THE ROLE OF ARTHROPOD SECOND INTERMEDIATE HOSTS AS AVENUES FOR AND CONSTRAINTS ON THE TRANSMISSION OF FROG LUNG FLUKES (DIGENEA: HAEMATOLOECHIDAE)

By

Matthew G. Bolek

A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Biological Sciences

Under the Supervision of Professor John Janovy Jr.

Lincoln, Nebraska

May, 2006

THE ROLE OF ARTHROPOD SECOND INTERMEDIATE HOSTS AS AVENUES FOR AND CONSTRAINTS ON THE TRANSMISSION OF FROG LUNG FLUKES (DIGENEA: HAEMATOLOECHIDAE)

Matthew G. Bolek, Ph.D.

University of Nebraska, 2006

Advisor: John Janovy Jr.

Avenues for and constraints on the transmission of frog lung flukes to frog definitive hosts were studied through examination of the role and fate of parasite metacercariae stages. This study was based on four species of North American frog lung flukes, *Haematoloechus coloradensis* (Cort, 1915), *H. complexus* (Seely, 1906), *H. longiplexus* Stafford, 1902 and *H. parviplexus* Stafford, 1902. Semi-terrestrial leopard frogs are commonly infected with *Haematoloechus coloradensis* and *H. complexus* but exhibit ecological host specificity and are rarely infected with *H. parviplexus* and *H. longiplexus*. The latter two lung flukes infect aquatic bullfrogs. Field and experimental infection data indicated that leopard frogs became infected with *H. coloradensis* and *H. complexus* by feeding on small non-odonate arthropods that served as second intermediate hosts for these parasites. Comparative experimental examinations of metacercarial survival patterns and distribution in second intermediate odonate hosts revealed that most metacercariae of *H. longiplexus* were lost during dragonfly metamorphosis, but most metacercariae of *H. coloradensis, H. complexus*, and *H. parviplexus* survived dragonfly metamorphosis. These observations suggest that the ecological host specificity of *H. longiplexus* in semi-terrestrial leopard frogs is due to few

metacercariae of *H. longiplexus* reaching these frogs in a terrestrial environment. Experimental infections of leopard frogs and bullfrogs with *H. parviplexus* indicate that leopard frogs are resistant to infection with this species. This result indicates that the synonymy of worms from leopard frogs and bullfrogs is not warranted and species from leopard frogs and bullfrogs are distinct. A phylogenetic study of 12 species of North American and European frog lung flukes was conducted using the internal transcribed spacer region. The phylogenetic analysis revealed that these different lineages shared similar patterns of arthropod host specificity distinct from patterns found in the other lineages. These results suggest that second intermediate host specificity may be a trait that has been conserved through evolutionary time. The phylogenetic data presented in this study reveal the importance of second intermediate host specificity among the evolutionary lineages of frog lung flukes because second intermediate hosts serve as avenues for and constraints on the movement of these parasites to their respective definitive amphibian hosts.

ACKNOWLEDGMENTS

Foremost, I would like to thank to my major professor, Dr. John Janovy Jr., for his guidance and friendship throughout this project and his acceptance of my many other side projects. He allowed me the absolute freedom to conduct studies on numerous amphibian parasite life cycles even though at times these studies must have seemed chaotic. I will always be grateful for his patience and support. Secondly, I would like to thank him for giving me the opportunity and constant guidance in preparing lectures for Invertebrate Zoology, Parasitology, Bios 101, and Introductory Zoology while teaching lectures at UNL. John is truly one of a kind teacher that always has a significant and effective lesson plan in mind for students. Finally, I credit Dr. Janovy as having not only shaped me as a biologist and a teacher but as a human being and a professional. I received my best learning experiences from him in the classroom, the field, and the Coffee House.

I also thank the other members of my committee: Drs. Brent B. Nickol, George E. Veomett, Gwen Bachman, and William E. Wagner. All of these individuals have influenced my career as a graduate student and added greatly to my studies and research project.

I greatly acknowledge Dr. Scott Snyder, University of Nebraska at Omaha for all of his help with the molecular aspect of this study. Scott welcomed me into his lab and allowed me the use of all of his equipment and his time to complete the molecular part of this work. He also freely shared all of his knowledge and his specimen collection of frog lung flukes that made this study possible. His help is greatly appreciated and his enthusiasm for the use of molecular tools to study trematode evolution was contagious.

Many thanks are due to the faculty and staff of Cedar Point Biological Station and the many land owners of Nebraska who readily gave their permission to collect frogs, arthropods, and snails. CPBS is an outstanding place to teach and conduct biological research. Dr. Rich Alward, Rob Anderson, and Roy and Kathy Bailey were always willing to help and provide me with resources and space I needed to conduct my field and laboratory work.

Completion of a Doctoral dissertation in parasitology is an endeavor which begins long before entrance into a Ph.D. program. My interests in parasitology and amphibian biology were fostered during my undergraduate career at Carroll College. I thank my undergraduate mentors Drs. John Mellen and Leslie Zettergren for introducing me to these critters and taking the time to discuss numerous projects on their biology. I was fortunate during my graduate education to learn from the best in the business including Dr. James R. Coggins, my masters advisor at the University of Wisconsin-Milwaukee, Drs. Kevin R. Kazacos and John MacDonald at Purdue University, and most importantly Drs. John Janovy Jr. and Brent Nickol at UNL. These individuals have taught me the professionalism and passion for parasitology.

I thank the many graduate students whose input and help over the last few years were invaluable to the completion of this study and my understanding of scientific investigations. During my first few years, Ben Hanelt, Jaclyn Helt, AKA the secretary, Jill Detwiler, Samana Schwank, Michelle Steinauer, and Sara Brant helped me get started. The peer group, with whom I shared the last portion of my Ph.D. study, include Alaine Knipes, Gabriel Langford, Laura Duclose, Terry Haverkost, and Dave Tinnin. These individuals have influenced and refined my thought processes on parasite life cycle

evolution and have helped me physically and mentally over the years. They have served as field assistants, consultants, editors, and most importantly my friends.

Dr. Agustín Jiménez-Ruiz is a close friend who made my life in Lincoln an outstanding experience. Our discussions about parasitology and science have always been provocative and stimulating. His input as a reviewer on numerous manuscripts, and kind suggestions about research and the business of parasitology, are greatly appreciated.

I would like to acknowledge my parents, Margaret and George Bolek, and my brother Luke Bolek for their support and understanding during my years in school. Without their support this work would have been impossible.

Finally, I would like to thank my wife, Melissa Bolek, for her continuous moral support, understanding and sacrifices. She has been my most ardent supporter and was always there for me during the bad and good times. Mel was a great field assistant on those cold rainy nights in Wisconsin and Indiana and hot dusty nights in Western Nebraska. This work is dedicated to her sacrifices over the years.

TABLE OF CONTENTS

LIST OF FIGURES

Overview:

Chapter 1:

Chapter 2:

Chapter 3:

Conclusion

LIST OF TABLES

Overview:

Chapter 1:

Chapter 2:

Chapter 3:

OVERVIEW

"Experiences with life-cycles are unique in one way: I do not think the average biologist or even some parasitologists have any idea of the amount of confining work that is necessary in completing one." Wendell Krull (Letter to Miriam Rothschild, 1953)

BACKGROUND

Parasitism represents a close association between a parasite and its host. The most widely used definition states that it is a symbiotic and intimate relationship between two organisms with one living on, or inside of, and at the expense of the other (Roberts and Janovy, 2005). Although, this definition implies harm, Smyth (1962) and others (Esch and Fernandez, 1993) have stated that whether parasitic organisms are harmful or not is irrelevant to the metabolic concept of parasitism. To the parasite, the host represents a resource and a habitat where the parasite can grow and reproduce. In the case of helminth parasites eggs are released from the host into the external environment where they hatch and undergo development; subsequent life cycle stages must find their way back in to another host. One of the major problems for parasites is for individuals of a particular species to find the correct host to propagate the next generation and complete the life cycle. This problem is a statistical one of colonization,

where parasites face spatial and temporal difficulties of transfer from one host to another, difficulties that must be overcome by enormous reproductive outputs and/or by exploiting complex ecological associations between successive hosts.

One common strategy parasites have used in order to infect their respective host is by exploiting food web relationships, using intermediate hosts, where the parasite develops but does not reach sexual maturity. These intermediate hosts are preyed upon by the definitive host where the parasite becomes sexually mature and reproduces, completing the life cycle. In this way intermediate hosts may act as potential avenues for parasite flow and successful colonization of appropriate definitive hosts. These complex life cycles that utilize multiple hosts have appeared and disappeared multiple times during the evolution of numerous parasite species (Combes, 2001). Although life cycle diagrams are usually drawn as static representations (Olsen, 1986), some have argued that life cycles are only fixed when perceived over the lifetime of human investigators (Combes, 2001). Parasitic helminthes have an astounding diversity of life cycles, using up to four hosts and a diverse array of transmission modes (Cribb et al., 2003). Clearly the diversity and complexity of parasite life cycles in related and unrelated parasite species indicates that such cycles have evolved numerous times (Olsen, 1986). Therefore, unlike the depictions in most textbooks, parasite life cycles are dynamic and fluid over evolutionary time.

A widespread and large group of parasites with complex life cycles are the digenetic trematodes or flukes. Ecological studies on the life cycles of digenetic trematodes indicate that these life cycles are tightly linked to the distribution of their hosts, particularly to their intermediate hosts (Olsen, 1986). Recent studies on

trematode life cycle evolution indicate that the presence of intermediate hosts in these complex life cycles is critical for parasites to find mates and increase transmission rates to definitive hosts (Rauch et al., 2005). The distribution of these intermediate hosts can therefore play a pivotal role in the distribution of parasites and their transmission to their definitive hosts where the parasite can reproduce and complete its life cycle. More importantly, intermediate hosts can act as filters (Combes, 2001), where the definitive hosts' feeding behavior, and the environment where the definitive host encounters the appropriate intermediate host, can affect the presence or absence of a particular parasite species in that definitive host. The diversity of intermediate hosts in a particular parasite life cycle should have an effect on the encounter rate with potential definitive hosts. This diversity is particularly important from an evolutionary perspective, where related species of parasites vary in their host specificity at one or more life cycle stage.

THE HOST-PARASITE SYSTEM

Frog lung flukes in the genus *Haematoloechus* Looss, 1899 comprise more than 50 species of amphibian lung flukes found worldwide (León-Règagnon and Brooks, 2003). Sixteen species have been described from the United States of America and Canada. Morphological characters used to differentiate these species are problematic (Cort, 1915; Prokopič and Křivanec, 1974; Kennedy, 1980a; 1980b, 1981). This situation has led to a major revision of North American representatives of the genus by Kennedy (1981) who declared only six of these species to be valid: *Haematoloechus breviplexus* Stafford, 1902, *H. complexus* (Seely, 1906), *H. kernensis* Ingles, 1932, *H.*

longiplexus Stafford, 1902, *H. medioplexus* Stafford, 1902 and *H. varioplexus* Stafford, 1902.

The life cycles of frog lung flukes that have been described are typical of many trematodes in that they involve obligatory molluscan, arthropod and vertebrate hosts. The life cycles of the few *Haematoloechus* species that are known are quite similar to one another (Krull, 1930; 1931; 1932; 1933; 1934; van Theil, 1930; Ingles, 1932; Dollfus et al., 1960; Grabda, 1960; Combes, 1968; Dronen, 1975; Bourgat and Kulo, 1979; Snyder and Janovy, 1994; 1996; Figure 1). Adult flukes are found in the lungs of numerous species of frogs, toads, and rarely salamanders, in the lungs they release eggs with fully formed miracidia, which are then swallowed and voided in the feces of the definitive amphibian host. If deposited in water, the first intermediate molluscan hosts may ingest the eggs. The miracidia give rise by asexual reproduction to two generations of sporocysts, and eventually multiple cercariae, which leave the first intermediate snail host and actively swim in the water column, until they come into contact with a suitable aquatic arthropod, usually a dragonfly, second intermediate host. Cercariae enter the dragonfly second intermediate host passively, encyst, and develop into the metacercaria stage. The metacercaria enters a definitive host when an amphibian ingests an infected second intermediate host. The worm is digested out in the stomach, migrates up the esophagus, and enters the lungs where it begins to feed on blood, eventually maturing sexually. This set of events is a common example of parasite flow through the exploitation of predator-prey relationships. However, when considered from an ecological and evolutionary perspective and the potential number of species that may be involved, this system can become quite complex.

Anuran host specificity among the six species of North American *Haematoloechus* has been primarily inferred from amphibian surveys. *Haematoloechus breviplexus* has been reported from five species of true frogs (*Rana catesbeiana*, *Rana clamitans*, *Rana grylio*, *Rana pipiens*, and *Rana utricularia*). *Haematoloechus complexus* has been reported from six species of true frogs (*Rana aurora*, *Rana blairi*, *R. clamitans*, *Rana pipiens*, *Rana sphenocephala*, *Rana sylvatica*), as well as a single species of toad (*Bufo woodhousii*) and single species of tree frog (*Hyla chrysoscelis*) (Ingles, 1933; Ulmer, 1970; Brooks, 1976; Catalono and White, 1977; Dronen, 1977; Underwood and Dronen, 1977). *Haematoloechus kernensis* has been reported from a single species of true frog *R. aurora*. *Haematoloechus longiplexus* has been reported from six species of true frogs (*R. blairi, R. catesbeiana, R. clamitans, R. grylio, R. pipiens, Rana septentrionalis*) and one species of toad (*B. woodhousii*) (Parker, 1941; Bouchard, 1951; Brooks, 1976; Williams and Taft, 1980; Muzall, 1991; McAlpine ,1997). *Haematoloechus medioplexus* has been reported from seven species of true frogs (*R. blairi, R. catesbeiana, R. clamitans, Rana palustris, R. pipiens, R. septentrionalis, R. sylvatica*), and two toads (*Bufo americanus* and *B. woodhousii*). Finally, *H. varioplexus* has been reported from six species of true frogs (*R. blairi*, *R. catesbeiana*, *R. clamitans*, *R. pipiens*, *R. palustris*, *Rana pretiosa*, *R. sylvatica*) and two species of toads (*B. americanus*, and *B. woodhousii*) (Waitz, 1961; Campbell, 1968; Brooks, 1976; Wiliams and Taft, 1981; Muzzall, 1991; Muzzall and Peebles, 1991, Russell and Wallace, 1992; Snyder, 1996; Bolek and Coggins, 2003).

These observations indicate that some frog lung flukes can infect a wide range of amphibian hosts, but the absence of a particular parasite species from a natural frog

population indicates little about the host specificity of that particular parasite species. More importantly, because of the lack of voucher specimens deposited in accredited museums for some of these records, difficulties in identifications of certain species of frog lung flukes, and conflicting results from experimental infections (Krull, 1930; 1931; Kennedy, 1980a) indicate that some of these records can not be trusted without conducting experimental anuran host specificity studies along with careful field surveys.

Few experimental studies have determined the amphibian host specificity of frog lung fluke. The paucity of experimental infections in these parasites is likely attributable to the logistical difficulties of maintaining the large number of molluscan, arthropod, and anuran hosts necessary to complete the life cycles. Five comparative experimental studies on anuran host specificity of frog lung flukes exist in the literature. Krull (1933) was able to show that *H. complexus* infected green frogs, *R. clamitans*, whereas bullfrogs, *R. catesbeiana*, were refractory to infection with this species. Krull (1930; 1931) also showed that *H. medioplexus* infected northern leopard frogs, *R. pipiens* but would not infect green frogs, *R. clamitans* or wood frogs, *R. sylvatica*. Dronen (1975) in a later study demonstrated that northern leopard frogs, *R. pipiens* were suitable host for *H. coloradensis* (a synonym for *H. complexus*) whereas bullfrogs were resistant to this parasite. Kennedy (1980a) was able to infect the spotted frog, *Rana pretiosa,* with *H. buttensis,* which he later synonymized with *H. complexus* but was not able to infect the northern leopard frog, *R. pipiens,* with this species (Kennedy, 1981). Finally, Snyder (1996) showed that Woodhouse's toads, *B. woodhousii*, and northern leopard frogs were susceptible to *H. complexus* whereas bullfrogs were refractory to this species. Snyder (1996) also showed that bullfrogs, northern leopard frogs, plains leopard frogs, *R. blairi*,

and Woodhouse's toads were all susceptible to *H. longiplexus*. This experimental work demonstrated that adult *H. complexus*, *H. longiplexus*, *H. medioplexus*, and *H. varioplexus* are capable of parasitizing a range of anuran hosts. However, not all anurans are susceptible to infections with all species of frog lung flukes.

Field data from Nebraska on population structure of *Haematoloechus* species among aquatic and semi-terrestrial anurans suggest that ecological differences also affect lung fluke distribution in the definitive hosts, perhaps as much as does definitive host specificity. Studies by Brooks (1976) and Snyder (1996) indicate that in Nebraska three *Rana* species are infected with four species of frog lung flukes (Table I). Bullfrogs, *Rana catesbeiana*, are large aquatic frogs that are commonly infected with two species (*H. varioplexus* and *H. longiplexus*), but are resistant to infections with *H. complexus* and *H. medioplexus*. *Rana blairi,* the plains leopard frog, and *Rana pipiens,* the northern leopard frog, both medium sized semi-terrestrial anurans, are infected with *H. complexus* and *H. medioplexus*. However, both leopard frog species are rarely infected by *H. longiplexus* and *H. varioplexus* suggesting ecological host specificity. Both Brooks (1976) and Snyder (1996) found over 40 % of bullfrogs infected with *H. longiplexus*, but 0% of northern leopard frogs and 1-2 % of plains leopard frogs were infected in nature (Table I). This observation is in contrast to an experimental study by Snyder (1996), where he infected seven out of 10 (70%) bullfrogs, two out of five (40%) plains leopard frogs, and three out of six (50%) northern leopard frogs with *H. longiplexus*, indicating that these frogs are suitable hosts for *H. longiplexus*. *Haematoloechus varioplexus*, on the other hand has been reported from all three of these hosts in Nebraska, however because its synonymy with *H. parviplexus* a parasite of bullfrogs by Kennedy (1981), it has not been

found in northern leopard frogs and plains leopard frogs in Nebraska when collected from the same locations as bullfrogs that harbor this species (Table I).

This ecological host specificity at the definitive host level is particularly interesting when considering that recent studies by Snyder and Janovy (1994, 1996) have shown that the cercarial behavior patterns of these four North American *Haematoloechus* species dictate host specificity at the second intermediate host level. In their studies, *Haematoloechus complexus* was identified as a generalist within the arthropod host; metacercariae of this species were able to develop in a wide range of aquatic arthropod hosts including dragonflies (anisopteran), damselflies (zygopterans) as well as non-odonate arthropods. Metacercariae of *H. medioplexus* and *H. varioplexus* only developed in dragonflies and were considered specialists. Finally metacercariae of *H. longiplexus* developed in both dragonflies and damselflies and were considered to have intermediate arthropod host specificity. Thus this difference in second intermediate host specificity and second intermediate host life histories may play important roles in parasite movement, distribution, and the observed host specificity in definitive frog hosts.

The generalist nature of *H. complexus* at the second intermediate host level suggests that this species may colonize leopard frogs more commonly than the three species of frog lung flukes that strictly utilize dragonflies, or dragonflies and damselflies as second intermediate hosts. Studies by Brooks (1976) and Snyder (1996) indicate that *H. medioplexus,* the second intermediate host specialist that strictly utilized dragonflies, infects leopard frogs less commonly than does *H. complexus,* the second intermediate host generalist (Table I). However, it does not explain why leopard frogs in Nebraska

are never or rarely infected with *H. varioplexus* and *H. longiplexus* when collected from the same location as bullfrogs infected with these two species.

Diet studies on leopard frogs and bullfrogs indicate that they consume odonates with similar frequencies (Knowlton, 1944; Kilby, 1945; Korschgen and Baskett, 1963; Whitaker, 1961; Fulk and Whitaker, 1968). Frogs of these species are opportunistic feeders that take food indiscriminately, choice being dictated largely by their size and the habitat that they occur in. However, semi-terrestrial leopard frogs spend a large proportion of their lives away from water and predominantly feed on adult damselflies and dragonflies, which make up to 15 % of the invertebrates reported in the frogs' diet (Knowlton, 1944; Linzey, 1967). Bullfrogs, which are restricted to aquatic habitats throughout their life history, predominantly feed on larval dragonflies and damselflies, which consist of up to 16% of the frogs' diet, with adult dragonflies being less commonly reported (Stewart and Sandison, 1972). In a study of stomach contents of bullfrogs and leopard frogs collected from the same location in New Mexico, Dronen (1977) showed that approximately 14% of ingesta by volume of bullfrogs was composed of odonates, of which 83% were larvae, whereas in leopard frogs, 22% of ingesta by volume was composed of odonates, of which only about 10% were larvae. Because of this overlap in host diet there should be some overlap in the parasite occurrence in these hosts, if the parasites are not host specific. Yet field studies by Brooks (1976) and Snyder (1996) on northern leopard frogs, plains leopard frogs and bullfrogs indicated that some strict host specificity exists among these three anuran species and the lung flukes infecting these frogs, suggesting that second intermediate hosts may act as filters, with only certain fluke species being able to pass through all filters and end up in the appropriate host.

This group of congeneric flukes provides an excellent opportunity to explore parasite flow through an ecosystem, particularly by addressing the question: how do second intermediate host life histories influence transmission dynamics, particularly movement of parasites to the definitive host? The specific objective of this study was to determine the role of second intermediate hosts in the transmission of frog lung flukes to their respective definitive amphibian hosts.

This work had three main goals:

1) To determine the role of non-odonate arthropod second intermediate hosts in the recruitment of frog lung flukes by conducting field work and experimental infections.
2) To determine the role of odonate intermediate hosts in the movement of frog lung flukes to aquatic and semi-terrestrial frogs by conducting field work and experimental infections.
3) To determine the phylogenetic relationships of some Holarctic frog lung flukes using recent DNA techniques, and to examine the evolutionary of life history patterns of this subset of the genus using the life cycle data generated from this study and from the literature.

LITERATURE CITED

Bolek, M. G., and J. R. Coggins. 2003. Helminth community structure of sympatric eastern American toad, *Bufo a. americanus*, northern leopard frog, *Rana pipiens*, and blue-spotted salamander, *Ambystoma laterale*, from southeastern Wisconsin. Journal of Parasitology **89**: 673-680.

