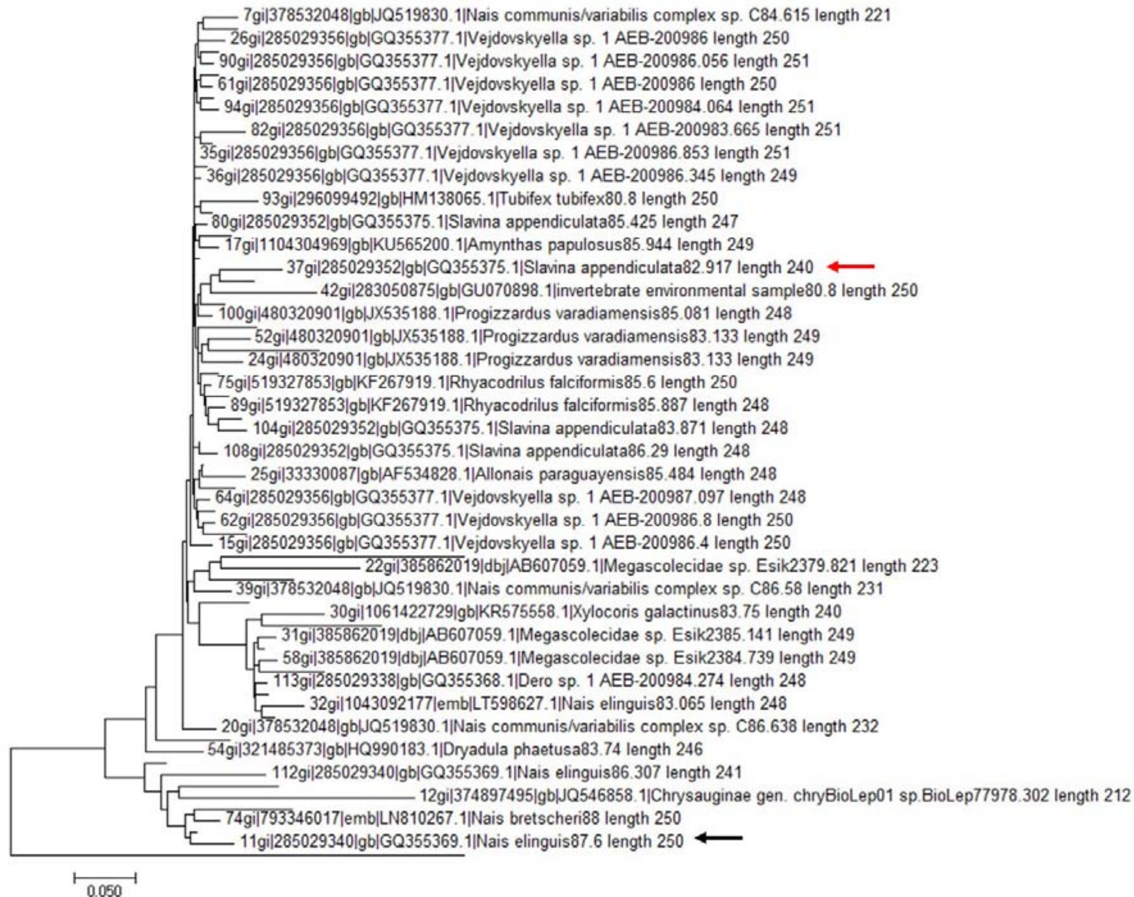



**Figure 52: Compressed neighbor joining tree of DNA sequences amplified by mLep-TAG/Folmer LCOI-TAG primers, from *Lebertia quinquemaculosa* (NG8BHL101516)**. The sequences are mainly oligochaetes and *Slavina appendiculata* is indicated by red arrow. The tree was computed using the number of differences method (base differences per sequence). The analysis involved 74 nucleotide sequences and there were a total of 200 positions in the final dataset. The branch names have the following format: gi|GenBank identifier|gb|GenBank accession number|name of sequence, percent identity (up to 5 digits with decimal point) length number of bases in the SEED.

### *Non-mite DNA associated with other genera:* **Arrenurus** *as an ostracod predator*

Figure 53 is a condensed tree of an *Arrenurus* mite (108BHL72216) whose associated DNA sequences mostly matched *Podocopida* sp., an ostrocod. However, when filtered for above 95% identity matches in the GenBank database only two sequences fulfilled that criterion, both matching to *Chironomus riparius*. The *Podocopida* sp. match hits were in the 90% – 94% range. Another *Arrenurus* sp. specimen had predominantly chironomid DNA but may represent a different species of *Arrenurus* as both these specimens were deuteronymphs and could not be

differentiated morphologically.

**Figure 53: Compressed neighbor joining tree of *Arrenurus* (108HL72216) diet composition**. The sequences are mainly *Podocopida* sp. (ostracods) highlighted by the bracket and a 97% *Chironomus riparius* match indicated by red arrow. The tree was computed using the number of differences method (base differences per sequence). The analysis involved 68 nucleotide sequences with a total of 210 positions in the final dataset. The format of the branch names for this tree is: gi|Genbank identifier|gb|GenBank accession ID|:name of sequence (usually genus or genus species):GenBank E value:length of sequence:percent identity. Arrows highlight several different commonly occurring high percentage matches.

### *Non-mite DNA associated with other genera:* **Limnesia** *as a worm predator*

Figure 54 is a condensed tree of the mLep-TAG:LCOI-TAG amplicon products from a *Limnesia* mite. Most sequences were putative oligochaete matches in the 80% - 90% range; however, this specimen yielded one oligochaete species with sequences with >97% identity: *Chaetogaster diastrophus*, and 7 matches in the 95% - 97% match range. *Limnesia* was previously reported to feed only on cladocera, copepod and insect larvae (Smith et al. 2010). This was the only *Limnesia* specimen subjected to next generation sequence analysis in the present study.

