GENETIC AND LIFE HISTORY DIFFERENCES AMONG LABORATORY AND WILD POPULATIONS OF *HYALELLA AZTECA*

BY

KALEY M. MAJOR

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Natural Resources and Environmental Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2012

Urbana, Illinois

Adviser:

Adjunct Assistant Professor David J. Soucek

ABSTRACT

The epibenthic amphipod *Hyalella azteca* has long been used as a model organism for toxicity testing. However, current morphological identification of this amphipod has proven to be insufficiently descriptive. Recent studies using allozymes and/or mitochondrial cytochrome *c* oxidase subunit I (COI) gene 'barcoding' sequences have provided evidence for the existence of numerous cryptic species within wild North American populations of *H. azteca*. Despite its long history of laboratory culture and use, very few studies have focused on species-level genetic diversity that may be present among different laboratory populations. Furthermore, the extent to which the genetic diversity of *H. azteca* found in laboratory cultures is represented in the wild has not been established. In the present study, *H. azteca* samples were collected from 22 field sites in the eastern US and Canada as well as from 15 laboratory populations from commercial sources, US and Canadian regulatory agency laboratories, and academic research groups. Genetic variation among these populations was measured by sequencing the entire COI gene. Pairwise distance comparisons and Bayesian analysis of the nucleotide sequences of 108 individuals yielded six distinct clades. Each of the six clades exhibited low within-clade pairwise divergence (< 5%), indicating that members of the same clade were conspecific. However, high across-clade pairwise divergences (20-25%) indicated that all clades exhibited species-level divergence from one another based on mean COI divergences documented among conspecific members of the sister genus *Gammarus*. Although most of the laboratory populations in the US and Canada were members of the same clade, individuals from one Canadian laboratory population grouped into a separate clade, indicating that all North American laboratories are not using the same provisional species to perform toxicity tests. Further, most of the individuals from field collected populations were genetically-distinct at a species-level from

either laboratory provisional species. An assessment was made of the ability of the COI 'barcoding' region (680 bp in *H. azteca*) and a subset fragment (335 bp) within the 'barcoding' region to resolve the genetic relationships established by the complete COI nucleotide sequences. Although genetic analysis with the COI fragments effectively delineated members of very divergent provisional species, the separation of more closely-related provisional species was not strongly supported by these fragments. Further, an analysis of the translated COI amino acid sequences yielded low protein-level divergence among two groups that were considered separate provisional species using nucleotide data, indicating that a lack of biologically relevant data (i.e. reproductive isolation) may prevent COI from being an effective tool in classifying *H. azteca* that have recently diverged. Incongruence between the Bayesian tree topologies of the nucleotide and amino acid sequence datasets indicates that Bayesian methods employing saturated nucleotide data may not be effective in reconstructing phylogenetic relationships among provisional species within *H. azteca*. Although a single gene may not be sufficient for the establishment of the true phylogenetic relationships among these provisional species, more weight should be given to the amino acid sequence data than nucleotide data when attempting to understand patterns of evolution within *H. azteca* in future. Given that sensitivity to select chemicals has been shown to have a genetic basis in *H. azteca* on a population-level, species-level genetic diversity among *H. azteca* populations could be particularly problematic in the context of toxicity testing. Because life history characteristics are the basis for chronic toxicity test endpoints, identifying potential differences in these characteristics among genetically-distinct populations is critical before differences in chemical sensitivities can be assessed among these groups in a laboratory setting. The life history characteristics of populations from two laboratory and two wild clades were quantified in a laboratory setting in the context of a 42-day

chronic water-only toxicity test. In addition to genetic differences among these clades, life history characteristics, namely body size and reproductive rates, differed by clade, for the most part in accordance with comparable life history data among these groups in the published literature. Although reproductive measurements deviated slightly from other studies in which it was quantified for members of some of the clades, establishment of optimal laboratory culturing conditions for each of the clades may reconcile these discrepancies in the future. Given the genetic and life history characteristic differences that occur on a clade-basis, laboratories using separate provisional species to perform toxicity tests should not directly combine results to establish water quality regulations for the protection of aquatic life. Further, the effectiveness of using *H. azteca* laboratory populations to act as surrogates for wild populations of this species complex is called into question.

ACKNOWLEDGEMENTS

This thesis would not have been possible without the instruction, guidance, and assistance of many individuals. I would first like to thank my advisor David Soucek for his support and guidance during this process. His patience, knowledge, and feedback were instrumental to every aspect of this project, as was his willingness to carefully edit every single draft of this thesis. I would also like to thank Rosanna Giordano for teaching me how to effectively obtain and analyze COI sequences for the *H. azteca* species complex – a feat that was not easily accomplished given the divergence among provisional species groups in this study. Her patience, expertise, resourcefulness, and commitment to obtaining quality sequence data made the genetic analysis of these sequences possible. I would to thank Felipe Soto for his help in executing the Bayesian methods used to establish phylogenetic relationships in this study, as well as his advice on the interpretation of those results. For serving on my committee and providing me with feedback, I would like to thank Chris Taylor and Michael Ward. I also owe a great deal of thanks to Amy Dickinson - her planning, preparedness, and assistance was vital to the maintenance and health of laboratory cultures as well as the completion of life history tests.

In addition, I would like to thank those who were willing to make collections of *H. azteca* and/or send laboratory samples and live populations. Particularly, the field collections of Mark J. Wetzel, R. Edward Dewalt, Massimo Pessino, Steve Fiske, and Warren Norwood served to further the geographic sampling range of this study significantly. As a result of these sampling efforts, the results of this study were far more interesting than they would have been without these collections. The willingness of several laboratories to send their populations for genetic and life history testing was also greatly appreciated.

TABLE OF CONTENTS

CHAPTER 1: SPECIES-LEVEL DIFFERENCES IN COMPLETE CYTOCHROME C OXIDASE SUBUNIT I (COI) SEQUENCES FOR *HYALELLA* SP. FROM LABORATORY AND WILD POPULATIONS

Introduction

The freshwater amphipod *Hyalella azteca* (Saussure 1858) (Amphipoda: Hyalellidae) is found in many different lentic and lotic habitats across North and Central America and is considered to be one of the most widely dispersed invertebrates in North America (Witt and Hebert 2000). It was originally described as *Amphithoe azteca* in 1858 in Mexico (Saussure 1858) and, according to Baldinger (2004), it can be distinguished from other North American and Caribbean species of *Hyalella* by the presence of dorsal mucronations, its narrow inner plate of the first maxilla lacking medial plumose setae (*H. montezuma*), its subequal (length) primary and secondary antennae, and a normal first pleonal plate (*H. texana*). However, organisms that fit this description vary widely in size and life history characteristics (Strong 1972; Wellborn 1993), and while as many as six different described species have been lumped into *H. azteca*, it is likely that this species name has been assigned to organisms across North and Central America (Gonzalez and Watling 2002) too liberally. Thus, it is not surprising that a host of recent studies (see below) have documented phenotypic and genetic diversity both within and among wild and laboratory-reared populations of this amphipod, indicating that *H. azteca* is a complex of many cryptic species.

Although mechanisms driving genetic divergence within *H. azteca* are not yet well understood, documentation of heritable phenotypic diversity among these organisms in the wild is abundant. Strong (1972) documented differences in life history traits, particularly size class differences, among eight wild populations of *H. azteca* in Oregon. Wellborn (1993) confirmed this observation and also documented similar differences in populations from southeastern

Michigan (Wellborn 1993, 1994a,b, 1995a,b) and Oklahoma (Wellborn et al. 2005). Essentially, the presence of fish predators is thought to have created a selection pressure for small body size in *H. azteca*, while the absence of fish predators removed that selection pressure, allowing for larger organisms in areas devoid of significant fish predation (Strong 1972; Wellborn 1993). Although these ecological studies were either performed in an era before genomics or without the use of direct sequencing methods, multi-generational laboratory acclimation (Strong 1972), predator-free pond acclimation (Wellborn 1994b), and allozyme variation (Wellborn and Cothran 2004; Wellborn et al. 2005) showed that these phenotypes were indeed heritable and nonplastic.

In addition to phenotypic diversity, noteworthy genetic diversity has been documented among these cryptic species, using allozyme methodology and direct sequencing of 'barcoding' fragments located in the cytochrome *c* oxidase subunit I (COI) mitochondrial gene region. A study by Wellborn and Cothran (2004) showed allozyme profile differences and significant COI divergence among three phenotypically similar populations found in the littoral area of a small Michigan lake, indicating that size phenotype alone did not necessarily determine whether a group of organisms was conspecific. Cytochrome *c* oxidase subunit I sequences among these populations differed by 16 to 20% (Wellborn and Broughton 2008). Among other amphipods, within the sister genus *Gammarus*, members of the same species typically have COI sequences that diverge by less than 4% (Costa et al. 2009). Thus, even by conservative measures, the three phenotypically similar populations could be considered separate species. Additional genetic studies of wild populations of *H. azteca* in other geographic locations across North America have documented levels of COI divergence among haplotypes on the order of 9-28% in the Mississippi and Great Lakes drainages and habitats in the Yukon Territory and New Brunswick

(Witt and Hebert 2000) and 4-29% in the Great Basin of California and Nevada (Witt et al. 2006). Witt and Hebert (2000) also noted significant genetic differentiation in organisms sampled from the same location, with 15 of field 24 sites harboring at least two different species. The co-occurrence of multiple provisional species at a single site has also been documented by Wellborn (1995b) and Wellborn and Cothran (2004) in Michigan, as well as Dionne et al. (2011) in Eastern Quebec, Canada.

In the laboratory, *H. azteca* is commonly used in toxicity tests because of its sensitivity to a wide variety of chemicals, its adaptability to laboratory culturing methods, and its importance to many aquatic food webs (Borgmann et al. 1989; Duan et al. 1997). Results of *H. azteca* acute and chronic toxicity bioassays are frequently used to determine state or federal regulatory water quality standards for the protection of aquatic life, including wild populations of *H. azteca*. However, the extent to which the phenotypic and species-level genetic variation found in wild populations of *H. azteca* may also exist in laboratory populations is unknown, especially when considering that not all North American laboratory cultures can be traced back to the same wild population. A few studies have used allozymes to quantify genetic variation among select laboratory populations (Hogg et al. 1998; Eisenhauer et al. 199; Duan et al. 1997, 2000a-c, 2001), although no studies exist that employ direct sequencing methods to test whether laboratory organisms in North American regulatory agency and academic laboratories are members of the same species. However, the available allozyme data for a limited number of laboratories suggest that species-level differences exist (Duan et al. 1997). The lack of sequence data for laboratory organisms prevents a comparison between these organisms and existing sequence data, published in the last 12 years, from a host of ecological studies of wild

populations of *H. azteca.* Further, not knowing the identity of *H. azteca* from laboratory populations prevents the confident extrapolation of toxicity test results across laboratories.

The first objective of the present study was to quantify the genetic variation among North American laboratory populations of *H. azteca* using complete mitochondrial COI sequences to determine whether North American laboratories are using organisms of the same species to perform toxicity tests. The advantages of sequencing this gene are numerous, including that it is a more robust quantification of genetic variation at the species level than provided by allozyme analysis, and it will provide the first complete, direct mitochondrial gene sequence data for laboratory populations of these organisms. The second objective of this study was to determine if organisms collected from various sites in the wild are the same species as those being used in laboratory tests. To make this determination, COI was sequenced in a select group of wild populations across North America including Ontario, Canada, and Illinois, Florida, Michigan, New Mexico, Wisconsin, and Vermont, USA. No documented COI data exist for any of these wild populations. Further, all of the complete COI sequence data from this study were compiled to create a phylogeny including both the laboratory and wild populations sampled in this study. Finally, all of the partial COI data on GenBank for members of the *H. azteca* species complex were added to the COI sequences generated from this project to better-understand the phylogeny and geographic distribution of the identified provisional species, particularly in relation to other members of the genus *Hyalella.* A comparison was also made between the phylogenetic relationships obtained among taxa when using the entire COI gene versus the fragments that have been utilized by other authors to delineate relationships among members of this species complex.

Methods

Source populations

Hyalella azteca specimens were obtained from 22 wild and 15 laboratory-maintained populations (Tables 1.1 and 1.2) between November 2009 and May 2011. Wild specimens were collected from sites in Florida, Illinois, Michigan, Wisconsin, and Vermont, USA and Ontario, Canada (Table 1.1). Laboratory populations came from a variety of academic, regulatory, and consulting agency laboratories as well as commercial sources from the US and Canada (Table 1.2). Organisms from Illinois were collected using a D-framed net to disturb submerged or emergent macrophytes and/or algal mats on rocks. Contents of the net were then emptied into a plastic tray, and a plastic transfer pipette was used to separate *H. azteca* adults from other organisms. Collection sites in Illinois were selected from separate drainages whenever possible to eliminate the potential for population mixing. Organisms from other field sites were collected in a similar manner, although methods may have varied slightly by individual collector as samples from outside of Illinois were obtained by other researchers. All samples from Florida, Michigan, and New Mexico (see below) were collected by Mark J. Wetzel (Illinois Natural History Survey, (INHS)) except for a single Michigan sample (TR-MI) collected by R. Edward DeWalt and Massimo Pessino (INHS), who also collected all the samples from Wisconsin. Samples from Vermont were collected by Steve Fiske (Vermont Department of Environmental Conservation). All organisms were transferred into 95% ethanol and stored at -20 ˚C as soon as possible (usually within one to two weeks and never more than three months after being fixed in ethanol). Before freezing specimens, ethanol was decanted and replaced

three times to ensure that the sample was well-preserved for DNA extraction. Laboratory-maintained population specimens and some wild population specimens from the United States and Canada were depurated for at least 12 hours when possible before fixation in ethanol to reduce the likelihood of amplifying remnants of material in the digestive tracts. All amphipods were morphologically identified as *H. azteca* based on Stevenson and Peden (1973) and Baldinger (2004) prior to DNA extraction. An additional collection from New Mexico (Table 1.1) was identified as *Hyalella* sp. using the key in Baldinger (2004), although its characters did not match any of the species exactly. In that key, the specimens most closely resembled *H. inermis*, but did not fit the original description by Smith (1875). Thus, these individuals were preserved as stated above and included in the analyses as a congeneric.

DNA extraction, gene amplification, and sequencing

Prior to DNA extraction, all specimens were sexed. For the majority of the individuals, photographs of the whole body, antennae, uropods, and gnathopods (males only) were taken to document subtle differences in morphology for a majority of extracted individuals. Each individual was then dissected and the head and uropods were preserved in 95% ethanol at -20 ˚C. DNA was extracted from the rest of the body using the Qiagen DNA Micro Kit (Qiagen Inc., Valencia, CA) and stored at 4 ˚C. DNA extraction followed the manufacturer's protocol except for the addition of a 10 minute, 70 ˚C incubation period after the addition of the AL buffer and the use of Wizard® SV Minicolumns (Promega Corporation, Madison, WI). Prior to the initial incubation step, each sample was macerated with a pestle (Bel-Art Products, Pequannock, NJ). Amplification of the desired COI region was accomplished using the extracted DNA and illustra PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare, Buckinghamshire, UK) (25 μL-total-

volume PCR: 1 µL 10 µM forward primer and reverse primer, 2 µL genomic DNA, and 21 µL water). PCR conditions consisted of 2 minutes at 95 ˚C, then 40 cycles of 95 ˚C for 30 seconds, 50-55 ˚C (depending on primer combination, see Tables 1.3 and 1.4) for 30 seconds, and 72˚C for 2 minutes. Agarose gel electrophoresis with GelGreen™ (Biotium Inc., Hayward, CA) nucleic acid stain was used to assess whether amplification occurred. If clean, singular bands were visualized on a gel, and the PCR product was cleaned using the QIAquick® PCR Purification Kit (Qiagen Inc., Valencia, CA). If multiple banding occurred, PCR was repeated and products were combined, run on an agarose gel, and the desired band was gel excised using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corporation, Irvine, CA). Sequencing was conducted using Big Dye v3.1 (Applied Biosystems, Foster City, CA) and a thermocycler setting of 5 minutes at 95 ˚C, followed by 30 cycles of 98 ˚C for 10 seconds, 50 ˚C for 5 seconds, and 6 ˚C for 4 minutes. Sequencing reactions of 21 µL consisted of: 1.6 µL 2 µM primer, 3 µL Big Dye v3.1, 1 µL DMSO, and variable amounts of DNA and water, depending on DNA concentration. DNA concentrations of cleaned PCR products were read with a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE), and the amount of DNA used in each sequencing reaction was calculated based on a rate of 5 ng of DNA per 100 bp of product to be sequenced. Ready-to-load reactions were brought to the Keck Center (University of Illinois at Urbana-Champaign, Urbana, IL) for running on an Applied Biosystems 3730xl DNA Analyzer (Life Technologies Corporation, Carlsbad, CA). Sequences were compiled using Sequencher® version v4.9 (Gene Codes Corporation, Ann Arbor, MI) and aligned using PAUP version 4.0b10 (Swafford 2002).

Primer development

Although universal primer sets first documented by Folmer et al. (1994) and then by others were available to sequence a 680 bp 'barcoding' fragment in the five prime region of COI of *H. azteca*, this study aimed to sequence the entire gene to attain better resolution, thus requiring novel primers. Degenerate primers TRNA-1F-D and COII-1R-D (Table 1.3) were developed based on the most conserved regions flanking COI in the most closely-related gammarids *Metacrangonyx longipes* (accession number NC_013032, Bauza-Ribot et al. 2009) and *Onisimus nanseni* (accession number NC_013819, Ki et al. 2010) for which the entire mitochondrial genomes had been sequenced. Primers were first tested on Illinois Natural History Survey (INHS) laboratory individuals and although the desired ~1810 bp segment was amplified, sequencing with degenerate primers proved unsuccessful. Therefore, PCR fragments were cloned using the TOPO® TA Kit (Invitrogen, Carlsbad, CA) according to manufacturer's directions. Cloned sequences of *H. azteca* were aligned to available sequences from *M. longipes* and *O. nanseni* using PAUP and used to design additional primers based on conserved tRNA and tRNA/COII regions that flank COI. To amplify the desired COI region in all remaining individuals, available external primers for laboratory populations were used, and if amplification occurred, specific internal primers were developed to ensure complete, bidirectional sequence coverage and establish more specific external primers for that group. For most individuals, a band (approx. 1800 bp) that represented the entire COI gene was amplified and sequenced. For organisms in two groups (See Results, Burlington and Pine Lake clades), amplification and sequencing was only successful when two overlapping fragments (5' 980 bp fragment and 3' 1150 bp fragment with roughly 450 bp of sequence overlap) were amplified and sequenced in turn. New primers were generated by comparing the conserved sequences among all clades of *Hyalella* sequenced thus far, as well as primers from Wellborn and Broughton (2008) (Table

1.3). Table 1.3 provides a listing of the all the primers used in this study as well as the clades for which they were most successful. Table 1.4 gives a listing of the most useful primer combinations (with annealing temperatures) for band amplification and the genetic groups (See Results section) for which they were used.

Statistical sequence analysis

After the sequences were aligned in PAUP, the start codon for the COI gene was inferred by comparing this study's *Hyalella* sequences with that of *M. longipes.* The start codon (ATT, non-canonical) in *Hyalella* was repeated in tandem for most organisms in this study, except for organisms from JP-VT (Table 1.1), which produced the sequence 'ATT CTT' (5' to 3'). Thus the upstream 'ATT' was treated as the start codon for all organisms in this study. Subsequent amino acid translation (*Drosophila* mitochondrial code), using MacClade version 4.05 (Maddison and Maddison 2000) with the primary 'ATT' codon as the first codon, produced continuous translations with no stop codons except for a single one at the 3' terminus (TAA). No other reading frames produced continuous translations. Based on this translation, a 1569 bp alignment was used for the entire COI gene and a 523 amino acid (aa) alignment was used as the corresponding translated amino acid product.