Bouchard, J. L. 1951. The platyhelminths parasitizing some northern Maine Amphibia. Transactions of the American Microscopical Society **70**: 245-250.

Bourgat, R., and S. D. Kulo. 1979. Cycle biologique d' *Haematoloechus johnsoni* Bourgat, 1977, (Trèmatode) parasite pulmonaire de *Dicroglossus occipitalis* (Günther, 1858) (Amphibien, Anoure) au Togo. Revue Suisse de Zoologie **86**: 467-472.

Brooks, D. R. 1976. Parasites of amphibians of the Great Plains: Part 2. Platyhelminths of amphibians in Nebraska. Bulletin of the University of Nebraska State Museum **10**: 65-92.

Campbell, R. A. 1968. A comparative study of the parasites of certain salientia from Pocahontas state park, Virginia. The Virginia Journal of Science **19**: 13-29.

Catalono, P. A., and A. M. White. 1977. New host records of *Haematoloechus complexus* (Seely) Krull, 1933 from *Hyla crucifer* and *Rana sylvatica*. Ohio Journal of Science **77**: 99.

Combes, C. 1968. Biologie, écologie des cycles et biogeography de digénes et monogénes d'amphibiens dans l'est des Pyrénéés. Mémoires du Muséum National d'Histoire Naturelle **51**: 1-195

Combes, C. 2001. Parasitism: the ecology and evolution of intimate interactions. University of Chicago Press, 728 p.

Cort, W. W. 1915. North American frog lung flukes. Transactions of the American Microscopical Society **34**: 203-243.

Cribb, T. H., Bray, R. A., P. D., Olson, D. Timothy, and J. Littlewood. 2003. Life cycle evolution in the Digenea: a new perspective from phylogeny. Advances in Parasitology **54**: 197-254.

Dollfus, R. P., J. M. Doby, and P. Laurent. 1960. Su rune xiphidiocercaire parasitant Limnaea trunculata (O. F. Müller) en haute-savoiet et s'enkystant dans des laves de moustiques. Bulletin de la Societe Zoologique de France **85**: 331-347.

Dronen, N. O. Jr. 1975. The life cycle of *Haematoloechus coloradensis* Cort 1915 (Digenea: Plagiorchiidae), with emphasis on host susceptibility to infection. Journal of Parasitology **61**: 657-660.

Dronen, N. O. Jr. 1977. Studies on the population structure of two species of *Haematoloechus* Looss, 1899 (Digenea: Plagiorchiidae) in raniid frogs in New Mexico. Proceedings of the Helminthological Society of Washington **44**: 68-72.

Esch, G. W., and J. C. Fernandez. 1993. A functional biology of parasitism. Chapman & Hall, New York, New York, 337 p.

Fulk, F. D., and J. O. Whitaker, Jr. 1968. The food of *Rana catesbeiana* in three habitats in Owen County, Indiana. Proceedings of the Indiana Academy of Sciences **3**: 491-496.

Grabda, B. 1960. Life cycle of *Haematoloechus similis* (Looss, 1899) (Trematoda: Plagiorchiidae). Acta Parasitologica Polonica **8**: 357-367.

Ingles, L. G. 1933. Studies on the structure and life history of *Ostiolum oxyorchis* (Ingles) from the California red-legged frog *Rana aurora draytoni*. University of California Publications in Zoology **39**: 135-161.

Kennedy, M. J. 1980a. Host-induced variation in *Haematoloechus buttensis* (Trematoda: Haematoloechidae). Canadian Journal of Zoology **58**: 427-442.

Kennedy, M. J. 1980b. Geographical variation in some representatives of *Haematoloechus* Looss, 1899 (Trematoda: Haematoloechidae) from Canada and the United States. Canadian Journal of Zoology **58**: 1151-1167.

Kennedy, M. J. 1981. A revision of the genus *Haematoloechus* Looss, 1899 (Trematoda: Haematoloechidae) from Canada and the United States. Canadian Journal of Zoology **59**: 1836-1846.

Kilby, J. D. 1945. A biological analysis of the food and feeding habits of two frogs, *Hyla cinerea cinerea* and *Rana pipiens sphenocephala*. Journal of the Florida Academy of Sciences **8**: 71-104.

Knowlton, G. F. 1944. Some insect food of *Rana pipiens*. Copeia **1944**: 119.

Korschgen, L. J., and T. S. Baskett. 1963. Foods of impoundment- and stream-dwelling bullfrogs in Missouri. Herpetologica **19**: 89-99

Krull, W. H. 1930. The life history of two North American frog lung flukes. Journal of Parasitology **16**: 207-212.

Krull, W. H. 1931. Life history studies on two frog lung flukes, *Pneumonoeces medioplexus* and *Pneumobites parviplexus*. Transactions of the American Microscopical Society **50**: 215-277.

Krull, W. H. 1932. Studies on the life history of *Haematoloechus longiplexus* (Stafford). Zoologischer Anzeiger **99**: 231-239.

Krull, W. H. 1933. Studies on the life history of a frog lung fluke, *Haematoloechus complexus* (Seely, 1906) Krull, n. comb. Zeitschrift für Parasitenkunde **6**: 193-206.

Krull, W. H. 1934. Some additional notes on the life history of a frog lung fluke, *Haematoloechus complexus* (Seely, 1906) Krull. Transactions of the American Microscopical Society **53**: 196-199.

León-Règagnon, V., and D. R. Brooks. 2003: Molecular phylogeny of *Haematoloechus* Looss, 1899 (Digenea: Plagiorchiidae), with emphasis on North American species. Journal of Parasitology **89**: 1206–1211.

Linzey, D. W. 1967. Food of the leopard frog, *Rana p. pipiens*, in Central New York. Herpetologica **23**: 11-17.

McAlpine, D. F. 1997. Helminth communities in bullfrogs (*Rana catesbeiana*), green frogs (*Rana clamitans*), and leopard frogs (*Rana pipiens*) from New Brunswick, Canada. Canadian Journal of Zoology **75**: 1883-1890.

Muzzall, P. M. 1991. Helminth infracommunities of the frogs *Rana catesbeiana* and *Rana clamitans* from Turkey Marsh, Michigan. Journal of Parasitology **77**: 366-371.

Muzzall, P. M., and C. R. Peebles. 1991. Helminths of the wood frog, *Rana sylvatica*, and the spring peeper, *Pseudacris c. crucifer*, from Southern Michigan. Journal of the Helminthological Society of Washington **58**: 263-265.

Olsen, O. W. 1986. Animal parasites: their life cycles and ecology. Dover, New York, 562 p.

Parker, M. V. 1941. The trematode parasites from a collection of amphibians and reptiles. Journal of the Tennessee Academy of Sciences **16**: 27-45.

Prokopič, J., and K. Křivanec. 1974. Trematodes of the genus *Haematoloechus* Looss, and their variability. Helminthologia **15**: 779-802.

Rauch, G., M. Kalbe, and T. B. H. Reusch. 2005. How a complex life cycle can improve a parasite's sex life. Journal of Evolutionary Biology **18**: 1,069-1,075.

Roberts, L. S., and J. Janovy Jr. 2005. Gerald D. Schmidt & Larry S. Roberts' foundations of parasitology, 7th ed. McGraw Hill, Boston, Massachusetts, 702 p.

Russell, K. R., and R. L. Wallace. 1992. Occurrence of *Halipegus occidualis* (digenea: Derogeniidae) and other trematodes in *Rana pretiosa* (Anura: Ranidae) from Idaho, U.S.A. Transactions of the American Microscopical Society **111**: 122-127.

Smyth, J. D. 1962. Introduction to animal parasitology. Charles C. Thomas, Springfield, Illinois, 470 p.

Snyder, S. D. 1996. Host specificity among species of *Haematoloechus* (Digenea: Haematoloechidae). Unpublished Ph.D. Thesis. University of Nebraska-Lincoln, 112 p.

Snyder, S. D., and J. Janovy Jr. 1994. Second intermediate host-specificity of *Haematoloechus complexus* and *Haematoloechus medioplexus* (Digenea: Haematoloechidae). Journal of Parasitology **80**: 1052-1055.

Snyder, S. D., and J. Janovy Jr. 1996. Behavioral basis of second intermediate host specificity among four species of *Haematoloechus* (Digenea: Haematoloechidae). Journal of Parasitology **82**: 94-99.

Stewart, M. M., and P. Sandison. 1972. Comparative food habits of sympatric mink frogs, bullfrogs and green frogs. Journal of Herpetology **6**: 241-244.

Ulmer, M. J. 1970. Studies on the helminth fauna of Iowa. I. Trematodes of Amphibians. American Midland Naturalist **83**: 38-64.

Underwood H. T., and N. O. Dronen. 1977. The molluscan intermediate hosts for species of *Haematoloechus* Looss 1899 (Digenea: Plagiorchiidae) from rannid frogs of Texas. Journal of Parasitology **63**: 122.

van Theil, P. H. 1930. Die Entwicklung von *Agamodistomum anophelis* zum *Pneumonoecues variegates* Rud. Zentralblatt für Backteriologie, Parasitenkunde, und Infektionskrankheiten **117**: 103-112.

Waitz, J. A. 1961. Parasitic helminths as aids in studying the distribution of species of *Rana* in Idaho. Transactions of the Illinois State Academy of Sciences **54**: 152-156.

Williams, D. D., and S. J. Taft. 1980. Helminths of anurans from NW Wisconsin. Proceedings of the Helminthological Society of Washington **47**: 278.

Whitaker, J. O. Jr. 1961. Habitat and food of mousetrapped young *Rana pipiens* and *Rana clamitans*. Herpetologica **17**: 173-179.

Figure 1. Life cycle of *Haematoloechus varioplexus*. A. Thirty two day old adult *Haematoloechus varioplexus* from the lungs of the bullfrog, *Rana catesbeiana*. B. Bullfrog, showing the escape of the metacercariae from the dragonfly in the stomach and their migration to the lungs; adult worms depositing eggs and the route of the eggs to the external environment. C. Egg. D. *Gyraulus parvus* eating eggs; releasing cercariae. E. Sporocyst from the digestive gland of *Gyraulus parvus* with cercariae in various stages of development. F. Cercaria showing body, tail, stylet, oral sucker, pharynx and cecae, ventral sucker, and excretory bladder. G. Larva of the eastern pondhawk dragonfly, *Erythemis simplicicollis*, showing the swimming cercariae being taken into the branchial basket respiratory organ of the larva. H. Lamella from the branchial basket of eastern pondhawk dragonfly larva containing two encysted metacercariae. I. Teneral eastern pondhawk dragonfly with encysted metacercariae within the vestige of the branchial basket of the larva. J. Encysted metacercaria. All drawings are not to scale.

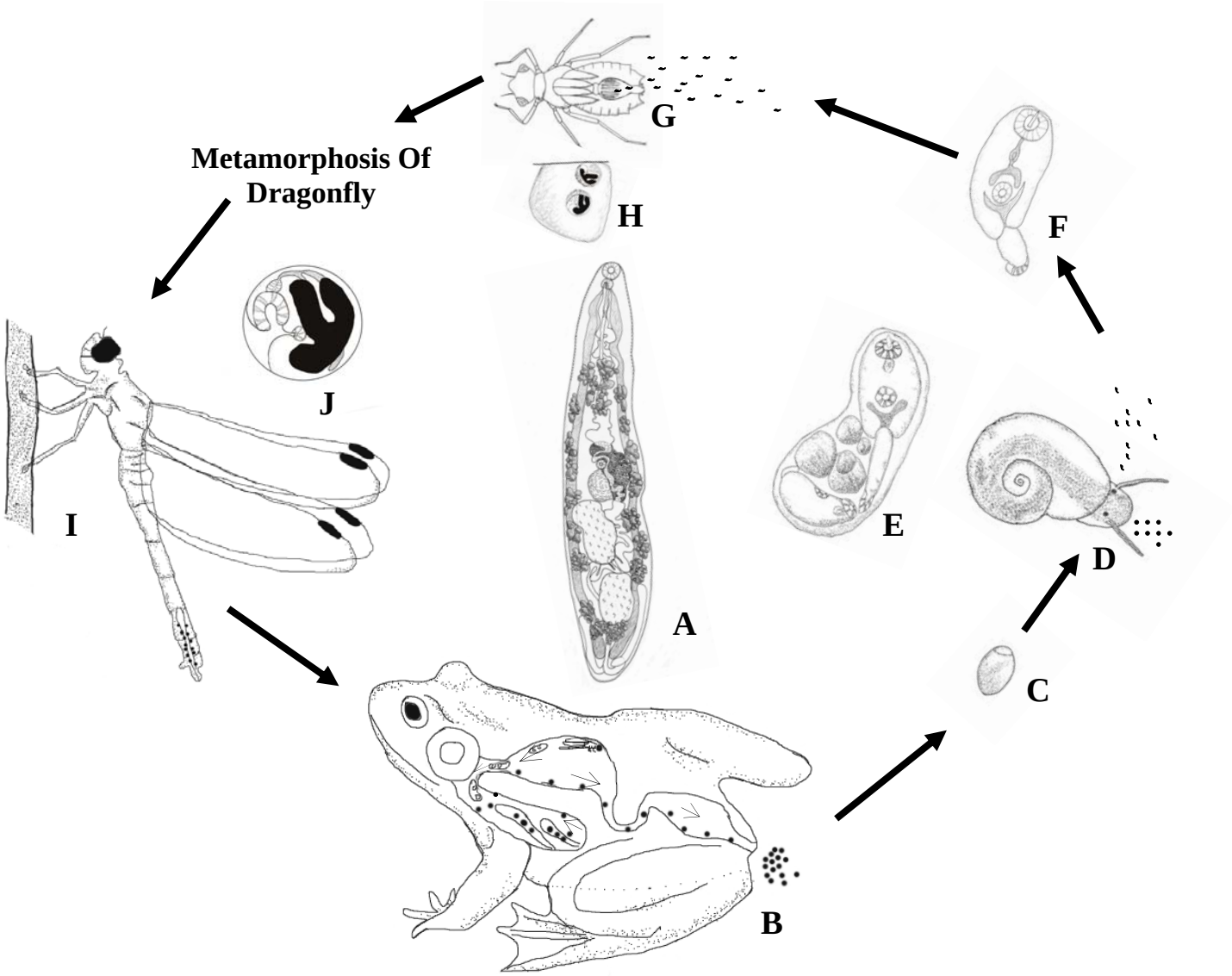

Metamorphosis Of
Dragonfly
G
H
F
J
I
A
E
D
C
B

Table I. Prevalence of *Haematoloechus* species in *Rana catesbeiana*, *R. pipiens* and *R. blairi* from Nebraska collected by Brooks from 27 localities throughout the state during 1973-1975, and Snyder from 25 localities throughout the state during 1993-1995.

		Brooks (1976)	Snyder (1996)
H. longiplexus			
	Rana catesbeiana	40.6 % (54/133)	41.3 % (66/166)
	Rana pipiens	0 % (0/152)	0 % (0/73)
	Rana blairi	2 % (2/98)	1 % (1/103)
H. varioplexus			
	Rana catesbeiana	9.8 % (13/133)*	6.9 % (11/160)
	Rana pipiens	8.55 % (13/152)	0 % (0/73)
	Rana blairi	9.2 % (9/98)	0 % (0/103)
H. complexus			
	Rana catesbeiana	0 % (0/133)	0 % (0/166)
	Rana pipiens	11.1 % (17/153)†	57.5 % (42/73)
	Rana blairi	18.4 % (18/98)†	48.5 % (50/103)
H. medioplexus			
	Rana catesbeiana	0 % (0/133)	0 % (0/160)
	Rana pipiens	6 % (9/152)	17.8 % (13/73)
	Rana blairi	2 % (2/98)	4.8 % (5/103)

* Identified as *H. parviplexus* by Brooks (1976); † identified as *H. coloradensis* and *H. complexus* by Brooks (1976).

CHAPTER ONE: THE ROLE OF NON-ODONATE ARTHROPODS IN THE RECRIUTMENT OF *HAEMATOLOECHUS COLORADENSIS* AND *HAEMATOLOECHUS COMPLEXUS* IN NEWLY METAMORPHOSED NORTHERN LEOPARD FROGS, *RANA PIPIENS*, AND WOODHOUSE'S TOADS, *BUFO WOODHOUSII*

"Some of the small frogs just after metamorphosis have difficulty eating adult dragonflies, but the larger ones do not…." Krull (1931)

Abstract: Studies on the life cycles and epizootiology of North American frog lung flukes indicate that most species utilize odonates as second intermediate hosts; adult frogs become infected by ingesting odonate intermediate hosts. Newly metamorphosed frogs are rarely infected with these parasites predominantly because they are gape limited predators that can not feed on large intermediate hosts such as dragonflies. I examined the role of the frog diet and potential intermediate hosts in the recruitment of the frog lung fluke, *Haematoloechus coloradensis,* to metamorphosed northern leopard frogs, Woodhouse's toads, and bullfrogs from western Nebraska. Because of the uncertain validity of *H. coloradensis* as a distinct species from *H. complexus* morphological characters of both species were reevaluated and the life cycles of both species were completed in the laboratory. The morphological data on *H. coloradensis* and *H. complexus* indicate that they differ in their oral sucker to pharynx ratio, uterine loop

distribution, and placement of vitelline follicles. However, in terms of their life cycles both species are quite similar, utilizing physid snails as first intermediate hosts, a wide range of non-odonate and odonate arthropods as second intermediate hosts, and leopard frogs and toads as definitive hosts. These results indicate that *Haematoloechus coloradensis* and *H. complexus* are generalists at the second intermediate host level and may be able to infect newly metamorphosed leopard frogs and toads by using small non-odonate arthropods more commonly than other frog lung fluke species. Comparasions of population structure of adult flukes in newly metamorphosed leopard frogs indicate that the generalist nature of *H. coloradensis* metacercariae enables it to colonize young of the year leopard frogs more commonly than other *Haematoloechus* species that only use odonates as second intermediate hosts. This comparative approach to life cycle studies within single species and multiple species of hosts allows us to generate hypotheses about the mechanisms that drive the evolution of parasite life cycles.

INTRODUCTION

Amphibian parasites are good model systems to address questions of parasite life cycle diversity and evolution. Recent comparative studies on amphibian parasite life cycles, recruitment, and community structure in anuran hosts by Bolek and Coggins, (1998; 2000; 2001; 2003), Hardin and Janovy (1988), McAlpine (1997), Muzzall and coworkers (Gillilland and Muzzall, 1999; Muzzall, 1991; Muzzall and Peebles, 1991; Muzzall et al., 2002), and Snyder and Janovy (1994; 1996) have provided base line data on the distribution, demography, field host specificity and life history of amphibian parasites. These studies indicate that the parasite communities of aquatic and semi-aquatic anurans are dominated by digenetic trematodes with complex life cycles, whereas the parasite communities of terrestrial anurans are dominated by nematodes which are acquired by direct penetration. Semi-terrestrial frogs have fewer adult digenetic trematodes and direct life cycle nematodes than do aquatic or semi-aquatic and terrestrial anurans. More important, these studies also indicate that within individual anuran species, newly metamorphosed and juvenile anurans are less commonly infected with parasites than are larger adult frogs.

Numerous hypotheses have been advanced for the lack of parasites in small anurans and include such causal factors as lack of time for exposure to parasites, a small body size, which affects the surface area available for skin penetrating parasites, and small gape size, which affects the size of potential intermediate hosts that can be ingested by these frogs. The few studies that exist on newly metamorphosed northern leopard frogs, *Rana pipiens,* a semi-terrestrial anuran and terrestrial toads (*Bufo* spp.) from Canada, the upper Midwest and New Mexico indicate that young of the year frogs and

toads are rarely if ever infected with adult trematodes such as lung flukes (Dronen, 1977; McAlpine, 1997 Gillilland and Muzzall, 1999; Bolek, and Coggins, 2003). Contrary to these studies, my observations from Nebraska indicate that in an arid environment, newly metamorphosed northern leopard frogs are commonly infected with *Haematoloechus coloradensis* (Cort, 1915), with prevalence reaching over 50%. These observations are intriguing from the perspective of complex life cycle evolution, due to the fact that this trematode is acquired through the frogs' diet and therefore should not be expected in newly metamorphosed leopard frogs that are gape limited predators (Dronen, 1975). These observations suggest that differences in host and or parasite life histories may be important in parasite population structure and influence selective pressures on parasite life cycle evolution.

Studies on the life cycles and epizootiology of North American frog lung flukes indicate that, in general, adult frogs become infected by ingesting odonate intermediate hosts (Krull, 1930; 1931; 1932; 1933; 1934; Ingles, 1933; Schell, 1965; Dronen, 1975; Kennedy, 1980). More recently, studies by Snyder and Janovy (1994; 1996) on four common frog lung flukes from Nebraska show that host specificity at the first and second intermediate host level can be variable among these parasite congeners, indicating that life cycles of closely related species may differ in evolutionarily significant ways. Due to recent confusion in the literature on the taxonomy of *H. coloradensis* and *H. complexus*, as seen in papers by Kennedy (1981) and León-Règagnon and Brooks (2003), it is unclear whether Snyder and Janovy (1994) were dealing with *H. complexus* or *H. coloradensis*. The life cycles of these parasites therefore must be re-evaluated.

The present study has five main goals: (1.) to determine the population structure of frog lung flukes in newly metamorphosed northern leopard frogs, newly metamorphosed Woodhouse's toads and three other anuran species, (2.) to determine the second intermediate arthropod hosts that serve as a route of infection for *H. coloradensis* to newly metamorphosed leopard frogs and toads in the field, (3.) to re-evaluate the second intermediate hosts specificity of *H. complexus*, (4.) to test whether non-odonate arthropods serve as a viable route of infection for *Haematoloechus coloradensis* and *H. complexus* to newly metamorphosed frogs and toads, and (5.) to re-evaluate the diagnostic characteristics *H. coloradensis* and *H. complexus*.