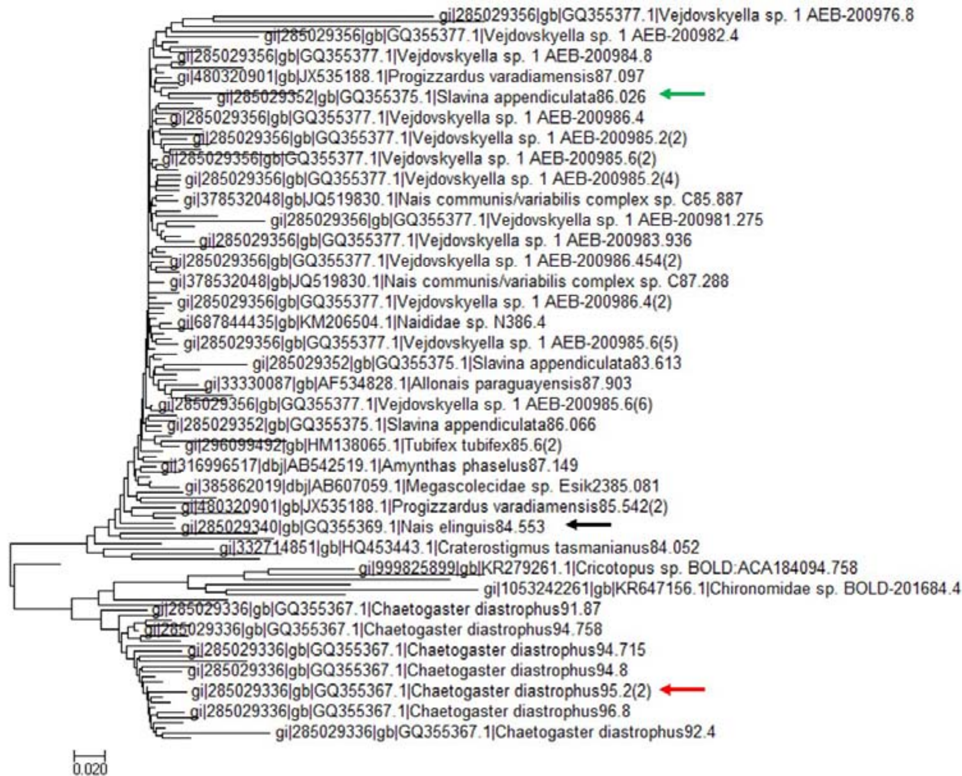



**Figure 54: Compressed neighbor joining tree of *Limnesia* (NG11101516) diet composition**. The sequences are comprised of oligochaetes. *Nais elinguis* match is indicated by black arrow and *Chaetogaster diastrophus* by red arrow. The tree was computed using the number of differences method (base differences per sequence). The analysis involved 142 nucleotide sequences and there were a total of 209 positions in the final dataset. MEGA6 was used to carry out this analysis.

### *Predominant genera in* **Lebertia quinquemaculosa** *and* **Lebertia davidcooki** *gut DNA*

Sequences of the most predominant genera of chironomids and oligochaetes (worms) were compared in *L. quinquemaculosa* and *L. davidcooki.* Table 11 summarizes for a selection of 10 specimens (5 *L. quinquemaculosa* and 5 *L. davidcooki*) the sequences that matched with chironomid sequences in the GenBank database above 97%. Oligochaetes and related phyla are also listed but these were in the range of 83% to 90% identity. Table 12 summarizes the number of *Lebertia* specimens out of all that were analyzed that had sequences of chironomids matching reference genera at >90% identity, suggested in the Discussion as reliably identifying chironomids at the genus level.

**Table 11. Predominant taxa in *Lebertia* mLep amplicons.**

| Mite ID | Chironomids (>97%) | Oligochaetes & related phyla (83% - 90%) |
|---|---|---|
| *L. quinquemaculosa* (NG1BHL110116) | *Chironomidae sp.*<br>*Cricotopus sp.*<br>*Chironominae sp.*<br>*Orthocladiinae sp.*<br>*Dicrotendipes tritomus*<br>*Paratanytarsus sp.* | *Progizzardus varadiamensis*<br>*Chaetogaster limnaei*<br>*Amynthas phaselus*<br>*Pheretima camiguinensis* |
| *L. quinquemaculosa* (NG2BHL110116) | *Chironomidae sp.*<br>*Cricotopus sp.*<br>*Dicrotendipes tritomus*<br>*Paratanytarsus* sp.<br>*Chironmus riparius* | *Vejdovskyella sp.*<br>*Slavina appendiculata*<br>*A. phaselus*<br>*Nais communis* |
| *L. quinquemaculosa* (NG3BHL110116) | *Chironomidae sp.*<br>*Chironominae sp.*<br>*Cricotopus sp.*<br>*D. tritomus*<br>*Paratanytarsus sp.* | *Chaetogaster diastrophus*<br>*Vejdovskyella sp.*<br>*S. appendiculata*<br>*N. communis*<br>*Henlea ventriculosa* |
| *L. quinquemaculosa* (NG8BHL101516) | | *Nais bretscheri*<br>*Nais elinguis*<br>*N. communis*<br>*Vejdovskyella sp.*<br>*S. appendiculata*<br>*Amynthas papulosos*<br>*P. varadiamensis* |
| *L. quinquemaculosa* (NG9BHL101516) | *Cricotopus sp.* | *S. appendiculata*<br>*Vejdovskyella sp.*<br>*P. varadiamensis*<br>*Rhyacodrilus falciformis*<br>*P. camiguinensis* |
| *L. davidcooki* (1BHL101516) | *Chironominae sp.*<br>*Paratanytarsus sp.*<br>*C. riparius* | |
| *L. davidcooki* (2BHL101516) | *Chironominae sp.*<br>*Paratanytarsus sp.*<br>*C. riparius*<br>*Tanyponinae sp.* | |
| *L. davidcooki* (2BHL111116) | *Cricotopus sp.*<br>*D. tritomus*<br>*Chironominae sp.*<br>*Chironomidae sp.*<br>*Paratanytarsus sp.* | *C. diastrophus*<br>*Amphichaeta raptisae*<br>*Vejdovskyella sp.*<br>*S. appendiculata*<br>*P. varadiamensis*<br>*Amynthas sp.*<br>*Rhyacodilus sp.*<br>*Nais christinae* |
| *L. davidcooki* (147BHL110116) | *Cricotopus sp.*<br>*D. tritomus*<br>*Chironominae sp.*<br>*Orthocladiinae sp.*<br>*Chironomidae sp.*<br>*Paratanytarsus sp.*<br>*C. riparius*<br>*Psectrocladius sp.*<br>*Cricotopus trifasciatus*<br>*Dicrotendipes sp.* | *Amynthas taiwumontis*<br>*Amynthas sp.*<br>*C. diastrophus* |
| *L. davidcooki* (123BHL40916) | *C. trifasciatus*<br>*C. riparius*<br>*Chironomidae sp.* | |

**Table 12. Sequences with >90% identity to chironomid genera or families in water mite DNA**

| **Chironomid genus or family** | ***L. quinquemaculosa* (out of 12 specimens)** | ***L. davidcooki* (out of 11 specimens)** |
|---|---|---|
| *Chironomus* | x | x |
| *Cricotopus* | x | x |
| *Dicrotendipes* | x | x |
| *Paratanytarsus* | x | x |
| *Psectrocladius* | x | x |
| *Orthocladiinae* | x | x |
| *Chironomidae* | x | x |
| *Chironominae* | x | x |
| *Coelotanypus* | | x |
| *Tanypodinae* | | x |
| *Cryptochironomus* | | x |
| *Polypedilum* | | x |
| *Phaenopsectra* | | x |
| *Tanytarsus* | | x |
| *Glyptotendipes* | | x |

***Additional species of mite-gut DNA, identified using the RamLab chironomid database***

While most analyses have been conducted so far using bioinformatic software able to access GenBank and automatically run and download BLAST results, selected datasets have been subjected to comparisons with the RamLab chironomid database (see Chapter 5A in this dissertation). Figure 55 represents the curated reference tree used for mite prey identification.

Figures 56 through 59 are several subtrees with close matches of *Lebertia* prey SEED sequences from specimens of *L. davidcooki* (123BHL40917), *L. quinquemaculosa* (NG9101516), *L. quinquemaculosa* (NG2BHL110116), and *L. davidcooki* (1BHL101516) compared against our curated chironomid database. Individual sequence pairs compared with a 2-sequence BLAST both confirmed specific relationships in the trees and yielded data on the percent identity of the SEED sequences to chironomid database sequences to which they were “neighbor-joined” by very short branches.