To conduct a genetic comparison of *Hyalella* individuals, full COI nucleotide sequence and amino acid sequence alignments were created. The data generated in this study were also used to generate a subset nucleotide alignment of the 680 bp 'barcoding' region primarily amplified by other studies including Witt and Hebert (2000) and Witt et al. (2003, 2006). Another alignment was also produced by using a 335 bp region (a subset within the 'barcoding' region) representative of the fragments used by Stutz et al. (2010), Dionne et al. (2011), and

Stanley (2011) to delineate provisional species in *H. azteca.* A final alignment of the present study's 335 bp region and all other available overlapping COI sequence data for *Hyalella* from GenBank was created. The 335 bp region was selected for this comparison in order to include as many published studies as possible, and as a result, several of Wellborn and Broughton's (2008) sequences that did not overlap with this fragment of COI were not included in this analysis because they were positioned at the 3' end of the gene.

Pairwise distances were generated using PAUP for all taxa with the entire COI dataset and its corresponding translated amino acid sequence as well as the 680 and 335 bp DNA fragments. Distance values between nucleotide sequences were reported as both uncorrected *p*-distances (See Appendix A.1-A.3) and corrected distances using the Kimura-2-Parameter (K2P) (Kimura 1980) model in the interest of making direct comparisons with other published COI datasets. Distance values between amino acid sequences were uncorrected *p*-distances. For nucleotide pairwise distances, Wellborn and Broughton (2008) reported uncorrected *p*-distances among members of the *H. azteca* species complex, while Witt and Hebert 2000, Witt et al. (2003, 2006), and Costa et al. (2009; *Gammarus*) reported corrected (K2P) pairwise distances. Thus, for purposes of comparison, this study primarily used K2P corrected pairwise distances. The discrimination of different groups was based on values derived by Costa et al. (2009), although values provided by Witt et al. (2006) were also considered. Within the sister genus *Gammarus*, conspecific divergences at COI (658 bp 'barcoding' fragment) range from 0 to 4.30% with congeneric divergences ranging from 5.19 to 34.23% (Costa et al. 2009; 17 putative species, 169 sequences). Within *Hyalella*, Witt et al. (2006) used a species screening threshold of 3.75% to distinguish provisional species. Although this value was not based on a comprehensive survey of diversity in all *Hyalella*, it was established using the mean intra-

population variation observed within members of *H. azteca* species complex in the Great Basin of southern California and Nevada (Witt et al. 2006). Because the *Gammarus* data were more taxonomically defined than the available *H. azteca* data from Witt et al. (2006), the Costa et al. (2009) values were primarily considered in this analysis.

A comparison of the sequence data generated in this study was made with GenBank COI sequence data for *Hyalella* using the 335 bp fragment region to include as many complete datasets as possible (Witt and Heber 2000; Witt et al. 2003, 2006; Wellborn and Broughton 2008, Stutz et al. 2010; Baird et al. 2011; Dionne et al. 2011). After aligning all sequences in PAUP and removing identical sequences, a Neighbor Joining tree was generated based on corrected (K2P) pairwise distances. Based on this analysis, *Hyalella* sequences that grouped closely with organisms in this study were noted. Included sequences (113 haplotypes) consisted of the following species: *H. azteca, H. montezuma, H. texana, H. muerta, H. sandra, and H. simplex*.

The following Bayesian analyses using *M. longipes* as an outgroup were conducted on the sequence data generated from the 108 individuals sequenced in this study: 1) The nucleotide analysis of the 61 unique haplotypes using the entire 1569 bp gene, 2) the complete COI protein sequence (523 aa, 35 unique haplotypes), 3) the 680 bp 'barcoding' region fragment (48 unique haplotypes), and 4) The 335 bp fragment within the barcoding region (38 unique haplotypes).

For Bayesian analysis of DNA sequences, ModelTest v3.7 (Posada and Crandall 1998) was used for each dataset to determine the best-fit nucleotide substitution model of 56 possible models by the Akaike Information Criterion. Models and parameters differed for each analysis (Table 1.5). For the 1569 bp nucleotide dataset, saturation was monitored by plotting uncorrected pairwise distances against pairwise sequence divergence as adjusted by the

appropriate model of evolution. Saturation was assessed by comparing the resulting slope of the linear relationship to the theoretical slope of an unsaturated dataset (m = 1) as established by Jeffroy et al. (2006). For the amino acid sequence dataset, a mixed model was used. In general, Bayesian analysis using the ModelTest parameters and nucleotide substitution model were employed using MRBAYES (Huelsenbeck and Ronquist 2001) with Markov Chain Monte Carlo (MCMC) methods consisting of four Markov chains (two cold, two hot) until the split-chain deviations reached $p < 0.01$. All majority-rule consensus trees were computed by removing the first 20% of trees as the burn-in.

Results

Complete COI sequence (1569 bp) variation and phylogeny

Sequencing the complete COI gene (1569 bp) of 108 individuals of *Hyalella* sp. from laboratories and field sites yielded 61 unique haplotypes. Six provisional species groups were assigned, each exhibiting within group percent divergences of < 5% (K2P, Table 1.6). These were based on the maximum COI barcoding region divergence (4.30%) among different species for the sister genus *Gammarus* (Costa et al. 2009). The outgroup, *M. longipes*, differed from the six *Hyalella* groups by a range of 33.70 to 39.66%. Bayesian analysis showed that each of the six *Hyalella* clades exhibited a posterior-probability (pp) of 100, indicating very strong support for these clade divisions (Fig. 1.1). Clades were named for their origin and will be referred to as 1) US Laboratory, 2) Flat Branch, 3) Clear Pond, 4) Burlington, 5) Pine Lake, and 6) Joe's Pond. Bayesian analysis produced a well-supported branching pattern that separated the US Laboratory clade from all other clades. After the separation of the US Laboratory clade, Joe's Pond grouped distinctly from the remaining clades. Within the remaining clades, Flat Branch formed a

separate clade from Clear Pond, which in turn was separated from the Pine Lake and Burlington clades (Fig. 1.1). Nucleotide saturation was documented in this dataset by the resulting slope between uncorrected pairwise distances and the corrected distances established with the model used in the Bayesian analysis (m = 0.32; Fig. 1.2).

Variation among laboratory organisms

Among the 15 laboratory sources sampled in this study, two provisional species were identified, but each of the 15 laboratories contained only one provisional species. Of the 40 laboratory organisms sequenced, 38 were grouped into the US Laboratory clade and two were grouped in the Burlington clade. Laboratories with organisms belonging to the US Laboratory clade included all those in Table 1.2 except for CCIW. No individuals belonging to the Flat Branch, Clear Pond, Pine Lake, or Joe's Pond clades were found in laboratory populations.

Four unique haplotypes were identified in the laboratory organisms (n = 38) in the US Laboratory clade. However, a majority of organisms (36) expressed one of two haplotypes that diverged by 0.19%. The third and fourth haplotypes were identified in a single individual from PYLET and one from TTU, respectively, which increased the maximum divergence among laboratory individuals in this clade to 1.55%. Two laboratory cultures had individuals of both most common haplotypes (ARO, INHS). The COI sequences for two individuals from the CCIW laboratory (Burlington clade) were identical but divergent from US Laboratory organisms by 23.14 to 24.90% (Table 1.6).

Variation among wild organisms

A total of 47 organisms collected from laboratories and field sites were grouped into the US Laboratory clade. In addition to US and most Canadian laboratory organisms, the US Laboratory clade included organisms from four sites in Florida (PS-FL, LS-FL, CS-FL, TL-FL, Fig. 1.1). Within the entire clade, 12 unique haplotypes were identified. Mean divergence within the US Laboratory clade, including laboratory and wild samples was 0.49% with a maximum divergence of 2.21% (Table 1.6).

The Burlington clade included 12 organisms, 10 from wild populations primarily from field sites in Michigan, with two from Wisconsin (WL-WI), and one (morphologically identified as *Hyalella* sp.) from New Mexico (RC-NM, Fig. 1.1). Of the 10 unique haplotypes within this clade, nine were from field sites (the remaining haplotype was from the CCIW laboratory population). Divergence in this clade averaged 1.77% with a maximum of 4.11% (Table 1.6).

The Flat Branch clade was comprised of 25 organisms with 20 unique haplotypes. These organisms originated from field sites in Illinois and Michigan (Fig. 1.1). Mean divergence among organisms in this clade was 0.93% with a maximum of 3.14% (Table 1.6).

The Clear Pond clade included 18 organisms primarily from field sites in Michigan, but also from one site in Illinois (CP-IL), one site in Ontario (VR-ON), and two sites in Wisconsin (LR-WI, WL-WI, Fig. 1.1). Within this clade, 16 unique haplotypes were identified. Mean divergence within the Clear Pond clade was 1.10% with a maximum of 2.34% (Table 1.6).

The Pine Lake clade included four organisms with two unique haplotypes. These organisms were collected from two sites in Michigan (MP-MI, PL-MI, Fig. 1.1), and represented the most diverse clade in this study with a mean divergence of 2.35% and a maximum of 4.71% (Table 1.6).

The Joe's Pond clade included two individuals collected from a field site in Vermont (JP-VT) that were identical. Although the divergence between these two organisms was 0%, they represented a distinct clade from all other organisms in this study from which they varied by 19.41 to 23.80% (Table 1.6).

Individuals from wild populations were present in each of the six clades. Of the 23 field sites sampled, seven sites harbored members of multiple clades, with two sites each having representatives from three different clades (Table 1.7). Across-clade sequence divergences ranged from 6.98% to 25.60% with most of the clade comparisons yielding divergences on the order of 19 to 25% (Table 1.6). Two clades, Burlington and Pine Lake, varied significantly less from one another, with a mean percent divergence of 8.02% and a range from 6.98 to 9.27% (Table 1.6).

COI protein translation variation and phylogeny

Among the 108 individuals sequenced in this study, 35 unique amino acid (aa) sequences were identified. Although most individuals had sequences of 523 aa in length, 14 of these organisms displayed nucleotide sequence three codons shorter. The missing amino acids were located at the 3' end. Of these organisms with shortened aa chains, 12 were grouped into the Burlington clade (all organisms in this clade), and two were grouped in a small clade within the larger Flat Branch clade in the 1569 bp analysis. The within-clade amino acid translations ranged from 0 to 1.35% although mean values were typically less than or equal to 0.5% (Table 1.8). Between-clade amino acid differences were higher in most cases, ranging from 0.19 to 5.35%, with most mean values greater than or equal to 2.93% (Table 1.8). The between-clade

comparison that yielded the minimum percent difference was a comparison between members of the Burlington and Pine Lake clades. Mean between-clade amino acid percent divergence for the Burlington and Pine Lake clades was 0.57%, a value comparable to the within-clade percent divergence of the Burlington clade 0.52%. This reduced variation in amino acid sequences between the Burlington and Pine Lake clades suggested that they comprise a single clade. Bayesian phylogenetic analysis supported this suggestion and grouped members of the Burlington and Pine Lake clades together into what will be referred to as the Michigan group (Fig. 1.3). Five of the six clades established in the complete gene analysis were maintained with strong support in the amino acid analysis, although the newly-formed Michigan clade was not strongly supported (pp = 83). However, the amino acid tree topology differed significantly from the topology established by the entire gene analysis. First, the Michigan group (previously Burlington and Pine Lake clades) was separated from the rest of the clades, unlike the entire gene analysis that showed the US Laboratory clade as being most divergent from all other clades (Fig. 1.1 and 1.3). Next, the Clear Pond clade was separated from all remaining clades, and then the Flat Branch clade. Finally, the Joe's Pond clade was separated from the US Laboratory clade (Fig. 1.3). Further, the individuals within the Flat Branch and Michigan clades with shortened amino acid chains did not form sub-groups with one another in either clade, but were dispersed within the clades (Fig. 1.3).

Partial COI 'barcoding' region (680 bp) and subset (335 bp) variation and phylogenies

An analysis of the 'barcoding' region (680 bp) was performed for the 108 individuals sequenced in this study. Sequence divergences generated using the 680 bp fragment were comparable to those generated with the 1569 bp fragment, except for the Pine Lake clade, where

distances ranged from 0 to 5.56% (Table 1.9) instead of 0 to 4.71% calculated in the 1569 bp analysis (Table 1.6). This increase in range suggested that the Pine Lake clade should be further subdivided into two discrete clades (provisional species) based on the maximum noted within-species divergences among sister genus *Gammarus* (Costa et al. 2009). Using this reduced data set, Bayesian posterior probability values for three of the six major clade divisions from the 1569 bp analysis were reduced, two only marginally and the third below the level of statistical significance (Pine Lake, pp = 58, Fig. 1.4). The resulting tree also maintained the general clade branching pattern observed in the 1569 bp analysis. However, the separation of the two Flat Branch clade haplotypes with shortened amino acid chains (see branch with terminal nodes IC-IL and EC-IL, pp = 100, Fig. 1.1) was not maintained in the 680 bp analysis, which nested these two haplotypes within the Flat Branch clade, although their relationship to one another was still supported (pp = 99, Fig. 1.4).

An analysis of the 335 bp subset of the barcoding region was conducted separately on the 108 individuals sequenced in this study. Sequence divergences generated with 38 unique haplotypes of the 335 bp fragment were comparable to those generated with the 1569 bp fragment (Table 1.6), except that, similarly to the 680 bp analysis (Table 1.9), distance range within the Pine Lake clade increased (maximum 5.62%, Table 1.10). This increase in range suggested that the Pine Lake clade should be further subdivided into two discrete clades (provisional species) based on the maximum noted within-species divergences among sister taxon *Gammarus* (Costa et al. 2009). The Bayesian analysis of the 335 bp sequences maintained five of the six clade divisions reported from the 1569 bp analysis, but posterior probability values were reduced below a level of significance for the US Laboratory clade (pp = 90, Fig. 1.5) in the 335 bp dataset. The tree topology of this shortened dataset differed slightly from the 1569 bp

and 680 bp datasets, particularly with regard to Joe's Pond, which was sister to the sub-divided Flat Branch, Burlington, Pine Lake, and Clear Pond clades in the larger bp analyses (Fig. 1.1, 1.4), but sister to only the Flat Branch clade in the 335 bp analysis (Fig. 1.5). Furthermore, the branch containing all of the remaining clades except the US Laboratory clade was poorly supported in the 335 bp dataset (pp = 79, Fig. 1.5, compared to pp = 100 and pp = 98 in the 1569 bp and 680 bp analyses, Fig. 1.1 and 1.4). In addition, the 335 bp analysis failed to resolve the Burlington clade and the Pine Lake clade from one another, although the grouping of the two haplotypes in the original Pine Lake clade was maintained (pp = 99). In addition, the two Flat Branch individuals that yielded shortened amino acid chains were nested within the Flat Branch clade in the 335 bp analysis, similarly to the 680 bp analysis, but in the 335 bp analysis, these two haplotypes were reduced to a single haplotype, and the resulting node was unresolved compared to other Flat Branch haplotypes (Fig. 1.5).

The 680 bp and 335 bp datasets had fewer unique haplotypes when compared to the 1569 bp dataset (Table 1.11). Reduction in number of unique haplotypes resulted in a reduction in the mean within-clade percent divergence for the US Laboratory, Flat Branch and Burlington clades but an increase for the Clear Pond and Pine Lake clades (Table 1.6 compared to Table 1.9 and Table 1.10).

A final genetic analysis combined data from this study and all closely-related GenBank data for *Hyalella* using the 335 bp fragment region. *H. azteca* sequences from several published studies grouped within the clades established in this study (Witt et al. 2003, 2006; Wellborn and Broughton 2008; Baird et al. 2011; Dionne et al. 2011), although the majority of sequences from some studies (Witt et al. 2006; Stutz et al. 2010) were more distantly related to those in this study. For all *H. azteca* GenBank sequences that grouped within the clades of the present study,

pairwise divergences (K2P) between sequences and their respective clade were less than 5.7% (Table 1.12). Wellborn and Broughton's (2008) OK-L Clade grouped within the US Laboratory clade. Members of the C Clade from the same study grouped with the Flat Branch clade, as did Haplotype 5 of Dionne et al. (2011), and Clade 6 of Witt et al. (2003). The Clear Pond clade encompassed members of Clade 3 of Witt et al. (2003) and A Clade (Wellborn and Broughton 2008). Members of Clade 1 (Witt et al. 2003), OR/MI-L Clade (Wellborn and Broughton 2008), Haplotype 1 (Dionne et al. 2011), and an unclassified group of organisms from Baird et al. (2011) grouped within the Burlington clade. Clade 2 (Witt et al. 2003), and provisional species 16 (HaPS16) from Witt et al. (2006) grouped closely with the members of the Pine Lake clade. Clade 5 (Witt et al. 2003), B Clade (Wellborn and Broughton 2008), and Haplotypes 2-4 (Dionne et al. 2011), grouped within the Joe's Pond clade. While all other sequences in the genus *Hyalella* grouped very distantly (> 25% K2P) from all the clades determined in this study, the sequences of *H. montezuma* (Witt et al. 2003) grouped relatively closely to those of the Pine Lake (6.97 - 7.32%) and Burlington clades (6.61 - 9.72%; Table 1.12).

Discussion

Genetic analysis of *H. azteca* individuals from 15 laboratories in the US and Canada and 22 field sites located east of the Mississippi River yielded six strongly divergent clades. Using the entire 1569 bp COI gene sequence, the classification of these six clades as separate provisional species was well-supported by both Bayesian phylogenetic analysis and pairwise distance comparisons. The 6.98% to 25.60% percent divergence observed between any two of the clades suggests that all clades be classified into the same genus (*Hyalella*) but as separate species based on a comparison to the minimum and maximum within-genus percent divergence

(5.19 and 34.23%, respectively) for the sister genus *Gammarus* at COI (Costa et al. 2009). Overall within-clade percent divergences were low (average 0.68±0.77%) and also supported these species divisions, and although the Pine Lake clade exhibited slightly higher within-clade divergence (4.71%) than the maximum within-species divergence observed in the COI barcoding region in *Gammarus* (4.30%), it was not as high as the 5.19% congeneric minimum (Cost et al. 2009). Witt et al. (2006) employed a 3.75% maximum within-species divergence for delineating relationships among *H. azteca* in the Great Basin of California and Nevada, but that cut-off was based on a calculation of intra-population divergence within that geographic area rather than being based on minimum and maximum known values of *Hyalella* species divergence, largely because diversity within the genus *Hyalella* appears to be poorly taxonomically characterized. If the 3.75% species-level cut-off suggested by Witt et al. (2006) is applied to the data in the present study, then the Burlington clade, with a maximum within-species divergence of 4.11%, may require further subdivision, as would the Pine Lake clade. Bayesian analysis did not show strong support for either of these subdivisions. For the Pine Lake clade, in particular, however, this subdivision in the Bayesian analysis would have been impossible given that only two haplotypes were found in this clade. Thus, based on the evidence from complete COI nucleotide pairwise distances and Bayesian analysis with consideration of the existence of some clades with very few haplotypes, this study indicated that only the six well-supported clades are sufficiently divergent for consideration as distinct provisional species.

The laboratory organisms sampled in this study belonged to two different clades (US laboratory and Burlington). All of the organisms sequenced from the US laboratories were placed in a single clade, primarily as one of two haplotypes that only differed from another by 0.19%. This low level of genetic diversity at COI among US laboratory sources indicated that all

the US laboratory *H. azteca* can be considered members of the same species. However, Canadian laboratory cultures of *H. azteca* fell into both previously-mentioned clades. Despite reports that the stocks of PYLET, ALET, OMOE, and ATOX were originally seeded by the CCIW stock (T. Watson-Leung, personal communication), our analysis suggested that the former laboratory cultures belonged to the US Laboratory clade, while the CCIW culture was distinctly part of the Burlington clade (roughly 24% divergent from the other laboratories), indicating that at some point, the US Laboratory type replaced the Burlington laboratory type in the previously-mentioned Canadian laboratories. No evidence was found of cultures containing both US Laboratory and Burlington clade representatives. While sample sizes for each laboratory were generally small (2-6 individuals per laboratory), the consistency of this finding over 15 laboratories supports the assertion that the laboratory cultures analyzed were all homogenous at the species-level. Duan et al. (1997, 2000c) used allozymes to delineate relationships among laboratory cultures, and they also observed that three US Laboratory populations used in the present study (EERD, MED, CERC) had profiles that were different at the species level from that of the Burlington population (CCIW).