MATERIALS AND METHODS

Haematoloechus coloradensis field studies

During July-August 2001, a total of 142 young of the year northern leopard frogs, *Rana pipiens,* were collected, from Cedar Creek, Keith County, Nebraska (41.18639, -101.36276), and examined for *Haematoloechus* species. Stomach content data were also obtained. All frogs were placed on ice as they were being collected, and brought into the laboratory. Frogs were euthanized, the snout vent length (SVL) was measured, and they were examined for parasites and stomach content data within 1-4 hr of collection. Trematodes were removed from the lungs, allowed to release eggs in water, and fixed in alcohol-formalin-acetic acid (AFA); representative specimens were stained with Seminchon's acetocarmine (Pritchard and Kruse, 1982). All lung flukes were identified based on the keys provided by Kennedy (1981) and descriptions of *H. complexus* by Krull (1933) and *H. coloradensis* by Cort (1915). All stomach contents were identified

as specifically as possible and all intact stomach content remains were measured to the nearest 0.5 mm. Stomach content data were grouped as frequencies of individuals ingested according to order, class, or subclass and aquatic or terrestrial ecological habitats. Invertebrates from stomach contents of frogs were identified to family, genus or species using keys in Borror et al., (1989), Merritt and Cummins (1996), Westfall and May (1996), Dunkle (2000), Needham et al., (2000), and Thorp and Covich (2001).

Additionally, 62 naturally infected frogs collected from August-September 2001 were maintained in the laboratory for four to six weeks in order to allow enough time for all immature specimens of *Haematoloechus* spp. naturally infecting these frogs to mature. Frogs were maintained in groups of five individuals in small plastic boxes (33 cm X 9 cm X 14 cm) on moist paper towels and fed commercially reared crickets three times per week.

Frogs were also sampled from Cedar Creek and Breen's Flyway (a pond adjacent to Cedar Creek) (41.18080, -101.57973) during 2002-2004, measured and examined for frog lung flukes. These specimens included 25 northern leopard frogs collected during September 2002, 40 northern leopard frogs and two bullfrogs collected during August-September 2003, and 20 northern leopard frogs, 25 metamorphosed Woodhouse's toads, *Bufo woodhousii*, and 10 metamorphosed and adult bullfrogs, *Rana catesbeiana* collected during June-September 2004.

In order to determine what second intermediate hosts served as reservoirs of infections for metamorphosed northern leopard frogs at Cedar Creek during 2001, numerous arthropods were sampled during June-August 2001 from this location. All aquatic and semi-aquatic arthropods were collected by the use of a dip-net, stored in

buckets without snails, and brought into the laboratory. Adult odonates were collected using a butterfly net, then immediately placed on ice in plastic jars, and brought back to the laboratory. All adult odonates and aquatic arthropods were isolated within one hr of collection, identified, and examined for the presence of metacercariae. Aquatic and semi-aquatic arthropods collected from Cedar Creek were identified to family, genus or species using keys in Borror et al., (1989), Merritt and Cummins (1996), Westfall and May (1996), Dunkle (2000), Needham et al., (2000), and Thorp and Covich (2001).

The following measures of parasitism were calculated for the various amphibians and invertebrates examined (Margolis et al., 1982): Prevalence, the percentage of infected organisms in a sample; mean intensity, the mean number of worms per infected host; and/or mean abundance, the mean number of individuals of a particular parasite species per organism of a particular species examined including infected and non-infected individuals. Values are reported as a mean $\pm$ one standard deviation.

***Haematoloechus coloradensis* snail first intermediate host infections**

Adult *H. coloradensis* flukes were obtained from wild-caught northern leopard frogs from Cedar Creek. Worms were placed in 70 ml plastic containers containing tap water and allowed to release their eggs. Worms were then fixed in AFA, stained, and identified to species. Colonies of *Physa (Physella) gyrina* snails were established in the laboratory from wild strains collected from Nickol Pond in Cass County, Nebraska (40.81412, -96.46000). Snails were maintained on a diet of frozen mustard greens and Tetra Min® fish food. Snails were reared from eggs for a period of six weeks in the laboratory, and then infected with *H. coloradensis* eggs, by placing individual snails into 70 ml plastic containers with *H. coloradensis* eggs and Tetra Min® fish food, for 5 min.

All snail feces were then checked for hatched *H. coloradensis* eggs. Exposed snails were maintained for a period of 30 days and all survivors were isolated in 1.5 ml well plates filled with aged tap water and observed daily for shedding cercariae.

Haematoloechus coloradensis non-odonate arthropod second intermediate host infections

Adult male giant water bugs (Hemiptera: *Belostoma* sp.) covered with eggs were collected from Nickol Pond and brought into the laboratory and placed in individual white 22.7 L buckets. Once hatched, young belostomatid bugs were individually isolated in 1.5 ml well plates filled with aged tap water, and fed chironomid larvae daily. Three additional non-odonate arthropod species (Diptera: *Tanytarsus* sp., Ephemeroptera: *Callibaetis* sp., and Crustacea: *Hyalella azteca*) used in the second intermediate host infections came from a variety of natural populations including the toe drains of Lake McConaughy, Keith County, Nebraska (41.23218, -101.66973), and Dunwoody Pond, Keith County, Nebraska, (41.21527, -101.5784). Larval eastern pondhawk dragonflies, *Erythemis simplicicollis,* collected from Dunwoody Pond were also exposed to *H. coloradensis* cercariae as positive controls. Non-odonate arthropods and dragonflies were divided into three equal groups and designated as time-0 controls, experimentals, or time-T controls. Non-odonate arthropods were isolated in 1.5 ml well plates, while dragonflies were isolated in 5 ml well plates filled with aged tap water for 24 hr before exposure. Time-0 controls were dissected at the beginning of the experimental infections, whereas time-T controls were maintained throughout the duration of the experiment and dissected along with the experimental group. For infections,

approximately 20-50 cercariae of *H. coloradensis* from lab reared and infected *P. gyrina* snails were pipetted into each well that contained an experimental non-odonate and odonate arthropod. After exposure to cercariae, water was changed daily for a period of four days, after which time all surviving experimentally exposed arthropods and time-T control arthropods were dissected in insect saline and inspected for the presence of *H. coloradensis* metacercariae. Cercarial attachment and penetration behavior was observed on a number of non-odonate and odonate arthropods including dragonfly and damselfly larvae.

***Haematoloechus coloradensis* frog definitive host infections**

Young tadpoles (Gosner stage 26-30) of northern leopard frogs were collected from Cedar Creek and maintained in the laboratory in 45.5 L tanks filled with aged tap water for a period of six weeks through metamorphosis. Tadpoles were maintained on a diet of frozen mustard greens and Tetra Min® fish food, whereas metamorphosed frogs were fed commercial lab reared crickets *Gryllus firmus*, and tenebrionid beetles *Tenebrio molitor* adults and larvae. Lab reared northern leopard frogs were each exposed to *H. coloradensis* metacercariae reared in non-odonate arthropods. All arthropods were dissected in insect saline (Hoar and Hickman, 1967). Upon removal from the non-odonate arthropod host metacercariae were divided into groups of 10-15 in insect saline. Ten to 15 metacercariae were drawn into a pipette and placed into the esophagus of an experimental frog and forced down its throat. The pipette was then examined under a dissecting microscope to confirm that no metacercariae remained. Infected frogs along with non infected time-T controls were maintained in groups of 2-4 individuals in 45.5 L tanks on moist sand or gravel. They were fed commercial crickets and tenebrionid

beetles daily for a period of up to 30 days at which time they were euthanized, necropsied and examined for frog lung flukes.

Additionally, three lab reared northern leopard frogs, three lab reared bullfrogs, and two field collected Woodhouse's toads, along with 10 time-0 controls, and 10 time-T controls collected from Beckius Pond, Keith County, Nebraska (41.20835, -101.61777) were used for experimental infections with *H. coloradensis*. Amphibians were infected with 5-10 metacercariae of *H. coloradensis* from laboratory infected dragonflies, *Erythemis simplicicollis,* collected from Dunwoody Pond in Keith County, Nebraska. Frogs and toads were maintained in the laboratory on a diet of commercial crickets for 20-30 days when they were euthanized, and examined for *H. coloradensis* infections.

***Haematoloechus complexus* life cycle studies**

Due to high mortality of experimentally infected snails, naturally infected snails were collected from Pawnee Lake, Lancaster County, Nebraska (40.84310, -96.85700). During 2001-2005, 399 frogs and toads of six species were examined from this location and *H. coloradensis* was never in these amphibians. The only frog lung fluke that uses physid snails as first intermediate hosts at this location is *H. complexus*. Fifty *Physa (Physella) gyrina* snails were collected by dip-net and were individually isolated in 1.5 ml well plates filled with aged tap water. Of these one snail shed *Haematoloechus* cercaria. Three non-odonate arthropod species (Diptera: *Tanytarsus* sp., Ephemeroptera: *Callibaetis* sp., and Crustacea: *Hyalella azteca*) along with eastern pondhawk dragonflies, *Erythemis simplicicollis,* used in the second intermediate host infections came from a variety of natural populations collected from toe drains of Lake McConaughy, Keith County, Nebraska, and Dunwoody Pond Keith County, Nebraska.

These arthropods were divided into three equal groups and designated as time-0 controls, experimentals, or time-T control controls and were isolated in 1.5 ml well plates filled with aged tap water for 24 hr before exposure. All arthropod infections followed the same procedure as previously described for *H. coloradensis*. In order to be sure that cercaria shed by the single *P. gyrina* were *H. complexus*, four newly metamorphosed Woodhouse's toads along with 10 time-0 and 10 time-T controls collected from Beckius Pond, Keith County, Nebraska were used for definitive host infections. The four experimental toads were each given 5-10 *H. complexus* metacercaria from laboratory infected non-odonate arthropods. Toads were maintained in the laboratory on a diet of commercial crickets and beetle larvae for 20-30 days when they were euthanized, and examined for *H. complexus* infections.

Other amphibian field surveys

During May-September of 2000-2004 an additional 57 adult bullfrogs, and two adult plains leopard frogs, *Rana blairi*, were collected from Nevens Pond, Keith County, Nebraska (41.20710, -101.40850) and three adult plains leopard frogs and 10 adult plains spadefoot toads, *Spea bombifrons*, were collected from Cedar Point Biological Station, Keith County, Nebraska (41.21051, -101.52220), and examined for frog lung flukes.

Morphological studies

Morphological data were collected on 20 *H. coloradensis* worms from northern leopard frogs, plains leopard frogs, and Woodhouse's toads, and 20 *H. complexus* worms from northern leopard frogs, plains leopard frogs, Woodhouse's toads, and green frogs, *Rana clamitans*. Worms used for morphological analysis were collected from a number of locations in Indiana, Nebraska, and Wisconsin. These included three *H. coloradensis*

collected from plains leopard frogs from Nevens Pond and Cedar Point Biological Station, Keith County, Nebraska, and seven *H. coloradensis* collected from northern leopard frogs from Cedar Creek, Keith County, Nebraska; one *H. complexus* collected from a northern leopard frog from West Lafayette, Tippecanoe County, Indiana (40.44472, -86.99024), 15 *H. complexus* from plains leopard frogs collected from Pawnee Lake, Nebraska, one *H. complexus* from an experimentally infected Woodhouse's toads from Nebraska, and two *H. complexus* worms from green frogs, *Rana clamitans,* collected from Genesse Depot, Waukesha County, Wisconsin (42.98984, -88.36634). Additionally, in order to get a better geographical representation *H. coloradensis* and *H. complexus*, voucher specimens collected by Dan Brooks were borrowed from the H. W. Manter Laboratory, University of Nebraska State Museum. These included, *H. coloradensis* HWML 20134: from a northern leopard frog from Antelope County, Nebraska (42.14764, -98.048055), two slides, HWML 20135: from a northern leopard frog from Saunders County, Nebraska (41.3125, - 96.69583), two slides; HWML 20136: from a northern leopard frog from Dawes County, Nebraska (42.773055, -103.066385), three slides; HWML 20137: from a northern leopard frog from Dawes County, Nebraska (42.699865, -103.272775), three slides; *H. complexus* HWML 20132: from a plains leopard frog from Lancaster County, Nebraska (40.791665, -96.675), one slide; HWML 20170: from a plains leopard frog from Jefferson County, Nebraska (40.234865, -97.069305), one slide. Based on recent and past *Haematoloechus* species description by Brooks, (1976) and León-Règagnon et al., (2001; 2002) the following characters were recorded: 1) body shape, body length, and body width, 2) oral sucker location, length, and width, 3) pharynx length and width, 4) oral sucker/ pharynx ratio OS/PH, 5)

acetabulum length from anterior end, and acetabulum length and width, 6) oral sucker/acetabulum ratio OS/AC, 7) testes morphology and location, 8) ovary morphology and location, 9) cirrus sac position and location, 10) uterus and uterine loop morphology, 11) vitellaria number, and location, 12) egg length and width. Student's two tailed *t*-test was used to compare morphological characteristics between *H. coloradensis* and *H. complexus*. An approximate t'_s -test was calculated when variances were heteroscedastic (Sokal and Rohlf, 1981). Figures were drawn of representative worms with the aid of a camera lucida.

RESULTS

Haematoloechus coloradensis field studies

Mean SVL of the 142 frogs collected during July-August 2001 was 3.85 $\pm$ 0.72 cm; range 2.3-8.3 cm. Seventy-five of 142 (53%) frogs were infected with frog lung flukes with a mean abundance of 3.7 $\pm$ 7.3 (range = 0-44). A total of 530 worms were recovered; 491 immature and 39 mature, indicating that frogs were recruiting parasites during the collection period. Seasonally, worms became gravid by mid August. There was a statistically significant difference in the mean intensity of worms recovered from frogs that contained immature worms verses frogs infected with gravid worms. Most frogs contained non-gravid worms during July 24-August 8, with a single frog having one gravid worm, whereas most frogs contained gravid worms on August 16, with a single frog having three immature worms (Figure 1; $t'_s = 3.41$, $P < 0.001$). Additionally, of the 62 frogs maintained in the laboratory for a period of 4-6 wk, 30 of 62 frogs (48%) were infected with four immature and 60 mature (MI = 2.13 $\pm$ 1.33) *H. coloradensis* indicating

that only one species of frog lung fluke infected this frog population. Stomach contents data were obtained from 139 of 142 (98%) frogs, with a total of 576 individual invertebrates recovered. Sixteen different groups of aerial, terrestrial, and aquatic invertebrates were recovered with odonates making up 0.7% of the total diet (Figure 2). The average size of invertebrates ingested by these frogs was 6.8 mm (range 0.5-18 mm) with all large invertebrates ingested being soft bodied oligochaetes or lepidopteran larvae.

A total of 320 aquatic and semi-aquatic arthropods was collected, including larval and adult dragonflies and damselflies, adult coleoptera, larval diptera, larval ephemeroptera, adult hemiptera, and adult amphipoda. Of these, larval and adult dragonflies and damselflies were infected, but coleoptera, ephemeroptera, hemiptera, and amphipoda were also infected with *Haematoloechus* spp. metacercariae. All metacercariae were located in the head, legs, and hemocoel of the arthropods sampled, with prevalence ranging from as high as 94% in larval dragonflies to as low as 0% in dipteran larvae (Table I). None of the metacercariae were encapsulated by the arthropod host. These naturally infected arthropods potentially made up 11.5% of the stomach content data of frogs sampled from this location (Figure 2).

Mean SVL of the 25 northern leopard frogs collected during September 2002 was 4.05 $\pm$ 0.59 cm (range 3.3-6.5 cm), of these 22 of 25 (88%) frogs were infected with *H. coloradensis* with a mean abundance of 5 $\pm$ 4.46 (range 0-16). Mean SVL of the 40 northern leopard frogs collected during May-September of 2003 was 4.45 $\pm$ 0.77 cm (range 3.5–6.5 cm) of these 27 of 40 (68%) northern leopard frogs were infected with *H. coloradensis* with a mean abundance of 2.83 $\pm$ 3.35 (range 0-16). Two of 40 (5%) northern leopard frogs were infected with *H. medioplexus* with a mean abundance of 0.5

± 3 (range 0-19). Additionally, one of two (50%) bullfrogs SVL (5.5-6.0 cm) was infected with 19 *H. varioplexus*. Mean SVL of the 20 northern leopard frogs, 25 Woodhouse's toads, and 10 bullfrogs collected during July-September 2004 was 4.67 ± 1 (2.6-6.2) cm, 1.6 ± 0.5 (1-2,8) cm, and 7.6 ± 2.1 (4.5-10) cm, respectively. Three *Haematoloechus* species were recovered from these anurans. Northern Leopard frogs shared *H. coloradensis* with Woodhouse's toads, whereas bullfrogs were infected with *H. varioplexus* and shared *H. longiplexus* with northern leopard frogs (Table II).

***Haematoloechus coloradensis* laboratory life cycle studies**

Surviving lab reared and infected snails began shedding cercariae after a period of 30 days and continued to shed cercariae for up to two weeks, when observations were stopped. Metacercariae of *H. coloradensis* developed in all four species of non-odonate arthropod hosts exposed, although not all exposed individuals became infected. Prevalence ranged from a high of 80% for ephemeropterans to a low of 20% for amphipods (Table III). Ten of 10 (100%) dragonflies became infected with *H. coloradensis.* Additionally, two chironomid larvae metamorphosed on the day of necropsy; one of these insects was infected with a single *H. coloradensis* metacercaria. None of the metacercariae were encapsulated by any of the arthropod hosts. No *Haematoloechus* metacercariae were observed in any of the time-0 or time-T control groups. Four out of six (67%) lab reared northern leopard frogs given 10-15 *H. coloradensis* metacercariae from non-odonate hosts became infected with a mean intensity of 1.75 ± 1 (range 1-3), while none of the six time-T control lab reared northern leopard frogs were infected.

Additionally, when given metacercariae from experimentally infected dragonfly larvae three out of three (100%) northern leopard frogs became infected with one, two and three *H. coloradensis* and one out of two (50%) Woodhouse's toads with three *H. coloradensis*, but none of the three (0%) bullfrogs became infected.

Observations on the behavior of *H. coloradensis* cercariae indicated that cercariae stopped swimming upon contact with the arthropod host. Individual cercaria attached to the arthropod with their ventral sucker and began to crawl along the surface of the arthropods body with the aid of their ventral and oral suckers (Figure 3). Once cercariae encountered an intersegmental membrane they began to thrust the stylet into the membrane. Some of these were observed to drop their tails, pierce the intersegmental membrane, enter and develop to the metacercarial stage (Figure 3).

Haematoloechus complexus life cycle studies

Metacercariae of *H. complexus* developed in all three species of non-odonate arthropod hosts exposed, although not all exposed individuals became infected. Prevalence ranged from a high of 67% for chironomids to a low of 21% for amphipods. Metacercariae were located in the head, haemocoel, prolegs, legs, and anal gills of the arthropod hosts. Metacercariae were not encapsulated in any of the arthropod hosts except for a single *Callibaetis* sp. individual, which encapsulated and destroyed all of the metacercariae (Figure 4). Additionally four of 10 (40%) dragonflies became infected with *H. complexus* (Table IV). Two out of four (50%) toads became infected with a total of five *H. complexus*, two of which were adults. No time-0 or time-T control arthropods or amphibians were infected.

Other amphibian field surveys

Of 57 bullfrogs, five plains leopard frogs, and 10 plains spadefoot toads examined from Nevens Pond, and Cedar Point Biological Station, 42 of 57 (74%) of bullfrogs and three of five (60%) plains leopard frogs were infected with lung flukes, but none of the 10 (0%) plains spadefoot toads were infected. Thirty three of 57 (58%) bullfrogs were infected with *H. longiplexus* with a mean intensity of 18.6 $\pm$ 18.4, and 19 of 57 (12%) bullfrogs were infected with *H. varioplexus* with a mean intensity of 19.9 $\pm$ 25.5, whereas all infected plains leopard frogs were infected with *H. coloradensis* with a mean intensity of 1.3 $\pm$ 0.6. Overall prevalence and mean intensity of *Haematoloechus* species recovered from the five anurans sampled from Keith County, Nebraska, during 2000-2004 are given in Table V.

Morphological analysis

Diagnostic characteristics

Haematoloechus coloradensis (Cort, 1915) Ingles, 1932 (Figure 5)

Based on 20 mature specimens: Body elongate, 4.180 to 7.280 mm long by 0.820 to 1.550 mm wide. Oral sucker subterminal and oval 300 to 500 µm long by 220 to 420 µm wide. Pharynx 180 to 340 µm long by 190 to 330 µm wide. Oral sucker/pharynx width ratio 1.04 to 1.43. Oral sucker/pharynx length ratio 1.2 to 1.77. Acetabulum 24-50% body length from anterior end, round to oval 230 to 340 µm long by 200 to 380 µm wide. Oral sucker/acetabulum ratio 0.88 to 1.31. Testes round to oval positioned in tandem in midhindbody, 400 to 650 µm long by 380 to 720 µm wide. Cirrus sac long, extending to level of acetabulum. Genital pore ventral to pharynx. Ovary oval, rarely round posterior or dorsolateral to acetabulum 130 to 580 µm long by 70 to 450 µm wide.

Uterus with intercecal loops, never extending past ceca at posterior testis. Vitellaria acinous, follicular forming clusters, distribution differing on each side of body. On ovarian side seven to 10 extracecal clusters, rarely one intracecal cluster in anterior part of body. On opposite side of body seven to 12 extracecal clusters, rarely one to two intracecal clusters in posterior end of body. Eggs 30 to 37.5 µm long by 15 to 21 µm wide.

Haematoloechus complexus (Seely, 1906) Krull, 1933 (Figure 6)

Based on 20 mature specimens: Body elongate, 1.220 to 6.080 mm long by 0.820 to 1.190 mm wide. Oral sucker subterminal and oval 110 to 420 µm long by 110 to 420 µm wide. Pharynx 60 to 220 µm long by 70 to 240 µm wide. Oral sucker/pharynx width ratio 1.47 to 2.1. Oral sucker/pharynx length ratio 1.67 to 2.63. Acetabulum 33 to 48% body length from anterior end, round to oval 80 to 330 µm long by 80 to 310 µm wide. Oral sucker/acetabulum ratio 1.1 to 1.68. Testes round to oval positioned in tandem in midhindbody, 160 to 980 µm long by 150 to 850 µm wide. Cirrus sac long, extending to level of acetabulum. Genital pore ventral to pharynx. Ovary oval, rarely round posterior or dorsolateral to acetabulum, 300 to 600 µm long by 200 to 450 µm wide. Uterus with extracecal loops, always extending past ceca on left and/or right side of body past posterior testis. Vitellaria acinous, follicular forming clusters, distribution differing on each side of body. On ovarian side three to six extracecal clusters and zero to four intracecal clusters located pre acetabulum and one to two intracecal post posterior testis. On opposite side of body four to nine extracecal clusters, zero to four intracecal clusters pre acetabulum and zero to two intracecal clusters post posterior testis. Eggs 27.5 to 35 µm long by 15 to 17.5 µm wide.