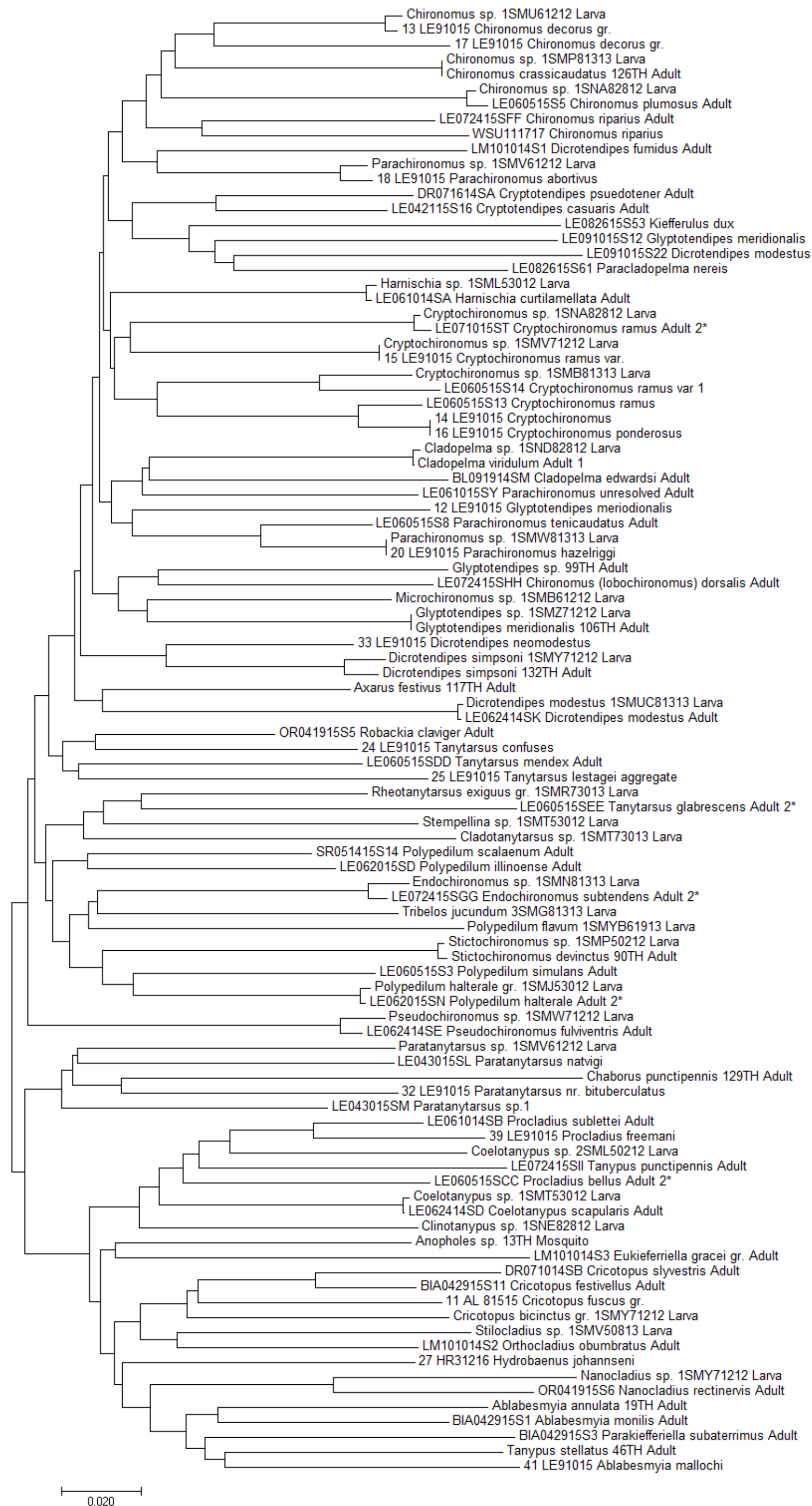



**Figure 55: Chironomid curated reference tree.** The tree was computed using the number of differences method (base differences per sequence). The analysis involved 98 nucleotide sequences and there were a total of 539 positions in the final dataset. MEGA6 was used to carry out this analysis.

Based on our chironomid reference database we were able to confirm identities of *L. davidcooki* (123BHL40917) prey DNA sequences (Figure 56C, in which the SEED sequence for KR756187.1 *C. riparius* matched our sequence WSU111717 at a 98% identity) and shed light on the identity of at least 2 of its prey items albeit not to the 97% identity match needed to definitively identify the prey (Figure 56 A and B, (A) the SEED sequence for KR625977.1 *Paratanytarsus* sp. aligned with LE043015SL *P. natvigi* at a 92% identity and (B) the SEED sequence for KR764064.1 *Paratanytarsus* sp. to LE043015SM *Paratanytarsus* sp. 1 with a similar percent identity).

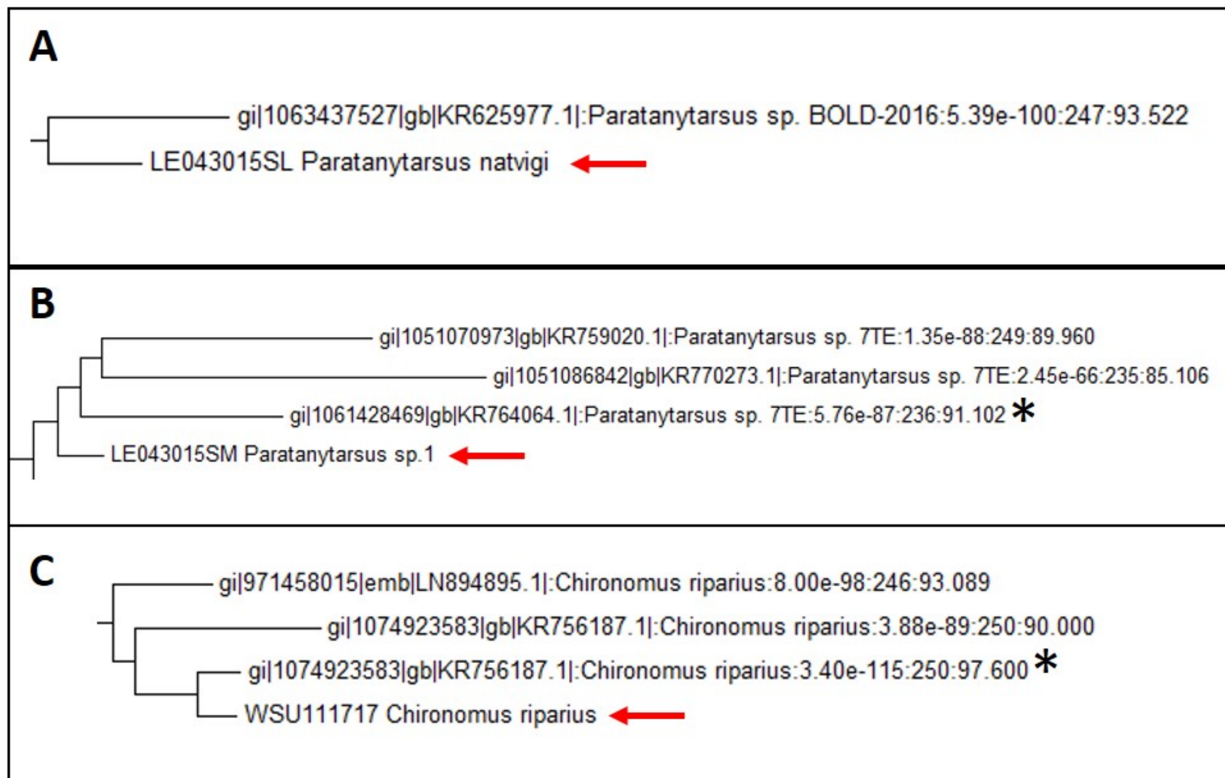



**Figure 56: Subtrees from analysis comparing *L. davidcooki* (123BHL40916) prey DNA sequences to our curated chironomid reference database.** Our reference sequences are indicated by red arrow. The analysis involved nucleotide 189 sequences and there were a total of 206 positions in the final dataset. Asterisk indicates sequence from diet analysis that was used for 2 sequence BLAST comparison to our reference sequence. See text for description of the magnitude of the pairwise differences.