To some degree, the differences found among the US Laboratory clade and the CCIW cultures was anticipated, as original collections from the US stocks originated from Corvallis, OR, (circa 1982, Duan et al. 2000c) and the CCIW stock from Valens Reservoir, Ontario (circa 1986, W. Norwood, personal communication). Thus, while all sampled US laboratories and most Canadian laboratories employing the same *H. azteca* provisional species, results from this study indicate that CCIW is employing a provisional species that is roughly 24% divergent from the other laboratory strains to conduct their toxicity tests. Given this high level of divergence, and considering that others have shown that even sub-species-level genetic differences can affect

the outcome of toxicity testing with a variety of toxicants (Duan et al. 2000a, 2000b, 2001, but see Eisenhauer et al. 1999), toxicity test data generated using the two provisional species should be compared with caution until more is known about the relative sensitivity of the two provisional species.

Although other studies have documented species-level divergence between laboratory and field-collected populations of *H. azteca* using allozyme analysis (Hogg et al. 1998; Duan et al. 2000c), only one study using allozymes (Duan et al. 2000c) identified laboratory organisms with same-species representatives in a single wild population. In the present study, both provisional species found in laboratory cultures (US laboratory and Burlington clades) were found in wild populations as well, but most (49 out of 68) wild individuals grouped into one of the other four clades that were not closely related to either laboratory group. Few wild representatives of the US Laboratory clade were found. Only four of the 22 *H. azteca* collection sites contained individuals from the US Laboratory clade, and all of these individuals came from springs in Florida. Thus, no representatives of US Laboratory type organisms were identified in any of the remaining locations in Illinois, Michigan, Wisconsin, Vermont or Ontario, although the sampling in the present study was admittedly not systematic. Although only one laboratory (CCIW) harbored members of the Burlington clade, individuals belonging to this clade were identified from four sites in Michigan and one site in Wisconsin. Overall, the US and some Canadian laboratories are using a provisional species that may be more limited in its geographic distribution than the provisional species employed by the Burlington (CCIW) laboratory. Further, neither laboratory provisional species accounts for the genetic diversity found in four of the six clades of *H. azteca* provisional species in the wild, indicating that these laboratory

provisional species are not accurate, species-level representatives of most wild populations of *H. azteca* found in the eastern United States.

Aside from its inclusion as a laboratory provisional species, two interesting points arise when considering the Burlington clade. First, a collection of *Hyalella* sp. from New Mexico (RC-NM) also grouped within this clade. This individual did not possess the dorsal mucronations usually characteristic of *H. azteca*, although Witt and Hebert (2000) also documented *H. azteca* forms without the same mucronations. Contrary to our results, however, the Witt and Hebert (2000) study produced none of these *Hyalella* sp. that grouped closely with any *H. azteca* provisional species containing dorsal mucronations. In this study, the *Hyalella* specimen's placement within the Burlington clade shows noteworthy geographic distribution and morphological variation among members of this clade. Second, although the laboratory organisms from within the Burlington clade (CCIW) were collected from Valens Reservoir, Ontario (circa 1986), recent collections in 2010 made at the same site yielded no representatives from the Burlington clade, but only representatives from the roughly 22% divergent Clear Pond clade (W. Norwood, personal communication). It is possible that this recent wild collection either failed to subsample the original provisional species existing at this site, or that the geographic distribution of these two species have changed in the past two decades. A number of other studies observed the existence of multiple cryptic provisional species of *H. azteca* within a given field site in regions of the US and Canada using COI sequences (Witt et al. 2000, 2006; Stutz et al. 2010; Dionne et al. 2011) and allozymes (Wellborn and Cothran 2004). In this study, multiple (two and sometimes three) provisional species were found at seven different sites across Michigan, Wisconsin, and Illinois indicating that these provisional species often exist in sympatry.

Although for many taxa, COI 'barcoding' regions have proven to effectively delineate taxonomic relationships (Hebert et al. 2003; Costa et al. 2007, 2009) the resolution with which this gene region can separate the *H. azteca* species complex has not been well established given that very little morphological and biological data have accompanied many of the COI-based provisional species distinctions. As evidenced by this study and others (Witt and Hebert 2000; Witt et al. 2003, 2006; Wellborn and Broughton 2008) little is understood about the geographic range of select members of the *H. azteca* species complex. Our analysis of the 680 bp 'barcoding' region of individuals within this study has shown that some species-level distinctions and relationships may not be well-supported by these barcoding COI fragments. Comparison of the genetic data associated with the 680 bp analysis and the entire gene analysis yielded somewhat conflicting results for at least one of the clade divisions supported by this study, although most relationships were supported overall. Focusing on pairwise distance range comparisons, the range of percent divergence within the Pine Lake clade increased from 4.71% to 5.56% when the dataset was reduced to 680 bp. By either species-distinction threshold (*Gammarus* from Costa et al. 2009 or *Hyalella* from Witt et al. 2006), this increase in divergence suggests that the two haplotypes of the Pine Lake clade be subdivided to into two separate provisional species. However, Bayesian analysis with the 680 bp dataset did not provide strong support for the relationship of the two haplotypes within the Pine Lake compared to the entire gene analysis. It is important to add that both species-distinction divergences (Witt et al. 2006 and Costa et al. 2009) were established based on roughly this same 680 bp region analysis. Thus, these cut-off values should be most meaningful to the present study's analysis at a resolution of 680 bp. However, the discrepancies between these species-definition thresholds and the results of the Bayesian analysis at this resolution indicate that caution should be used

when attempting to discriminate closely-related provisional species while using the traditional 'barcoding' region in *Hyalella*.

While some studies have further reduced the resolution at which they attempt to discriminate species-level differences in *Hyalella* using smaller fragments (< 400 bp) within the COI barcoding region (Stutz et al. 2010; Dionne et al. 2011; Stanley 2011), the 335 bp analysis of the present study's data showed that these fragments do not provide strong support for the overall relationships established in *Hyalella* by using the entire gene or the 680 bp 'barcoding' dataset. For example, the 335 bp analysis suggested that the Pine Lake clade be subdivided into two separate provisional species according to either the Costa et al. (2009) or the Witt et al (2006) standards of 4.30 and 3.75% divergence respectively. Furthermore, according to the stricter Witt et al. (2006) standard, the Clear Pond clade would be further subdivided into two provisional species. The Bayesian analysis using 335 bp did not lend support to either of these suggestions, and in fact, failed to resolve the Pine Lake clade from the Burlington clade, although it still supported relationship between the two Pine Lake haplotypes. While the entire gene Bayesian analysis produced six well-supported clades, and the 680 bp analysis produced five, the 335 bp analysis only produced three well-supported clades, and failed to support the major overall branching patterns achieved by the two larger datasets. Aside from failing to resolve the Burlington and Pine Lake clades, the 335 bp analysis also failed to support the separation of the US Laboratory and Joe's Pond clades, and changed the placement of the Joe's Pond clade in relation to the other clades. Although shorter segments within the barcoding region may be effective in determining whether two *H. azteca* individuals are likely to be members of the same provisional species, they do not appear to be effective in delineating the

relationships among the resulting clades that were established in the larger segment Bayesian analyses.

Although the amino acid translation of the COI fragment has been commonly used to ensure that the mitochondrial region of the gene has been successfully sequenced without stop codons (Witt and Hebert 2000; Witt et al. 2003, 2006; Wellborn and Broughton 2008), this is the first study to use the protein translation data to generate phylogenetic relationships among provisional species. The amino acid data were effective in delineating most of the clades established by the entire nucleotide-based gene analysis, but they did not separate the Pine Lake and Burlington clades. While the pairwise DNA sequence comparisons consistently suggested that these two groups be considered separate provisional species, the low amino acid sequence divergence between these two groups instead suggests that perhaps, at a functional level, these two clades should not be separated. It is unknown whether these organisms are reproductively isolated in the locations in which they co-occur. Thus, DNA sequence divergence measurements used to delineate provisional species within *H. azteca* should be used with caution among closely-related groups.

Previous studies involving COI and *H. azteca* have relied on Maximum Likelihood and/or Bayesian Inference to establish relationships among groups within this species complex (Witt et al. 2003, 2006; Wellborn and Broughton 2008). In the present study, while all of the DNA sequence Bayesian analyses produced trees with very similar topologies, the Bayesian analysis of amino acid sequences yielded a very different tree topology. The most likely explanation for this incongruence between the DNA and amino acid datasets lies in the considerable DNA sequence saturation that was observed among *H. azteca* from different clades in this study. As DNA becomes saturated, the phylogenetic signal within the dataset is reduced

significantly, also reducing the likelihood of delineating the true evolutionary relationships among clades when using methods such as Maximum Likelihood and Bayesian Inference to construct a phylogeny (Jeffroy et al. 2006). Thus, the high level of DNA sequence saturation observed in these DNA datasets likely prevented elucidation of the true phylogeny among the six clades of *H. azteca* established by pairwise distance data in this study. As a result of the degeneracy of the genetic code, DNA sequences become saturated much more quickly than amino acid sequences (Jeffroy et al. 2006). Therefore, the most parsimonious approach is to consider the amino acid tree topology produced by the Bayesian analysis as being the most accurate representation of the phylogenetic relationships among the clades rather than those from the complete or fragment nucleotide-sequence-based Bayesian analyses. Despite the advantages of using amino acid sequences to construct phylogenies among divergent datasets, no other amino acid tree topologies have been created by others studying the *H. azteca* species complex. The phylogenetic relationships in previously published studies were established using saturated DNA sequences in conjunction with Maximum Likelihood or Bayesian Inference methods (Witt et al 2003, 2006; Wellborn and Broughton 2008). Although, Witt et al. (2006) also noted DNA sequence saturation at levels of roughly 20% sequence divergence (K2P), they showed congruence between the tree topologies established using Maximum Likelihood methods with highly divergent COI haplotypes and the more conserved nuclear 28S ribosomal corresponding sequences. It is unclear why Witt et al. (2006) observed congruence among their mitochondrial and nuclear datasets. However, data from this study suggest that caution should be used when attempting to recreate phylogenies within *H. azteca* using highly saturated datasets and Maximum Likelihood and Bayesian Inference.

Despite the uncertainty surrounding the use of COI sequence fragments to generate accurate phylogenies in *H. azteca*, the increased use of this gene as a means to delineate provisional species has produced a wealth of GenBank data for *H. azteca* from diverse geographic areas. By analyzing only a 335 bp fragment of the gene and comparing data from the present study with other published sequences, it was possible to identify haplotypes from other studies that grouped within clades established in this study based on pairwise divergence data. In doing so, this study increased the known geographic range of these provisional species. For example, although members of the US Laboratory clade were only identified in Florida springs by the present study, this provisional species is also present in Oklahoma (Wellborn and Broughton 2008). Members of the Burlington clade were collected from Michigan, Wisconsin, and New Mexico in the present study, but GenBank sequences showed that this provisional species has also been found in Ontario, Quebec, and the Yukon Territory, Canada (Witt et al. 2003; Dionne et al. 2011). Although members of the Flat Branch clade were only identified in Illinois and Michigan in our study, other studies have identified this provisional species in Wisconsin, Oklahoma, USA, as well as Ontario, Quebec, and New Brunswick, Canada (Witt et al. 2003; Wellborn and Broughton 2008; Dionne et al. 2011). The Pine Lake provisional species was obtained from Michigan in this study, but other studies have published closely-related sequences obtained from sites in Nevada, Wisconsin, USA, and Ontario, Canada (Witt et al. 2003, 2006). Although our study only identified two individuals from Joe's Pond in Vermont, other members of this provisional species have been documented in Michigan, USA, and Ontario, Quebec, and New Brunswick, Canada (Witt et al. 2003; Wellborn and Broughton 2008; Dionne et al. 2011). Our study also increased the known range of the Clear Pond provisional species from sites in Michigan and Wisconsin, USA, and Ontario, Canada to include sites in

Illinois. Although taxonomic classification of these provisional species is lagging behind the generation of COI sequence data, a more complete understanding of the geographic ranges of members of the *H. azteca* species complex will likely aid in the taxonomic identification of these species in the future. Further, inclusion of existing GenBank data into this study showed Burlington and Pine Lake clades were closely-related to *H. montezuma*. Witt et al. (2006) showed a similar relationship between these sequences and Clade 2, and suggested divergence of *H. montezuma* from *H. azteca* was recent. This study supports that conclusion as evidenced by the low sequence divergences found between *H. montezuma* and the Burlington and Pine Lake clades established in this study.

The present study has shown that although most US and Canadian laboratory stocks of *H. azteca* are homogenous at the species-level, the cultures of one Canadian laboratory population are sufficiently divergent from all other laboratory populations to be considered distinct species. Wild representatives from each of the two laboratory provisional species are present in select wild populations of *H. azteca* across the Eastern US and Canada. However, at least four provisional species in the wild identified by this study and others are not present in laboratory cultures, indicating that the diversity of the *H. azteca* species complex in the wild is not accurately represented in these laboratory cultures. This finding raises questions regarding ability of toxic responses measured in *H. azteca* laboratory organisms to accurately predict the responses of divergent wild populations. Thus, although toxicity testing data generated with laboratory organisms may be applied for the protection of select wild populations, it is uncertain whether these regulations are protective of the rest of the members of the *H. azteca* species complex documented in this and other studies. Given the great diversity found within this cryptic species complex, the COI sequences used in this study were generally effective in

assigning organisms to a given provisional species group, even at the 335 bp resolution, although primarily only when provisional species were distantly related. However, confidence in Bayesian analysis clade support was not strong at this resolution, although the entire COI sequence provided strong support for clades established by pairwise distances. Considering the conflicting bodies of evidence that proposed either further separation (pairwise distances) or grouping (amino acid similarity) of the Pine Lake and Burlington clades, caution should be used when attempting to define provisional species of *H. azteca* that are closely-related given the lack of biological information (i.e. reproductive isolation) that accompanies these separate 'species' living in sympatry. While a single gene is generally insufficient to delineate accurate phylogenetic relationships (Jeffroy et al. 2006), the level of DNA saturation exhibited in these and other *Hyalella* nucleotide datasets (Witt et al. 2003; Wellborn and Broughton 2008) indicates that the most commonly employed Maximum Likelihood and Bayesian Inference methods to reconstruct phylogenies with DNA sequences may lead to inconsistency among the relationships of the clades. Thus, more weight should be given to less saturated datasets in reconstructing phylogenies, such as the amino acid sequences used in this study.

References

Baird DJ, Pascoe TJ, Zhou X, and Hajibabaei M (2011). Building freshwater macroinvertebrate DNA-barcode libraries from reference collection material: formalin preservation vs specimen age. *Journal of the North American Benthological Society,* **30**(1): 125-130.

Baldinger AJ (2004). A new species of *Hyalella* (Crustacea: Amphipoda: Hyalellidae) from Ash Springs, Lincoln County, Nevada, USA, with a key to the species of genus in North America and the Caribbean region. *Journal of Natural History*, **38**: 1087-1096.

Bauza-Ribot MM, Jaume D, Juan C, and Pons J (2009). The complete mitochondrial genome of the subterranean crustacean *Metacrangonyx longipes* (Amphipoda): A unique gene order and extremely short control region. *Mitochondrial DNA,* **20**(4): 88-99.

Borgmann U, Ralph KM, and Norwood WP (1989). Toxicity test procedures for *Hyalella azteca,* and chronic toxicity of cadmium and pentachlorophenol to *H. azteca, Gammarus fasciatus,* and *Daphnia magna*. *Archives of Environmental Contamination and Toxicology,* **18**: 756-764.

Costa FO, deWaard JR, Boutillier J, Ratnasingham S, Dooh RT, Hajibabaei M, and Hebert PDN (2007). Biological identifications through DNA barcodes: the case of the Crustacea. *Canadian Journal of Fisheries and Aquatic Sciences*, **64**: 272-295.

Costa FO, Henzler CM, Lunt DH, Whiteley NM and Rock J (2009). Probing marine *Gammarus* (Amphipoda) taxonomy with DNA barcodes. *Systematics and Biodiversity*, **7**(4): 365-379.

Dionne K, Vergilino R, Dufresne F, Charles F, and Nozais C (2011). No evidence for temporal variation in a cruptic species community of freshwater amphipods of the *Hyalella azteca* species complex. *Diversity*, **3**: 390-404.

Duan Y, Guttman SI, and Oris JT (1997). Genetic differentiation among laboratory populations of *Hyalella azteca*: Implications for toxicology. *Environmental Toxicology & Chemistry,* **16**(4): 691-695.

Duan Y, Guttman SI, Oris JT, and Bailer AJ (2000a). Genotype and toxicity relationships among *Hyalella azteca*: I. Acute exposure to metals and low pH. *Environmental Toxicology & Chemistry,* **19**(5): 1414-1421.

Duan Y, Guttman SI, Oris JT, Huang XD, and Burton GA (2000b). Genotype and toxicity relationships among *Hyalella azteca*: II. Acute exposure to fluoranthene-contaminated sediment. *Environmental Toxicology & Chemistry,* **19**(5): 1422-1426.

Duan Y, Guttman SI, Oris JT, and Bailer AJ (2000c). Genetic structure and relationships among populations of *Hyalella azteca* and *H. Montezuma* (Crustacea:Amphipoda). *Journal of the North American Benthological Society,* **19**(2): 308-320.

Duan Y, Guttman SI, Oris JT, and Bailer AJ (2001). Differential survivorship among allozyme genotypes of *Hyalella azteca* exposed to cadmium, zinc, or low pH. *Aquatic Toxicology*, **54**: 15-28.

Eisenhauer JB, Sullivan KB, and Lydy MJ (1999). Responses of genotypes of *Hyalella azteca* to zinc toxicity. *Bulletin of Environmental Contamination and Toxicology* **63**: 125-132.

Folmer O, Black M, Hoeh W, Lutz R, and Vrijenhoek R (1994). DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, **3**(5): 294-299.

Gonzalez ER and Watling L (2002). Redescription of *Hyalella azteca* from its type locality, Vera Cruz, Mexico (Amphipoda: Hyalellidae). *Journal of Crustacean Biology*, **22**(1): 173-183.

Hebert PDN, Cywinska A, Ball SL, and de Waard JR (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London Series B- Biological Sciences*, **270**(1512): 313-321.

Hogg ID, Larose C, de Lafontaine Y, and Doe KG (1998). Genetic evidence for a *Hyalella* species complex within the Great Lakes – St. Lawrence River drainage basin: implications for ecotoxicology and conservation biology. *Canadian Journal of Zoology*, **76**: 1134-1140.

Huelsenbeck JP and Ronquist F (2001). MRBAYES: Bayesian inference of phylogeny. *Bioinformatics*, **17**: 754-755.

Jeffroy O, Brinkmann H, Delsuc F, and Philippe H (2006). Phylogenomics: the beginning of incongruence? *TRENDS in Genetics*, **22**(4): 225-231.

Ki JS, Hop H, Kim SJ, Kim IC, Park HG, and Lee JS (2010). Complete mitochondrial genome sequence of the Arctic gammarid, *Onisimus nanseni* (Crustacea; Amphipoda): Novel gene structures and unusual control region features. *Comparative Biochemistry and Physiology D-Genomics & Proteomics*, **5**(2): 105-115.

Kimura M (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**: 111-120.

Maddison DR and Maddison WP (2000). MacClade 4: Analysis of phylogeny and character evolution. Version 4.0. Sinauer Associates, Sunderland, Massachusetts.

Posada D and Crandall KA (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics*, **14**(9): 817-818.

Saussure, H (1858). Memoire sur divers crustaces nouveaux des Antilles et du Mexique. *Mémoires de la Société de Physique et d'Histoire Naturelle de Genève*, **14**:417-496.

Smith SI (1875). Report on the amphipod crustaceans of Colorado. Annual Report of the United States Geological and Geographic Survey of the Territories for 1873, 1875:608-611.

Stanley CE (2011). Effects of long-term isolation and metal exposure on the common test species *Hyalella azteca*. Thesis. Oklahoma State University, Stillwater, OK, USA.