Morphological comparisons among species

Morphological comparisons between *H. complexus* and *H. coloradensis* are presented in Table VI. Statistically significant differences were observed in body length, pharynx length and width, OS/PH width and length ratios, acetabulum length and width, OS/AC width ratios, testis length, and vitellaria number among *H. complexus* and *H. coloradensis*. Although these differences were statistically significant, there was overlap among all of these characteristics except for the oral sucker/pharynx width ratio. However, *H. coloradensis* had a uterus with intercecal loops that never extended past the cecae at the posterior testis level, whereas in *H. complexus* the uterus extracecal loops always extended past the cecae on the left and/or right side of the body past the posterior testis. Finally, the distribution of vitelline follicles differed among these two species. *Haematoloechus coloradensis* always had rows of extracecal vitelline follicles on each side of the body with rarely one group being intracecal in the anterior part of body located pre acetabular and rarely one to two intracecal clusters in the posterior end of the body located posterior of the posterior testis. *Haematoloechus complexus* on the other hand always had three to five groups of intracecal vitelline follicles in the anterior part of the body located pre acetabulum, and one to three intracecal vitelline follicles in the posterior part of the body located posterior of the posterior testis.

DISSCUSION

***Haematoloechus coloradensis* recruitment**

Field surveys of the five amphibian species in Keith County indicate that *H. coloradensis* is the dominant lung fluke in northern leopard frogs and plains leopard

frogs, whereas northern leopard frogs are rarely infected with *H. medioplexus* and *H. longiplexus*. Bullfrogs collected from the same locations as northern leopard frogs and plains leopard frogs are commonly infected with both *H. longiplexus* and *H. varioplexus*. These data support previous surveys on these frogs in Nebraska by Brooks (1976) and Snyder (1996) indicating that *H. longiplexus*, and *H. varioplexus* rarely or never infects northern leopard frogs or plains leopard frogs in Nebraska.

The field and laboratory studies I conducted show that in Nebraska, *H. coloradensis* as a generalist at the second intermediate host level and predominantly infects newly metamorphosed leopard frogs by using small non-odonate aquatic and semi-aquatic arthropods**.** Until recently, the limits of second intermediate host specificity among species of frog lung flukes were considered to be relatively strict, with most species infecting odonate second intermediate hosts. The life cycle of *H. coloradensis* was initially described from New Mexico by Dronen (1975), who demonstrated that this species also infected dragonflies and damselflies as second intermediate hosts and northern leopard frogs as definitive hosts. No attempt was made to infect non-odonate arthropods.

Reports of non-odonate second intermediate hosts for frog lung flukes are rare and restricted to dipteran and plecopteran larvae (van Thiel, 1930; Combes, 1968). However, all other studies on the life histories of frog lung flukes were conducted on odonates as second intermediate hosts, and it is unclear if non-odonate arthropod infections were attempted in these studies (Krull, 1930; 1933; 1934; Ingles, 1933; Grabda, 1960; Schell, 1965; Dronen, 1975; 1977; 1978; Bourgat and Kulo, 1979; Kennedy, 1980). Studies by Snyder and Janovy (1994, 1996), on second intermediate

host specificity of four Nebraska *Haematoloechus* species were the first to show that cercarial behavior patterns dictate host specificity at the second intermediate host level among the frog lung flukes. Their studies showed that *Haematoloechus* species from Nebraska varied from being generalists (*H. complexus*), infecting dragonflies and damselflies as well as non-odonate insects and crustacean species, to specialists (*H. medioplexus* and *H. varioplexus*), infecting only dragonflies. The various parasite species therefore may have different avenues for host colonization. *Haematoloechus medioplexus* and *H. varioplexus* cercariae are passive host invaders and must be drawn into the unique branchial basket respiratory apparatus of dragonflies, where they encyst and are restricted to this region of the host. *Haematoloechus complexus* cercariae are active host invaders; these cercariae stop upon contact with the arthropod host and penetrate the hosts at any inter-segmental membrane by the use of a stylet, and therefore may be found in all parts of the body, occurring in a thin hyaline cyst in the abdominal cavity, legs and head of the second intermediate host. Like *H. complexus, H. longiplexus* is also an active host invader, but can only penetrate damselflies naiads at the base of the caudal gills; *H. longiplexus* cercariae are also drawn into the branchial basket respiratory apparatus of dragonflies. Interestingly, Snyder (1996) also showed with a rather elegant injection experiment that cercariae of species that only use dragonfly second intermediate hosts can develop in damselfly arthropod hosts, showing that cercariae behavior is the main factor or avenue controlling second intermediate host specificity in these frog lung flukes. More importantly, phylogenetic studies on some North American and European frog lung flukes by Snyder and Tkach (2001) indicate that the use of odonates as second intermediate hosts may be the ancestral condition within this genus. From these and

other studies on frog lung fluke life cycles, it is clear that frog lung fluke species may vary in their host specificity at the second intermediate host level.

The present study clearly indicates that non-odonate arthropods are a viable avenue for the transmission of *H. coloradensis* and *H. complexus* to newly metamorphosed leopard frogs and toads in Nebraska. These observations, that *H. coloradensis* and *H. complexus* may colonize young of the year leopard frogs more commonly than other *Haematoloechus* species (*H. longiplexus*, *H. medioplexus*, and *H. varioplexus*) that only use odonates as second intermediate hosts, are important for two reasons: 1) the large number of species of second intermediate hosts utilized that are represented in these frogs' diet, and 2) the range of sizes of second intermediate hosts utilized.

These observations reveal that this second intermediate host infection pattern is the main reason why newly metamorphosed leopard frogs at Cedar Creek are commonly infected with *H. coloradensis* compared to other studies in which newly metamorphosed frogs are never or rarely infected with *Haematoloechus* species (Krull, 1930; 1931; Dronen, 1977; Gillilland and Muzzall, 1999; Muzzall et al., 2001; Bolek and Coggins, 2003). Because small frogs prey primarily on small invertebrates due to gape size limitations, the use of small, non-odonate arthropods as second intermediate hosts allows *H. coloradensis* and *H. complexus* to colonize newly metamorphosed frogs. In fact, of the 289 newly metamorphosed leopard frogs sampled at Cedar Creek during 2001-2004 168 (58%) were infected with *H. coloradensis*, whereas only one (0.3%) and two (0.7%) were infected with *H. longiplexus* and *H. medioplexus* respectively, and none (0%) were infected with *H. varioplexus*, all of which use dragonflies and damselflies, and

dragonflies as second intermediate hosts. Therefore, this study demonstrates that use of a diversity of small arthropod species as second intermediate hosts by *H. coloradensis* and *H. complexus* provides an avenue for colonization of small frogs, which prey primarily on small invertebrates presumably due to their limitations in gape size.

Among the five anurans sampled from Keith County, Nebraska both leopard frog species, and to al lesser degree Woodhouse's toads became infected with *H. coloradensis* in nature as well as in the laboratory, whereas bullfrogs were resistant to this species in nature and in the laboratory, confirming previous studies on definitive host specificity of *H. coloradensis* by Dronen (1975).

Haematoloechus coloradensis and *H. complexus* taxonomy and distribution

The morphological data on *H. coloradensis* and *H. complexus* suggest that although morphologically similar, these two species are distinct, differing in their oral sucker to pharynx width and length ratios, uterine loop distribution, and placement of vitelline follicles. However, in terms of their life cycles both species are quite similar. Both utilize physid snails as the first intermediate hosts, the cercariae of both of these species are active host invaders and can infect a wide range of non-odonate and odonate arthropods (generalists at the second intermediate host level), and both may be able to infect newly metamorphosed leopard frogs more commonly than other lung flukes that use only odonates as second intermediate hosts. As with *H. complexus*, upon contact with a potential arthropod host, the cercariae of *H. coloradensis* attach to the arthropod and are able to penetrate the host at any intersegmental membrane. Finally, both of these species infect northern leopard frogs, plains leopard frogs, and Woodhouse's toads, but cannot infect bullfrogs (Krull, 1933; Dronen, 1975).

The similarities in the life history of *H. coloradensis* and *H. complexus* suggest that they may be closely related species. In previous phylogenetic studies on other European and North American species of frog lung flukes (which did not include *H. coloradensis*), host specificity at the first and second intermediate host level have been shown by Snyder and Tkach (2001) to be conserved among related species of *Haematoloechus*. However, recent molecular phylogenetic studies by León-Règagnon and Brooks (2003) on African, European and North American species of frog lung flukes indicate that *H. complexus* and *H. coloradensis* form unrelated distinct lineages. I have examined the voucher specimens identified as *H. coloradensis* by León-Règagnon and Brooks (2003) (CNHE4661: six slides) and determined that it actually belongs to an undescribed species of *Haematoloechus*. It differs from *H. complexus* by its oral sucker to pharynx width ratio, and it differs from *H. coloradensis* by its distribution of vitelline follicles.

Haematoloechus coloradensis appears to be a western species, having been reported from five anuran species in the western United States: the Chiricahua leopard frog, *Rana chiricahuensis*, in Arizona, the northern leopard frog, *R. pipiens*, from Colorado, Idaho, Montana, and New Mexico; Woodhouse's toad, *B. woodhousii*, northern leopard frog, *R. pipiens*, and the plains leopard frog, *R. blairi*, in Nebraska, and Woodhouse's toad, *B. woodhousii*, southwestern toad, *Bufo microscaphus*, and northern leopard frog, *R. pipiens* in Utah (Cort, 1915; Frandsen and Grundman, 1960; Parry and Grundman, 1965; Dronen, 1975; Brooks, 1976). *Haematoloechus complexus* is an eastern species, being reported from seven species of anurans: the northern leopard frog, *R. pipiens*, in Iowa, and North Carolina, the green frog, *R. clamitans*, in Maryland and

Wisconsin, northern leopard frog, *R. pipiens*, wood frog, *Rana sylvatica*, and spring peeper, *Pseudacris crucifer*, in Ohio, northern leopard frog, *R. pipiens*, green frog, *R. clamitans*, and southern leopard frog, *Rana utricularia*, in Indiana and Kentucky, and northern leopard frog, *R. pipiens*, plains leopard frog, *R. blairi*, Woodhouse's toad, *B. woodhousii*, and copes gray treefrog, *Hyla chrysocelies*, in Nebraska (Brooks, 1976; Catalano and White, 1977; Cort, 1915; Krull, 1933; 1934; Odlaug, 1954; Seely, 1906; Ulmer, 1970; Whitehouse, 2002; personal observations). There are also reports of *H. complexus* from leopard frogs and other true frogs (*R. pipiens*, *Rana montezumae*, and *Rana vaillanti*) in Mexico (Caballero, 1942), however recent molecular phylogenetic studies suggest that these "*H. complexus*" may also be distinct and undescribed species (León-Règagnon and Brooks, 2003). Nebraska appears to be the eastern geographic limit for *H. coloradensis* and the western geographic limit for *H. complexus*.

It is unclear why these two species exhibit eastern versus western distributions, because their life cycles are so similar. However, except for northern leopard frogs, *R. pipiens*, *H. coloradensis* has been reported from species of frogs and toads that primarily have a distribution west of the Mississippi River (Brown, and Morris, 1990; Gergus, 1998; Platz and Mecham, 1979; Sullivan, et al., 1996). Recent biogeographical studies on the northern leopard frog, *R. pipiens*, using mitochondrial DNA also indicate that there are eastern and western lineages of these frogs genetically isolated by the Mississippi River (Hoffman and Blouin, 2004). *Haematoloechus complexus* on the other hand has been reported from frogs and toads that overlap in their distribution east and west of the Mississippi River and appears to be found in some strictly western species such as Woodhouse's toads (Conant and Collins, 1998).

The northern leopard frog, *R. pipiens,* in Nebraska has a more arid distribution than its eastern lineage; it predominantly uses clear sand bottom streams for reproduction and hibernation (Lynch, 1978; Vogt, 1981). During spring and fall, adult northern leopard frogs remain quite close to water but disperse into grassland during the summer. Importantly, newly metamorphosed northern leopard frogs are found closer to water than are the adults and therefore have different habitats than do the adults during the summer months (Vogt, 1981). My field observations support these findings and over the last four years during the summer I have never observed adult northern leopard frogs at Cedar Creek or any of the other small artificial ponds in the general area. In fact the only adult leopard frogs I have observed during the summer are the occasional individual plains leopard frog, *Rana blairi,* at some of the small artificial ponds. Studies by Kruse (1978) on the life histories of *R. blairi* and *R. pipiens* from Nebraska indicate that *R. pipiens* can readily replenish body moisture from soil capillary water, and has a much faster rater of rehydration than does the plains leopard frog, *R. blairi,* indicating that *R. pipiens* may be more adapted to a semi-terrestrial existence in western Nebraska than is *R. blairi*. In fact the plains leopard frog is not found west of the confluence of the North and South Platte Rivers in Nebraska except as an isolate along Lake McConaughy in Keith County (Lynch, 1978; 1985).

The only anurans commonly observed at Cedar Creek during the summer are an occasional bullfrog, *R. catesbeiana*, newly metamorphosed young of the year northern leopard frogs, *R. pipiens*, and Woodhouse's toads, *B. woodhousii*, which metamorphose during late June and early July and are found along the margin of Cedar Creek throughout the fall when they go into hibernation. These observations suggest that at this

location the only commonly occurring frogs that can support adult *H. coloradensis* worms and release eggs of this species back into an aquatic environment are newly metamorphosed northern leopard frogs, *R. pipiens*, and newly metamorphosed Woodhouse's toads, *B. woodhousii*. These observations on the natural history of the anuran hosts in western Nebraska, along with the generalist nature of *H. coloradensis* at the second intermediate host level show this life cycle is well suited for the Western Nebraska environment. The present study suggests that a particular life cycle variant will be favored by regional environmental conditions and specific definitive host combinations because these factors influence probabilities of transmission at one or more stages during a complex life cycle. This comparative approach to life cycle studies within both single and multiple species of hosts allows us to generate hypotheses about mechanisms that drive evolution of parasite life cycles.

ACKNOWLEDGMENTS

The author greatly acknowledges Dr. Virginia León-Règagnon, Universidad Nacional Autonoma de Mexico, and Dr. Agustín Jiménez-Ruiz, Harold. W. Manter Laboratory of Parasitology, University of Nebraska State Museum for providing specimens of *H. coloradensis* and *H. complexus* for comparisons. Additionally, I thank my wife Melissa Bolek for help in collecting frogs and snails, Randy Peterson and Dr. Brent Nickol, for access to field sites, Dr. Brent Nickol for the use of his animal room, and Cedar Point Biological Station for providing facilities. This work was supported by grants from the Center for Great Plains Studies graduate student grant-in-aid, University of Nebraska-Lincoln, Initiative for Ecology and Evolutionary Analysis, University of

Nebraska-Lincoln, and The School of Biological Sciences, University of Nebraska-Lincoln.

LITERATURE CITED

Bolek, M. G. and J. R. Coggins. 1998. Endoparasites of Cope's gray treefrog, *Hyla chrysoscelis*, and western chorus frog, *Pseudacris t. triseriata*, from southeastern Wisconsin. Journal of the Helminthological Society of Washington **65**: 212-218.

Bolek, M. G. and J. R. Coggins. 2000. Seasonal occurrence and community structure of helminth parasites from the eastern American toad, *Bufo a. americanus*, from southeastern Wisconsin, U.S.A. Comparative Parasitology **67**: 202-209.

Bolek, M. G. and J. R. Coggins. 2001. Seasonal occurrence and community structure of helminth parasites from the green frog, *Rana clamitans melanota*, from southeastern Wisconsin, U.S.A. Comparative Parasitology **68**: 164-172.

Bolek, M. G. and J. R. Coggins. 2003. Helminth community structure of sympatric eastern American toad, *Bufo a. americanus*, northern leopard frog, *Rana pipiens*, and blue-spotted salamander, *Ambystoma laterale*, from southeastern Wisconsin. Journal of Parasitology **89**: 673-680.

Bourgat, R., and S. D. Kulo. 1979. Cycle biologique d' *Haematoloechus johnsoni* Bourgat, 1977, (Trématode) parasite pulmonaire de *Dicroglossus occipitalis* (Günther, 1858) (Amphibien, Anoure) au Togo. Revue Suisse de Zoologie **86**: 467-472.

Borror, D. J., C. A. Triplehorn, and N. F. Johnson. 1989. An introduction to the study of insects. Sixth edition. Harcourt Brace College Publishers, Philadelphia. 875 p.

Brooks, D. R. 1976. Parasites of amphibians of the Great Plains: Part 2. Platyhelminths of amphibians in Nebraska. Bulletin of the University of Nebraska State Museum **10**: 65-92.

Brown, L. E., and M. A. Morris. 1990. Distribution, habitat, and zoogeography of the plains leopard frog (*Rana blairi*) in Illinois. Illinois Natural History Survey Biological Notes **136**: 1-6.

Caballero y C., E. 1942. Trematodos de las ranas de la cienaga de Lerma, Mexico. III. Redescripcion de una forma norteamerica de *Haematoloechus* y algunos consideraciones sobre *Glypthelmins californiensis* (Cort, 1919). Analis Instiutional Biologia Universidat Nacional de Mexico **13**: 71-79,

Catalono, P. A., and A. M. White. 1977. New host records of *Haematoloechus complexus* (Seely) Krull, 1933 from *Hyla crucifer* and *Rana sylvatica*. Ohio Journal of Science **77**: 99.

Combes, C. 1968. Biologie, écologie des cycles et biogeography de digénes et monogénes d'amphibiens dans l'est des Pyrénéés. Mémoires du Muséum National d'Histoire Naturelle **51**: 1-195

Conant, R., and J. T. Collins. 1998. A field guide to the reptiles and amphibians of eastern and central North America. Houghton-Mifflin, Boston, 616 p.

Cort, W. W. 1915. North American frog lung flukes. Transactions of the American Microscopical Society **34**: 203-243.

Dronen, N. O. Jr. 1975. The life cycle of *Haematoloechus coloradensis* Cort 1915 (Digenea: Plagiorchiidae), with emphasis on host susceptibility to infection. Journal of Parasitology **61**: 657-660.

Dronen, N. O. Jr. 1977. Studies on the population structure of two species of *Haematoloechus* Looss, 1899 (Digenea: Plagiorchiidae) in raniid frogs in New Mexico. Proceedings of the Helminthological Society of Washington **44**: 68-72.

Dronen, N. O. Jr. 1978. Host-parasite population dynamics of *Haematoloechus coloradensis* Cort, 1915 (Digenea: Plagiorchiidae). American Midland Naturalist **99**: 330-349.

Dunkle, S. W. 2000. Dragonflies through binoculars. A field guide to dragonflies of North America. Oxford University Press, 266 p.

Frandsen, J. C., and A. W. Grundman. 1960. The parasites of some amphibians of Utah. Journal of Parasitology **46**: 678.

Gergus, E. W. A. 1998. Systematics of the *Bufo microscaphus* complex: allozyme evidence. Herpetologica **54**: 317 - 325.

Gillilland, M. G., III, and P. M. Muzzall. 1999. Helminths infecting froglets of the northern leopard frog (*Rana pipiens*) from Foggy Bottom Marsh: Journal of the Helminthological Society of Washington **66**: 73–77.

Grabda, B. 1960. Life cycle of *Haematoloechus similis* (Looss, 1899) (Trematoda: Plagiorchiidae). Acta Parasitologica Polonica **8**: 357-367.

Hardin, E. L., and J. Janovy, Jr. 1988. Population dynamics of *Distoichometra bufonis* (Cestoda: Nematotaeniidae) in *Bufo woodhosii*. Journal of Parasitology **74**: 360-365.

Hoar, W. S., and C. P. Hickman, Jr. 1967. A laboratory companion for general and comparative physiology. Prentice-Hall, Inc. Englewood Cliffs, New Jersey, 296 p.

Hoffman, E. A., and M. S. Blouin. 2004. Evolutionary history of the northen leopard frog: Reconstruction of phylogeny, phylogeograpy, and historical changes in population demography from mitochondrial DNA. Evolution **58**: 145-159.

Ingles, L. G. 1933. Studies on the structure and life history of *Ostiolum oxyorchis* (Ingles) from the California red-legged frog *Rana aurora draytoni*. University of California Publications in Zoology **39**: 135-161.

Kennedy, M. J. 1980. Host-induced variations in *Haematoloechus buttensis* (Trematoda: Haematoloechidae). Canadian Journal of Zoology **58**: 427-442.

Kennedy, M. J. 1981. A revision of the genus *Haematoloechus* Looss, 1899 (Trematoda: Haematoloechidae) from Canada and the United States. Canadian Journal of Zoology **59**: 1836-1846.

Krull, W. H. 1930. The life history of two North American frog lung flukes. Journal of Parasitology **16**: 207-212.

Krull, W. H. 1931. Life history studies on two frog lung flukes, *Pneumonoeces medioplexus* and *Pneumobites parviplexus*. Transactions of the American Microscopical Society **50**: 215-277.

Krull, W. H. 1932. Studies on the life history of *Haematoloechus longiplexus* (Stafford). Zoologischer Anzeiger **99**: 231-239.

Krull, W. H. 1933. Studies on the life history of a frog lung fluke, *Haematoloechus complexus* (Seely, 1906) Krull, n. comb. Zeitschrift für Parasitenkunde **6**: 193-206.

Krull, W. H. 1934. Some additional notes on the life history of a frog lung fluke, *Haematoloechus complexus* (Seely, 1906) Krull. Transactions of the American Microscopical Society **53**: 196-199.

Kruse, K. C. 1978. Causal factors limiting the distribution of leopard frogs in eastern Nebraska. Unpublished Ph.D. Thesis. University of Nebraska-Lincoln, 95 p.

León-Règagnon, V., and D. R. Brooks. 2003: Molecular phylogeny of *Haematoloechus* Looss, 1899 (Digenea: Plagiorchiidae), with emphasis on North American species. Journal of Parasitology **89**: 1206–1211.