Comparison of *L. quinquemaculosa* (NG9BHL101516) SEED sequences to the chironomid reference database identified the species name of at least one prey item, as seen in Figure 57A where our reference sequence DR071014SB *Cricotopus sylvestris* matched 98% with the prey SEED sequence identified as DNA KR279261.1 *Cricotopus* sp., resolving several adjacent *Cricotopus* sp. branches (partially shown) as well. Figure 57B and 57D identified prey DNA sequences at least to genus level (see Discussion) with 91% matches. Figure 57C served as confirmation of *Chironomus riparius* with a 95% match.

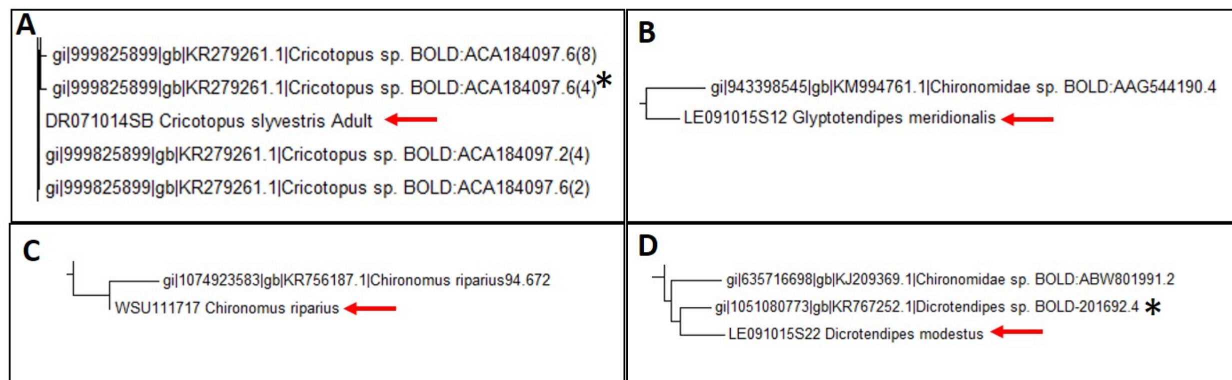



**Figure 57: Subtrees from analysis comparing *L. quinquemaculosa* (NG9BHL101516) prey DNA sequences to our curated chironomid reference database.** Our reference sequences are indicated by red arrow. The analysis involved 409 nucleotide sequences and there were a total of 191 positions in the final dataset. Asterisk indicates the full length SEED sequence from diet analysis that was used for 2 sequence BLAST comparison to our reference sequence. See text for description of the magnitude of the pairwise differences.

Analysis of *L. quinquemaculosa* (NG3BHL110116) sequences confirmed at least two prey DNA sequences at genus level by having matches above 90%. LE060515S13 *Cryptochironomus ramus* matched to the SEED sequence for KR271921.1 *Cryptochironomus* at 93% identity while BIA042915S11 *Cricotopus festivellus* matched the SEED sequence for KR279261.1 *Cricoptopus* sp. at 94%. LE060515SCC *Procladius bellus* matched with the SEED for KR291435.1 Orthocladiinae at only 86% identity, which was less than the SEED match percent to Orthocladiinae (89.958%). Since *Procladius* is in a different subfamily than Orthocladiinae, these paired branches probably do not indicate that the SEED was *Procladius.*

In other parts of the tree, matches of a SEED identified only as Chironomidae had 90% identity to a reference sequence for *Tanypus glabrescens*.

*L. davidcooki* (1BHL101516) sample had one prey DNA sequence (SEED sequence for KR623167.1 *Paratanytarsus* sp., Figure 59A) with a close match with LE043015SL *Paratanytarsus natvigi* at 96% identity and the SEED for KR085249.1 *Chironomus* sp. matched *Chironomus crassicaudatus* with 92% identity (Figure 59B). *Coelotanypus* sp. (SEED for KR085247.1) was confirmed as *Coelotanypus* sp. by a 95% identity to a *Coelotanypus* sp. larval sequence (Figure 59D). In Fig 59C, LE072415S11 *Tanypus punctipennis* and the SEED for KR282540.1 *Tanypodinae* sp. had an 86% identity.

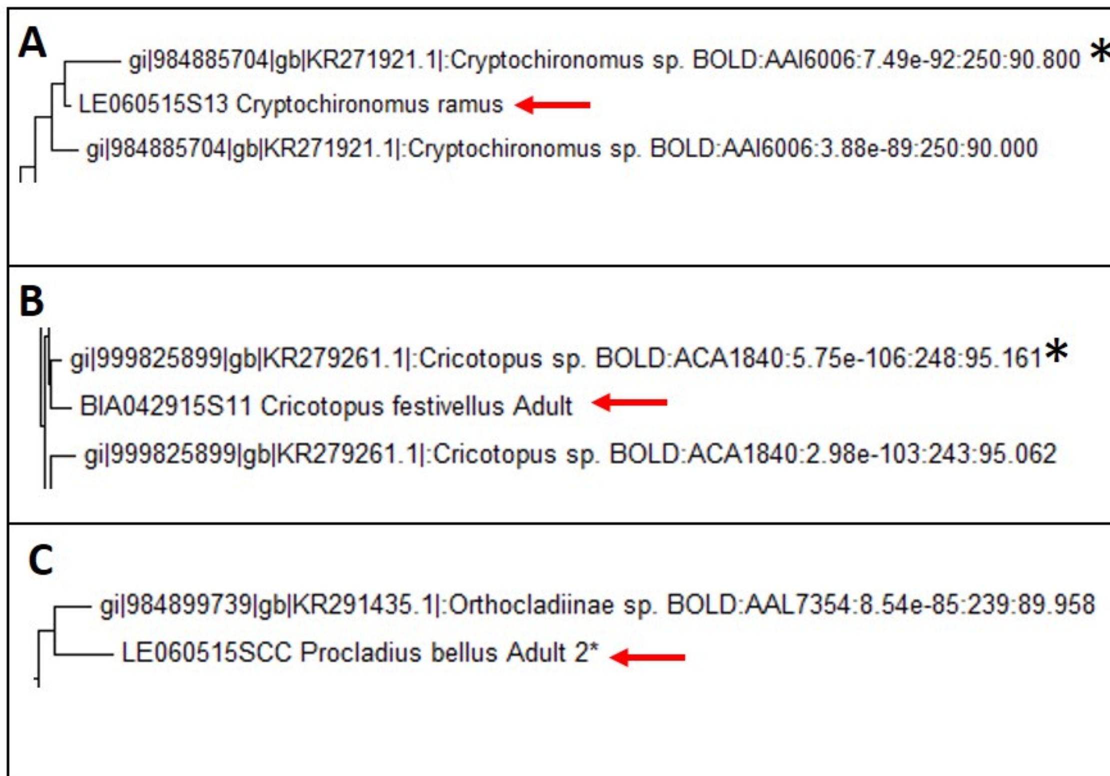



**Figure 58: Subtrees from analysis comparing *L. quinquemaculosa* (NG3BHL110116) prey DNA sequences to our curated chironomid reference database.** Our reference sequences are indicated by red arrow. The analysis involved 427 nucleotide sequences and a total of 185 positions analyzed in the final dataset. Asterisk indicates sequence from diet analysis that was used for 2 sequence BLAST comparison to our reference sequence. See text for description of the magnitude of the pairwise differences.