Stevenson MM and Peden AE (1973). Description and ecology of *Hyalella texana* n. sp. (Crustacea: Amphipoda) from the Edwards Plateau of Texas. *The American Midland Naturalist*, **89**(2): 426-436.

Strong DR Jr. (1972). Life history variation among populations of an amphipod (*Hyalella azteca*). *Ecology*, **53**(6): 1103-1111.

Stutz HL, Shiozawa DK, and Evans RP (2010). Inferring dispersal of aquatic invertebrates from genetic variation: a comparative study of an amphipod and mayfly in Great Basin springs. *Journal of the North American Benthological Society*, **29**(3): 1132-1147.

Swafford DL (2002). PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Wellborn GA (1993). Ecology and evolution of life history variation among populations of a freshwater amphipod, *Hyalella azteca*. Dissertation. University of Michigan, Ann Arbor, Michigan, USA.

Wellborn GA (1994a). The mechanistic basis of body-size differences between two *Hyalella* (Amphipoda) species. *Journal of Freshwater Ecology*, **9**(2): 159-168.

Wellborn GA (1994b). Size-biased predation and prey life histories – a comparative study of fresh-water amphipod populations. *Ecology*, **75**(7): 2104-2117.

Wellborn GA (1995a). Determinants of reproductive success in freshwater amphipod species that experience different mortality regimes. *Animal Behavior*, **50**: 353-363.

Wellborn GA (1995b). Predator community composition and patterns of variation in life history and morphology among *Hyalella* (Amphipoda) populations in Southeast Michigan. *The American Midland Naturalist*, **133**: 322-332.

Wellborn GA and Cothran RD (2004). Phenotypic similarity and differentiation among sympatric cryptic species in a freshwater amphipod species complex. *Freshwater Biology*, **49**: 1 -13.

Wellborn GA, Cothran R, and Bartholf S (2005). Life history and allozyme diversification in regional ecomorphs of the *Hyalella azteca* (Crustacea: Amphipoda) species complex. *Biological Journal of the Linnean Society*, **84**:161-175.

Wellborn GA and Broughton RE (2008). Diversification on an ecologically constrained adaptive landscape. *Molecular Ecology*, **17**: 2927-2936.

Witt JDS and Hebert PDN (2000). Cryptic species diversity and evolution in the amphipod genus *Hyalella* within central glaciated North America: a molecular phylogenetic approach. *Canadian Journal of Fish and Aquatic Science,* **15**:3073-3082.

Witt JDS, Blinn DW, an Hebert PDN (2003). The recent evolutionary origin of the phenotypically novel amphipod *Hyalella montezuma* offers an ecological explanation for morphological stasis in a closely allied species complex. *Molecular Ecology*, **12**: 405-413.

Witt JDS, Threloff DL, and Hebert PDN (2006). DNA barcoding reveals extraordinary cryptic diversity in an amphipod genus: implications for desert spring conservation. *Molecular Ecology,* **15**: 3073-3082.

Tables and Figures

Table 1.1. Wild *Hyalella* population source locations. *N* is the number of individuals sequenced in the genetic analysis from each site. All sites yielded samples of *H. azteca*, except for RC-NM, which yielded *Hyalella* sp.

Code	*N*	Waterbody	County	State/Province	Country	Latitude (N)	Longitude (W)
LS-FL	4	Lowry Park Spring Outflow	Hillsborough	FL	USA	28.0149	82.4676
TL-FL	2	Trout Lake Marsh	Hillsborough	FL	USA	28.0134	82.4664
PS-FL	2	Peacock Spring	Suwannee	FL	USA	30.1220	83.1322
CS-FL	1	Convict Springs	Suwannee	FL	USA	30.0885	83.0963
IC-IL	4	Indian Creek	Jackson	IL	USA	37.6557	89.1798
FB-IL	3	Flat Branch	Coles	IL	USA	39.5997	88.3214
PB-IL	2	Pope Branch	Edgar	IL	USA	39.8141	87.9273
CP-IL	4	Clear Pond	Vermilion	IL	USA	40.1381	87.7417
PC-IL	2	Panther Creek	Perry	IL	USA	38.0235	89.3337
EC-IL	2	Eagle Creek	Saline/Gallatin	IL	USA	37.6558	88.3743
JP-IL	2	Japan House Pond	Champaign	IL	USA	40.0932	88.2168
ER-IL	2	East Fork Embarras River	Champaign	IL	USA	39.9451	88.1231
RC-NM	1	Marsh/Pond of the Rio Cebolla Stream	Sandoval	NM	USA	35.8784	106.6570
IV-MI	7	Ives Lake	Marquette	MI	USA	46.8437	87.8548
TL-MI	2	Marsh/Pond of the Trout Lake Drainage	Marquette	MI	USA	46.8534	87.8884
PR-MI	4	Pine River	Marquette	MI	USA	46.8831	87.8687
PL-MI	3	Pine Lake	Marquette	MI	USA	46.8804	87.8677
MP-MI	6	Middle (2nd) Pine Lake	Marquette	MI	USA	46.8699	87.8559
TR-MI	5	Tittabawasee River	Saginaw	MI	USA	43.4812	84.0924
JP-VT	2	Joe's Pond	Washington/Caledonia	VT	USA	44.4069	72.2203
WL-WI	4	Winnebago County Lake	Calumet	WI	USA	44.1126	88.3253
LR-WI	2	La Crosse River	La Crosse	WI	USA	43.9008	90.9901
VR-ON	2	Valens Reservoir	Hamilton[1]	ON	Canada	43.3867	80.1333

[1] Hamilton is a city, as counties do not exist in this region of Canada.

Table 1.2. Laboratory population source locations. *N* is the number of individuals sequenced from each laboratory population.

Code	*N*	Population	City	State/Province	Country	Facility[1]
INHS	6	Illinois Natural History Survey	Champaign	IL	USA	AL
OSU	2	Oklahoma State University	Stillwater	OK	USA	AL
ASU	2	Arkansas State University[2]	Jonesboro	AK	USA	AL
SIU	2	Southern Illinois University	Carbondale	IL	USA	AL
TTU	3	Texas Tech University	Lubbock	TX	USA	AL
ABS	2	Aquatic BioSystems, Inc.	Fort Collins	CO	USA	CS
ARO	6	Aquatic Research Organisms, Inc.	Hampton	NH	USA	CS
EERD	2	USEPA[3] Ecological Exposure Research Division	Cincinnati	OH	USA	RAL
MED	2	USEPA[3] Mid-Continental Ecology Division	Duluth	MN	USA	RAL
CERC	3	US Geological Survey Columbia Environmental Research Center	Columbia	MO	USA	RAL
ATOX	2	AquaTox Testing & Consulting, Inc	Guelph	ON	Canada	CL
OMOE	2	Ontario Ministry of the Environment	Etobicoke	ON	Canada	RAL
PYLET	2	Environ. Can. Pacific and Yukon Laboratory for Environmental Testing	North Vancouver	BC	Canada	RAL
ALET	2	Environ. Can. Atlantic Laboratory for Environmental Testing	Moncton	NB	Canada	RAL
CCIW	2	Environ. Can. Canada Centre for Inland Waters	Burlington	ON	Canada	RAL

[1]AL = Academic Laboratory, CS = Commercial Source, RAL = Regulatory Agency Laboratory, and CL = Consulting Laboratory.
[2] Samples from Arkansas State University were sent indirectly through Oklahoma State University.
[3] USEPA = United States Environmental Protection Agency.

Table 1.3. Primers for used for entire COI gene amplification followed by clade-based success for select members of the *H. azteca* species complex. ~ = moderate success, X = consistent success, and blank spaces indicate a lack of data.

			Clade[1]					
Primer Name[2]	Position[3]	Sequence (5'-3')	US	FB	CP	BU	PL	JP
TRNA-1F-D	-130	TTTAAGTTATWWAAACTAAGARCCTTCAAAG	~					
COII-2R-D	+30	GTWCCYACATGATCTTCRYTMGGG		X	X	X	X	
COII-1R-D	+190	CTTGGTYTWTAWTWWAYTTTCAAGATAGA	~					
LCO1490F[4]	20	GGTCAACAAATCATAAAGATATTGG						
TRNA-2F-US	-81	TGTGTGATTAAGTCTAACAGATCTTAAG	X					
TRNA-5F-US	-71	GATCTTAAGTTATACAAACTATAAGCCTTCCAAGCT	X	X	X	X	X	
TRNA-6F-US	-71	GATCTTAAGTTATACAAACTATAAGCCTTCC	X	X	X	X	X	
TRNA-3F-US	-62	TTATACAAACTATAAGCCTTCCAAGCT	X					
TRNA-4F-US	-42	TATGAGCCTTCCAAGCT	X		X	X	X	
COI-370F-US, COI-370R-US	370	GGATGAACTGTCTACCCTCCATTG	X					
COI-516F-US	516	TCCAGGGATAAGAATGGAGCGAGTGCC	X					
COI-596F-US	596	CTGTCTTGGCCGGGGCTATTACGA	X					
COI-763F-US	763	CATATTGTGAGAGAGGAGTCTGGT	X					
COI-1099R-US	1099	CATGATACTTATTATGTAGTGGCACA	X					
COI-1136R-US	1136	GCTATCTATAGGAGCAGTATTCGG	X					
COI-1312F-US	1312	CGGTATTCTGATTATCCGGACTC	X					
COII-1R-US	+185	CCGGCTACTTCTGCAAGAGCAGGTAGTT	~	~				
COII-2R-US	+139	GGGCTTCTCCGAGAATAGAGCAG	X					
TRNA-1F-FB	-53	TTATAAAAACTATGAGCCTTCCAAGCT		X				
COI-517R-FB	517	CCAGGTATGACAATAGACCGAGTCCC		X				
COI-610F-FB, COI-610R-FB	610	GCAATTACTATGCTACTGACTGACCG		X				
COI-813F-FB	813	CATAATCTATGCTATACTGGCCCATC		X				
COI-884F-FB	884	GGATAGACGTAGACACACGAGCG		X				
COI-884F-EC-IL	884	GGATAGACGTAGACACGCGGGCG		X				
COI-1099R-FB	1099	TGAGCCACTACATAATAGGTGTCGTG		X				
COII-1R-FB	+99	GTTCCCACATGATCTTCGCTAGGGC		~	X			
COII-2R-FB	+139	TGGTTATTACGCTAATCACTGTGACGGGTCGC		X				X
COI-517R-CP	517	CCAGGAATGAGAATAGACCGAGTGCC			X			
COI-610F-CP, COI-610R-CP	610	GCTATTACTATGCTTCTTACTGACCG			X			
COI-883F-CP	883	GGATGGACGTAGACACACGAGCT			X			
H2ORMIL-CP	877	ACAGTAGGAATGGACGTAGACA			X			
COI-1139F-CP	1139	TGTCTATGGGGGCTGTATTCGGC			X			
L2v2[5]	448	CATTTGGCAGGTGCTTCTTC				~		
L2v2-BU	448	CATCTGGCAGGTGCGTCTTC				X		
L2v2-PR-MI	448	CATTTGGCAGGTGCCTCTTC				~	X	
L2v2-TR-MI	448	CATCTGGCCGGGGCCTCTTC				X	~	
L2v2-PL-MI	448	CATCTGGCGGGTGCGTCTTC					X	
COI-517R-BU	517	TCGGGGATAAGAATAGACCGAGTGCC				X		
H2ORMIL[5]	887	ACAGTGGGAATAGACGTTGACA				X	X	
COI-1079F-BU	1079	CGTCTATCGATGTAGTGCTTCA				X	X	
COI-1139F-BU	1139	TATCTATGGGGGCAGTATTCGGC				X	X	
COI-1557R-BU	1557	GTGACAGAGTTAAAGCCTG				~	~	
L2v2-JP	448	CATTTGGCAGGGGCTTCTTC						X
COI-517R-JP	517	CCTGGGATAAGAATGGAGCGAGTGCC						X
COI-610F-JP, COI-610R-JP	610	GCTATTACAATCCTGCTAACTGACCG						X
COI-884F-JP	884	GAATGGATGTAGACACTCGAGCG						X

[1] Clade abbreviations are as follows: US = US Laboratory, FB = Flat Branch, CP = Clear Pond, BU = Burlington, PL = Pine Lake, JP = Joe's Pond.

[2]Primers are named for the mitochondrial region in which they are located, followed by a primer number or the 5' placement of the primer within COI (if applicable), followed by their designation as either forward (F) or reverse (R), followed by the two letter abbreviation of the clade for which they were designed. If followed by a four letter, hyphenated location abbreviation (see Table 1.1 for location abbreviations), primer was developed for individuals from a specific location. Primers that do not follow this naming scheme were either created by or adapted from publications. If adapted from publications, original primer name is appended by the four letter location abbreviation for individuals for which they were modified or the two letter abbreviation of the clade for which they were modified.

[3]Position is relative to the 5' end of COI in *H. azteca* sp. (1569 bp). Negative values indicate approximate position upstream of COI. Positive values indicate approximate position downstream of the end of COI. Numbers without positive/negative designation indicate the starting position of the 5' end of each primer within COI.

[4]Folmer et al. (1994).

[5]Wellborn and Broughton (2008).

Table 1.4. Most successful primer combinations for amplifying the entire COI gene and flanking regions or fragments within COI in select members of the *H. azteca* species complex. ~ = moderate success, X = consistent success.

Primer Combination[1]			Approx. Fragment	Annealing	Clade[3]					
Forward	X	Reverse	Size[2]	Temperature (°C)	US	FB	CP	BU	PL	JP
TRNA-2F-US	X	COII-2R-US	1810	53.0	X					
TRNA-IF-FB	X	COII-2R-FB	1790	53.0		X	X			X
TRNA-5F	X	C01-610R-FB	710	55.0		X				
L2v2-PR-MI	X	COII-2R-D	1170	55.0		X				
TRNA-F5-US	X	H2ORMIL-CP	980	55.0			X			
TRNA-4F-US	X	H2ORMIL-CP	950	50.0			~			
L2v2-TR-MI	X	COII-2R-D	1150	55.0			X			
TRNA-5F-US	X	H2ORMIL	980	55.0				X	X	
TRNA-4F-US	X	H2ORMIL	950	50.0				~	~	
L2v2-PL-MI	X	COII-2R-D	1150	55.0				X	X	
L2v2-BU	X	COII-2R-D	1150	55.0				X	X	

[1]For a list of primer details, see Table 1.3.

[2]Fragment sizes have been approximated to the nearest 10 bp because of the uncertainty of base counts outside of the COI gene region.

[3] US = US Laboratory, FB = Flat Branch, CP = Clear Pond, BU = Burlington, PL = Pine Lake, JP = Joe's Pond.

Table 1.5. Models and parameter details for each of three Bayesian analyses on COI datasets of different lengths in *H. azteca*. All nucleotide substitution models were selected by Akaike Information Criterion using ModelTest as the best-fit of 56 models.

Dataset	Best-Fit	Base Frequencies				MCMC	Among-Site Rate Variation[1]		Rate Matrix of Substitution Model[2]					
	Model	A	C	G	T	Generations	I	G	R(a)	R(b)	R(c)	R(d)	R(e)	R(f)
1569 bp	GTR+I+G	0.2480	0.1761	0.2062	0.3698	$3x10^6$	0.4616	0.8557	0.6453	13.2539	0.7533	2.1770	15.9014	1.0000
680 bp	TVM+I+G	0.2407	0.1870	0.2110	0.3613	$3x10^6$	0.4811	0.8366	0.293	11.2236	0.6323	1.5122	11.2236	1.0000
335 bp	TIM+I+G	0.2566	0.1791	0.2258	0.3385	$2x10^6$	0.5363	0.8903	1.000	41.0144	4.9145	4.9145	70.1965	1.0000

[1] I = the proportion of invariable sites, G = gamma distribution shape parameter.
[2]R(a) = A-C, R(b) = A-G, R(c) = A-T, R(d) = C-G, R(e) = C-T, R(f) = G-T.

Table 1.6. Pairwise sequence divergence as K2P (mean%±sdev±stderr; (min, max)) within and among *H. azteca* clades using the entire COI gene sequence (1569 bp). Numbers immediately below the clade names are the number of individuals in each clade used to make comparisons.

Clade	US Laboratory	Flat Branch	Clear Pond	Burlington	Pine Lake	Joe's Pond
	47	25	18	12	4	2
US Laboratory	0.49±0.55±0.02 (0, 2.21)	21.40±0.25±0.01 (20.61, 22.25)	23.29±0.25±0.01 (22.16, 23.96)	24.13±0.37±0.02 (23.14, 24.90)	25.36±0.33±0.02 (24.19, 25.60)	19.94±0.11±0.01 (19.57, 20.11)
Flat Branch	- -	0.93±0.89±0.05 (0, 3.14)	22.12±0.38±0.02 (21.24, 23.16)	21.01±0.39±0.02 (20.25, 22.32)	22.35±0.29±0.03 (21.76, 23.04)	19.98±0.25±0.04 (19.41, 20.47)
Clear Pond	-	-	1.10±0.68±0.05 (0, 2.34)	21.59±0.32±0.02 (20.81, 22.50)	21.19±0.30±0.04 (20.70, 21.91)	22.34±0.31±0.05 (21.76, 23.05)
Burlington	-	-	-	1.77±1.54±0.19 (0, 4.11)	8.02±0.64±0.09 (6.98, 9.27)	23.00±0.38±0.08 (22.33, 23.80)
Pine Lake	-	-	-	-	2.35±2.58±1.05 (0, 4.71)	21.97±0.20±0.07 (21.65, 22.08)
Joe's Pond	-	-	-	-		0.00±0±0 (-)

Table 1.7. Field sites with *H. azteca* from multiple clades based on pairwise divergence (K2P) and the Bayesian analysis of the entire COI gene (1569 bp).

	Individuals in Each Clade			
Field Site[1]	Flat Branch	Clear Pond	Burlington	Pine Lake
CP-IL	2	2	-	-
TR-MI	2	3	-	-
IV-MI	2	2	3	-
PR-MI	2	2	-	-
WL-WI	-	2	2	-
PL-MI	-	-	1	2
MP-MI	-	3	1	2

[1]For field site abbreviations, see Table 1.1.

Table 1.8. Pairwise sequence divergence as uncorrected *p*-distance (mean%±sdev±stderr; (min, max)) within and among *H. azteca* clades using the entire translated COI amino acid sequence (523 aa). Numbers immediately below the clade names are the number of individuals in each clade used to make comparisons.

Clade	US Laboratory 47	Flat Branch 25	Clear Pond 18	Burlington 12	Pine Lake 4	Joe's Pond 2
US Laboratory	0.22±0.20±0.01 (0, 0.76)	3.36±0.19±0.01 (2.87, 3.85)	3.80±0.19±0.01 (3.25, 4.21)	4.63±0.17±0.01 (4.23, 5.00)	4.75±0.20±0.01 (4.40, 5.16)	2.93±0.11±0.01 (2.68, 3.06)
Flat Branch	-	0.19±0.18±0.01 (0, 0.77)	3.60±0.23±0.01 (3.06, 4.23)	4.19±0.21±0.01 (3.65, 4.81)	4.34±0.22±0.02 (4.02, 5.00)	3.27±0.12±0.02 (3.06, 3.65)
Clear Pond	-	-	0.27±0.19±0.02 (0, 0.76)	3.58±0.26±0.02 (3.08, 4.23)	3.61±0.18±0.02 (3.25, 4.02)	3.94±0.16±0.03 (3.63, 4.21)
Burlington	-	-	-	0.52±0.37±0.05 (0, 1.35)	0.57±0.36±0.05 (0.19, 1.54)	4.95±0.12±0.02 (4.81, 5.19)
Pine Lake	-	-	-	-	0.29±0.31±0.13 (0, 0.57)	5.07±0.18±0.06 (4.97, 5.35)
Joe's Pond	-	-	-	-	-	0.00±0±0 (-)

Table 1.9. Pairwise sequence divergence as K2P distance (mean%±sdev±stderr; (min, max)) within and among *H. azteca* clades using the 'barcoding' region of COI (680 bp). Numbers immediately below the clade names are the number of individuals in each clade used to make comparisons.