León-Règagnon, V., D. R. Brooks, amd D. A. Zelmer. 2001: Morphological and molecular description of *Haematoloechus meridionalis* n. sp. (Digenea: Plagiorchioidae: Haematoloechidae) from *Rana vaillanti* Brocchi of Guanacaste, Costa Rica. Journal of Parasitology **87**: 1423-1427.

León-Règagnon, V., Paredes-Calderón, and L. Erika. 2002: *Haematoloechus danbrooski* n. sp. (Digenea: Plagiorchioidea) from *Rana vaillanit* from Los Tuxtlas, Veracruz, Mexico. Journal of Parasitology **88**: 1215-1221.

Lynch, J. D. 1978. The distribution of leopard frogs (*Rana blairi* and *Rana pipiens*) (Amphibia, Anura, Ranidae) in Nebraska. Journal of Herpetology **12**: 157-162.

Lynch, J. D. 1985. Annotated checklist of the amphibians and reptiles of Nebraska. Transactions of the Nebraska Academy of Sciences **8**: 33-57.

Margolis, L., G. W. Esch, J. C. Holmes, A. M. Kuris, and G. A. Schad. 1982. The use of ecological terms in parasitology (report of an ad hoc committee of the Amercian Society of Parasitologists). Journal of Parasitology **68**: 131-133.

McAlpine, D. F. 1997. Helminth communities in bullfrogs (*Rana catesbeiana*), green frogs (*Rana clamitans*), and leopard frogs (*Rana pipiens*) from New Brunswick, Canada. Canadian Journal of Zoology **75**: 1883-1890.

Merritt, R. W., and K. W. Cummins. 1996. An introduction to the aquatic insects of North America. Third edition. Kendall/Hunt Publishing Company, Dubuque, Iowa, 862 p.

Muzzall, P. M. 1991. Helminth infracommunities of the frogs *Rana catesbeiana* and *Rana clamitans* from Turkey Marsh, Michigan. Journal of Parasitology **77**: 366-371.

Muzzall, P. M., and C. R. Peebles. 1991. Helminths of the wood frog, *Rana sylvatica*, and the spring peeper, *Pseudacris c. crucifer*, from Southern Michigan. Journal of the Helminthological Society of Washington **58**: 263-265.

Muzzall, P. M., M. G. Gillilland, III, C. S. Summer, and C. J. Mehne. 2001. Helminth communities of green frogs *Rana clamitans* Latreille, from southwestern Michigan**.** Journal of Parasitology **87**: 962–968.

Needham, J. G., M. J. Westfall, Jr., and M. L. May. 2000. Dragonflies of North America. Revised edition. Scientific publishers, Gainesville, 939 p.

Odlaug, T. O. 1954. Parasites of some Ohio amphibia. Ohio Journal of Science **54**: 126-128.

Parry, J. E., and A. W. Grundman. 1965. Species composition and distribution of the parasites of some common amphibians of Iron and Washington counties, Utah. Proceedings of the Utah Academy of Sciences, Arts, and Letters **42**: 271-279.

Platz, J. E., and J. S. Mecham. 1979. *Rana chiricahuensis*, a new species of leopard frog (*Rana pipiens* complex) from Arizona. Copeia **1979**: 383-390.

Pritchard, M. H., and G. O. W. Kruse. 1982. The collection and preservation of animal parasites. University of Nebraska Press, Lincoln , Nebraska, 141 p.

Schell, S. C. 1965. The life history of *Haematoloechus breviplexus* Stafford, 1902, (Trematoda: Haplometridae McMullen, 1937), with emphasis on the development of the sporocysts. Journal of Parasitology **51**: 587-593.

Seely, L. B. 1906. Two distomes. Biological Bulletin **10**: 249-254.

Snyder, S. D. 1996. Host specificity among species of *Haematoloechus* (Digenea: Haematoloechidae). Unpublished Ph.D. Thesis. University of Nebraska-Lincoln, 112 p.

Snyder, S. D., and J. Janovy Jr. 1994. Second intermediate host-specificity of *Haematoloechus complexus* and *Haematoloechus medioplexus* (Digenea: Haematoloechidae). Journal of Parasitology **80**: 1052-1055.

Snyder, S. D., and J. Janovy Jr. 1996. Behavioral basis of second intermediate host specificity among four species of *Haematoloechus* (Digenea: Haematoloechidae). Journal of Parasitology **82**: 94-99.

Snyder, S. D., and V. V. Tkach. 2001. Phylogenetic and biogeographical relationships among some Holarctic frog lung flukes (Digenea: Haematoloechidae). Journal of Parasitology **87**: 1433-1440

Sokal, R. R., and J. F. Rohlf. 1981. Biometry. Second edition. W. H. Freeman and Company, New York, New York, 859 p.

Sullivan, B. K., K. B. Malmos, and M. F. Given. 1996. Systematics of the *Bufo woodhousii* complex (Anura: Bufonidae): Advertisement call variation. Copeia **1996**: 274-280.

Thorp, J. H., and A. P. Covich. 2001. Ecology and classification of North American freshwater invertebrates. Second edition. Academic press, San Diego, 1056 p.

Ulmer, M. J. 1970. Studies on the helminth fauna of Iowa. I. Trematodes of Amphibians. American Midland Naturalist **83**: 38-64.

van Theil, P. H. 1930. Die Entwicklung von *Agamodistomum anophelis* zum *Pneumonoecues variegates* Rud. Zentralblatt für Backteriologie, Parasitenkunde, und Infektionskrankheiten **117**: 103-112.

Vogt, R. C. 1981. Natural history of amphibians and reptiles of Wisconsin. The Milwaukee Public Museum, and Friends of the Museum, Inc., Milwaukee, Wisconsin, 205 p.

Westfall, M. J. Jr., and M. L. May. 1996. Damselflies of North America. Scientific Publishers, Gainesville, 649 p.

Whitehouse, C. A. 2002. A study of the frog lung flukes *Haematoloechus* (Trematoda: Haematoloechidae) collected from areas of Kentucky and Indiana. Proceedings of the Indiana Academy of Science **1**: 67-76.

Figure 1. Mean intensity of immature and gravid *Haematoloechus coloradensis* recovered from northern leopard frogs infected with predominantly immature worms during late July-early August and predominantly gravid worms during mid August 2001.

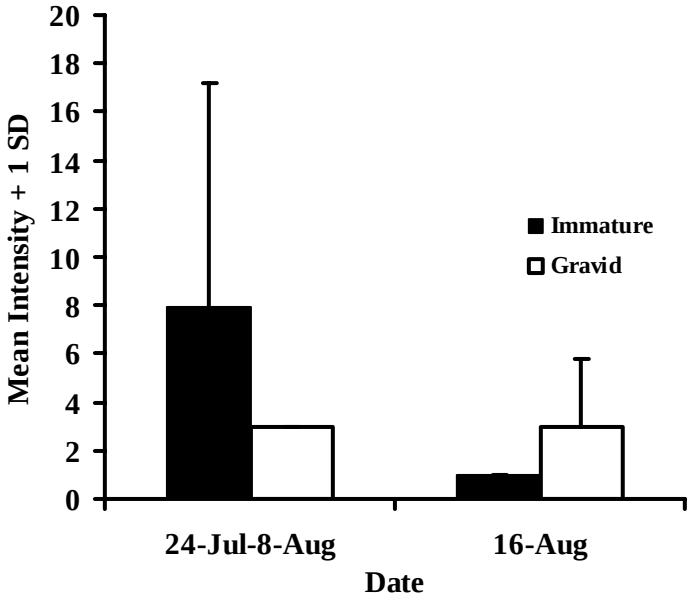
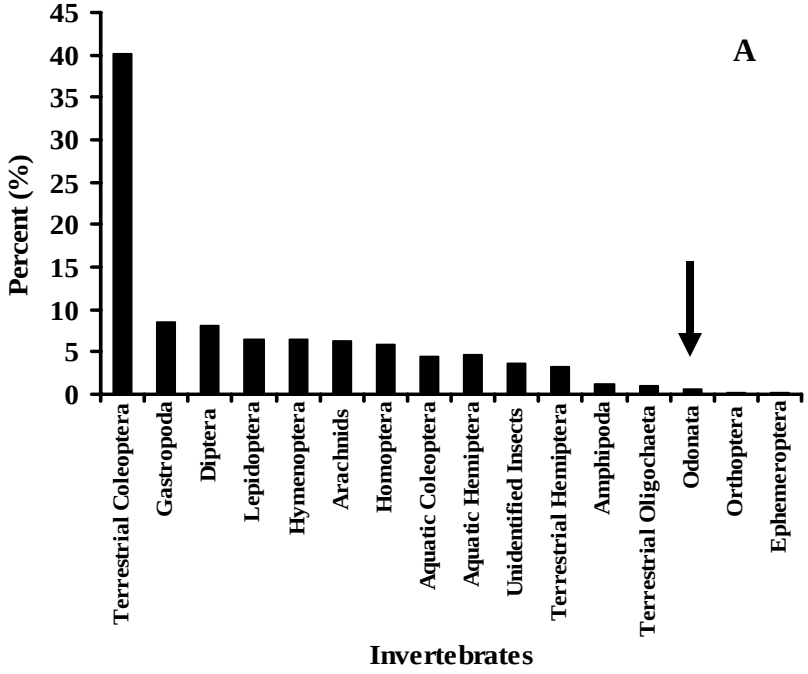

20
18
16
14
12
10
8
6
4
2
0
Mean Intensity + 1 SD
Immature
Gravid
24-Jul-8-Aug
16-Aug
Date

Figure 2. A. Frequency stomach contents data for 142 young of the year northern leopard frogs collected from Cedar Creek during 2001, note that odonates (arrow) make up only 0.7% of the diet. B. Frequency stomach contents data for 142 young of the year northern leopard frogs indicating the potential intermediate hosts for *Haematoloechus coloradensis*, recovered from Cedar Creek, Keith County Nebraska.


A
Percent (%)
45
40
35
30
25
20
15
10
5
0
Terrestrial Coleoptera
Gastropoda
Diptera
Lepidoptera
Hymenoptera
Arachnids
Homoptera
Aquatic Coleoptera
Aquatic Hemiptera
Unidentified Insects
Terrestrial Hemiptera
Amphipoda
Terrestrial Oligochaeta
Odonata
Orthoptera
Ephemeroptera
Invertebrates

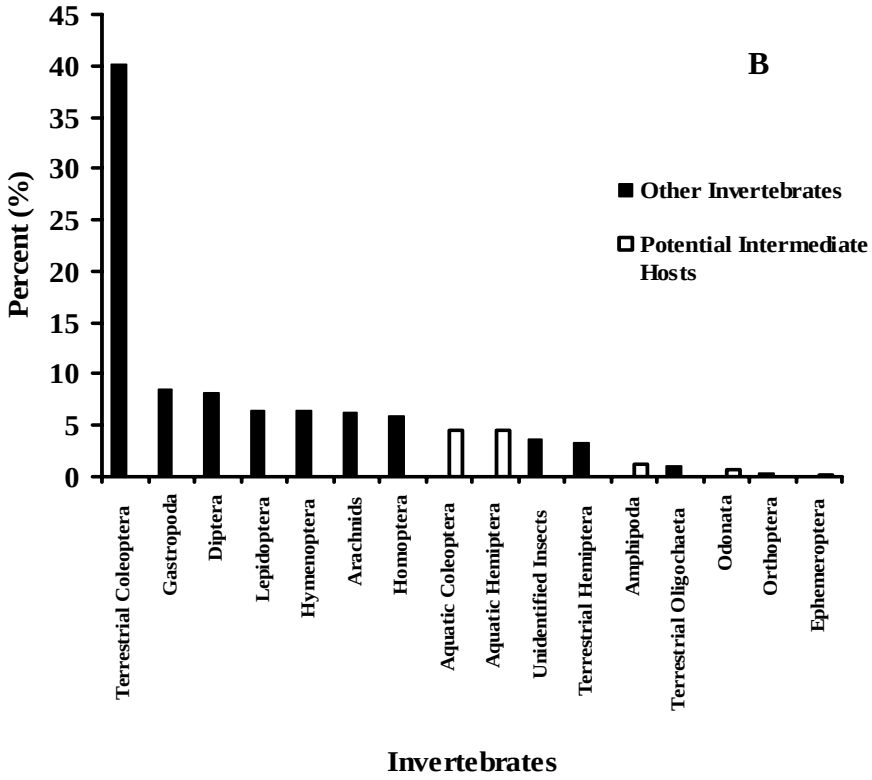

B
Percent (%)
45
40
35
30
25
20
15
10
5
0
Other Invertebrates
Potential Intermediate Hosts
Terrestrial Coleoptera
Gastropoda
Diptera
Lepidoptera
Hymenoptera
Arachnids
Homoptera
Aquatic Coleoptera
Aquatic Hemiptera
Unidentified Insects
Terrestrial Hemiptera
Amphipoda
Terrestrial Oligochaeta
Odonata
Orthoptera
Ephemeroptera
Invertebrates

Figure 3. *Haematoloechus coloradensis* cercarial attachment, creeping and penetration behavior on *Ischnura verticalis*. A. Cercaria attached with its ventral sucker (arrow) to the tibia of *Ischnura verticalis*. B. Cercaria attached with its oral sucker (arrow) to the tibia of *Ischnura verticalis* after one movement up the leg of the damselfly larva. C. Cercaria beginning to penetrate the intersegmental membrane of the thorax of *Ischnura verticalis*. D. Enlargement of C, note the penetrating cercaria and lost tail (arrows). Scale bars = 200 μm in A and B, 0.5 mm in C and 100 μm D.

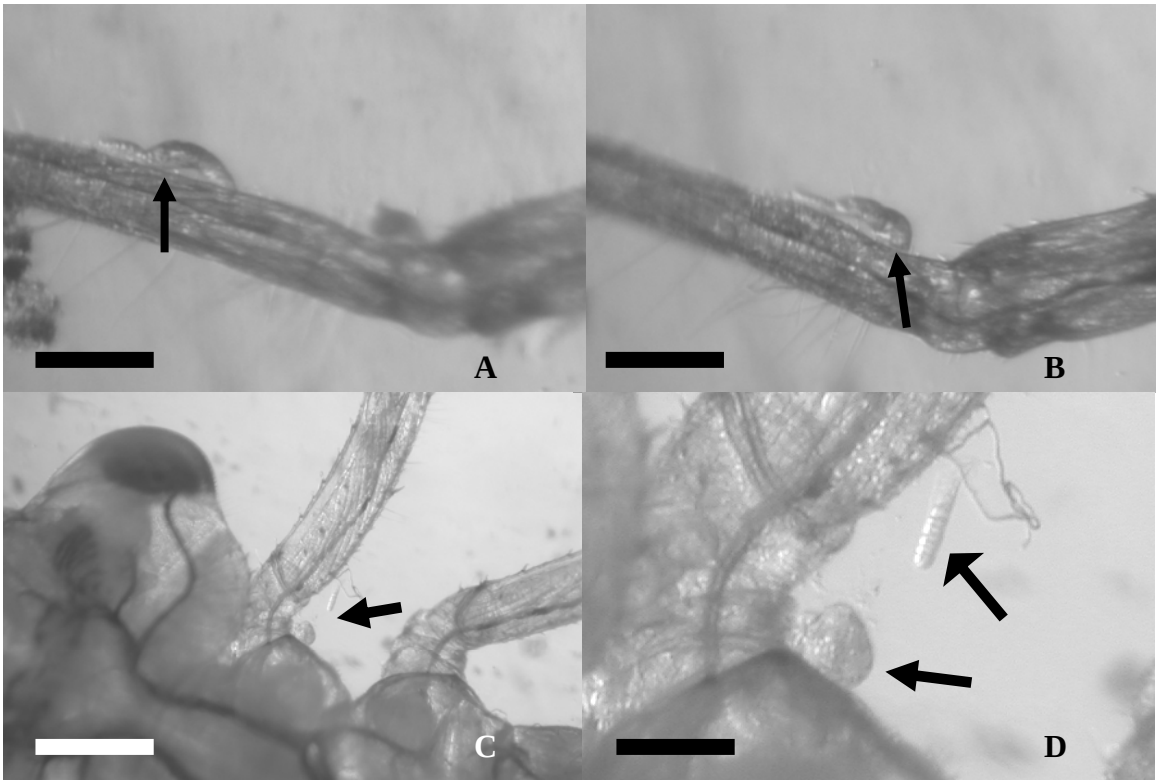

A
B
C
D

Figure 4. Metacercariae of *Haematoloechus complexus* in different body regions of non-odonate arthropods recovered four to six days post exposure. A. Metacercariae in prolegs of *Tanytarsus* sp. B. Metacercariae in anal gills of *Tanytarsus* sp. C. Metacercaria in thorax of *Tanytarsus* sp. D. Metacercaria in leg of *Hyalella azteca*. E. Encapsulated and dead metacercaria recovered from the thorax of *Callibaetis* sp. F. Note the stylet. Scale bars = 200 μm.

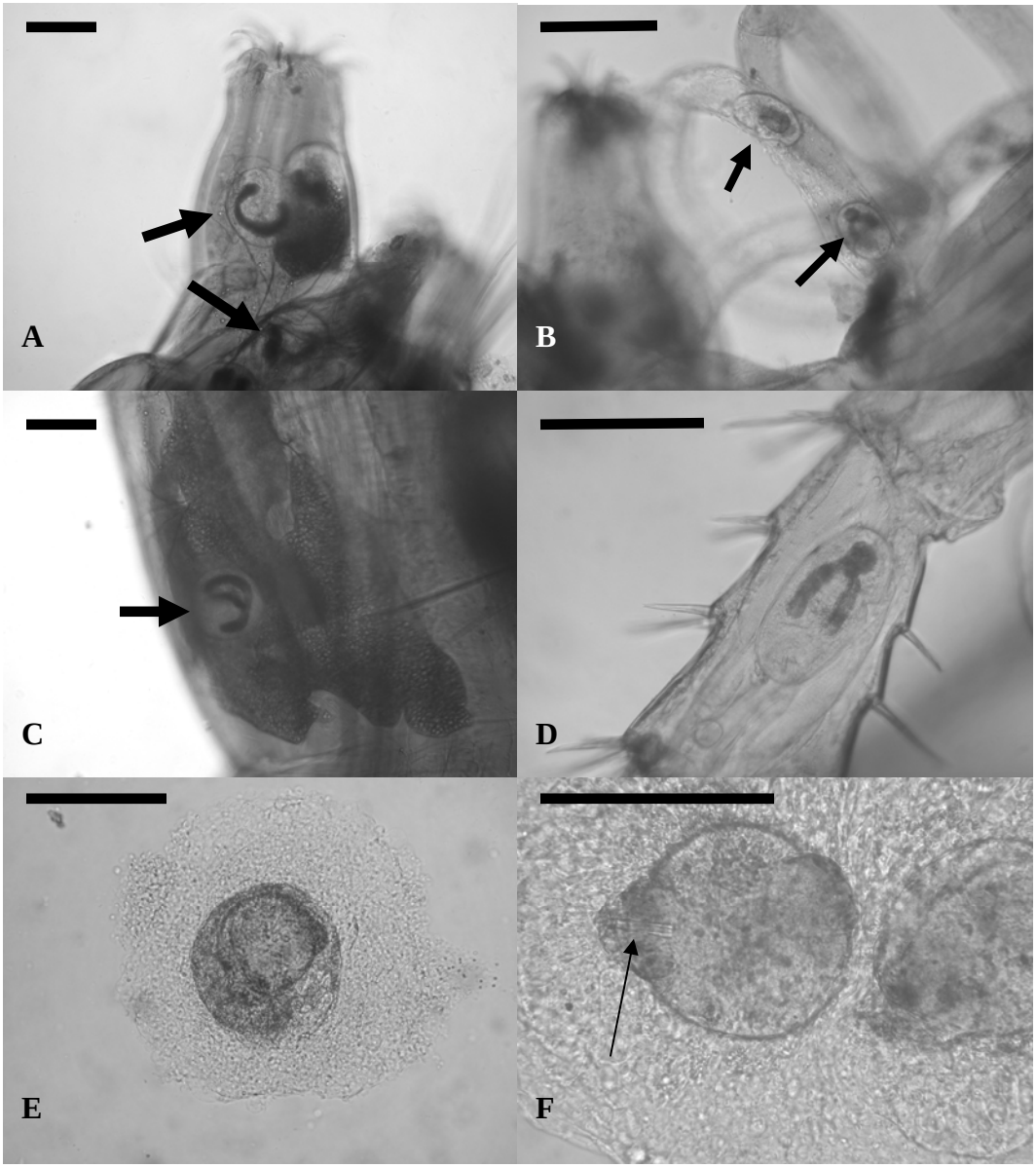

A
B
C
D
E
F

Figure 5. Line drawing of *Haematoloechus coloradensis* from the northern leopard frog, *Rana pipiens*, collected from Cedar Creek, Keith County, Nebraska. Scale bar = 1.5 mm.