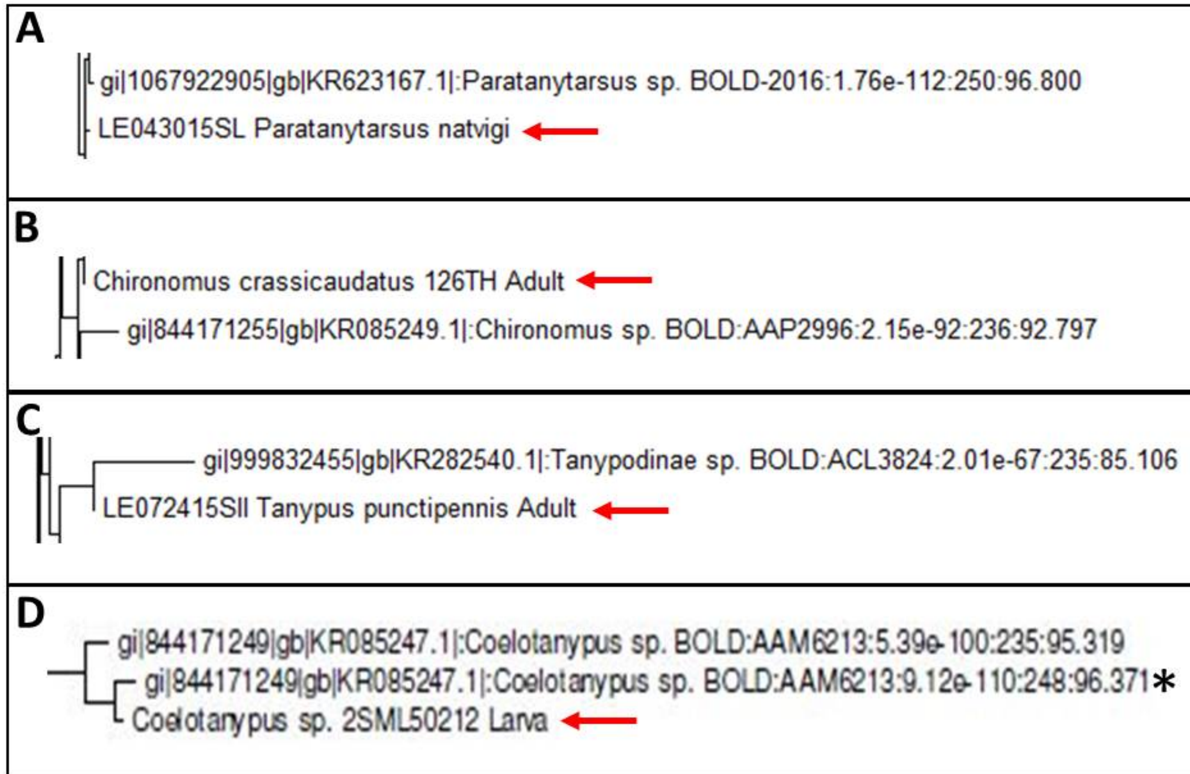



**Figure 59: Subtrees from analysis comparing *L. davidcooki* (1BHL101516) to our curated chironomid reference database.** Our reference sequences are indicated by red arrow. The analysis involved 353 nucleotide sequences analyzed at 183 positions in the final dataset. The sequence pair was used for 2 sequence BLAST comparison to our reference sequence. See text for description of the magnitude of the pairwise differences.

### Discussion and Conclusion

Previous knowledge on the diet of water mites had been based almost exclusively on laboratory experiments and observations in other artificial settings. In a summary of mite feeding strategies, Walter and Proctor (2013) suggests that laboratory experiments cannot replace field data on what water mites are eating. The work of Ten Winkel et al. (1989) was suggested as a good approach to understand water mite prey selection where laboratory feeding experiments were exhaustively done in tandem with measuring mite weights from the field and density of chironomid prey in the field. This paper builds on the foundation of DNA analysis of mite diets by Martin et al. (2015) who examined non-mite DNA of laboratory-fed *Hygrobates* mites using 18S primers. The present study expanded this approach to field-collected animals using broader

range primers (mLep), and next generation sequencing methods. Next generation sequencing has provided a powerful tool to analyze the molecular gut contents of species that would otherwise be impractical for diet composition studies using morphological analysis of gut contents, which is not possible in water mites which ingest liquefied prey tissues). New observations to be discussed in this paper include the diverse diets of *Lebertia* mites, the unprecedented evidence that oligochaetes may be a significant part of water mite diets, the need for expanded reference sequence databases for definitive identification of the majority of water mite associated prey DNA, and the potential of water mites to be "DNA detectives" for detecting rare or difficult to collect benthic microinvertebrates.

Starved water mites can be fed potential prey items in laboratory settings and then their gut contents tested to check for their prey DNA. This was done using the genus *Hygrobates*, and 18S-amplified DNA from prey was noted to persist up to 2 days in the gut (Martin et al. 2015). Our data analyzing the presence of mLep-amplified DNA after feeding with particular prey items supports this finding on the detectability of prey DNA in water mites after laboratory feeding. The next step they suggested was to test this method using mites collected from the field; however, our preliminary analysis using both 18S and mLep primer sets (see Table 9 and 10) revealed that field-collected water mites had a complex diet, necessitating the use of next generation sequencing to be able to tell all that water mites were feeding on in their natural setting.

Testing the primers listed in Table 7 led us to select the mLep + Folmer LCOI primer pair for our next generation sequencing experiments. The water mites of our choice, *Lebertia*, were observed to feed readily on Diptera larvae in the laboratory (see Figure 48). They were also observed to feed on previously frozen prey items (data not shown). Analysis of the gut contents of these laboratory fed mites gave 100% matches to the public database when BLAST was done

(Table 8). These preliminary experiments expanded on the foundation laid by Martin et al. (2015) by testing the specificity of 18S primers in not amplifying water mite DNA from other genera of water mites than *Hygrobates*, including *Lebertia*, *Arrenurus*, *Mideopsis*, and *Neumania*. All these mites were observed to feed on chironomids and using the 18S primers, we were able to amplify and sequence solely the prey DNA. However, because the database for reference COI sequences is much larger than for 18S sequences and many more are resolved to species level, we further expanded on the work of Martin et al. (2015) by showing similar results obtained with mLep primers, a primer set that targets the COI gene with a broader target species range than the 18S primers but still specifically not amplifying mites and other arachnids. Although the ostensible target range of the mLep primers is solely *Diptera* arthropods (Hamback et al. 2016) , the present study found that these primers will also amplify oligochaetes. Our analysis with the mLep primers using oligochaete DNA from our collections in Lake Erie was positive for amplification.