Clade	US Laboratory 47	Flat Branch 25	Clear Pond 18	Burlington 12	Pine Lake 4	Joe's Pond 2
US Laboratory	0.53±0.54±0.02 (0, 1.94)	22.00±0.34±0.01 (21.17, 22.97)	24.58±0.37±0.01 (22.94, 25.15)	25.35±0.43±0.02 (24.19, 26.18)	26.26±0.40±0.03 (24.81, 26.55)	19.58±0.22±0.02 (19.36, 20.19)
Flat Branch	- -	0.77±0.78±0.05 (0, 2.72)	23.31±0.50±0.02 (22.25, 24.84)	23.27±0.38±0.02 (22.34, 24.61)	23.79±0.28±0.03 (23.23, 24.53)	20.90±0.35±0.05 (20.03, 21.53)
Clear Pond	-	-	1.39±0.93±0.8 (0, 3.02)	22.36±0.51±0.03 (21.38, 23.89)	20.40±0.91±0.11 (19.06, 22.46)	24.03±0.39±0.06 (23.37, 24.92)
Burlington	-	-	-	1.58±1.44±0.18 (0, 3.80)	8.26±0.71±0.10 (6.87, 9.76)	23.55±0.44±0.09 (22.86, 24.50)
Pine Lake	-	-	-	-	2.78±3.04±1.24 (0, 5.56)	23.83±0.73±0.26 (22.65, 24.22)
Joe's Pond	-	-	-	-	-	0.00±0±0 (-)

Table 1.10. Pairwise sequence divergence as K2P (mean%±sdev±stderr; (min, max)) within and among *H. azteca* clades using a subset of the 'barcoding' region of COI (335 bp). Numbers immediately below the clade names are the number of individuals in each clade used to make comparisons.

Clade	US Laboratory 47	Flat Branch 25	Clear Pond 18	Burlington 12	Pine Lake 4	Joe's Pond 2
US Laboratory	0.43±0.44±0.01 (0, 1.52)	22.94±0.60±0.02 (21.10, 24.75)	27.10±0.91±0.03 (24.36, 28.82)	26.23±0.62±0.03 (24.55, 27.89)	29.59±0.88±0.06 (26.29, 30.41)	20.36±0.26±0.03 (19.77, 21.00)
Flat Branch	-	0.73±0.68±0.04 (0, 2.44)	28.82±0.71±0.03 (26.92, 30.41)	24.32±0.77±0.04 (22.02, 26.51)	26.51±1.05±0.10 (25.12, 28.77)	19.80±0.36±0.05 (18.94, 20.62)
Clear Pond	-	-	1.71±1.12±0.09 (0, 4.36)	22.59±0.96±0.07 (20.19, 25.08)	20.94±0.92±0.11 (19.68, 23.13)	25.67±0.83±0.14 (24.43, 27.25)
Burlington	-	-	-	1.44±1.39±0.17 (0, 3.40)	6.97±0.57±0.08 (6.29, 8.69)	23.10±0.41±0.08 (22.59, 23.92)
Pine Lake	-	-	-	-	2.81±3.08±1.26 (0, 5.62)	25.53±1.06±0.37 (23.82, 26.10)
Joe's Pond	-	-	-	-	-	0.00±0±0 (-)

Table 1.11. Unique *Hyalella* haplotypes identified within each clade considering the complete COI gene versus the number of unique haplotypes identified by fragments of the same gene.

		Unique haplotypes		
Clade	*N*	1569 bp	680 bp	335 bp
Overall	108	61	48	38
US Laboratory	47	12	11	8
Flat Branch	25	20	12	9
Clear Pond	18	16	14	12
Burlington	12	10	8	6[1]
Pine Lake	4	2	2	2[1]
Joe's Pond	2	1	1	1

[1]In the 335 bp Bayesian analysis, the Burlington and Pine Lake clades were actually grouped into the same clade. Values represent the number of unique haplotypes based on the separation of each clade established in the 1569 bp analysis.

Table 1.12. Relationships among the clades defined by the present study (at resolution of 1569 bp) and the closest COI sequences available for *Hyalella* on GenBank. Distances are corrected (K2P) pairwise comparisons made from the 335 bp fragments alignments.

Relationships of select H. azteca provisional species to the provisional species in the present study

Present Study's Clade	Source of Related *H. azteca* Genbank Sequences	Related Clade from Publication	GenBank Accession Number	Geographic Region State (US)/Province (Can)	Distances (Min - Max) to Present Study's Clade
US Laboratory	Wellborn and Broughton 2008	OK-L Clade	EU621727-EU621728	Oklahoma	0.00 - 3.06%
Flat Branch	Witt et al. 2003	C6	AY152770- AY152773; AY152797	Wisconsin, Ontario, New Brunswick	
	Wellborn and Broughton 2008	C Clade	EU621745, EU621748, EU621750, EU621751	Michigan, Oklahoma	
	Dionne et al. 2011	Haplotype 5	JN161817	Quebec	0.00 - 4.34%
Clear Pond	Witt et al. 2003	C3	AY152762-AY152764, AY152766	Wisconsin, Ontario	
	Wellborn and Broughton 2008	A Clade	EU621755, EU621758, EU621762	Michigan	0.00 - 4.18%
Burlington	Witt et al. 2003	C1	AY152752-53, AY152755-56. AY152757-58, AY152791, AY152798-99, AY152801, AY152804	Wisconsin, Ontario, Yukon Territory	
	Wellborn and Broughton 2008	OR/MI-L Clade	EU621738, EU621740	Michigan	
	Dionne et al. 2011	Haplotype 1	JN161821	Quebec	
	Baird et al. 2011	(unclassified)	HM138024, HM138031-32	-	0.00 - 4.68%
Pine Lake	Witt et al. 2003	C2	AY152759-AY152761, AY152794	Wisconsin, Ontario	
	Witt et al. 2006	HaPS16	DQ464660	Nevada	0.90 - 5.63%
Joe's Pond	Witt et al. 2003	C5	AY152769	Ontario, New Brunswick	
	Wellborn and Broughton 2008	B Clade	EU621730, EU621732	Michigan	
	Dionne et al. 2011	Haplotypes 2-4	JN161818-JN161819	Quebec	0.90 - 1.81%

Relationships of H. montezuma to the provisional species in the present study

Present Study's Clade	Source of Related *Hyalella* GenBank Sequences	GenBank Accession Numbers	Geographic Region State (US)/Province (Can)	Distances (Min - Max) to Present Study's Clade
Burlington	Witt et al. 2003	AY152805-AY152807	Arizona	6.61 - 9.72%
Pine Lake	Witt et al. 2003	AY152805-AY152807	Arizona	6.97 - 7.32%

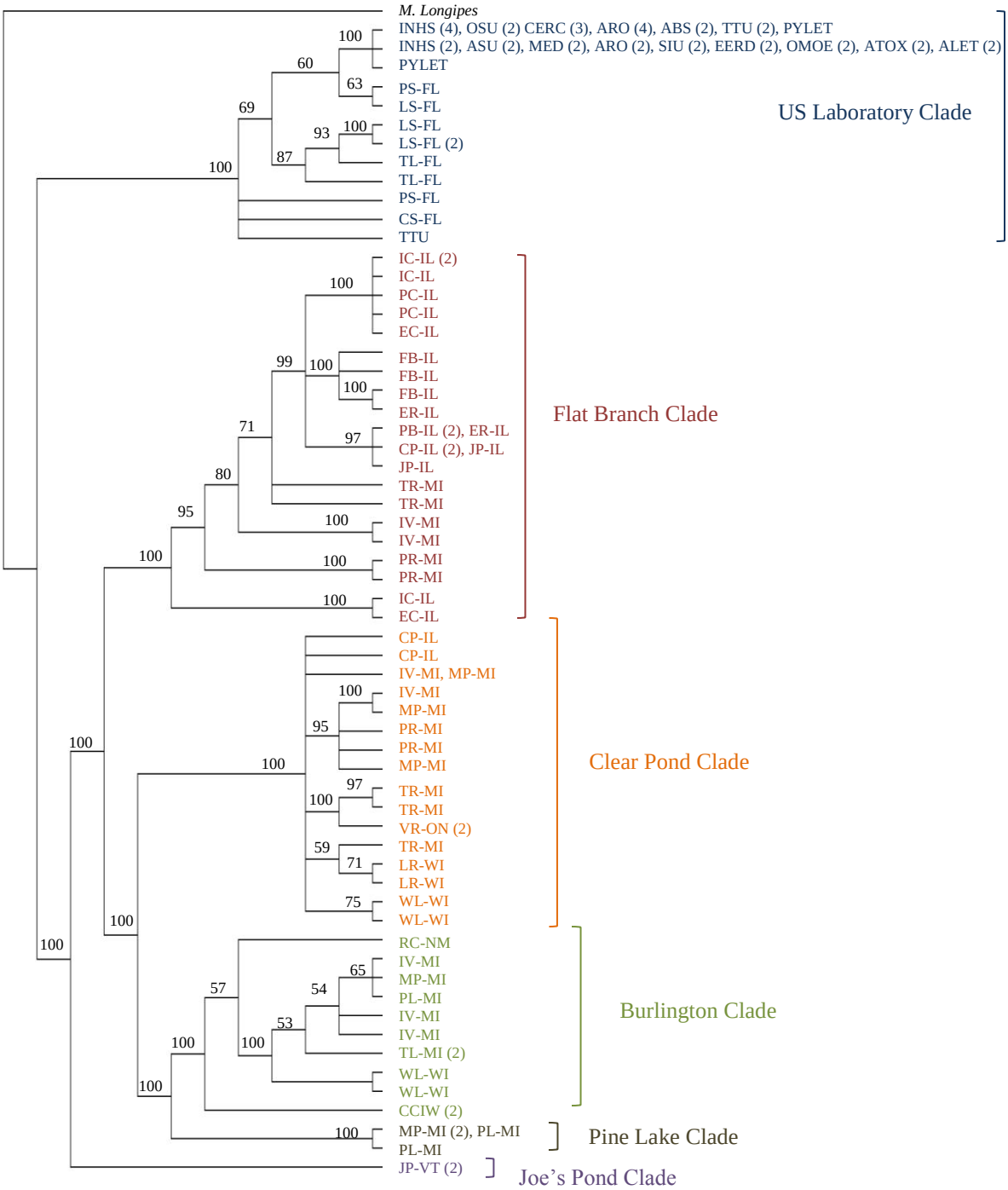

Figure 1.1. Bayesian tree resulting from the 61 unique *Hyalella* haplotypes of 108 entire COI sequences (1569 bp) with *M. longipes* as the outgroup. Individuals are indicated by field site or laboratory abbreviations (see Tables 1.1 and 1.2 for abbreviations). Numbers in parentheses immediately following abbreviations indicate multiple individuals from the same site or laboratory. All individuals are *H. azteca* except for the individual from RC-NM which is an unidentified *Hyalella* sp. Numbers at nodes indicate posterior probabilities, and posterior probabilities lower than 95 are not considered significant. Clades were established by a combination of pairwise distance comparisons (K2P) and Bayesian tree posterior probability support.

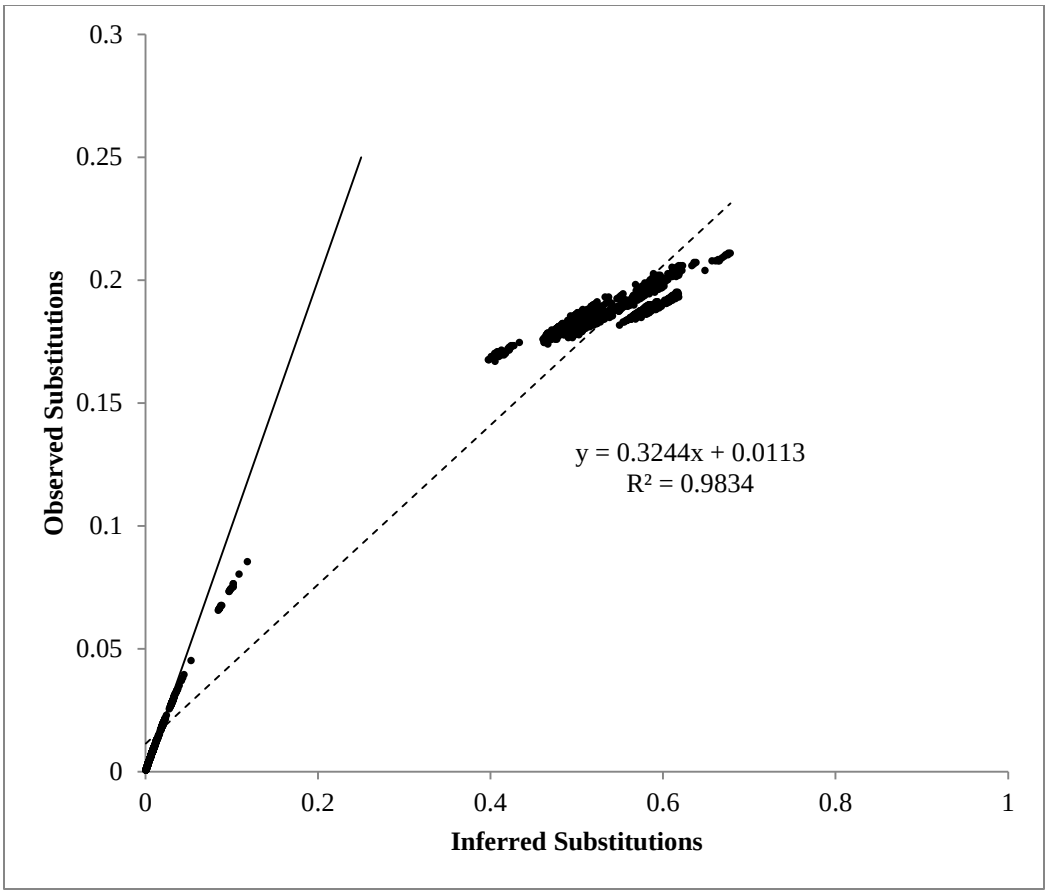

Figure 1.2. Observed substitutions established by uncorrected *p*-distances (y-axis) versus inferred substitutions established by pairwise distances corrected with the model used in the Bayesian analysis (x-axis) for 108 individuals using the entire COI gene (1569 bp). The solid line has a slope of 1 and represents sequence data that are not saturated. The dotted line is the linear relationship among the pairwise comparisons of sequences in this study. A slope of 0.32 indicates sequence saturation as established by Jeffroy et al. (2006).

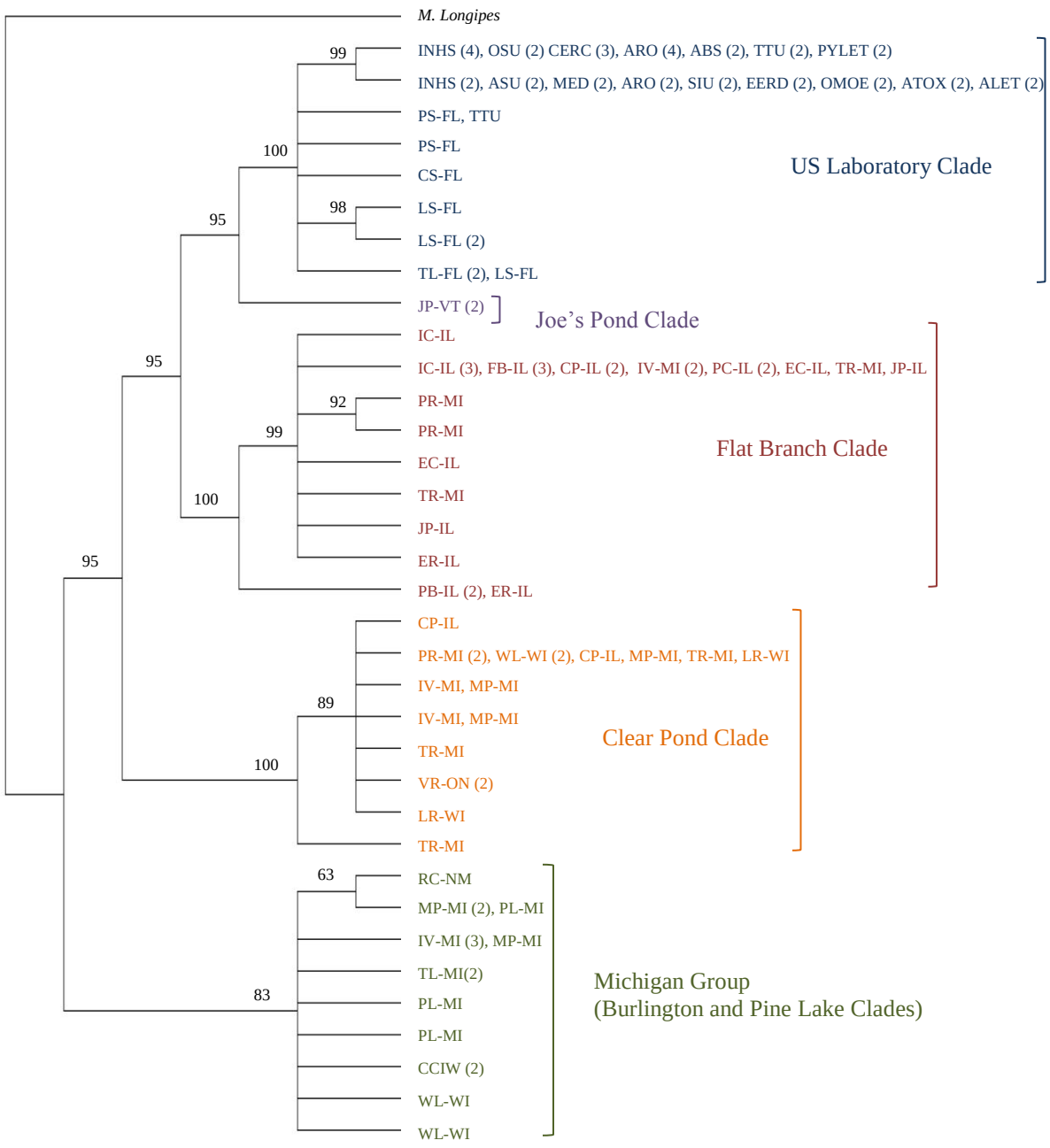

Figure 1.3. Bayesian tree resulting from the 35 unique *Hyalella* haplotypes of 108 translated COI amino acid sequences (523 aa) with *M. longipes* as the outgroup. Individuals are indicated by field site or laboratory abbreviations (see Tables 1.1 and 1.2 for abbreviations). Numbers in parentheses immediately following abbreviations indicate multiple individuals from the same site or laboratory. All individuals are *H. azteca* except for the individual from RC-NM which is an unidentified *Hyalella* sp. Numbers at nodes indicate posterior probabilities, and posterior probabilities lower than 95 are not considered significant. Clades were established by a combination of pairwise distance comparisons (K2P) and Bayesian tree posterior probability support.