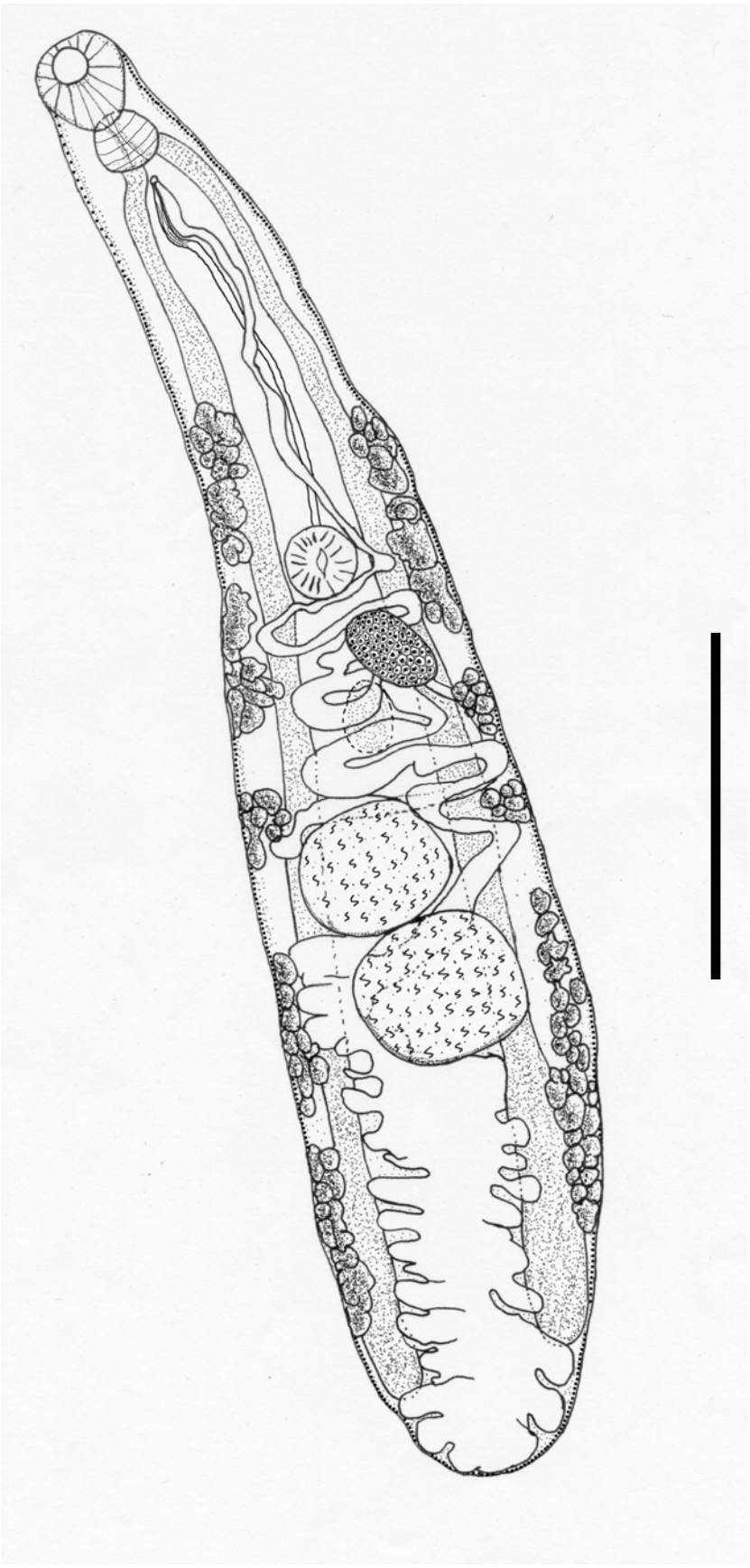


Figure 6. Line drawing of *Haematoloechus complexus* from the plains leopard frog, *Rana blairi*, collected from Pawnee Lake, Lancaster County, Nebraska. Scale bar = 1.5 mm.

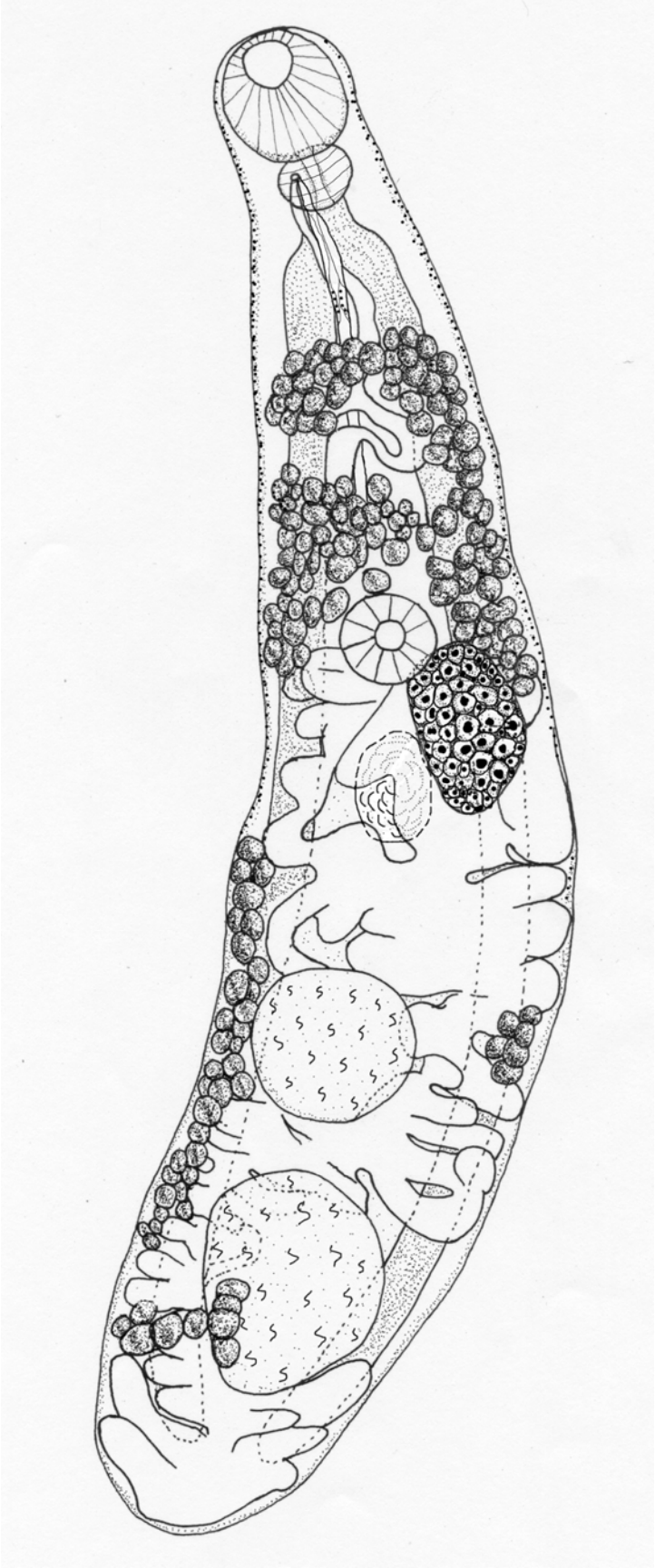


Table I. Prevalence, mean intensity, mean abundance, total number, and location of *Haematoloechus* spp. metacercariae in 320 arthropods collected from Cedar Creek, Keith County, Nebraska.

Family, genus or species of arthropods examined	Prevalence (no. infected/no. examined)	Mean intensity ± 1 SD (range)	Mean abundance ± 1 SD	No. of metacercariae recovered	Location in host
Insecta					
Odonata: Anisoptera					
Larva *Anax junius*	94 (15/16)	19.7 ± 11.3 (1-38)	18.5 ± 12	296	Head, thorax, leg
Adult Anisoptera					
Libellulidae*	7 (6/81)	4.1 ± 4.7 (1-12)	0.3 ± 1.6	25	Head, thorax, leg
Odonata: Zygoptera					
Larva Zygoptera					
Coenagrionidae†	67 (10/15)	3.9 ± 3.1 (1-11)	2.6 ± 3.1	39	Head, thorax, leg
Adult Zygoptera					
Hetaerina Americana	48 (13/27)	2.4 ± 2 (1-6)	1.1 ± 1.8	31	Head, thorax, leg
Coleoptera: Hydrophilidae	11 (3/27)	2.3 ± 1.5 (1-4)	0.3 ± 0.9	11	Thorax
Hemiptera: *Belostoma* sp.	9 (3/33)	1 ± 0 (1)	0.09 ± 0.3	3	Head and thorax
Ephemeroptera‡	10 (4/42)	3.5 ± 1.7 (1-5)	0.3 ± 1.1	14	Head, thorax, and gills
Diptera: Stratiomyidae larva	0 (0/9)	___	0 ± 0	0	Not found
Crustacea					
Amphipoda*:* *Hyalella azteca*	4 (3/70)	1.6 ± 0.6 (1-2)	0.07 ± 0.4	5	Cephalo-thorax, leg

* *Erythemis simplicicollis* (0/3), *Libellula luctuosa* (0/1), *Plathemis lydia* (0/3), *Sympetrum occidentale* (6/71), *S. semicinctum* (0/1), *S. rubicundulum* (0/1), and *S. vicinum* (0/1). † *Amphiagrion abbreviatum* (10/14), and *Ischnura verticalis* (0/1). ‡ *Callibaetis* sp (0/20), and *Caenis* sp. (4/22).

Table II. Prevalence (Pr), mean intensity (MI) and mean abundance (MA) of *Haematoloechus* species in newly metamorphosed and adults of northern leopard frogs, *Rana pipiens*, newly metamorphosed Woodhouse's toads, *Bufo woodhousii*, and newly metamorphosed and adult bullfrogs, *Rana catesbeiana,* from Cedar Creek and Breen's Flyway, Keith County, Nebraska during 2004.

	Rana pipiens N = 20			*Bufo woodhousii* N = 25			*Rana catesbeiana* N = 10		
	Pr	MI ± 1SD	MA ± 1SD	Pr	MI ± 1SD	MA ± 1SD	Pr	MI ± 1SD	MA ± 1SD
H. coloradensis	70%	6.64 ± 5.5	4.65 ± 5.5	4%	1	0.04 ± 0.2	0%	__	0 ± 0
H. longiplexus	5%	1	0.5 ± 0.22	0%	__	0 ± 0	30%	2.3 ± 1.6	0.7 ± 1.3
H. varioplexus	0%	__	0 ± 0	0%	__	0 ± 0	30%	37 ± 40.6	11.1 ± 26.2

Table III. Prevalence, mean intensity, mean abundance, total number, and location of *Haematoloechus coloradensis* metacercariae recovered four days post exposure in experimentally infected non-odonate arthropods.

Species of arthropods exposed	Prevalence (no. infected/no. exposed survivors)	Mean intensity ± 1 SD (range)	Mean abundance ± 1 SD	No. of metacercariae recovered	Location in host
Hemiptera:					
Belostomatidae					
Belostoma sp.	25 (5/20)	1.8 ± 1.3 (1-4)	0.45 ± 1	9	Head, thorax, leg
Diptera:					
Chironomidae					
Tanytarsus sp.	29 (16/55)	4.2 ± 4.0 (1-15)	1.2 ± 2.9	67	Head, thorax, anal gill
Ephemeroptera:					
Baetidae					
Callibaetis sp.	80 (8/10)	5.5 ± 4.5 (1-14)	4.4 ± 4.6	44	Head, thorax, leg, gill
Crustacea					
Amphipoda					
Hyalella azteca	20 (2/10)	1.5 ± 0.7 (1-2)	0.3 ± 0.7	3	Cephalothorax, leg

Table IV. Prevalence, mean intensity, mean abundance, total number, and location of *Haematoloechus complexus* metacercariae recovered four to six days post exposure in experimentally infected odonate and non-odonate arthropods.

Species of arthropods exposed	Prevalence (no. infected/no. exposed survivors)	Mean intensity ± 1 SD (range)	Mean abundance ± 1 SD	No. of metacercariae recovered	Location in host
Odonata					
Libellulidae					
Erythemis simplicicollis	40 (4/10)	22.3 ± 20.9 (3-50)	10 ± 16.2	89	Head, thorax, leg
Diptera:					
Chironomidae					
Tanytarsus sp.	67 (20/30)	6.2 ± 5.2 (1-18)	4.3 ± 5.2	124	Head, thorax, anal gill
Ephemeroptera:					
Baetidae					
Callibaetis sp.	40 (2/5)	6.5 ± 3.5 (4-9)	2.6 ± 4.0	13	Head, thorax, leg, gill
Crustacea					
Amphipoda					
Hyalella azteca	21 (5/24)	2.4 ± 2.1 (1-6)	0.5 ± 1.3	12	Cephalothorax, leg

Table V. Population structure of *Haematoloechus coloradensis*, *Haematoloechus longiplexus*, *Haematoloechus medioplexus*, and *Haematoloechus varioplexus* in five species of anurans collected in Keith County, Nebraska during July 2000 to September 2004.

Anuran Species (n)	*Haematoloechus* species	Prevalence (%)	Mean Intensity ± 1 SD
Bufo woodhousii (25)			
	H. coloradensis	4	1
	H. longiplexus	0	_
	H. medioplexus	0	_
	H. varioplexus	0	_
Rana blairi (5)			
	H. coloradensis	60	1.3 ± 0.6
	H. longiplexus	0	_
	H. medioplexus	0	_
	H. varioplexus	0	_
Rana catesbeiana (69)			
	H. coloradensis	0	_
	H. longiplexus	52	18.6 ± 18.4
	H. medioplexus	0	_
	H. varioplexus	33	22.3 ± 20.2
Rana pipiens (289)			
	H. coloradensis	58	5.5 ± 6.6
	H. longiplexus	0.3	1
	H. medioplexus	0.7	10 ± 12.7
	H. varioplexus	0	_
Spea bombifrons (10)			
	H. coloradensis	0	_
	H. longiplexus	0	_
	H. medioplexus	0	_
	H. varioplexus	0	_

Table VI. Morphological characteristics of adult *Haematoloechus coloradensis* and *Haematoloechus complexus*. Means and ranges (in parentheses) are given in µm unless otherwise noted.

	H. complexus n = 20 Mean (range)	*H. coloradensis* n = 20 Mean (range)	T-test	*P* /=/
Shape	Elongate	Elongate	__	__
Body length (mm)	4.910 mm (1.220-6.080)	5.644 mm (4.180-7.280)	-2.68	0.046
Body width at acetabulum (mm)	0.824 mm (0.260-1.100)	0.851 mm (0.550-1.350)	0.39	0.70
Greatest body width (mm)	0.982 mm (0.820-1.190)	1.067 mm (0.820-1.550)	1.33	0.20
Oral Sucker Length	332 (110-420)	364 (300-500)	1.65	0.11
Oral sucker width	335 (110-420)	304 (220-420)	-1.57	0.12
Pharnyx length	162 (60-200)	255 (180-340)	6.5	0.0001
Pharanyx width	189 (70-240)	241 (190-330)	4.45	0.0001
OS/PH width ratio	1.77 (1.47-2.10)	1.26 (1.04-1.43)	-10.28	0.0001
OS/PH length ratio	2.04 (1.67-2.63)	1.46 (1.20-1.77)	7.99	0.0001
Acetabulum location from anterior end	39% (33-48)	35% (24-50)	0.56	0.57

Table VI continued.

	H. complexus n = 20 Mean (range)	*H. coloradensis* n = 20 Mean (range)	T-test	*P* /=/
Acetabulum length	239 (80-330)	275 (230-340)	2.46	0.02
Acetabulum width	241 (80-310)	274 (200-380)	2.14	0.04
OS/AC width ratio	1.40 (1.10-1.68)	1.11 (0.88-1.31)	-6.52	0.0001
Testes length	685 (160-980)	507 (400-650)	-4.08*	0.0003
Testes width	540 (150-850)	520 (380-720)	-0.52	0.61
Ovary length	420 (300-600)	429 (130-580)	-0.30	0.76
Ovary width	314 (200-450)	310 (70-450)	0.15	0.88
Uterus	With loops extending posterior and lateral to cecal tips	With intercecal Loops	—	—
Vitellaria number	18.60 (16-21)	17.15 (15-21)	-2.67	0.011
Egg length	32.00 (27.50-35.00)	31.50 (28.00-35.00)	-0.63	0.53
Egg width	17.00 (15.00-17.50)	16.60 (15.00-21.00)	-0.91	0.37

* Approximate t'_s –test.

CHAPTER TWO: THE ROLE OF ODONATE METAMORPHOSIS IN THE SURVIVAL OF FOUR SPECIES OF FROG LUNG FLUKE METACERCARIAE

> "In nymphs the cysts, however, have never been found any distance from the branchial basket, and in the insect after transformation they have always been found loosely attached to the vestige of the respiratory organ in the posterior end of the abdomen." Krull (1931)

Abstract: Metacercarial survival patterns and their distribution in second intermediate odonate hosts were examined for four species of frog lung flukes. Surveys of aquatic larvae and recently emerged teneral dragonflies and damselflies indicated that prevalence and mean abundance of *Haematoloechus* metacercariae were significantly lower in teneral dragonflies than larval dragonflies, while there was no significant difference in prevalence or mean abundance of *Haematoloechus* metacercariae among larval and teneral damselflies. Experimental infections of dragonflies indicated that metacercariae of *H. coloradensis* and *H. complexus* were located in the head, thorax and branchial basket of dragonflies, whereas metacercariae of *H. longiplexus* and *H. varioplexus* were restricted to the branchial basket of these hosts. Metacercariae of *H. coloradensis*, *H. complexus* and *H. longiplexus* infected the head, thorax and abdomen of damselflies but these insects were resistant to infection with *H. varioplexus*. Subsequent metamorphosis experiments on experimentally infected dragonflies indicated that most metacercariae of

H. longiplexus were lost from the branchial basket during metamorphosis, but most metacercariae of *H. coloradensis*, *H. complexus*, and *H. varioplexus* survived dragonfly metamorphosis. These observations suggest that the observed ecological host specificity of *H. longiplexus* in semi-terrestrial leopard frogs may be due to few metacercariae of *H. longiplexus* reaching these frogs in a terrestrial environment. Because of the uncertain validity of *H. varioplexus* as a distinct species from its synonym *H. parviplexus* their morphological characters were reevaluated. The morphological data on *H. varioplexus* and *H. pariplexus* indicate that they differ in their acetabulum length and width, ovary shape, testes length, and egg length and width. Experimental infections of plains leopard frogs, northern leopard frogs, and bullfrogs with worms from bullfrogs, indicates that the synonymy of *H. parviplexus* with *H. varioplexus* was not warranted, and these flukes are distinct species: *H. parviplexus* in bullfrogs, and *H. varioplexus* in plains leopard frogs and northern leopard frogs.

INTRODUCTION

Field data on population structure of *Haematoloechus* species among aquatic and semi-terrestrial anurans suggests that ecological differences and diet of these hosts affect lung fluke distribution in the definitive hosts (Brooks, 1976; Dronen, 1977; Bolek and Coggins, 2003). Studies by Brooks (1976) and Snyder (1996) indicate that in Nebraska three *Rana* species commonly serve as definitive hosts for five species of frog lung flukes. Bullfrogs, *Rana catesbeiana*, are large aquatic species that are commonly infected with two species (*H. longiplexus* and *H. varioplexus*), but are resistant to infections with *H. medioplexus*, *H. coloradensis* and *H. complexus*. *Rana blairi,* the plains leopard frog, and *Rana pipiens,* the northern leopard frog, both of which are medium sized semi-terrestrial anurans, are infected with *H. coloradensis*, *H. complexus* and *H. medioplexus*. However, both of these leopard frogs exhibit ecological host specificity to *H. longiplexus* and *H. varioplexus*. Both Brooks (1976) and Snyder (1996) found over 40 % of bullfrogs infected with *H. longiplexus*, but only 1-2 % of plains leopard frogs and none of the northern leopard frogs were infected in nature. This observation is particularly interesting because Snyder (1996) in an experimental study infected seven out of 10 (70%) bullfrogs, two out of five (40%) plains leopard frogs, and three out of six (50%) northern leopard frogs with *H. longiplexus*, indicating that they are suitable hosts for this lung fluke. *Haematoloechus varioplexus* has been reported from all three of these frog species. However, since Kennedy (1981) synonymized *H. varioplexus* and *H. parviplexus* from bullfrogs and leopard frogs, *H. varioplexus* has not been found in northern leopard frogs and plains leopard frogs in Nebraska when collected from the same locations as bullfrogs that harbor this species (Snyder, 1996). These observations

suggest that the synonymy of *H. parviplexus* with *H. varioplexus* was not warranted, or that ecological factors are also effecting the distribution of *H. varioplexus* among aquatic bullfrogs and semi-terrestrial leopard frogs.

This ecological host specificity at the definitive host level is particularly interesting, considering that recent studies by Snyder and Janovy (1994, 1996), and Bolek (Chapter One) have shown that the cercarial behavior patterns of these five North American *Haematoloechus* species dictate host specificity at the second intermediate host level. *Haematoloechus coloradensis* and *H. complexus* are generalists within the arthropod host; metacercariae of these species are able to develop in a wide range of aquatic arthropod hosts including dragonflies (anisopteran), damselflies (zygopterans) as well as non-odonate arthropods. Metacercariae of *H. medioplexus* and *H. varioplexus* only develop in dragonflies and are considered specialists. Metacercariae of *H. longiplexus* develop in both dragonflies and damselflies and this species is considered to have intermediate arthropod host specificity.

The fact that leopard frogs are infected with *H. medioplexus* indicates that these frogs feed on odonates, but it does not explain why they are rarely or never infected with *H. longiplexus* and *H. varioplexus*, both of which that also utilize odonates as second intermediate hosts and infect bullfrogs. Studies on the diet of semi-terrestrial leopard frogs indicate that they commonly feed on terrestrial invertebrates including terrestrial stages of odonates, whereas aquatic bullfrogs commonly feed on aquatic stages of odonates (Dronen, 1977). These observations suggest that second intermediate odonate hosts may act as filters or sieves; only certain lung fluke species are able to pass through all filters and end up in the appropriate terrestrial environment and infect leopard frogs.

Therefore differences in second intermediate host specificity and second intermediate host life histories may play an important role in parasite movement, distribution, and the observed host specificity in definitive frog hosts.

Few studies have concentrated on frog lung flukes in invertebrate second intermediate hosts, and their role in movement of these parasites to their definitive hosts. Most surveys of odonates as second intermediate hosts for *Haematoloechus* species have concentrated on only the larval stages of odonates (Krull, 1930; 1933; 1934; Ingles, 1933; Grabda, 1960; Dronen, 1975; 1977; 1978; Bourgat and Kulo, 1979; Snyder and Janovy, 1996; Wetzel and Esch, 1996) and field evidence of *Haematoloechus* species in larval, teneral, and adult stages of the same species of odonate are lacking.

The present study has four main goals: (1.) to determine the population structure of frog lung flukes in six amphibian species from a single location, (2.) to determine the population structure of frog lung fluke metacercariae in different life stages of odonate second intermediate hosts from a single location, (3.) to test whether metacercariae of four common *Haematoloechus* species can survive metamorphosis in dragonflies and damselflies, and (4.) to re-evaluate the definitive host specificity and diagnostic characteristics of *H. varioplexus* in bullfrogs and leopard frogs.