The hypothesis on which the present work is based is that non-mite DNA amplified from field collected water mites represents DNA from organisms that the mite has ingested. An alternative interpretation that it is DNA that adheres externally to the mites is, we believe, less likely for several reasons: first, except for the oligochaete DNA that we have detected, the types of DNA that we have detected is mostly restricted to the known types of organisms thought to be in mite diets (especially chironomids); second, different species of mites would more likely have identical patterns of externally-adhering DNA, in contrast to our results showing clearly a diversity of patterns from different mites; third, the processing procedure for the mites in this study took great care to wash the outside of the mites thoroughly before processing for DNA extraction; and fourth, the laboratory feeding experiments followed by detection of the fed organism DNA demonstrates that non-mite DNA amplified from these mites indeed reflect the

DNA from organisms that the mites are known to have ingested. Therefore, for the remainder of this chapter, the organisms identified by the non-mite DNA associated with mite specimens will be referred to as water mite prey or diet.

The initial Sanger sequencing studies of mLep-amplified DNA from *Lebertia* and *Neumania* confirmed these species as feeding on chironomids, but they also produced the surprising result that oligochaetes were detected in two specimens, albeit with the query and ID percentages in the 80 percentile range (Table 10). We can find no precedent in the literature for oligochaetes being part of water mite diets. The fact that this observation is also strongly supported by results obtained from next generation sequencing (see Figures 51, 52, and 54 and Table 11) suggests that a significant part of water mite diets has previously been inadequately studied. In addition to oligochaetes, a cladoceran (*Diaphasoma* sp., a name that is also applied to species of *Diaphanosoma*, http://agris.fao.org/agris-search/search.do?recordID= US201302802840) was found in the diet of *L. quinquemaculosa* 8-BHL072216 and also identified as *Diaphanosoma brachyura* by a 97% ID in a next generation sequencing of a *L. quinquemaculosa* specimen (123BHL40917). The water flea *Diaphanasoma brachyurum* has most frequently been reported in European lakes but is also frequently seen in North America, including the Great Lakes (https://sv.wikipedia.org/wiki/Diaphanosoma_brachyurum, http://eol.org/pages/338857 /overview, https://www.glerl.noaa.gov/seagrant/GLWL/Zooplankton/Cladocera/CladoceraGallery4.html). Another mite (*Neumania* 11BHL070916) had copepod (*Macrocyclops* sp.) DNA as has also been reported in a summary of *Neumania* diet (Smith et al. 2010). Thus, the next generation sequencing results confirmed all of the “single” species identifications that were observed with Sanger sequencing and went further by demonstrating that multiple species could be identified in the diets of individual specimens.

Analysis of water mite diet composition by next generation sequencing revealed that many chironomid species could be definitively identified in the diets of *L. davidcooki* and *L. quinquemaculosa*. We have defined “definitive identification” as having a better than 97% match to a reference database sequence that has previously been identified to species. This standard of 97% match is based on previous work, reported in this dissertation (Chapter 2 and Chapter 5) that individual species of chironomids had pairwise differences within a 3% range (i.e., >97% identity). In that study, a “same species” barcode gap occurred at 3% pairwise difference; chironomid specimens with pairwise differences of 3% – 6% (i.e., 97% to 94% identity) were always of the same genus; and intragenus pairwise differences among chironomid sequences occurred even with differences as great as 16% (84% identity), though in many cases with <89% identity, specimens may be of different genera (examples with pairwise differences of 20% were cited). Therefore, chironomid sequence identities >97% almost certainly identify the correct species if the reference sequence is a species level identification, and sequence identities >89% almost certainly identify the genus of the chironomid. Family-level identifications are expected to be accurate in the 80 – 90% identity range, and in some cases they also identify the correct genus. Thus, the species-level identifications in Table 11, based on >97% identities to reference chironomid sequences are expected to be completely accurate, and matches in the 90% - 97% range (not shown, except in the details of the branch names of the representative trees in Figures 51 – 54) are expected to reliably identify the genus of chironomids that were ingested. Table 12, which lists which genera found in *L. quinquemaculosa* and *L. davidcooki*, respectively, with >90% identity to GenBank reference genera or families, is expected to be a reliable list of genera that the water mite had actually been ingesting. The greater richness of chironomid prey for *L. davidcooki* may indicate a dietary difference or might simply be related to somewhat different collection dates for the two species.

In some cases, BLAST comparisons to GenBank returned high identity (>97%) sequences that were identified only to genus, and in other cases, the best match to the reference database was a <97% match to a known species. These are indications of inadequate species coverage in GenBank of the chironomids that *Lebertia* are ingesting. To partially remedy this inadequacy of GenBank, the research described here (Chapter 4) also determined additional species- and genus-level chironomid barcodes that are, up to now, not yet uploaded to GenBank. This RamLab "improved chironomid reference database," described in Chapter 5 enabled additional definitive species and genus identifications that were not possible through the use of GenBank alone (Figure 55).

In contrast to the many excellent species and genus identities in these various databases for chironomids, hardly any of the oligochaete sequences that resulted from the BLAST analysis of various specimen sequences had identities >90%. The one exception was the identification of *Chaetogaster diastropus* in a specimen of *Limnesia* with a reference database identity as high as 96.8%. An unpublished intensive investigation of oligochaete barcodes of morphologically identified species from Toledo Harbor by the Ram Laboratory showed that the same species could have barcode pairwise differences as low as 85% identity and still be considered the same species (in that study, an example was *Limnodrilus hofmeisterei*). Thus, while the presence of matches in the 80 – 89% identity range certainly gives leeway for misidentification, the large numbers of specimens that provide such matches to a range of various oligochaetes suggest that at least up to the family level, these oligochaete "identifications" may be generically reliable. Preliminary tests of my high throughput sequencing data against the unpublished RamLab oligochaete data did not reveal any matches with the mite diet.

The lack of high identity species or genus level sequences for oligochaetes in this work and the failure of many chironomid sequences to be identified to species level point to the need

to develop more complete chironomid and oligochaete reference databases for definitive identification of the majority of water mite-associated prey DNA. Other future studies that are suggested by these data is the need to compare the diets as they change seasonally. Whereas these data indicate that *L. davidcooki* has greater preference for chironomids compared to oligochaetes, these differences in diet may also be related to the different times of the year that the illustrated water mite specimens were collected, At times we observed *L. quinquemaculosa* feeding solely on oligochaetes (see Figure 52). Whether this is due to seasonal shifts in prey availability or a biological selection warrants further study. It would also be interesting to determine the average size of these prey items since a distinctive feature of *L. quinquemaculosa* and *L. davidcooki* is size (Chapter 3) with the larger *L. quinquemaculosa* presumptively being able to handle larger prey. The use of next generation sequencing as in this study will surely bring to light many details about water mite ecology and predator-prey relationships that were unknown until now. Given the diversity of species found in water mite diets, water mites have the potential to be “DNA detectives” for detecting rare or difficult to collect benthic microinvertebrates.

# CHAPTER 7 - GENERAL DISCUSSION, SUMMARY OF RESULTS AND FUTURE DIRECTIONS

Research on water mites presents an excellent opportunity in the 21st century. Advances in both scientific technologies and global transportation have led to ease of access to many habitats that were previously inaccessible and increased the opportunities to advance the description of new species. The United Nations adopted a resolution on the 20th December 2010 and declared the period 2011-2020 as the United Nations Decade on Biodiversity (United Nations Decade on Biodiversity 2010). However, my work on water mites has shown the need for more interest in the biodiversity of "charismatic microfauna" since water mites are wholly understudied even in the Laurentian Great Lakes which make up over 80% of North America's fresh water and may represent one of the best studied lakes in the world (Allan et al. 2013).