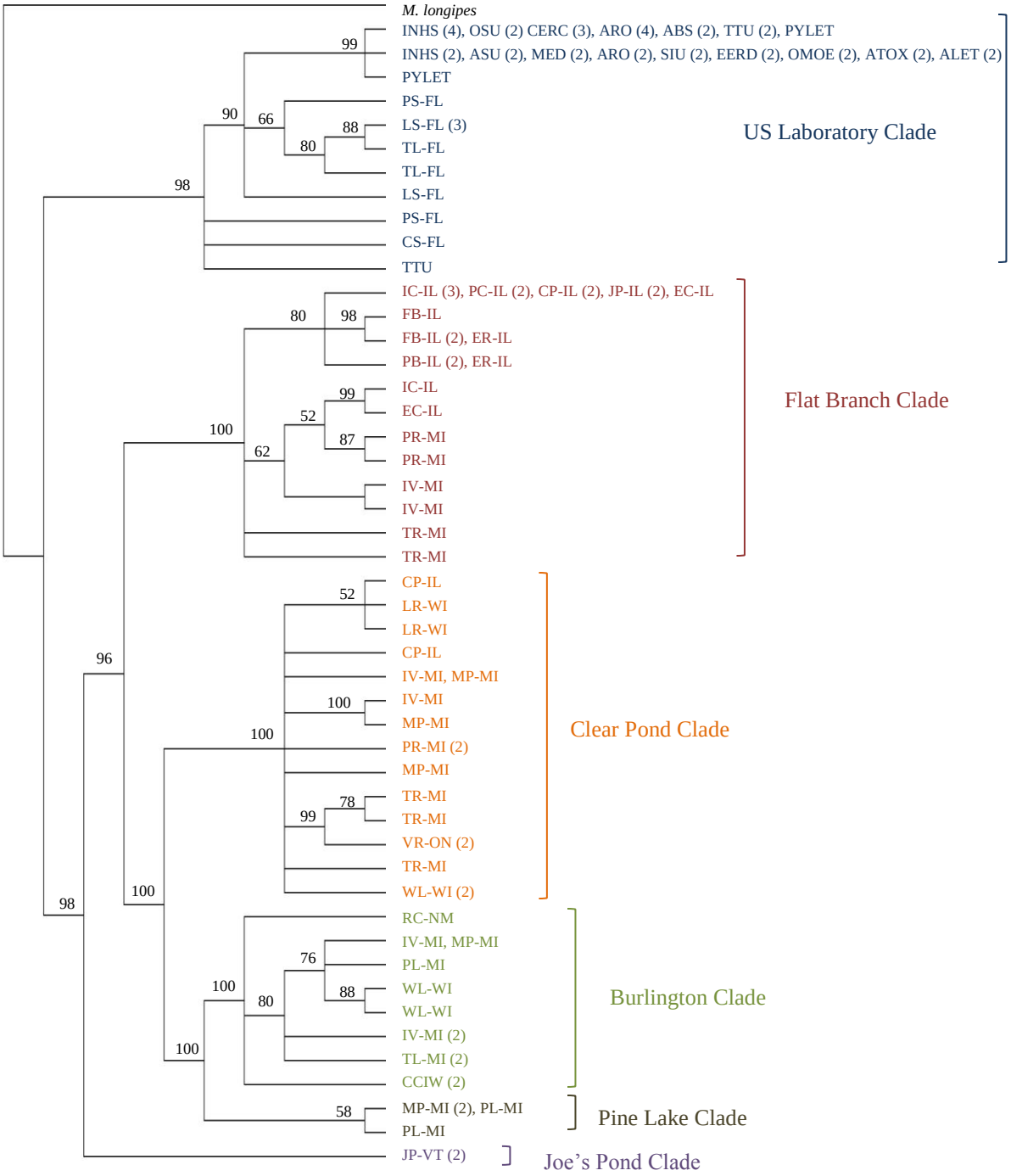

Figure 1.4. Bayesian tree resulting from the 48 unique *Hyalella* haplotypes of 108 COI 'barcoding' region nucleotide sequences (680 bp) with *M. longipes* as the outgroup. Individuals are indicated by field site or laboratory abbreviations (see Tables 1.1 and 1.2 for abbreviations). Numbers in parentheses immediately following abbreviations indicate multiple individuals from the same site or laboratory. All individuals are *H. azteca* except for the individual from RC-NM which is an unidentified *Hyalella* sp. Numbers at nodes indicate posterior probabilities, and posterior probabilities lower than 95 are not considered significant. Clades were established by a combination of pairwise distance comparisons (K2P) and Bayesian tree posterior probability support.

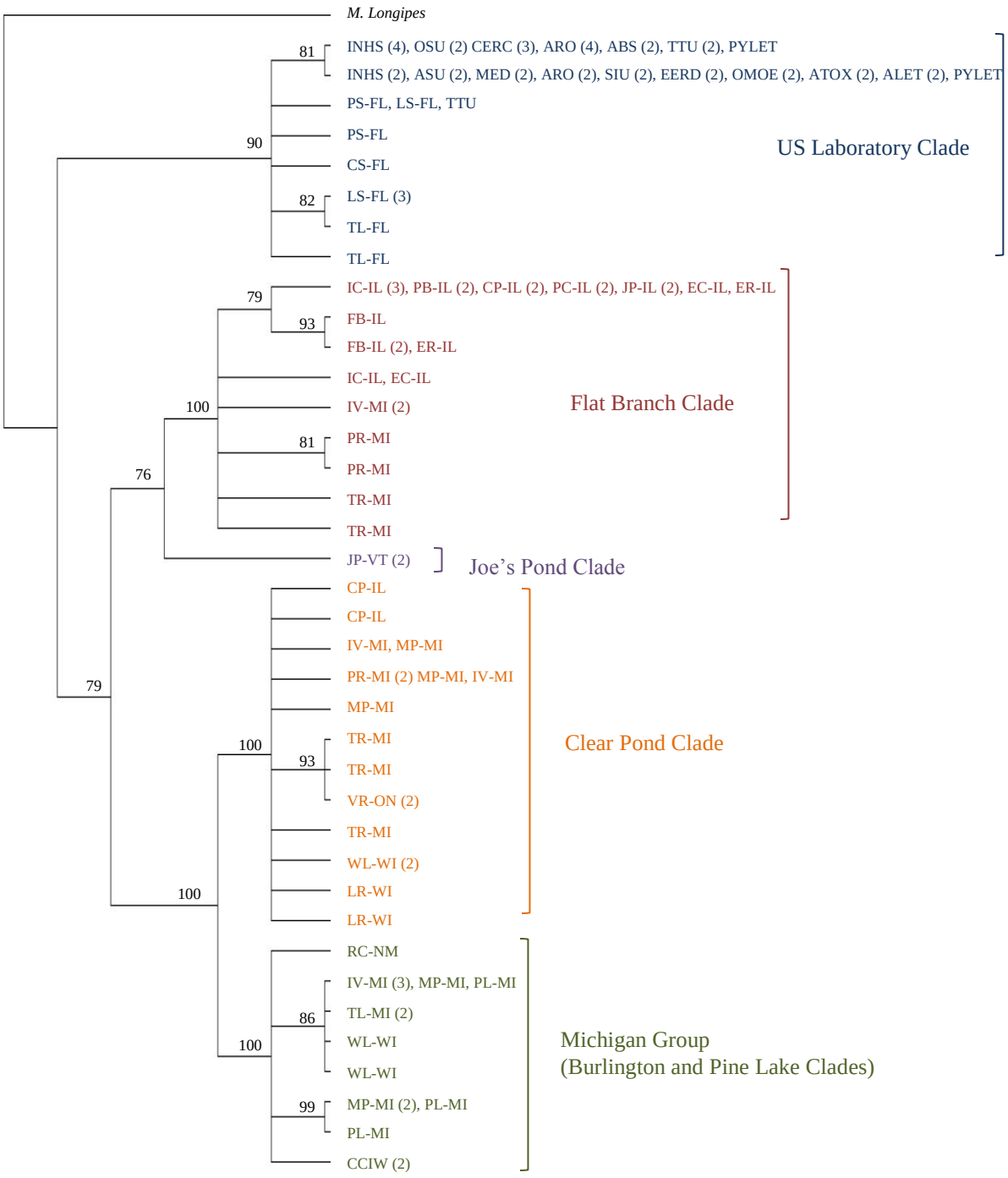

Figure 1.5. Bayesian tree resulting from the 38 unique *Hyalella* haplotypes of 108 COI 'barcoding' region subset nucleotide sequences (335 bp) with *M. longipes* as the outgroup. Individuals are indicated by field site or laboratory abbreviations (see Tables 1.1 and 1.2 for abbreviations). Numbers in parentheses immediately following abbreviations indicate multiple individuals from the same site or laboratory. All individuals are *H. azteca* except for the individual from RC-NM which is an unidentified *Hyalella* sp. Numbers at nodes indicate posterior probabilities, and posterior probabilities lower than 95 are not considered significant. Clades were established by a combination of pairwise distance comparisons (K2P) and Bayesian tree posterior probability support.

CHAPTER 2: USE OF WATER-ONLY CHRONIC TOXICITY TEST METHODS TO COMPARE LIFE HISTORY CHARACTERISTICS OF FOUR GENETICALLY-DISTINCT CLADES OF *HYALELLA* SP.

Introduction

Ecotoxicological studies frequently use laboratory-reared surrogate species to predict the effects of toxicants on wild species. The epibenthic amphipod *Hyalella azteca* is one of these laboratory surrogates, and is frequently used to test both water-column and sediment toxicity. However, strong phenotypic (Strong 1972; Wellborn 1993; Wellborn 1994a,b; Wellborn 1995 a,b; Wellborn et al. 2005) and species-level genetic variation (Duan et al. 1997; Hogg et al. 1998; Witt and Hebert 2000, Witt et al. 2003, 2006; Wellborn and Cothran 2004; Wellborn and Broughton 2008) suggest that *H. azteca* is in fact a species complex. In the last 12 years, multiple studies across the United States (US) and Canada have employed cytochrome *c* oxidase subunit I (COI) as a marker to document species-level differences among wild members of this complex. In these studies, sequence divergences ranged from 4-29% (Witt and Hebert 2000; Witt et al. 2003, 2006; Wellborn and Broughton 2008), often with a single location harboring multiple provisional species (Witt and Hebert 2000; Witt et al. 2006; Stutz et al. 2010; Dionne et al. 2011). In my own COI analysis of wild populations from sites previously unsampled in the US and Canada (Chapter 1), I found comparable levels of divergence among six different clades (provisional species), and on several occasions collected multiple provisional species from the same sampling site.

Although many ecological studies have shown that *H. azteca* is a species complex in the wild, few studies have focused on the relatedness and genetic composition of the laboratory stocks of *H. azteca* (Duan et al. 1997, 200a-c; Eisenhauer et al. 1999; Stanley 2011; also see Chapter 1). These laboratory stocks, although originally collected from wild populations, have

been maintained in state and federal agency and academic institution laboratories for many years. In addition, the stocks are maintained at several commercial sources that regularly distribute organisms to laboratories for toxicity testing. While Duan et al. (1997) observed population and even species-level differences among different laboratory cultures using allozymes, my own analysis of COI sequences of organisms from 15 different laboratories revealed that nearly all laboratory populations in the US and Canada can be considered the same species (< 1.6% divergence at COI, Chapter 1). However, a stock from one Canadian agency laboratory was composed of a provisional species roughly 24% divergent (at COI) from this US laboratory type.

While my data suggest that US and Canadian laboratory populations of *H. azteca* are rather uniform at COI (given the one Canadian exception), the laboratory populations differed from many of the wild populations. Using COI as a marker, I identified at least six distinct clades (provisional species) of *H. azteca*, and among 22 field sites sampled in the US and Canada. Only three sites in Florida were inhabited by the same provisional *H. azteca* species as the US laboratory type. More specifically, the US laboratory type was not found at any of the 16 sites sampled in the Midwestern US. In agreement with this finding, Hogg et al. (1998) compared allozyme polymorphisms in six wild populations from the Great Lakes – St. Lawrence River drainage to that of one laboratory population of *H. azteca* and found that the laboratory population was indeed different enough from wild populations to be considered a separate species. This means that, for example, in the Midwestern US, toxicants are being tested on a provisional species that has not been found in Midwestern surface waters (in this study's admittedly limited search), and any resulting regulations on those toxicants might not necessarily be protective of the organisms actually living in Midwestern lakes and streams. Conversely, although one Canadian laboratory population was significantly different from the US laboratory

type, that Canadian provisional laboratory species was prevalent in samples collected from field sites across the US and Canada (Chapter 1).

The variation documented among *H. azteca* populations in the wild and in the laboratory has the potential to be especially problematic in the context of toxicity testing. Although an understanding of the connection between traits and toxic response is lacking in these organisms, several studies have tested the relationships between allozyme polymorphisms in laboratory populations of *H. azteca* and toxic responses of these organisms. Studies with heavy metals and acidic pH demonstrated that significantly different mortality occurred based on allozyme genotype in each group of *H. azteca* exposed or that survival patterns after acute exposures were dependent on genotype (Duan et al. 200a,b, 2001, but see Eisenhauer et al. 1999). Thus, variation in genotype, even at a population-level, appears to affect an organism's response in toxicity tests.

Toxicity is assessed by measuring the deficits that occur in population survival, growth, and reproduction with exposure to a chemical when compared to its normal survival, growth, and reproductive capacities without that chemical stressor. These 'normal' levels of survival, growth, and reproduction under a given set of conditions can more generally be referred to as life history characteristics. Given the genetic divergence documented between the two laboratory provisional species in Chapter 1, it is expected that the sensitivities of these populations to a given toxicant may be different. Aside from expected variations in sensitivities to chemicals, it is also likely that the two laboratory provisional species also differ in growth and reproductive rates. Others have documented differences in life history characteristics among members of the *H. azteca* species complex, (Strong 1972; Wellborn 1993, 1994a,b; 1995 a,b; Wellborn and Cothran 2004; Wellborn et al. 2005; Wellborn and Broughton 2008), and some of these

populations were the same as the provisional species identified in each of the laboratory clades in Chapter 1 (US Laboratory clade and Burlington clade). Because so little is understood about the potential life history differences among these provisional species under the laboratory-rearing conditions that precede toxicity testing, it is important to quantify these differences before attempting to quantify toxicity on a population-level. Otherwise, differences among laboratories or between populations in endpoints measured in chronic toxicity tests may not indicate so much the effect of a given chemical as much as the inherent characteristics of the provisional species being used in the test. Comparison of the toxicity results obtained by testing two different "species" becomes misleading and inappropriate if the controls in those tests will not even produce organisms with similar 'normal' measures of survival, growth, and reproduction because the two populations possess different life history characteristics under a given set of conditions.

In an effort to understand how genetically-based differences in life-history characteristics might influence results of chronic toxicity tests conducted with different provisional species of *H. azteca*, the present study was designed to quantify the life history characteristics of genetically-distinct wild and laboratory populations. Test populations were chosen from four genetically distinct clades (20-25% divergent from each other at COI) identified in the previous genetic analysis generated with complete COI sequences (Chapter 1). The study groups included three populations from US laboratories, one population from a Canadian laboratory, and two wild populations from different sites in Illinois. The primary objective of this study was to acclimate all of these populations to a common set of controlled of laboratory culturing conditions for a minimum of three generations (to minimize environmental and maternal effects) and to test these populations/clades side-by-side as treatments, measuring all of the same endpoints that are regularly measured in chronic toxicity tests with *H. azteca*. The inclusion of

three different populations from one genetic clade was meant to determine how variable survival, growth, and reproduction are among different populations of the same clade or provisional species.

Methods

Test populations

To compare life history characteristics, populations of *H. azteca* were obtained from a variety of laboratory, commercial, and wild sources (Table 2.1), acclimated to Illinois Natural History Survey (INHS) (Champaign, IL) laboratory conditions, and allowed to reproduce for several generations prior to testing. The US laboratory populations included those maintained in the INHS laboratory (Champaign, IL, hereafter referred to as INHS), originally obtained from the commercial source Environmental Consulting & Testing (ECT), Inc. (Superior, WI), the US Environmental Protection Agency's Mid-Continent Ecology Division laboratory (Duluth, MN, hereafter referred to as MED), and the commercial source, Aquatic BioSystems (ABS) Inc. (Fort Collins, CO, hereafter referred to as ABS). In addition to the US laboratory populations, a population from Environment Canada's Canada Centre for Inland Waters Laboratory (Burlington, ON, hereafter referred to as CCIW) was also obtained and acclimated to INHS conditions. Finally, two wild populations of *H. azteca* were also included in this study and were collected from separate watersheds in Illinois: Flat Branch (FB-IL) near Humboldt, in Coles County, IL, and Clear Pond (CP-IL) in Kickapoo State Park, Vermilion County, IL (hereafter referred to as FB-IL and CP-IL, respectively). The wild populations were collected using a D-framed net to disturb submerged or emergent macrophytes and/or algal mats on rocks. Contents of the net were then emptied into a plastic tray, and a plastic transfer pipette was used to separate

H. azteca adults from other organisms. Roughly 100 adults were collected from each field site and brought back to the INHS laboratory to begin acclimation to laboratory conditions. Upon initiation of culture, representatives from each population included in this study were morphologically identified as *H. azteca* based on the keys provided by Stevenson and Peden (1973) and Baldinger (2004).

These test populations were chosen based on preliminary genetic analyses of the COI mitochondrial gene (Chapter 1). The three US laboratory clade populations (INHS, MED, and ABS) exhibited very little variation from one another at COI (< 0.2%). The CCIW stock (Burlington clade) diverged from the previously-mentioned US laboratory organisms by roughly 24% at COI. The two wild populations collected from FB-IL and CP-IL (Flat Branch and Clear Pond clades, respectively) each had a percent divergence on the order of 20-25% from each other and the INHS, MED, ABS, and CCIW populations. Because divergence at COI within the members of the same species of the sister genus *Gammarus* is typically under 4% (Costa et al. 2009), divergence on the order of 20-25% gives strong evidence that these populations comprise entirely separate species, and thus measurable differences in life history characteristics were expected among the highly divergent populations. Conversely, divergences observed among the US laboratory populations (< 0.2%) classify these organisms as members of the same species, and life history characteristics among these organisms were expected to vary less.

Culture and holding of test organisms

Upon receipt or collection, the six test populations used in this study were cultured for at least three generations under the same conditions to reduce potential variability due to environmental acclimation or maternal effects. Culture conditions were as follows: roughly 20-

40 *H. azteca* individuals were kept in 1L glass beakers filled with 1L of a reconstituted laboratory water developed by Borgmann (1996, hereafter referred to as Borgmann water), with nitex mesh as substrate. All beakers were kept in an environmental chamber with photoperiod of 16:8 L:D and a temperature of 25±1ºC. Each beaker received 2.5 to 5 mg daily (depending on organism age/body size) of Tetramin Tropical Flakes© (TetraWerke, Melle, Germany), hereafter referred to as Tetramin. Tetramin was ground with a mortar and pestle, sieved to < 500 µm, and then added to culture water at 1 mg solid per 1 ml Borgmann water immediately prior to feeding, and suspensions were never kept overnight. Cultures were also fed 2 ml of a wheatgrass suspension (2 mg dry wheatgrass per ml deionized water) daily. Wheatgrass was obtained from ABS Inc. (Fort Collins, CO). The nitex mesh screens used for substrate were "conditioned" before being placed in culture beakers based on preliminary results that screen conditioning promoted better organism survival, growth, and reproduction (D. Soucek personal communication). Conditioning was achieved by placing screens in a 1L beaker filled with 1L of Borgmann water and adding 5 mg of ground Tetramin and 2 ml of wheatgrass suspension daily until screens developed a green biolfilm coating. Once biofilm on a screen was well-established, it was used to seed a new group of conditioned screens. In culture, the feeding regime was designed so that food was not limiting, but also to avoid an excess of food that might degrade water quality.

Complete water changes were performed twice a week, and survival and reproduction were assessed at that time. Offspring were either discarded, used to start a subculture, or, once enough generations had been maintained under our laboratory conditions (at least three), collected for use in a life history test. When collected for test use, offspring were removed from mating adults and placed in a separate 1L beaker of water with a conditioned nitex screen when

they were 0-4 days old, although specific age range depended on the actual test (Table 2.2). They were then acclimated to a temperature of 23±1 ℃ (standard testing temperature, US EPA 2000) in a separate growth chamber for seven days until they reached an age range of roughly 6-11 days old, at which time the life history test was initiated.

Test conditions and measurement of life history characteristics

At 6-11 days old, one-week survival was assessed, and if found to be favorable (typically above 90%), these organisms were used in a life history test. To compare life history characteristics, a 42-day water column toxicity test without a toxicant was used to assess the survival, growth, and reproduction of these laboratory organisms under chronic test conditions. Using the USEPA (2000) 42-day sediment toxicity testing methods as a guide, test chambers consisted of 300 mL glass beakers filled with 200 mL of Borgmann water and a 2.5 x 5 cm conditioned nitex screen as substrate. Test conditions were held constant at 23±1 ºC and 16:8 LT:DK. Each test chamber constituted a replicate, and five to seven replicates existed for each treatment (Table 2.2), with ten individuals per replicate. Further details on common test conditions are provided in Table 2.3. Rather than a contaminant concentration or sediment source, treatments consisted of a population source (either from a US or Canadian laboratory or a wild Illinois population). Three separate 42-d tests were performed, with two to five population treatments used per test (Table 2.2).