MATERIALS AND METHODS

Amphibian field studies

During March 2001-June 2005, a total of 399 individual amphibians of six species were collected, from Pawnee Lake, Lancaster County, Nebraska (40.84310, -96.5261), and examined for *Haematoloechus* species. These included 50 Blanchard's cricket frogs,

Acris crepitans blanchardi, 50 Woodhouse's toads, *B. woodhousii*, 36 Cope's gray treefrogs, *Hyla chrysoscelis*, 93 western chorus frogs, *Pseudacris t. triseriata*, 70 plains leopard frogs, *R. blairi*, and 100 bullfrogs, *R. catesbeiana*. Frogs and toads were collected at night by hand, brought back to the laboratory, euthanized, the snout vent length (SVL) was measured, and all organs were examined for parasites within 1-2 days of collection. Trematodes were removed, allowed to release eggs in tap water, and fixed in alcohol-formalin-acetic acid (AFA). Representative specimens were stained with Seminchon's acetocarmine (Pritchard and Kruse, 1982). All lung flukes were initially identified based on the keys provided by Kennedy (1981) and descriptions of *H. complexus* by Krull (1933), and *H. coloradensis* by Cort (1915). The chi-square test for independence was calculated to compare differences in prevalence among frogs.

Odonate field studies

During June-July 2001 a total of 381 larval, teneral, and adult anisopteran and zygopteran odonates of 10 species (Table II) were collected from Nickol Pond, Cass County, Nebraska (40.81412, -96.46000) and examined for *Haematoloechus* species metacercariae. Larval odonates were collected by dip net, placed in buckets of water with no snails, and brought back to the laboratory. Teneral and adult odonates were collected with a butterfly net along the edges of Nickol Pond, placed in 3.78 L plastic containers, stored on ice and brought back to the laboratory. All odonates were identified according to Westfall and May (1996) and Needham et al., (2000) before being processed. Each individual zygopteran larva, teneral, and adult odonate was divided into three body regions: the head, the thorax including the legs, and the abdomen including the anal gills for the larvae, while all larva, teneral, and adult anisopteran odonates were

divided into four body regions: the head, the thorax including the legs, the abdomen, and the branchial basket for larvae or remnants of the branchial basket for the tenerals and adults. All odonates where then teased apart and examined for metacercariae of *Haematoloechus* species and their location. All *Haematoloechus* metacercariae were identified to genus based on descriptions provided by Krull (1930; 1931; 1932; 1933; 1934); species identification was not attempted. Due to low samples sizes of some species and the ability of adult dragonflies and damselflies to colonize ponds from locations other than where they metamorphosed (Conrad, et al., 1999) statistical comparisons of infection rates were only compared among larval and teneral stages of the two most commonly collected dragonfly and damselfly species. The chi-square test for independence was calculated to compare differences in prevalence of larval and teneral life stages of odonates, while Student's *t*-test was used to compare differences in mean abundance between larval and teneral life stages of odonates. Approximate t'_s -tests were calculated when variances were heteroscedastic (Sokal and Rohlf, 1981).

Odonate metamorphosis experiments

Two species of odonates that were commonly infected in nature with frog lung fluke metacercariae were chosen for experimental infections and metamorphosis experiments. These included the eastern forktail damselfly, *Ischnura verticalis,* and the eastern pondhawk dragonfly, *Erythemis simplicicollis*. Both of these species are commonly found in Nebraska, easily maintained in the laboratory and previous reports indicate that they serve as intermediate hosts for frog lung flukes in other locations throughout North America (Grieve, 1937; McVey, 1985; Snyder and Janovy, 1996; Westfall and May, 1996; Wetzel and Esch, 1996; Corbet, 1999; Needham, et al., 2000).

During June-July 2002, a total of 100 naturally exposed ultimate or penultimate instars of the eastern pondhawk dragonfly, *E. simplicicollis*, and 25 naturally exposed ultimate or penultimate instars of the eastern forktail damselfly, *I. verticalis*, were collected by dip-net at Nickol's Pond. Each odonate was placed in an individual 1,000 ml jar with 200 ml of aged tap water along with a single 15 cm wooden applicator stick as a perch, and covered with a screen lid. All odonates were fed field collected chironomid larvae, freshwater oligochaetes, or laboratory reared *Daphnia pulex* crustaceans three times a week; water was changed weekly. Cast exuviae were removed and examined for *Haematoloechus* metacercariae. After metamorphosis, all teneral damselflies and dragonflies along with their respective exuviae were divided into three or four body regions respectively and examined for *Haematoloechus* metacercariae. Because *Haematoloechus* metacercariae take up to four days to become infective to the definitive frog host, only dragonflies that were infected for at least one week before metamorphosis were used in data analysis (Krull, 1930; 1931; 1933). Larval prevalence and mean abundances were calculated by combining any metacercariae left in the exuviae and the metacercariae recovered from the teneral stage. Additionally, 10 *E. simplicicollis* exuviae were collected from Nickol Pond soaked in water and examined for *Haematoloechus* metacercariae in order to confirm that the loss of metacercariae during metamorphosis was not a laboratory artifact. Comparisons among mean abundance of larval and teneral life stages of *I. verticalis* and *E. simplicicollis* were compared with paired *t*-tests, while prevalence was compared with the chi-square test for independence. The Wilcoxon single rank test was calculated when variances were heteroscedastic (Sokal and Rohlf, 1981).

Odonate experimental infections

Gravid *H. coloradensis* flukes were obtained from wild-caught northern leopard frogs, *R. pipiens*, from Cedar Creek, Keith County, Nebraska (41.18639, -101.36276), gravid *H. complexus* flukes were obtained from wild-caught plains leopard frogs, *R. blairi*, from Pawnee Lake, Lancaster County, Nebraska and gravid *H. longiplexus* and *H. varioplexus* flukes were obtained from wild-caught bullfrogs, *R. catesbeiana*, from Pawnee Lake, Lancaster County and Nevens Pond, Keith County, Nebraska (41.20710, -101.40850) respectively. Individual worms were placed in 70 ml plastic containers containing aged tap water and allowed to release eggs. Worms were then fixed in AFA, stained, and identified to species. *Physa gyrina* snails used for *H. complexus* and *H. coloradensis* infections were laboratory reared, and infected using techniques described in Chapter One. Additionally, *Gyraulus parvus* snails were established in the laboratory from wild strains collected from Dunwoody Pond, Keith County, Nebraska (41.21527, -101.578610). Snails were maintained on a diet of frozen mustard greens, maple leaves, and Tetra Min® fish food. *Gyraulus parvus* snails were reared from eggs for a period of six weeks in the laboratory. Groups of laboratory reared *G. parvus* snails were exposed to eggs of *H. longiplexus* or *H. varioplexus* using the techniques described in Chapter One.

Due to high snail mortality of *G. parvus* experimentally infected with *H. varioplexus*, naturally infected snails infected with *H. varioplexus* were collected from Nevens Pond, Keith County, Nebraska. During 2000-2004, bullfrogs, plains leopard frogs, Woodhouse's toads and tiger salamanders, *Ambystoma tigrinum mavortium*, were collected from this location and the only amphibian species infected with *H. varioplexus*

was bullfrogs (Chapter One; personal observations). Two frog lung fluke species, *H. longiplexus* and *H. varioplexus,* use *G. parvus* snails as first intermediate hosts at Nevens Pond. Therefore, to be sure that snails were infected with *H. varioplexus*, cercariae were identified to species based on morphology, based on the inability to infect damselflies, (Krull, 1931; Snyder and Janovy, 1996), and by feeding developed metacercariae from dragonflies to bullfrogs, plains leopard frogs, and northern leopard frogs and recovering adult worms for identification.

Ultimate and penultimate larvae of *E. simplicicollis* and *I. verticalis*, dragonflies and damselflies used in the second intermediate host infections came from Nickol Pond, Cass County, Oak Lake, Lancaster County, (40.83056, -96.70778) and Dunwoody Pond, Keith County, Nebraska. Dragonflies and damselflies were divided into three equal groups and assigned to either time-0 controls, experimentals, or time-T controls and were isolated in 5 ml well plates filled with aged tap water for 24 hr before exposure. All odonate infections followed the same procedure as previously described in Chapter One.

Twenty-four hours after exposure, all exposed odonates and time-T controls were transferred to individual 1,000 ml jars with 200 ml of aged tap water along with a single 15 cm applicator stick as a perch, covered with a screen lid, and fed three times a week as previously described. Upon metamorphosis, individual tenerals along with their exuviae were examined for *Haematoloechus* metacercariae as previously described. Comparisons among infection rates in larval and teneral life stages of *E. simplicicollis* were compared with paired *t*-tests, and the chi-square test for independence. The Wilcoxon single rank test was calculated when variances were heteroscedastic (Sokal and Rohlf, 1981).

Haematoloechus varioplexus frog experimental infections

Anuran host specificity experiments were conducted in two separate trials. Three anuran species were used. Metacercariae assumed to be *H. varioplexus* recovered from *E. simplicicollis* dragonfly metamorphosis experiments were used in the infections. All metacercaraie from *E. simplicicollis* dragonflies were dissected into dishes of odonate saline (Fielden, 1960) and randomly mixed. Northern leopard frogs, *R. pipiens*, were reared from tadpoles collected at Cedar Creek, Keith County, Nebraska, and bullfrogs, *R. catesbeiana*, were reared from tadpoles collected from Nevens Pond, Keith County and Pawnee Lake, Lancaster County, Nebraska as described in Chapter One. In trial I, four laboratory reared northern leopard frogs, *R. pipiens*, and four laboratory reared bullfrogs, *R. catesbeiana*, were each given 10-40 *H. varioplexus* metacercariae. In trial II, three laboratory reared bullfrogs, *R. catesbeiana*, and three field collected young of the year plains leopard frogs, *R. blairi*, along with time-0 and time-T controls were collected from Pawnee Lake, Lancaster County, Nebraska. Frogs in the experimental group were each given 10-20 *H. varioplexus* metacercaria. For all infections, metacercariae were drawn into a pipette, and the pipette was placed into the esophagous of an experimental frog and the metacercariae were forced into the esophagous. The pipette then was examined under a dissecting microscope to confirm that no metacercariae remained. All exposed frogs along with time-T controls of *R. blairi*, were maintained individually in plastic shoe boxes (35 cm X 25 cm X 15 cm), and fed commercial crickets three times a week. Water was changed once or twice a week. Thirty to 35 days post exposure all exposed frogs along with the time-T controls *R. blairi* were euthanized and examined for frog lung flukes.

Frog odonate feeding trials

Eighteen newly metamorphosed bullfrogs, and five adult plains leopard frogs were collected from Pawnee Lake, Lancaster County, Nebraska. Frogs were housed in individual plastic shoe boxes (35 cm X 25 cm X 15 cm) containing 5 cm of water for 24 hr before introduction of a single ultimate *E. simplicicollis* larvae. Shoe boxes were checked every hour on the first day and once daily over a period of a week to see if frogs ingested dragonfly larvae.

Morphological studies

Due to recent confusion on the taxonomy of *H. varioplexus*, worms recovered from bullfrogs and plains leopard frogs were also compared and identified according to the descriptions of *H. parviplexus* by Irwin (1929), and *H. varioplexus* and *H. similiplexus* by Stafford (1902) and Cort (1915). Morphological data were collected on 20 *H. varioplexus* worms from northern leopard frogs, plains leopard frogs, Woodhouse's toads, and wood frogs, *Rana sylvatica*, and 20 *H. parviplexus* worms from bullfrogs, and green frogs, *Rana clamitans*. Worms used for morphological analysis were collected from a number of locations in Nebraska, New York, South Dakota, and Wisconsin. These included one *H. varioplexus* collected from a northern leopard frogs from Lake Preston, Kingsbury County, South Dakota (44.37581, -97.55479), and six *H. varioplexus* collected from wood frogs from the University of Wisconsin-Milwaukee Field Station, Ozaukee County, Wisconsin (43.38875, -88.02208); one *H. parviplexus* collected from a green frog from Queechy Lake, Colombia County, New York (42.40246, -73.42456), eight *H. parviplexus* collected from green frogs from Genesse Depot, Waukesha County, Wisconsin (42.98984, -88.36634), two *H. parviplexus* collected from a bullfrog from

Pawnee Lake, Lancaster County, Nebraska, and three *H. parviplexus* from bullfrog experimental infections from Nevens Pond, Keith County, Nebraska. Additionally, in order to get a better geographical representation *H. varioplexus* and *H. parviplexus* voucher specimens collected by Dan Brooks and Stewart C. Schell were borrowed from the H. W. Manter Laboratory, University of Nebraska State Museum. These included, *H. varioplexus* HWML 20151: from plains leopard frogs from Lancaster County, Nebraska (40.791665, -96.675), six slides, HWML 20153: from a northern leopard frog from Nance County, Nebraska (41.424305, -97.86542), one slide; HWML 20155: from a Woodhouse's toad from Nance County, Nebraska (41.424305, -97.86542), three slides; HWML 20157: from a northern leopard frog from Cherry County, Nebraska (42.71139, -100.82528), one slide, HWML 20158: from a northern leopard frog from Dawes County, Nebraska (42.699865, -103.272775), one slide, HWML 20159: from a northern leopard frog from Grant County, Nebraska (41.99597, -101.63667), one slide, and HWML 20160: from a plains leopard frog from Webster County, Nebraska (40.12778, -98.509865), one slide; *H. parviplexus* HWML 20142: from a bullfrog from Richardson County, Nebraska (40.156945, -95.82236), five slides; HWML 20143: from a bullfrog from Rock County, Nebraska (42.49153, -98.872225), one slide, and HWML 23879: from a green frog from Wisconsin, (no latitude or longitude date given) one slide. Morphological characters recorded for each worm were the same ones taken for *H. coloradensis* and *H. complexus* in Chapter One. Student's two tailed *t*-test was used to compare differences in morphology among morphological characteristics of *H. parviplexus* and *H. varioplexus*. An approximate t'_s-test was calculated when variances

were heteroscedastic (Sokal and Rohlf, 1981). Figures were drawn of representative worms with the aid of a camera lucida.

RESULTS

Amphibian field studies

Of the six amphibian species examined from Pawnee Lake, only bullfrogs and plains leopard frogs were infected with frog lung flukes (Table I). Bullfrogs were infected with *H. longiplexus* and *H. varioplexus*, whereas plains leopard frogs were predominantly infected with *H. complexus*, with a single plains leopard frog containing one individual *H. complexus* and *H. longiplexus* in each lung. Statistically significant differences were observed in prevalence among all three species of frog lung flukes infecting bullfrogs and plains leopard frogs: *H. complexus* $\chi 2 = 52.04$, df = 1, $P < 0.001$; *H. longiplexus* $\chi 2 = 16.72$, df = 1, $P < 0.001$; *H. varioplexus* $\chi 2 = 14.97$ df = 1, $P < 0.001$.

Odonate field studies

A total of 387 odonates (209 anisopterans and 178 zygopterans) of 10 species were collected from Nickol Pond, however not all life stages were collected for each species. Twenty-two of 209 (10.5%) anisopterans and 39 of 178 (22%) zygopterans were infected with *Haematoloechus* spp. metacercariae. Prevalence and mean intensity varied among the different species and life stages (Table II). All metacercariae recovered from zygopteran hosts were located in the head, thorax, and abdomen, while all metacercariae recovered from anisopteran hosts were located in the branchial basket of the larvae or the remnants of the branchial basket of tenerals and adult dragonflies. Of the two most commonly collected species, there was no significant difference in prevalence or mean

abundance of *Haematoloechus* metacercariae among larvae or tenerals of the eastern forktail damselfly, *I. verticalis* (Figure 1; $\chi 2 = 0.42$, df = 1, $P > 0.05$; $t'_s = -1.03$, df = 34, $P = 0.31$), whereas larval eastern pondhawk dragonflies, *E. simplicicollis* had a significantly higher prevalence and mean abundance of *Haematoloechus* metacercriae than the tenerals, *E. simplicicollis* (Figure 1; $\chi 2 = 6.16$, df = 1, $P < 0.025$; $t'_s = 2.07$, df = 107, $P = 0.04$).

Odonate metamorphosis experiments

Eleven of 25 (44%) laboratory metamorphosed eastern forktail damselflies, *I. verticalis,* were infected with *Haematoloechus* metacercariae with a mean intensity of 2.5 $\pm$ 1.6. All metacercariae were located in the head, thorax and abdomen and all survived metamorphosis. There were no statistically significant differences in prevalence or mean abundance between the larval and teneral stages of *I. verticalis* (Figure 2; $\chi 2 = 0.0$, df = 1, $P > 0.05$; Paired *t*-test = 0.0, df = 24, $P > 0.05$).

Sixty five of 100 (65%) eastern pondhawk dragonfly larvae, *E. simplicicollis,* were infected with *Haematoloechus* metacercariae. Most metacercariae were located in the branchial basket, however a few were located in the thorax. All metacercariae located in the thorax survived dragonfly metamorphosis, whereas a significant proportion of metacercariae located in the branchial basket were lost during metamorphosis. There was a statistically significant difference in prevalence and mean abundance of metacercariae located in the branchial basket region of the larvae and tenerals of eastern pondhawk dragonflies, *E. simplicicollis*, whereas there was no statistically significant difference in prevalence and mean abundance of metacercariae located in the thorax of the larvae or tenerals of the eastern pondhawk dragonflies, *E. simplicicollis* (Figure 2; metacercariae in

branchial basket: $\chi 2 = 32.84$, df = 1, $P < 0.0001$; Wilcoxon Signed Rank Test Z = -6.34, $P < 0.0001$; metacercariae in thorax: $\chi 2 = 0.0$, df = 1, $P > 0.05$; Paired *t*-test = 0.0, df = 99, $P > 0.05$).

Of 131 *Haematoloechus* metacercariae located in the branchial basket of larval *E. simplicicollis*, 99 (76%) were lost during metamorphosis. Metacercariae left behind in the remnants of the branchial basket of the exuviae of *E. simplicicollis* were unencysted or lightly encysted (Figure 3). Of the 32 metacercariae located in the remnants of the branchial basket region of tenerals *E. simplicicollis* that survived metamorphosis, 22 (68%) were encapsulated by the dragonfly (Figure 3). Additionally, two of 10 (20%) *E. simplicicollis* exuviae collected at Nickol Pond contained one and two *Haematoloechus* metacercariae, indicating that the loss of these metacercariae was not a laboratory artifact.

Odonate experimental infections

All four *Haematoloechus* species developed metacercariae in eastern pondhawks, *E. simplicicollis*, although not all exposed individuals became infected. *Haematoloechus coloradensis, H. complexus* and *H. longiplexus* metacercariae also developed in the damselfly *I. verticalis*, and were located in the head, thorax and abdomen of this host, whereas *H. varioplexus* was unable to infect this damselfly (Table III). None of the time-0 or time-T control dragonflies or damselflies was infected. None of the exposed damselflies survived through metamorphosis but most exposed dragonflies survived through metamorphosis.

Species specific differences were observed in the location of *Haematoloechus* metacercariae infecting dragonfly hosts among the four species of *Haematoloechus* (Table III). *Haematoloechus coloradensis* and *H. complexus* metacercariae were located

in the head, thorax and branchial basket of eastern pondhawk dragonflies, *E. simplicicollis,* whereas *H. longiplexus* and *H. varioplexus* were always restricted to the branchial basket of eastern pondhawk dragonflies, *E. simplicicollis*. During metamorphosis most metacercariae of *H. coloradensis*, *H. complexus*, and *H. varioplexus* survived metamorphosis in the eastern pondhawk, *E. simplicicollis*, whereas all but one metacercaria of *H. longiplexus* were lost during metamophosis (Figures 4 and 5). There was no significant difference in prevalence or mean abundance of *H. coloradensis* and *H. varioplexus* and prevalence of *H. complexus* among larval and teneral life stages of *E. simplicicollis* (*H. coloradensis*: $\chi 2 = 0.0$, df = 1, $P > 0.05$; Paired *t*-test = 2.22, df = 9, $P > 0.05$; *H. varioplexus*: $\chi 2 = 0.0$, df = 1, $P > 0.05$; Paired *t*-test = 1.00, df = 5, $P > 0.05$; *H. complexus*: $\chi 2 = 0.20$, df = 1, $P > 0.05$). However, statistically significant differences existed among the larval and teneral life stages of *E. simplicicollis* in prevalence and mean abundance for *H. longiplexus* ($\chi 2 = 5.84$, df = 1, $P < 0.05$; Wilcoxon Signed Rank Test $Z = -2.201$, $P < 0.05$) and mean abundance for *H. complexus* (Paired *t*-test = 2.3, df = 9, $P < 0.05$).

***Haematoloechus varioplexus* frog experimental infections**

In trial I, four out of four (100%) bullfrogs became infected whereas none of the northern leopard frogs (0%) became infected. In trial II, three out of three (100%) bullfrogs became infected whereas none of the plains leopard frogs (0%) became infected (Table IV). None of the time-0 or time-T control frogs were infected. All worms recovered from bullfrogs were gravid and most closely resembled the description of *H. parviplexus* (Irwin, 1929).

Frog odonate feeding trials

Of the 18 newly metamorphosed bullfrogs eight ingested larval *E. simplicicollis* within one hr of placing the larva dragonfly with frogs. Additionally five more newly metamorphosed bullfrogs ingested *E. simplicicollis* larvae within a week. Five bullfrogs did not ingest odonate larvae because dragonflies hid on or under the frogs for the duration of the trial. In total 13 of 18 bullfrogs ingested *E. simplicicollis* larvae. Of the five adult plains leopard frogs only a single individual ingested one *E. simplicicollis* larva within six days of placing the odonate larvae with plains leopard frogs.

Morphological analysis

Diagnostic characteristics

Haematoloechus parviplexus (Irwin, 1929) Harwood, 1932 (Figure 6)

Based on 20 mature specimens: Body elongate, 2.320 to 9.150 mm long by 0.400 to 1.500 mm wide. Oral sucker subterminal and round to oval 260 to 510 µm long by 220 to 480 µm wide. Pharynx 110 to 260 µm long by 110 to 250 µm wide. Oral sucker/pharynx width ratio 1.15 to 1.92. Oral sucker/pharynx length ratio 1.24 to 1.84. Acetabulum 37-53 % body length from anterior end, round to oval 50 to 150 µm long by 50 to 130 µm wide. Oral sucker/acetabulum ratio 1.78 to 4.00. Testes oval positioned in tandem in midhindbody, 440 to 1,800 µm long by 150 to 880 µm wide. Cirrus sac long, extending to level of acetabulum. Genital pore ventral to pharynx. Ovary dorsolateral to acetabulum, deeply lobed 350 to 1,180 µm long by 120 to 500 µm wide. Longitudinal extracecal uterine loops reaching intertesticular level. Vitellaria acinous, follicular forming clusters, distribution differing on each side of body. On ovarian side six to eight extracecal clusters, and zero to three intracecal clusters located pre acetabulum and zero

to three intracecal post posterior testis. On opposite side of body five to 10 extracecal clusters, zero to one intracecal clusters pre acetabulum and zero to three intracecal clusters post posterior testis Eggs 20 to 25 µm long by 12.5 to 18 µm wide.