***Summary of results***

An initial survey of water mite biodiversity was presented in Chapter 2 A and B. These two studies differed in biogeographic location with the Toledo Harbor study looking at the populations of water mites in Lake Erie and the Blue Heron Lagoon looking at populations of a recently altered island lagoon. Collectively, these two studies demonstrated the importance of water mite biodiversity research with the contribution of the first molecular DNA barcodes for 5 genus level water mite identifications namely: *Krendowskia*, *Koenikea, Albia*, *Hydrochoreutes* and *Madawaska*. The paucity of genetic information led us to publish the first molecular barcodes for *Krendowskia* and *Koenikea* genera (Vasquez et al. 2017) and with our Blue Heron Lagoon chapter prepared for submission we will contribute a total of >50 new molecular DNA barcodes that will contribute to populating the reportedly deficient Great Lakes water mite genetic representation in public databases (Trebitz et al. 2015). One genus of water mites that stood out in our collections was *Lebertia*, and this was due to their predatory behavior and wide

choice of prey items (discussed in Chapters 4 and 6). However, we first determined what species were present in Blue Heron Lagoon. This led us on a quest to meet with the leading authority on water mites in North America, former Wayne State University faculty member Dr. David Cook. With his help we were able to identify the larger species *Lebertia quinquemaculosa* Marshall in our collections (Marshall 1928). Further analysis of the different types of *Lebertia* led us to describe a new species which we plan to name *L. davidcooki* after Dr. David Cook as a tribute to his unending leadership and goodwill to all water mite workers around the world. Chapter 3 focuses on differentiating the two *Lebertia* types used in my subsequent analysis.

**Figure 60: Biodiversity of water mites of Blue Heron Lagoon**, Detroit Michigan. Panels represent dorsal and ventral views of representative water mites that were studied in this work.

Water mite experts agree that knowledge on physiological mechanisms used by these cryptic organisms is generally inadequate and in regards to the digestive system only one reference from 1938 was listed in the authoritative work that includes the only known North American taxonomic key for water mites (Smith et al. 2010). Some new work has been done on anatomy and structural descriptions of the digestive system but we have not found any detailed

study on water mite structure and function of feeding and digestion of water mites. This is addressed in Chapter 4 where we selected the two species of *Lebertia* identified in Chapter 3 and did a comparative study of the digestive system of these two mites. To our knowledge this is the first kind of work done on *Lebertia* and the first of this type of research on any water mite from North America. *L. quinquemaculosa* was observed to predate on chironomids, mosquito larvae and even *Drosophila* larvae. These observations and their unique adaptations led us to study the external and internal structures that are used for feeding and digestion. Several important differentiating features were observed when the two *Lebertia* were compared and contrasted including the chelicerae and their overall size. The midgut and excretory organ structures were studied by experimenting with feeding studies. Our lab has previously used the vital dye fluorescein diacetate (FDA) with aquatic invertebrates (Adams et al. 2014). We exposed chironomids to FDA and then fed them to *Lebertia*. In both species we were able to document the movement of fluorescent food contents by viewing through the integument of the mite. This led us to study the internal microstructure using transmission electron microscopy which revealed large digestive cells and no connection between the midgut and excretory organ as previously reported (Mitchell 1970, Shatrov 2010a). We postulate that these digestive cells are playing a key role in water mite digestion and further studies are warranted.

This work also contributed valuable baseline biodiversity data for potential prey organisms of water mites, and this is the focus of Chapter 5. The work presented in Chapter 5A proved to be especially valuable to my subsequent analysis of the diet composition of water mites. Chapter 5A is the published work on a chironomid reference database that we developed along with our collaborators and represents one of the first type of studies of this kind (Failla et al. 2016). We used adult taxonomically identified chironomids from the Great Lakes region to identify the larvae from our collections in the Toledo Harbor region (Failla et al. 2016).

However, we expanded the database by adding more taxonomically identified adults that were captured closer to Blue Heron Lagoon (Supplement to Chapter 5A) and this new curated chironomid reference database was used in Chapter 6 to assist with identification of prey sequences that were obtained from water mite molecular gut contents. Chapter 5B continues to increase our knowledge on aquatic biodiversity of the Great Lakes by surveying the zooplankton populations which are prey for some species of water mites. This work led to the re-description of an invasive copepod from the Great Lakes, *Eurytemora carolleeae* (Vasquez et al. 2016). This study is important since it was carried out in Toledo harbor a major port that releases many tons of ballast water that may bring in unwanted invasive species. We have contributed valuable biodiversity data, with both studies already in the public domain providing better understanding of Great Lakes aquatic biodiversity.

Sequencing the molecular gut contents of individual water mites revealed a wide variety of prey items being consumed, such as copepods, chironomids, oligohcaetes and cladocerans. This is the focus of Chapter 6 which was to conduct a DNA analysis of mite diets. An initial analysis of individual water mite diet composition using "Diptera" specific primers demonstrated that water mite diets were complex as they involved more than one prey item. This necessitated the use of next generation sequencing to elucidate the diverse identities of in the gut contents of water mites studied. The results of next generation sequencing identified multiple chironomid prey of *Lebertia* water mites. A subset of these included *Cricotopus*, *Paratanytarsus*, *Chironomus riparius*, *Chironomus maturus* and *Dicrotendipes tritomus* with approximately 8 other genus and family level identifications. The RamLab curated chironomid reference database presented in the supplement to Chapter 5A further assisted the identification of some of the prey sequences beyond what the public databases could do. Our curated chironomid reference database enabled confident identification of certain *Cricotopus* sp. prey sequences to

*Cricotopus sylvestris* and *Critcotopus festivellus*. In other cases the RamLab curated chironomid database enabled a genus level identification for a sequence that GenBank had identified only a family level identification in the prey sequence such as a *Chironomidae* sp. prey sequence resolved to the genus level *Glyptotendipes* sp.

***Future directions: public interest and potential of water mites in science education***

Science education begins when a child takes a hike through a forest or plays beside a stream. The diversity of arthropods (in kid's language "bugs") has captured the attention of many children around the world and in many cases these are the first experiences that scientists talk about when they speak about the beginnings of their interest in science. These memories are what instills public interest in nature and can form important components of a future career choice. Water mites have the potential and charisma to be used by public institutions like aquariums and museums to generate interest in biodiversity and science education. This makes them extremely useful to generate interest in science-related fields especially in urban settings that may attract young students from marginalized populations.

Water mites are easy to collect, are comprised of striking, beautiful colors and are found in practically any aquatic ecosystem in both rural and urban areas (Cook and Mitchell 1953). They are also voracious predators in their microscopic context and can be considered the "lion of the microscopic aquatic Serengeti"! When viewed by all levels of students or the general public, water mites evoke a feeling of excitement and intrigue which is why we call these organisms "charismatic microfauna". When their beauty and behavior can be observed, any child or adult can appreciate these singularly, specious arachnids that play such a key role in aquatic systems worldwide.

Little detailed knowledge is available about most North American water mite species and "over half of the species expected to occur in North America have not been named" (Smith et al.

2010). This presents an attractive incentive to citizen scientists that could help with collecting and identifying water mites with the possibility of even naming a few new species!