At the beginning of each test, ten organisms from each population were pulled from the group of potential test organisms and frozen in 95% ethanol for later determination of a mean starting dry weight of organisms from each population, to be used for growth calculations. Complete water changes were performed three times per week (Monday, Wednesday, and

Friday), and feeding occurred on scheduled intervals with known amounts of Tetramin and wheatgrass. Tetramin feeding rates were modified from those utilized by the Environment Canada Laboratory (Burlington, ON) for chronic sediment toxicity testing. This general feeding regime, per replicate chamber, was as follows: Week 1: 1.0 mg Tetramin 7x per week, weeks 2-3: 1.25 mg Tetramin 7x per week, weeks 4-6: 2.5 mg Tetramin 7x per week. The wheatgrass feeding rate was 1 ml of a 2mg/ml suspension per replicate every Monday, Wednesday and Friday. This rate schedule was designed to increase food ration as the organisms grew without contributing to significant deterioration of water quality parameters including pH and dissolved oxygen, and to mimic rates used in laboratory-rearing conditions. However, feeding regimes in the life history tests varied slightly by test (Table 2.2) due to the accumulation of excess food in some treatments, largely attributed to the disparity among mean organism size across the different populations.

With each water change, survival and reproduction (number of surviving offspring) were assessed. Outgoing water chemistry parameters including pH, conductivity, and dissolved oxygen concentration of the test water were also measured at this time. The pH measurements were made using an Accumet® (Fisher Scientific, Pittsburgh, PA) model AB15 pH meter equipped with an Accumet® gel-filled combination electrode (accuracy $< \pm 0.05$ pH at 25 °C). Dissolved oxygen was measured using an air-calibrated Yellow Springs Instruments (RDP, Dayton, OH) model 55 meter. Conductivity measurements were made using a Mettler Toledo® (Fisher Scientific, Pittsburgh, PA) model MC226 conductivity/TDS meter. On day 42, at the conclusion of each test, survival and reproduction were assessed, and all test organisms were fixed in 95% ethanol, sexed, and dried for no less than 3 days at 75 ºC as were the 6-11-day-old organisms subsampled at test initiation from each population. Individuals were then weighed to

the nearest 0.001 mg using a Cahn C-35 microbalance (range: 1.0 µg – 250 mg). Growth was measured as the difference between the dry weight of each individual and the mean dry weight of the ten organisms taken from each population at the time of test initiation.

Statistical analysis

At the end of each test, life history characteristics encompassing survival (proportion survival, proportion surviving females, proportion surviving males), growth (mean growth, taken as mean final dry weight less mean starting weight), biomass (total dry weight of organisms per beaker multiplied by number of surviving individuals) and reproduction (young per surviving adult, young per surviving female, and total reproduction as the total offspring produced per replicate) were calculated for each replicate. Each chronic test was separately analyzed using the TOXSTAT® statistical program (WEST, INC. and Gulley 1996), following the recommendations for chronic toxicity testing statistical analysis provided by US EPA (2000). First, each variable distribution was checked for normality using the Shapiro-Wilks test. If the distribution passed normality, then the variances were checked for homogeneity using the F-test (Test #1 with only two treatments) or Bartlett's Test (Tests #2 and 3 with more than two treatments). Upon passing normality and variance homogeneity (alpha = 0.01), a Two-Sample t-Test (two treatments) or Tukey's Test (three or more treatments) were used to determine whether differences existed among treatment means. If test variable distributions were not normally distributed, then the Wilcoxon Rank-Sum Test with Bonferonni's adjustment (two treatments) or the Kruskal-Wallis (three or more treatments) nonparametric test were used to determine differences in treatment means. All means were tested at a significance level of alpha = 0.05.

The relationship between size (both growth and biomass) and reproduction was assessed via linear regression, but no significant positive correlations were noted.

Results

Forty-two-day life history Tests #1-3

Within all three life history tests, treatments did not vary significantly in mean survival, proportion males, or proportion females. Total reproduction had very high variability and was not included in the statistical analysis. No major differences in water chemistry were observed during any of the tests, and mean values for measured water chemistry parameters may be found in Table 2.4.

At the end of life history Test #1, test populations INHS and FB-IL had survival means of 95.7% and 90.0%, respectively (Table 2.5), but the INHS treatment had mean 42-d growth and biomass approximately quadruple that of the FB-IL treatment. In addition, reproduction for the INHS population was nearly double that of FB-IL in terms of both the mean #young/adult and the mean #young/female (Table 2.5). Reproduction began on test day 24 for INHS and test day 31 for FB-IL.

Life history Test #2 included five treatments (INHS, ABS, MED, CCIW, and FB-IL), and mean survival ranged from 82.0% to 94.0% (Table 2.5). Analysis of mean growth values yielded four significantly different groups ranked from largest to smallest: INHS, ABS, MED and CCIW, and FB-IL (Table 2.5). Mean biomass analysis values were ranked similarly, with INHS being significantly greater than MED and CCIW, but biomass for ABS was not significantly different from either INHS or MED and CCIW. The FB-IL population had significantly lower biomass than all of the other treatments. Mean #young/female was

significantly higher for INHS than both CCIW and FB-IL, with ABS and MED falling in between these two groups (Table 2.5). Although mean #young/adult did not differ significantly between treatments, the general nominal trends mirrored those of #young/female. First reproduction occurred at test day 24 for INHS, test day 26 for ABS, MED, FB-IL, and test day 28 for CCIW.

Life history Test #3 included 5 treatments (INHS, ABS, MED, CCIW, and CP-IL), with survival means ranging from 70.0% to 94.0% (Table 2.5). Despite the increased survival range, survival did not vary significantly among treatments. Analysis of mean growth among the treatments indicated that ABS, INHS, CCIW, and MED were all significantly greater than CP-IL. In addition, ABS had significantly greater growth than MED (Table 2.5). Similar trends were observed for biomass, with ABS, INHS, and CCIW all being significantly greater than MED and CP-IL. For mean #young/adult, ABS was significantly higher than both MED and CP-IL and was nominally higher than both INHS and CCIW. The #young/female trends were similar, with ABS being nominally greater than INHS, and statistically greater than CCIW, MED, and CP-IL (Table 2.5). However, in the analysis of #young/female, one replicate from each of CCIW and CP-IL was removed from the analysis because although reproduction occurred in those beakers, the end of the test yielded no surviving females, thus leaving the parameter without a denominator. Day of first reproduction varied from test day 23 for INHS, test day 25 for ABS, MED, and CCIW, and test day 28 for CP-IL.

Pooled life history tests by population and by clade

Although life history endpoints for common populations from separate tests could not be combined for statistical analysis, means over the three tests were calculated to observe general

trends (Table 2.6). Because CP-IL was included only in Test #3, no standard deviations of the means were calculated for this group. Unless otherwise noted in this section, it is assumed that all life history endpoints are mean values of the mean values from all similar treatments within the three life history tests previously described. The lowest survival percentage was from CP-IL (70.0%) and the highest mean was for ABS (94.0%). Aside from CP-IL, all mean survival values were greater than 80% (Table 2.6).

By population, growth measurements suggested a division into three general size-groups, with INHS and ABS comprising the first, CCIW and MED being the intermediate group, and CP-IL and FB-IL being the smallest (Table 2.6). Based on the clade classifications established in Chapter 1, grouping the US laboratory types (INHS, ABS, MED) together yielded a mean growth of 0.986 mg (Table 2.6). Because CCIW, FB-IL, and CP-IL populations each represent their own distinct clades (Burlington, Flat Branch, and Clear Pond, respectively), growth measurements for these treatments did not change based on population or clade analysis. Thus, grouping US Laboratory types together reduced the division of size classes established by the population grouping from 3 to 2, with US Laboratory and Burlington in a size class generally two-to-three-fold larger than FB-IL and CP-IL in the smaller size class. Biomass trends mirrored those of growth for both population- and clade-based pooling (Table 2.6).

Initial samples of 6-11 day-old organisms indicated that, on a population basis, CCIW was the largest of all the groups at that age, followed by MED, INHS, ABS, CP-IL, and FB-IL. When grouping these initial dry weights by clade, Burlington remained the largest, followed by the US Laboratory and then Clear Pond and Flat Branch (Table 2.6).

Reproductive capacity on a population basis revealed that INHS and ABS had higher reproductive rates than all of the other populations (MED, CCIW, FB-IL, CP-IL) by roughly double in terms of #young/adult and by two-to three-fold larger in terms of #young/female. On a clade basis, the US Laboratory Clade reproduced (in terms of #young/female) at roughly double the rate of Burlington, Flat Branch, and Clear Pond. However, a different pattern was observed for #young/adult, with the Burlington clade being closer to the US Laboratory clade than either Flat Branch or Clear Pond (Table 2.6).

Discussion

A 42-d chronic toxicity testing framework showed that the four provisional species of the *H. azteca* complex had different life history characteristics. In general, the US and Canadian laboratory populations had 6-to-11-day-old-dry weight and adult growth and biomass measurements that were two- to four-times larger than those of either wild population collected from Illinois. Reproduction of the US Laboratory populations was higher than that of the Canadian laboratory population, which was in turn higher than reproduction of the wild Flat Branch and Clear Pond populations.

Several authors have focused on the genetic and life history characteristic differences that abound within wild members of the *H. azteca* complex (Wellborn 1993, 1994ab, 1995ab, Wellborn and Cothran 2004; Wellborn et al. 2005; Wellborn and Broughton 2008). These studies have supported the repeated evolution of provisional species with either small or large-bodied phenotypes as a result of the selection pressure (or release) associated with fish predation risk. In Chapter 1, my genetic analysis indicated that several provisional species, particularly those studied by Wellborn and Cothran (2004), Wellborn et al. (2005), and Wellborn and

Broughton (2008), represented the same provisional species used in this life history study. While some of the provisional species documented by other authors (Witt and Hebert 2000; Witt et al. 2003, 2006; Baird et al. 2011; Dionne et al. 2011) were also closely related to organisms in this study, those from Wellborn and Broughton's (2008) genetic dataset were particularly valuable because select life history characteristics were evaluated for these organisms as well. For the US Laboratory and Burlington clades (Chapter 1) the Wellborn and Broughton (2008) representatives (OK-L Clade and OR/MI-L Clade, respectively) were both characterized as having a large-bodied adult phenotype. Comparatively, the representatives from Wellborn and Broughton (2008) in the 'C Clade' (corresponding to the Flat Branch clade) and the 'A Clade' (corresponding to the Clear Pond clade) displayed a small-bodied phenotype. These results are consistent with my findings that the populations within the US Laboratory and Burlington clades are larger than those in the Flat Branch or Clear Pond clade populations.

Regarding reproductive life history characteristics, Wellborn et al. (2005) found that both the large species (OK-L/US Laboratory clade and OR/MI-L/Burlington clade) had clutch sizes roughly twice as large as the small bodied species (C/Flat Branch clade and A/Clear Pond clade). In the present study, although reproduction was measured in terms of #young/adult and #young/female, higher reproduction was noted in the US Laboratory clade (OK-L Clade) compared to all other clades. Both reproductive measurements were lower among the Burlington (OR/MI-L Clade), Flat Branch (C clade) and Clear Pond (A Clade) populations. Further, the Flat Branch (C Clade) population had reproductive measurements that were higher than those from the Clear Pond (A Clade) population, although these measurements were not directly comparable on a statistical level because these populations were never included within the same test.

Wellborn and Cothran (2004) characterized the 'A Clade' and the 'C Clade' as being generally indistinguishable from one another with no significant differences in reproduction (clutch size).

Reasons for the discrepancy among the reproductive trends in this study and those reported in Wellborn and Cothran (2004) and Wellborn et al. (2005) are unclear, but could include: 1) differences in maturation period, 2) incomplete acclimation to test conditions, and 3) sub-optimal food and/or culture water. Previous studies by Strong (1972) with wild populations from Oregon, suggested that although adult body size and growth rates differed among cryptic provisional species of *H. azteca*, maturation period did not. It was reasonable to assume that the present study's design could sufficiently characterize differences among cryptic species' reproductive capacities without considering differences in reproductive schedules, because maturation period appears to be a conserved trait among other very divergent groups within *H. azteca*. If maturation periods were different among species, then the relatively short time span of the 42-d chronic test would have skewed higher reproductive values towards populations with earlier reproductive maturity. The seven-day reproductive lag noted in the FB-IL population compared to the INHS population in Test #1 may indicate that the FB-IL organisms develop on a different schedule than INHS organisms, although a lag of that magnitude did not occur in subsequent tests. In Tests #2 and 3, the first day of reproduction was similar for all populations tested (days 24-28 and 23-28, respectively), with a majority of the populations first reproducing on the same day within that range. In each test, however, the last populations to reproduce were populations in which lower reproductive capacities were measured.

In addition to the possibility of the provisional species having different reproductive schedules, another explanation for the differences in reproduction among populations and could be that the acclimation period for these wild and originally-external laboratory populations was

not sufficient. In general, the range of environmental conditions (i.e. temperature, water quality, light and dark regimes, etc.) experienced by wild populations of the *H. azteca* species complex is likely to be greater than that experienced by INHS laboratory populations. Although less variable, the conditions experienced by other laboratory populations also differ from those of the INHS laboratory, as permitted by a variety of recommended methods for culturing *H. azteca* USEPA (2000). Although many life-history differences among populations from different environmental regimes are assumed to be adaptive, environmental heterogeneity may contribute to these measurable differences through mechanisms such as gene regulation and maternal effects (Lam and Calow 1989). Despite the present study's multi-generational (at minimum three) laboratory acclimation to control for prior environmental heterogeneity, it is possible that these acclimation periods were not sufficient for complete removal of these effects.

A third explanation for the reproductive discrepancies among the present study and the study by Wellborn and Cothran (2004) is sub-optimal laboratory culturing conditions. It is likely that the present study's feeding regime and/or food source was not the most favorable for members of the small-bodied clades, as evidenced by the need to alter feeding schedules during the tests to prevent water quality deterioration in the Flat Branch and Clear Pond treatments. Because the details of the laboratory acclimation conditions used by Wellborn and Cothran (2004) were not reported, it is possible that they differed significantly from those employed in this study. However, the precedent of laboratory conditions having an effect on life history measurements among different members within this species complex has been recorded by Strong (1972), particularly regarding food source. Therefore, it is possible that these variations could have had an impact on measurable life history characteristics in this study.

Aside from measuring the differences in body size and reproduction in divergent populations, my goal was to test genetically similar populations to determine whether divergence at COI (Chapter 1) was a good predictor of life history endpoints. For this assessment, three genetically similar (< 0.2% at COI, Chapter 1) populations were included as members of the US Laboratory clade. Among these populations (INHS, ABS, MED), size and reproductive measurements were generally similar, although MED ranked lowest in growth, biomass, #young/male, and #young/female than either of the other US Laboratory populations. Instead of grouping statistically with other US Laboratory populations, MED grouped with the other clades for six of the eight size and reproductive measurements taken in this study. Shortly after this population was received by the INHS laboratory, the original stock population was terminated because of the presence of a parasite that appeared to be affecting survival, growth, and reproduction. Although this parasite was not documented in the sample received from the MED population, it is possible that this population had underlying health issues prior to arriving in the INHS laboratory. The remaining members of the US Laboratory clade showed consistent life history measurements, statistically grouping with one another for seven of the eight size and reproductive measurements taken in this study. Thus, even with all US Laboratory organisms nearly genetically indistinguishable at the species level, measurable differences in life history characteristics could be detected for reasons that are not entirely clear. Considering that culture conditions (i.e. food source, water quality, container size, light/dark regime, temperature, etc.) for *H. azteca* vary on a per-laboratory basis, it is possible that the laboratory acclimation period for external laboratory populations was not sufficient (as described above). However, these laboratory-rearing regimes may also have contributed to sub-species level genetic effects that possibly reduced fitness in select populations. Stanley (2011) used AFLP methodology to show

noteworthy population-level genetic variation among select US Laboratory clade populations, indicating that measureable population-level genetic differences were present among different US Laboratory cultures. Thus, caution should be used in assuming that conspecific laboratory populations will produce similar life history characteristic measurements, especially when considering that the contribution of genetic composition and environmental factors to life history characteristics are not well understood in *H. azteca* (US) laboratory individuals.

The present study showed that the genetic divergence documented among select laboratory and wild provisional species of *H. azteca* corresponded to measurable life history characteristic differences in a laboratory-acclimated toxicity testing context. Under the laboratory conditions employed by this study, any attempt to quantify and understand toxic responses by comparing results obtained between any two of the provisional species populations will be likely confounded by inherent differences in their basic life history characteristics. Thus, toxicity tests performed using the members of the US Laboratory and Burlington clades should not be directly pooled for the creation of toxicity standards because of the marked genetic and life history differences that occur between these two provisional species, particularly in reproductive rate. The efficacy of using toxicity tests performed with either laboratory group to infer population-level effects in and protect divergent wild populations is also further drawn in question based on the life history differences observed in this study. Although the reproductive measurements among some clades did not entirely correspond to comparable measurements for these genetic groups in published literature (Wellborn and Cothran 2004; Wellborn et al. 2005), finding the optimal food for each of the provisional species might may yield reproductive measurements that better-correspond to the published studies. These deviations from anticipated results among divergent wild populations as well as very similar US Laboratory populations also

indicates that the contribution of genetic and environmental factors that govern the outcome of survival, growth, and reproduction in wild and laboratory-reared *H. azteca* provisional species should further be delineated before attempting to quantify population-level effects of a given chemical through toxicity testing.

References

Baird DJ, Pascoe TJ, Zhou X, and Hajibabaei M (2011). Building freshwater macroinvertebrate DNA-barcode libraries from reference collection material: formalin preservation vs specimen age. *Journal of the North American Benthological Society,* **30**(1): 125-130.

Baldinger AJ (2004). A new species of *Hyalella* (Crustacea: Amphipoda: Hyalellidae) from Ash Springs, Lincoln County, Nevada, USA, with a key to the species of genus in North America and the Caribbean region. *Journal of Natural History*, **38**: 1087-1096.

Borgmann U (1996). Systematic analysis of aqueous ion requirements of *Hyalella azteca*: A standard medium including the essential bromide ion. *Archives of Environmental Contamination and Toxicology*, **30**(3): 356-363.

Costa FO, Henzler CM, Lunt DH, Whiteley NM and Rock J (2009). Probing marine *Gammarus* (Amphipoda) taxonomy with DNA barcodes. *Systematics and Biodiversity*, **7**(4): 365-379.

Dionne K, Vergilino R, Dufresne F, Charles F, and Nozais C (2011). No evidence for temporal variation in a cruptic species community of freshwater amphipods of the *Hyalella azteca* species complex. *Diversity*, **3**: 390-404.

Duan Y, Guttman SI, and Oris JT (1997). Genetic differentiation among laboratory populations of *Hyalella azteca*: Implications for toxicology. *Environmental Toxicology & Chemistry,* **16**(4): 691-695.

Duan Y, Guttman SI, Oris JT, and Bailer AJ (2000a). Genotype and toxicity relationships among *Hyalella azteca*: I. Acute exposure to metals and low pH. *Environmental Toxicology & Chemistry*, **19**(5): 1414-1421.

Duan Y, Guttman SI, Oris JT, Huang XD, and Burton GA (2000b). Genotype and toxicity relationships among *Hyalella azteca*: II. Acute exposure to fluoranthene-contaminated sediment. *Environmental Toxicology & Chemistry,* **19**(5): 1422-1426.

Duan Y, Guttman SI, Oris JT, and Bailer AJ (2000c). Genetic structure and relationships among populations of *Hyalella azteca* and *H. Montezuma* (Crustacea:Amphipoda). *Journal of the North American Benthological Society,* **19**(2): 308-320.

Duan Y, Guttman SI, Oris JT, and Bailer AJ (2001). Differential survivorship among allozyme genotypes of *Hyalella azteca* exposed to cadmium, zinc, or low pH. *Aquatic Toxicology,* **54**: 15-28.

Eisenhauer JB, Sullivan KB, and Lydy MJ (1999). Responses of genotypes of *Hyalella azteca* to zinc toxicity. *Bulletin of Environmental Contamination and Toxicology* **63**: 125-132.

Hogg ID, Larose C, de Lafontaine Y, and Doe KG (1998). Genetic evidence for a *Hyalella* species complex within the Great Lakes – St. Lawrence River drainage basin: implications for ecotoxicology and conservation biology. *Canadian Journal of Zoology,* **76**: 1134-1140.

Lam PKS and Calow P (1989). Intraspecific life-history variation in *Lymnaea peregra* (Gastropoda: Pulmonata). II. Environmental or genetic variance? *Journal of Animal Ecology*, **58** 589-602.

Stanley CE (2011). Effects of long-term isolation and metal exposure on the common test species *Hyalella azteca*. Thesis. Oklahoma State University, Stillwater, OK, USA.

Stevenson MM and Peden AE (1973). Description and ecology of *Hyalella texana* n. sp. (Crustacea: Amphipoda) from the Edwards Plateau of Texas. *The American Midland Naturalist*, **89**(2): 426-436.

Strong DR Jr. (1972). Life history variation among populations of an amphipod (*Hyalella azteca*). *Ecology*, **53**(6): 1103-1111.

Stutz HL, Shiozawa DK, and Evans RP (2010). Inferring dispersal of aquatic invertebrates from genetic variation: a comparative study of an amphipod and mayfly in Great Basin springs. *Journal of the North American Benthological Society,* **29**(3): 1132-1147.

U.S. Environmental Protection Agency (2000) Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates, second edition. EPA 823-B-99-007, Duluth, MN and Washington, DC.

Wellborn GA (1993). Ecology and evolution of life history variation among populations of a freshwater amphipod, *Hyalella azteca*. Dissertation. University of Michigan, Ann Arbor, Michigan, USA.

Wellborn GA (1994a). The mechanistic basis of body-size differences between two *Hyalella* (Amphipoda) species. *Journal of Freshwater Ecology,* **9**(2): 159-168.

Wellborn GA (1994b). Size-biased predation and prey life histories – a comparative study of fresh-water amphipod populations. *Ecology*, **75**(7): 2104-2117.

Wellborn GA (1995a). Determinants of reproductive success in freshwater amphipod species that experience different mortality regimes. *Animal Behavior*, **50**: 353-363.

Wellborn GA (1995b). Predator community composition and patterns of variation in life history and morphology among *Hyalella* (Amphipoda) populations in Southeast Michigan. *The American Midland Naturalist,* **133**: 322-332.

Wellborn GA and Cothran RD (2004). Phenotypic similarity and differentiation among sympatric cryptic species in a freshwater amphipod species complex. *Freshwater Biology*, **49**: 1 -13.

Wellborn GA, Cothran R, and Bartholf S (2005). Life history and allozyme diversification in regional ecomorphs of the *Hyalella azteca* (Crustacea: Amphipoda) species complex. *Biological Journal of the Linnean Society*, **84**:161-175.

Wellborn GA and Broughton RE (2008). Diversification on an ecologically constrained adaptive landscape. *Molecular Ecology*, **17**: 2927-2936.

WEST Inc., Gulley DD (1996) Toxstat version 3.5. University of Wyoming, Department of Zoology and Physiology, Laramie, WY.

Witt JDS and Hebert PDN (2000). Cryptic species diversity and evolution in the amphipod genus *Hyalella* within central glaciated North America: a molecular phylogenetic approach. *Canadian Journal of Fish and Aquatic Science,* **15**:3073-3082.

Witt JDS, Blinn DW, an Hebert PDN (2003). The recent evolutionary origin of the phenotypically novel amphipod *Hyalella montezuma* offers an ecological explanation for morphological stasis in a closely allied species complex. *Molecular Ecology*, **12**: 405-413.

Witt JDS, Threloff DL, and Hebert PDN (2006). DNA barcoding reveals extraordinary cryptic diversity in an amphipod genus: implications for desert spring conservation. *Molecular Ecology*, **15**: 3073-3082.

Tables

Table 2.1. Life history test population source, abbreviation, and clade placement as established by the genetic analysis of cytochrome *c* oxidase I (COI) sequences in Chapter 1.

Laboratory populations

Clade	Abbreviation	Population	City	State/Province	Country
US Laboratory	INHS	Illinois Natural History Survey	Champaign	IL	USA
US Laboratory	ABS	Aquatic BioSystems, Inc.	Fort Collins	CO	USA
US Laboratory	MED	USEPA[1] Mid-Continental Ecology Division	Duluth	MN	USA
Burlington	CCIW	Environ. Can. Canada Centre for Inland Waters	Burlington	ON	Canada

Wild populations

Clade	Abbreviation	Field Site	Latitude (N)/Longitude (W)	State/Province	Country
Flat Branch	FB-IL	Flat Branch, Coles County	39.5997/88.3214	IL	USA
Clear Pond	CP-IL	Clear Pond, Vermilion County	40.1381/87.7417	IL	USA

[1]USEPA = United States Environmental Protection Agency.

Table 2.2. Treatment details for 42-d population life history tests #1-3. T = Tetramin, W = wheatgrass

Test #	Age at Test Initiation	Feeding Rate	Replicates	Treatment[1, 2]
1	7-10 d	(Full T + Full W)[3]	7	A) INHS (Generation 3) B) FB-IL (Generation 5)
2	7-11 d	(Reduced T + Full W)[4]	5	A) INHS (Generation 4) B) ABS (Generation 5) C) MED (Generation 5) D) CCIW (Generation 3) E) FB-IL (Generation 7)
3	6-10 d	(Reduced T + Full W)	5	A) INHS (Generation 5) B) ABS (Generation 6) C) MED (Generation 5) D) CCIW (Generation 4) E) CP-IL (Generation 5)

[1]Treatment population abbreviations can be found in Table 2.1.
[2] 'Generation' refers to the number of generations of acclimation to INHS laboratory conditions.
[3] [1.0 mg T Wk.1, 1.25 mg T Wk. 2&3, 1.75 T mg Wk. 4-6] 7x per week + [1.0 ml W] 3x per week.
[4] [1.0 mg T Wk.1, 1.25 mg T Wk. 2&3, 1.25 T mg Wk. 4-6] 7x per week + [1.0 m1 W] 3x per week.

Table 2.3. Common test conditions for population treatment 42-d Tests #1-3 with *H. azteca*.

1. Temperature (˚C)	23 ± 1
2. Test chamber size	300 ml
3. Test solution volume	200 ml
5. Overlying water	Borgmann (1996)
6. Substrate	nitex mesh, conditioned
7. # organisms per chamber	10
9. Food	Tetramin (T) ground to <500 µm, ground wheatgrass (W)
11. Aeration	none
12. Test type	static/renewal
13. Renewal frequency	MWF
14. Test duration	42 days
15. Endpoints	survival, growth (mg), biomass, #young/adult, #young/female, start d.w. (mg)

Table 2.4. Life history test water chemistry measurements (mean ± stdev, (min-max)). All values were measured on outgoing water across all treatments on water change days.

Water Chemistry Parameter	Test #1	Test #2	Test #3
Total measurements taken	4	85	80
Temperature (ºC)	23 ± 0 (23-23)	23.0 ± 0.1(22.9-23.2)	23 ± 0 (23-23)
pH	7.6 ± 0 (7.6-7.6)	7.6 ± 0.1 (7.4-7.8)	7.6 ± 0.1 (7.4-7.8)
Conductivity (µS/cm)	385 ± 5 (380-389)	376 ± 9 (351-405)	375 ± 20 (327-401)
Dissolved Oxygen (mg/L)	7.00 ± 0.30 (5.01-8.12)	6.64 ± 0.55 (5.01-8.12)	6.37 ± 0.47 (5.52-7.26)

Table 2.5. Mean (standard deviation) values for 42-d life history test endpoints by population (treatment). Values followed by different letters indicate statistical significance (alpha = 0.05).

Test #1												
Treatment	%survival		growth (mg)		biomass (mg)		#young/adult		#young/female		start d.w.(mg)
INHS	95.7 (5.3)	A	1.032 (0.049)	A	10.180 (0.800)	A	3.1 (1.3)	A	6.7 (2.9)	A	0.031 (0.015)
FB-IL	90.0 (10.0)	A	0.261 (0.042)	B	2.440 (0.407)	B	1.7 (0.8)	B	3.2 (1.3)	B	0.012 (0.003)
Test #2												
Treatment	%survival		growth (mg)		biomass (mg)		#young/adult		#young/female		start d.w.(mg)
INHS	84.0 (11.4)	A	1.147 (0.066)	A	9.877 (1.252)	A	5.0 (2.4)	A	13.1 (2.8)	A	0.032 (0.009)
ABS	94.0 (8.9)	A	0.961 (0.074)	B	8.998 (0.961)	AB	5.1 (2.0)	A	10.2 (4.0)	AB	0.021 (0.005)
MED	90.0 (14.1)	A	0.811 (0.086)	C	7.496 (0.816)	B	3.6 (2.2)	A	7.2 (3.5)	AB	0.030 (0.006)
CCIW	92.0 (4.4)	A	0.828 (0.052)	C	7.946 (0.192)	B	3.6 (1.3)	A	5.5 (1.4)	B	0.038 (0.005)
FB-IL	82.0 (11.0)	A	0.395 (0.047)	D	3.332 (0.542)	C	2.9 (2.1)	A	5.5 (4.7)	B	0.012 (0.003)
Test #3												
Treatment	%survival		growth (mg)		biomass (mg)		#young/adult		#young/female		start d.w.(mg)
INHS	84.0 (15.2)	A	1.059 (0.111)	AB	9.123 (1.719)	AB	4.0 (3.0)	AB	7.1 (5.5)	AB	0.031 (0.006)
ABS	94.0 (5.5)	A	1.073 (0.056)	A	10.320 (0.444)	A	6.2 (4.0)	A	14.7 (5.7)	A	0.027 (0.010)
MED	76.0 (15.2)	A	0.820 (0.123)	C	6.394 (0.973)	C	1.1 (0.9)	B	3.9 (3.0)	B	0.034 (0.006)
CCIW	90.0 (17.3)	A	0.932 (0.131)	ABC	8.651 (1.106)	AB	2.7 (0.9)	AB	5.5 (2.9)	B	0.047 (0.015)
CP-IL	70.0 (14.1)	A	0.371 (0.115)	D	2.639 (0.535)	C	1.2 (1.1)	B	2.7 (1.4)	B	0.018 (0.007)

Table 2.6. Summary mean (standard deviation) values for 42-d life history test endpoints by population source (treatment) and by clade (see Table 2.1). *N* is the number of pooled tests for each population or clade.

Means of Tests #1-3, By Population							
Treatment	*N*	%survival	growth (mg)	biomass (mg)	#young/adult	#young/female	start d.w.(mg)
INHS	3	87.9 (6.8)	1.079 (0.060)	9.727 (0.545)	4.0 (1.0)	9.0 (3.6)	0.031 (0.000)
ABS	2	94.0 (0.0)	1.017 (0.080)	9.659 (0.935)	5.7 (0.8)	12.5 (3.2)	0.024 (0.004)
MED	2	83.0 (9.9)	0.816 (0.006)	6.945 (0.779)	2.3 (1.8)	5.5 (2.3)	0.032 (0.003)
CCIW	2	91.0 (1.4)	0.880 (0.074)	8.298 (0.498)	3.2 (0.6)	5.5 (0.1)	0.043 (0.006)
FB-IL	2	86.0 (5.7)	0.328 (0.095)	2.886 (0.630)	2.3 (0.9)	4.4 (1.6)	0.012 (0.000)
CP-IL	1	70.0 (-)	0.371 (-)	2.639 (-)	1.2 (-)	2.7 (-)	0.018 (-)
Means of Tests #1-3, By Clade							
Clade	*N*	%survival	growth (mg)	biomass (mg)	#young/adult	#young/female	start d.w.(mg)
US Lab	7	88.2 (7.2)	0.986 (0.129)	8.913 (1.468)	4.0 (1.7)	9.0 (3.9)	0.029 (0.004)
Burlington	2	91.0 (1.4)	0.880 (0.074)	8.298 (0.498)	3.2 (0.6)	5.5 (0.1)	0.043 (0.006)
Flat Branch	2	86.0 (5.7)	0.328 (0.095)	2.886 (0.630)	2.3 (0.9)	4.4 (1.6)	0.012 (0.000)
Clear Pond	1	70.0 (-)	0.371 (-)	2.639 (-)	1.2 (-)	2.7 (-)	0.018 (-)

SUMMARY

Species-level genetic diversity was documented among *H. azteca* sampled from 22 field sites in the eastern US and Canada and 15 US and Canadian laboratory populations by using the entire cytochrome *c* oxidase subunit I (COI) gene as a marker. Pairwise distance and Bayesian analyses supported the establishment of six genetically-distinct clades among the 108 wild and laboratory individuals sampled in this study. Although most laboratory individuals in the US and Canada were genetically similar enough to be considered members of the same provisional species, individuals from one Canadian laboratory population were sufficiently divergent from that clade to be considered members of a separate species. Further, although individuals from wild populations grouped within both laboratory clades, a majority of the field-collected individuals in this study grouped into the remaining four clades, indicating that the genetic diversity among wild populations is not accurately represented by the laboratory populations in the US and Canada. An assessment of the ability of fragments of and within the COI 'barcoding' region to distinguish provisional species within *H. azteca* was made in comparison to the resolution achieved with the entire COI gene sequences. Although the fragments were effective in discriminating among members of divergent provisional species, they were less effective in consistently separating more closely-related provisional species. Because of relatively low amino acid sequence divergence values between select groups, amino acid sequence analysis also failed to resolve some closely-related provisional species established in the full gene analysis. Incongruence between the Bayesian tree topologies produced by the nucleotide and amino acid analyses indicated that that amino acid data should be used as a preferred method of identifying the evolutionary relationships among members of the *H. azteca* species complex because the nucleotide sequences of these very divergent provisional species are saturated.

The life history characteristics of representative populations from two laboratory and two wild provisional species established in the genetic analysis were assessed in the context of a laboratory-acclimated 42-d chronic water-only toxicity test. Genetically-distinct populations displayed different life history characteristics particularly with respect to body size and reproductive capacities. Life history characteristic endpoints among the clades in the present study were in accordance with comparable life history characteristics measured by others, although discrepancies among reproductive measurements were observed. Longer laboratory acclimation periods and/or the establishment of more favorable laboratory culturing conditions for each of the provisional species may serve to make the observed discrepancies among the present study and published literature more congruent. The direct comparison of toxicity test results obtained with laboratory populations displaying species-level divergence from one another for the creation of water quality regulations for the protection of aquatic life is discouraged by the results of the present study. In addition, the ability of laboratory populations of *H. azteca* to act as surrogates for wild populations of this species complex in the context of toxicity testing is further called into question given the species-level divergence and life history characteristic differences measured among laboratory and wild populations.

APPENDIX A

Table A.1. Pairwise sequence divergence as uncorrected p-distance (mean%±sdev±stderr; (min, max)) within and among *H. azteca* clades using the entire COI gene sequence (1569 bp). Numbers immediately below the clade names are the number of individuals in each clade used to make comparisons.

Clade	US Laboratory	Flat Branch	Clear Pond	Burlington	Pine Lake	Joe's Pond
	47	25	18	12	4	2
US Laboratory	0.49±0.54±0.02 (0, 2.27)	18.23±0.18±0.01 (17.65, 18.80)	19.51±0.16±0.06 (18.74, 19.95)	20.01±0.25±0.01 (19.44, 20.65)	20.93±0.023±0.03 (20.14, 21.16)	17.02±0.08±0.01 (16.76, 17.14)
Flat Branch	- -	0.92±0.87±0.05 (0, 3.06)	18.78±0.26±0.01 (18.16, 19.50)	17.91±0.26±0.02 (17.40, 18.80)	18.85±0.20±0.02 (18.48, 19.31)	17.11±0.18±0.03 (16.70, 17.46)
Clear Pond	-	-	1.09±0.54±0.05 (0, 2.29)	18.33±0.22±0.02 (17.78, 18.99)	18.06±0.21±0.02 (17.72, 18.55)	18.89±0.21±0.04 (18.48, 19.38)
Burlington	-	-	-	1.72±1.49±0.18 (0, 3.95)	7.47±0.55±0.08 (6.56, 8.54)	19.30±0.25±0.05 (18.87, 19.82)
Pine Lake	-	-	-	-	2.26±2.48±1.01 (0, 4.53)	18.61±0.12±0.04 (18.42, 18.67)
Joe's Pond	-	-	-	-		0.00±0±0 (-)

Table A.2. Pairwise sequence divergence as uncorrected *p*-distance (mean%±sdev±stderr; (min, max)) within and among *H. azteca* clades using the 'barcoding' region of COI (680 bp). Numbers immediately below the clade names are the number of individuals in each clade used to make comparisons.

Clade	US Laboratory 47	Flat Branch 25	Clear Pond 18	Burlington 12	Pine Lake 4	Joe's Pond 2
US Laboratory	0.52±0.53±0.02 (0, 1.91)	18.63±0.23±0.01 (18.09,19.26)	20.37±0.24±0.01 (19.26, 20.74))	20.91±0.29±0.01 (20.15, 21.47)	21.56±0.27±0.02 (20.59, 21.76)	16.78±0.15±0.02 (16.62, 17.21)
Flat Branch	- -	0.76±0.76±0.04 (0, 2.64)	19.70±0.34±0.02 (18.97, 20.74)	19.57±0.24±0.01 (18.97, 20.44)	19.94±0.19±0.02 (19.56, 20.44)	17.80±0.24±0.03 (17.21, 18.24)
Clear Pond	-	-	1.36±0.90±0.07 (0, 2.94)	18.94±0.36±0.02 (18.24, 20.00)	17.58±0.63±0.07 (16.62, 18.97)	20.16±0.27±0.04 (19.71, 20.74)
Burlington	-	-	-	1.54±1.39±0.17 (0, 3.68)	7.69±0.62±0.09 (6.47, 8.97)	19.69±0.28±0.04 (19.26, 20.29)
Pine Lake	-	-	-	-	2.65±2.90±1.18 (0, 5.29)	19.89±0.48±0.17 (19.12, 20.15)
Joe's Pond	-	-	-	-	-	0.00±0±0 (-)

Table A.3. Pairwise sequence divergence as uncorrected *p*-distance (mean%±sdev±stderr; (min, max)) within and among *H. azteca* clades using a subset of the 'barcoding' region of COI (335 bp). Numbers immediately below the clade names are the number of individuals in each clade used to make comparisons.

Clade	US Laboratory 47	Flat Branch 25	Clear Pond 18	Burlington 12	Pine Lake 4	Joe's Pond 2
US Laboratory	0.43±0.43±0.01 (0, 1.49)	19.15±0.39±0.00 (17.91, 20.30)	21.66±0.55±0.02 (20.00, 22.69)	21.37±0.39±0.02 (20.30, 22.39)	23.64±0.60±0.07 (21.49, 24.18)	17.43±0.18±0.02 (17.01, 17.91)
Flat Branch	-	0.72±0.67±0.04 (0, 2.39)	23.27±0.43±0.02 (22.09, 24.18)	20.37±0.51±0.03 (18.81, 21.79)	21.81±0.69±0.07 (20.90, 23.28)	17.04±0.25±0.04 (16.42, 17.61)
Clear Pond	-	-	1.67±1.08±0.09 (0, 4.18)	18.96±0.64±0.04 (17.31, 20.60)	17.88±0.60±0.08 (17.01, 19.40)	21.09±0.52±0.09 (20.30, 22.09)
Burlington	-	-	-	1.40±1.35±0.17 (0, 3.28)	6.59±0.50±0.07 (5.97, 8.06)	19.45±0.27±0.06 (19.10, 20.00)
Pine Lake	-	-	-	-	2.69±2.94±1.20 (0, 5.37)	21.11±0.69±0.24 (20.00, 21.49)
Joe's Pond	-	-	-	-	-	0.00±0±0 (-)