**Figure 61: Water mites are charismatic microfauna.** (A-B) Water mite preying on chironomid and pest mosquito larvae. (C-E) Collecting water mites and other aquatic "bugs" is easy and can be done with young scientists.

Water mites are not known to pose any threats to humans and can be considered extremely beneficial since they prey on and parasitize a real pest - mosquito larvae. Exhibits can be created with water mites, including a multi-media cart with a video-microscope for hands-on activities that can help youngsters view and manipulate swimming mites and their bright red prey, chironomids. Cross-curricular activities could encourage students in the area of taxonomy and identification by making photographs and drawings of what they observe in the video-microscope. Water mites are already beginning to make an impact in an urban public aquarium here in Detroit (see Figure 61). We are developing displays, citizen science initiatives and portable teaching aquatic labs to promote science enthusiasm in students and adults of all ages. Being that we are in the "decade on biodiversity" we hope that our work will have lasting impact and produce a new biodiversity tool in water mites that will stimulate and sustain increasing interest in the natural world around us.

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**Author:** Adrian A. Vasquez, Milad S. Qazazi, J. Ray Fisher, et al

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## ABSTRACT

**THE DIGESTIVE COMPOSITION AND PHYSIOLOGY OF WATER MITES**

**by**

**ADRIAN AMELIO VASQUEZ**

**December 2017**

**Advisor**: Jeffrey Ram, Ph.D.

**Major**: Physiology

**Degree**: Doctor of Philosophy

Water mites are a diverse group of arachnids that inhabit aquatic habitats and have been studied in the past for their biodiversity, unique lifecycle, bioindicator species use and for their impact as parasites on insects of human pathological significance such as the mosquito. Water mites are critical in their environment as possible apex predators however, their life cycle and morphological complexity has made taxonomy and description of water mites difficult. Although water mite species richness is estimated at over 6000 species described to date, descriptions of extant North American water mite species are estimated to be only 50% of the existing species. Water mite digestive physiology is also virtually unknown even though water mites are known to be efficient predators and parasites of dipteran pest such as chironomids. With the use of microscopic, biochemical and molecular genetic technologies this work aims to improve water mite knowledge in both digestive physiology and diversity of North American water mite populations.

Water mites from Blue Heron Lagoon at Belle Isle, Detroit were collected and processed for assessment of both species diversity and gut molecular contents. Using genetic and morphological methods, water mites and their prey were identified. Water mites in different genera are observed to be generalists as we did not see any water mite genera feeding exclusively

on only one type of prey. Gut molecular contents were assessed using primers targeting the COI gene that has been used for molecular barcoding. Dipteran "specific" primers (mLep) were used to elucidate what prey were being consumed. These sequences were obtained by Sanger Sequencing and by Next Generation Sequencing. These sequences were compared to a large database of chironomid species that were generated from the same biogeographic region. The conclusion is that *Lebertia* water mites are generalist and opportunistic predators who consume a large diversity of chironomids, including various species of *Cricotopus*, *Chironomus*, and *Paratanytarsus*. A novel finding of this study is that for some mites the nearest matches to the DNA sequences of gut-associated DNA were sequences from oligochaetes, albeit in most cases the percentage identity to any GenBank sequence of oligochaetes was in the range of 80 – 90%..

Water mite diversity in the Blue Heron Lagoon is also reported here with a new record for *Lebertia quinquemaculosa* Marshall and the possibility of new *Lebertia* species descriptions from Blue Heron Lagoon. Scanning electron microscopy was used to verify morphological characters and to aid in describing the new *Lebertia*, which we are proposing to name *L. davidcooki*. *L. quinquemaculosa* and *L. davidcooki* were also studied to characterize the structures that facilitate digestive passage of ingested food. Fluorescein, a fluorescent metabolic product from fluorescein diacetate (FDA), was used to visualize the gut structures of the water mites by feeding them fluorescent chironomid larval prey that had been exposed to FDA. Water mites were also examined using confocal fluorescent microscopy to describe gut structures. Transmission electron (TE) microscopy was used to visualize the internal microstructures of *L. quinquemaculosa* and *L. davidcooki* for the first time. Digestive structures such as the excretory organ and mid-gut were observed from water mite dissections and further analyzed by toluidine blue staining of mite sagittal sections.

This work represents the first ever digestive physiology experiments on *Lebertia*. The

results of this work have also contributed new North American DNA barcode genetic representation of water mites, chironomids, and a morphological and molecular description of a Great Lakes invasive copepod *Eurytemora carolleae* to the public databases. The importance and contribution of water mites to aquatic ecosystems validates this study which begins to fill in knowledge gaps on water mite physiology and biodiversity.

# AUTOBIOGRAPHICAL STATEMENT

## Adrian Amelio Vasquez

### Education

**Ph.D. in Physiology,** Wayne State University, School of Medicine, Detroit, MI (2017)
**M.S. Molecular Developmental Biology,** Wayne State University, Detroit, MI (2013)
**M.S. in Cellular and Molecular Biology**, Oakland University, Rochester, MI (2006)
**B.S. in Biology**, Oakland University, Rochester, MI (2003)
**A.S. in Biology and English Literature**, Corozal Junior College, Belize, (2001)

### Honors and Awards (selected)

- Sharon L. Ram Aquatic Sciences Fund Award for research on water mites of Blue Heron Lagoon, Belle Isle, Belle Isle Aquarium, Detroit, Michigan, 2016
- Marion I Barnhart Graduate Student Award for Academic Distinction & Research Progress. 2016.
- Sharon L. Ram Aquatic Sciences Fund Award for research on Gar species in the Belle Isle Aquarium. Detroit, Michigan, 2015
- Travel Award (to Belize & Mexico), Office of the Vice President for Research, Wayne State University. 2015.
- Enhancement Graduate Research Assistantship, Wayne State University. 2012
- Enhancement Summer Graduate Research Award. Wayne State University. 2012
- Graduate Research Enhancement Award, Wayne State University. 2010
- Research Excellence Award. Oakland University Center for Biomedical Research, 2004-2006
- Academic Excellence Award, International Students & Scholars of Oakland University 2005.
- International Students and Scholars Office Service Scholarship, Oakland University, 2003
- Provost Award for Undergraduate Student Research Scholar November 2002. Oakland University, Rochester, MI
- Recipient of a two week terrestrial and marine fellowship by Leading Conservation and Ecological Research Driven Belizean NGO: Programme for Belize. July 1995. Belize Central America.

### Peer Reviewed Publications (selected)

1. Farley NJ, **Vasquez AA**, Kik R IV, David SR, Katailiha AS, Ram JL. 2017. Primer designs for identification and eDNA detection of gars (Lepisosteidae). ***In press***.
2. **Vasquez AA***, Qazazi MS*, Failla AJ, Rama S, Ram JL. 2017. Molecular barcodes of water mites (Hydrachnidiae) from the Toledo Harbor region of Western Lake Erie U.S.A. International Journal of Acarology, DOI: 10.1080/01647954.2017.1349178. *Both authors contributed equally to this work.
3. **Vasquez AA***, Ram JL*, Qazazi MS, Sun J, Kato I. 2016. Oral microbiome; Potential link to systemic disease and oral cancer. Book chapter. ***In review***. *Both authors contributed equally to this work.
4. **Vasquez AA**, Hudson PL, Keeler K, Armenio PM, Fujimoto M, Ram JL. 2016. Eurytemora carolleeae in the Laurentian Great Lakes revealed by phylogenetic and morphological analysis. 42: 802-811.
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