Haematoloechus varioplexus Stafford, 1902 (Figure 7)

Syn: *Haematoloechus similiplexus* Stafford, 1902; Cort, 1915

Based on 20 mature specimens: Body elongate, 3.200 to 7.830 mm long by 0.760 to 1.630 mm wide. Oral sucker subterminal and oval 260 to 510 µm long by 220 to 480 µm wide. Pharynx 160 to 260 µm long by 150 to 280 µm wide. Oral sucker/pharynx width ratio 1.37 to 2.00. Oral sucker/pharynx length ratio 1.62 to 2.73. Acetabulum 32 to 52 % body length from anterior end, round to oval 180 to 380 µm long by 180 to 410 µm wide. Oral sucker/acetabulum ratio 0.85 to 1.66. Testes round to oval positioned in tandem in midhindbody, 380 to 760 µm long by 240 to 550 µm wide. Cirrus sac long, extending to level of acetabulum. Genital pore ventral to pharynx. Ovary oval posterior or dorsolateral to acetabulum, 280 to 640 µm long by 190 to 370 µm wide. Longitudinal extracecal uterine loops reaching intertesticular level. Vitellaria acinous, follicular forming clusters, distribution differing on each side of body. On ovarian side five to eight extracecal clusters, and one to three intracecal clusters located pre acetabulum and zero to three intracecal post posterior testis. On opposite side of body four to 10 extracecal clusters, zero to two intracecal clusters pre acetabulum and zero to two intracecal clusters post posterior testis. Eggs 30 to 42.5 µm long by 15 to 22.5 µm wide.

Morphological comparisons among species

Morphological comparisons among *H. varioplexus* and *H. parviplexus* are presented in Table V. Statistically significant differences were observed between *H.*

varioplexus and *H. parviplexus* in oral sucker length and width, OS/PH length and width ratios, acetabulum length and width, OS/AC width ratio, testis length and width, ovary length and width, and egg length and width. Although these differences were statistically significant there was overlap among these characteristics in all cases except for the acetabulum length and width, OS/AC width ratio, and egg length. Additionally, *H. parviplexus* had a distinctly lobed ovary while the ovary was never lobed in *H. varioplexus*.

DISSCUSION

Frog lung fluke distribution in frog hosts

Population structure of frog lung flukes from the six species of anurans collected from Pawnee Lake, Lancaster County, Nebraska, indicates that *H. complexus* is restricted to plains leopard frogs, *H. varioplexus* is restricted to bullfrogs, and *H. longiplexus* predominantly infects bullfrogs and is rarely found in plains leopard frogs. Previous studies on the life history of *H. complexus* by Krull (1933) indicate that bullfrogs are resistant to this species, explaining the strict host specificity in plains leopard frogs observed at this site. Morphological studies on frog lung flukes currently known as *H. varioplexus* and experimental infections of bullfrogs, northern leopard frogs, and plains leopard frogs indicate that the synonymy by Kennedy (1981), of *H. parviplexus* from bullfrogs, green frogs, and toads with *H. varioplexus* from leopard frogs, wood frogs and toads was not warranted and that these worms represent distinct species. The reason that northern and plains leopard frogs are never infected with *H. varioplexus* when collected from the same location as bullfrogs infected with this species is that frog lung flukes from

bullfrogs are actually a distinct species, *H. parviplexus*, which does not establish in Nebraska leopard frogs.

Haematoloechus parviplexus and *H. varioplexus* taxonomy

Examination of voucher material or drawings from recent publications indicates that studies on bullfrogs and/or green frogs by Snyder and Janovy (1996), McAlpine and Burt (1998), Bolek and Coggins (2001), Muzzall, et al. (2001), Snyder and Tkach (2001), Yoder et al. (2001), Whitehouse (2002), and León-Règagnon and Brooks (2003) were actually dealing with *H. parviplexus* and not *H. varioplexus*, whereas the study on wood frogs by Muzzall and Peebles (1991) was actually dealing with *H. varioplexus* and not *H. parviplexus*. Although superficially similar, these two species differ in their acetabulum length and width, ovary shape, testes length, and egg length and width. *Haematoloechus parviplexus* has a small acetabulum compared to the oral sucker (OS/AC width ratio 2.68; range = 1.78-4.00), the ovary is lobed, and the testes are elliptical in shape, whereas *H. varioplexus* has a acetabulum that is large and comparable in size to its oral sucker (OS/AC width ration 1.25; range = 0.85-1.66), the ovary is never lobed, and the testes are round to elliptical in shape. Egg length and width also differ among these two species being smaller 24.6 (range = 20-25) X 15.9 (range = 12.5-18) in *H. parviplexus*, and larger 36.4 (range = 30-42.5) X 19.1 (range = 15-22.5) in *H. varioplexus*.

The taxonomy of *H. varioplexus* and *H. parviplexus* has been problematic, with confusion in their taxonomy dating back to their original descriptions. Stafford (1902) described *H. varioplexus* from bullfrogs from Toronto and Montreal Canada, and *H. similiplexus* from northern leopard frogs and American toads, *Bufo americanus*, from numerous locations in Canada, but did not deposit any type specimens in any accredited

museum. Cort (1915), in a later study on North American frog lung flukes, emended the description of *H. similiplexus* and considered *H. varioplexus* a species inquirenda. Irwin (1929) described *H. parviplexus* from the green frog, *R. clamitans*, from Minnesota, but did not compare her specimens to the descriptions of *H. varioplexus* and *H. similiplexus* by Stafford (1902). From her description, and from the description and drawing of *H. varioplexus* by Stafford (1902), it is clear that both authors were dealing with the same species of frog lung fluke.

Manter (1938), in a review of amphibian trematodes, synonymized Stafford's (1902) *H. varioplexus* from bullfrogs with Stafford's (1902) *H. similiplexus* from northern leopard frogs and American toads, without giving any justification for this synonymy. Brooks (1976) used Manter's synonymy and reported *H. varioplexus* (originally described as *H. similiplexus*) from northern leopard frogs, plains leopard frogs, and Woodhouse's toads, and *H. parviplexus* from bullfrogs and Woodhouse's toads in Nebraska and deposited voucher specimens of both species. Finally, Kennedy (1981) synonymized *H. parviplexus* with *H. varioplexus* and three other *Haematoloechus* species, based on his own specimens and vouchers deposited by Brooks (1976) and others. Examination of voucher specimens used by Kennedy (1980; 1981) that were available and his drawings in the revision (Kennedy, 1980; 1981) indicates that a number of these were misidentified and apparently Kennedy (1981) based his synonymy on these identifications. Most North American workers dealing with *Haematoloechus* species have used Kennedy's (1981) descriptions for *Haematoloechus* identifications, and as a result, these reports cannot be trusted. This study on infections of laboratory reared frogs, and previous surveys of anurans in Nebraska by Brooks (1976) and Snyder (1996),

clearly indicate that bullfrogs and leopard frogs in Nebraska are infected with two distinct species, *H. parviplexus* in bullfrogs and *H. varioplexus* in northern leopard frogs and plains leopard frogs.

Review of the literature, my personal collections of frog lung flukes, and voucher specimens indicate that *H. parviplexus* has been reported from bullfrogs, *R. catesbeiana*, and green frogs, *R. clamitans*, in Indiana, Kentucky, Louisiana, Michigan, Virginia, and Wisconsin, from green frogs, *R. clamitans*, in Minnesota and New York, from bullfrogs, *R. catesbeiana*, in Toronto, Montreal, and New Burnswick Canada, Connecticut, and Idaho, and from bullfrogs, *R. catesbeiana*, and Woodhouse's toad, *B. woodhousii* in Nebraska (Stafford, 1902; Irwin, 1929; Bennett, 1938; Waitz, 1961; Campbell, 1968; Babero and Golling, 1974; Brooks, 1976; Snyder, 1996; McAlpine and Burt, 1998; Yoder et al., 2001; personal observations). There is also a report of *H. parviplexus* infecting a single spotted frog, *R. pretiosa*, and spotted frog-wood frog hybrids, *R. pretiosa* X *R. sylvatica,* from Idaho by Waitz (1961). However, no voucher specimens were deposited and Waitz (1961) indicated that these worms were immature and not gravid, suggesting that they may not mature in these hosts.

Haematoloechus varioplexus has been reported from northern leopard frogs, *R. pipiens*, from Illinois, Indiana, Michigan, North Dakota, and South Dakota, from wood frogs, *R. sylvatica* from Michigan and Wisconsin, from the spring peeper, *Pseudacris crucifer*, from Wisconsin, from northern leopard frogs, *R. pipiens* and American toads, *B. americanus*, from Toronto and Montreal, Canada, and Wisconsin, and from northern leopard frogs, *R. pipiens*, plains leopard frogs, *R. blairi*, and Woodhouse's toads, *B. woodhousii*, from Nebraska (Stafford, 1902; Cort, 1915; Fortner, 1923; Brooks, 1976;

Muzzall and Peebles, 1991; Yoder and Coggins, 1996; Yoder, 1998; Goldberg, et al., 2001; Bolek and Coggins, 2003). The reports of *H. varioplexus* from bullfrogs and green frogs by Campbell (1968) and from green frogs by Bouchard (1951), can not be verified because voucher specimens were not deposited. However, both investigators used Stafford's (1902) original descriptions for species identification which suggests that they were dealing with *H. parviplexus*. It is unclear if bullfrogs and green frogs can become infected with *H. varioplexus*. During this study, over 200 *P. gyrina*, 100 *G. parvus* and one *Planorbella (Helisoma) trivolvis* laboratory reared snails were exposed to *H. varioplexus* eggs recovered from wood frogs, but none became infected, thus experimental infections of arthropods and frogs could not be attempted.

Metacercarial survival study

Observations on difference in metacercaria survival during dragonfly metamorphosis indicate that certain dragonfly species may act as filters or sieves. Only certain lung fluke species are able to pass from an aquatic environment through all filters and end up in the appropriate terrestrial environment and encounter semi-terrestrial leopard frogs. My study indicates that metacercariae of *H. longiplexus* are more commonly lost during metamorphosis of eastern pondhawk dragonflies than are the other three species of *Haematoloechus*. These observations may be important in terms of the observed ecological host specificity of *H. longiplexus*. Both northern leopard frogs and plains leopard frogs are rarely infected with this species in nature but can be infected in the laboratory (Brooks, 1976; Snyder, 1996; Chapter One; Table I). Diet studies on leopard frogs and bullfrogs indicate that semi-terrestrial leopard frogs feed predominantly on adult damselflies and dragonflies, which make up to 15 % of the invertebrates

reported in their diet (Linzey, 1967; Dronen 1977), whereas aquatic bullfrogs feed predominantly on larval dragonflies and damselflies, which consist of up to 10-16% of the frequency of their diet, with adult dragonflies being less commonly reported (Korschgen and Baskett, 1963; Fulk and Whitaker, 1969; Stewart and Sandison, 1972). These dietary studies indicate that even though larval *E. simplicicollis* lose most *H. longiplexus* metacercariae during metamorphosis they can still act as potential intermediate hosts for flukes of aquatic bullfrogs, but rarely for worms infecting semi-terrestrial leopard frogs. Clearly, in the laboratory, bullfrogs will commonly ingest larval *E. simplicicollis* when given the chance, indicating that they potentially feed on these insects in nature, whereas semi-terrestrial leopard frogs rarely come in contact with aquatic larval odonates in nature.

Previous life history and ecological studies on *H. longiplexus* by Krull (1932) and Wetzel and Esch (1996) in the swamp spreadwing damselfly, *Lestes vigilax,* and eastern pondhawk dragonfly, *E. simplicicollis*, indicate that the metacercariae of this lung fluke were free or only lightly encysted in the head, thorax, and abdomen of damselflies and branchial basket of dragonflies. These observations are in contrast to studies on four other species of North American frog lung flukes. Both *H. coloradensis* and *H. complexus* can infect any body region of dragonflies, damselflies, and numerous aquatic arthropods. Life history studies on these two species by Krull (1933; 1934), Snyder and Janovy (1994; 1996), Dronen (1975), and Bolek (Chapter One) indicate that metacercariae are always encysted in the branchial basket of dragonflies or are found in the body cavity of odonates and other arthropod. Further more, the present study indicates that most survive metamorphosis in dragonfly hosts. Life history studies on *H.*

medioplexus, and *H. parviplexus* by Krull (1930; 1931) and Snyder and Janovy (1994; 1996), also indicate that metacercariae of these species are always found in the branchial basket of dragonflies where they are covered by a cuticula of uniform thickness and encysted in the lamella of the branchial basket. My study clearly shows that metacercariae of *H. parviplexus* also survive metamorphosis in dragonfly hosts, however, leopard frogs are resistant to infection with this species.

Metamorphosis survival studies on *H. medioplexus*, which infects semi-terrestrial leopard frogs, were not conducted as part of this study because too few gravid *H. medioplexus* worms were collected for snail infections. However, Krull (1930; 1931) showed that the metacercariae of *H. medioplexus* survive metamorphosis in two dragonfly species, the white-face meadowhawk, *Sympetrum obrususm,* and the ruby meadowhawk, *S. rubicundulum,* suggesting that the worms should also survive metamorphosis in *E. simplicicollis*. My data indicate that different *Haematoloechus* species' ability to survive odonate metamorphosis is determined by infection site and the ability to encyst, and that such difference in the location of metacercariae within dragonflies or damselflies consequently determine whether the flukes can gain access to terrestrial and semi-terrestrial frogs.

Other European *Haematoloechus* species are known to have metacercariae that do not encyst. One such species is *Haematoloechus similis* (Looss, 1899). Interestingly, enough it has only been reported to infect three species of zygopteran hosts (*Coenagrion hastulatum*, *C. aramtum*, and *C. plchellum*). Although no experimental infections were attempted with anisopterans, Grabda (1960) reported that only zygopterans in the genus *Coenagrion* were infected in nature. Her laboratory life cycle studies indicate that

cercariae of this species creep along the body of damselflies and penetrate at the base of the head or appendages, and the metacercariae are located in the anterior part of the abdomen where they remain unencysted (Grabda, 1960). These life history observations suggest that the inability to form encysted metacercariae may preclude some *Haematoloechus* species from infecting and/or surviving anisopteran metamorphosis. The similarities in the life history of *H. longiplexus* and *H. similis* also suggest that they may be closely related species. Similarities in host specificity at the first and second intermediate host level have been shown to be conserved among related species of *Haematoloechus* species in previous phylogenetic studies on other European and North American species of frog lung flukes, although those studies did not include *H. similis* (Snyder and Tkach, 2001).

Finally, it is unclear what role adult damselflies play in the recruitment of *H. longiplexus* to semi-terrestrial leopard frogs. My experimental infections clearly indicate that *H. longiplexus* metacercariae are located in the head, thorax and abdomen of damselfly hosts, and all *Haematoloechus* metacercariae located in these regions of naturally infected damselflies survived metamorphosis. Field data from Nebraska, however, indicate that adult damselflies are rarely infected with *Haematoloechus* metacercariae. Of the 51 adult damselflies collected from Nickol Pond only one was infected. Additionally of the 64 adult lyre-tipped spreadwing damselflies, *Lestes unguiculatus*, collected from Nevens Pond none were infected with *Haematoloechus* metacercariae.

Metacercariae of *H. longiplexus* are commonly found in the head, including the brain, of larval and teneral damselflies, but adult damselflies are rarely infected with

Haematoloechus in nature, suggesting that infected damselflies may not survive long after infections, or that other factors, such as odonate dispersal to other ponds and feeding sites, reduces the prevalence of *Haematoloechus* metacercariae in adult damselflies. Whatever the reason, it suggests that frogs become infected with *H. longiplexus* close to aquatic habitats where they can feed on infected larvae dragonflies and damselflies or teneral damselflies. Of the three *Rana* species in Nebraska, the bullfrog is strictly aquatic (Hudson, 1942); however, among the two semi-terrestrial leopard frogs in Nebraska, studies by Kruse (1978) indicate that plains leopard frogs are more commonly associated with aquatic habitats than northern leopard frogs. Field data by Brooks (1976) and Snyder (1996), and the present study, indicate that plains leopard frogs are more commonly infected with *H. longiplexus* than northern leopard frogs. Of 271 plains leopard frogs and 514 northern leopard frogs sampled from Nebraska by Brooks (1976), Snyder (1996), Bolek (Chapter One) and as part of the current study four plains leopard frogs (1.5%) were infected with *H. longiplexus*, whereas only one juvenile northern leopard frog (0.2%) associated with an aquatic habitat was infected with *H. longiplexus*.

ACKNOWLEDGMENTS

The author greatly acknowledges Dr. Eric Hoberg, U.S. National Parasite Collection, Beltsville Agricultural Research Center Beltsville, MD, Dr. Agustín Jiménez-Ruiz, Harold. W. Manter Laboratory of Parasitology, University of Nebraska State Museum, and Dr. Randal H. Yoder, Lamar University, for providing specimens of *H. parviplexus* and *H. varioplexus* for comparisons. Additionally, I thank my wife Melissa Bolek for help in collecting frogs, Randy Peterson, Dwaine Dunwoody, and Dr. Brent

Nickol, for access to field sites, Dr. Brent Nickol for the use of his animal room, and Cedar Point Biological Station for providing facilities. This work was supported by grants from the Initiative for Ecology and Evolutionary Analysis, University of Nebraska-Lincoln, and The School of Biological Sciences, University of Nebraska-Lincoln.

LITERATURE CITED

Barbero, B. B., and K. Golling. 1974. Some helminth parasites of Nevada bullfrogs, *Rana catesbeiana* Shaw. Revista de Biología Tropical **21**: 207-220.

Bennett, H. J. 1938. A partial checklist of the trematodes of Louisiana vertebrates. Proceedings of the Louisiana Academy of Science **4**: 178-181.

Bolek, M. G., and J. R. Coggins. 2001. Seasonal occurrence and community structure of helminth parasites from the green frog, *Rana clamitans melanota*, from southeastern Wisconsin, U.S.A. Comparative Parasitology **68**: 164-172.

Bolek, M. G., and J. R. Coggins. 2003. Helminth community structure of sympatric eastern American toad, *Bufo a. americanus*, northern leopard frog, *Rana pipiens*, and blue-spotted salamander, *Ambystoma laterale*, from southeastern Wisconsin. Journal of Parasitology **89**: 673-680.

Bouchard, J. L. 1951. The platyhelminths parasitizing some northern Maine Amphibia. Transactions of the American Microscopical Society **70**: 245-250.

Bourgat, R., and S. D. Kulo. 1979. Cycle biologique d' *Haematoloechus johnsoni* Bourgat, 1977, (Trématode) parasite pulmonaire de *Dicroglossus occipitalis* (Günther, 1858) (Amphibien, Anoure) au Togo. Revue Suisse de Zoologie **86**: 467-472.

Brooks, D. R. 1976. Parasites of amphibians of the Great Plains: Part 2. Platyhelminths of amphibians in Nebraska. Bulletin of the University of Nebraska State Museum **10**: 65-92.

Campbell, R. A. 1968. A comparative study of the parasites of certain salientia from Pocahontas state park, Virginia. The Virginia Journal of Science **19**: 13-29.

Conrad, K. F., K. H. Wilson, I. F. Harvey, C. J. Thomas, and T. N. Sherratt. 1999. Dispersal characteristics of seven odonate species in an agricultural landscape. Ecography **22**: 524-531.

Corbet, P. H. 1999. Dragonflies: Behavior and Ecology of Odonata. Comstoch Publishing Associates, Cornell University Press, New York. 829 p.

Cort, W. W. 1915. North American frog lung flukes. Transactions of the American Microscopical Society **34**: 203-243.

Dronen, N. O. Jr. 1975. The life cycle of *Haematoloechus coloradensis* Cort 1915 (Digenea: Plagiorchiidae), with emphasis on host susceptibility to infection. Journal of Parasitology **61**: 657-660.

Dronen, N. O. Jr. 1977. Studies on the population structure of two species of *Haematoloechus* Looss, 1899 (Digenea: Plagiorchiidae) in raniid frogs in New Mexico. Proceedings of the Helminthological Society of Washington **44**: 68-72.

Dronen, N. O. Jr. 1978. Host-parasite population dynamics of *Haematoloechus coloradensis* Cort, 1915 (Digenea: Plagiorchiidae). American Midland Naturalist **99**: 330-349.

Fielden, A. 1960. Transmission through the last abdominal ganglion of the dragonfly *Anax imperator*. Journal of Experimental Biology **37**: 832-844.

Fortner, H. C. 1923. The distribution of frog parasites of Douglas Lake region, Michigan. Transactions of the American Microscopical Society **42**: 79-90.

Fulk, J. D., and J. O. Whitaker. 1969. The food of *Rana catesbeiana* in three habitats in Owen County, Indiana. Proceedings of the Indiana Academy of Science **78**: 491-496.

Grabda, B. 1960. Life cycle of *Haematoloechus similis* (Looss, 1899) (Trematoda: Plagiorchiidae). Acta Parasitologica Polonica **8**: 357-367.

Grieve, E. G. 1937. Studies on the biology of the damselfly *Ischnura verticalis* Say, with notes on certain parasites. Entomologica America **17**: 121-153.

Goldberg, S. R., C. R. Bursey, R. G. McKinnell, and I. S. Tan. 2001. Helminths of northern leopard frogs, *Rana pipiens* (Ranidae), from North and South Dakota. Western North American Naturalist **61**: 248-251.

Harwood, P. D. 1932. The helminths parasitic in the amphibia and reptilia of Houston, Texas and vicinity. Proceedings of the United States National Museum **81**: 1-71.

Hudson, G. E. 1942. The amphibians and reptiles of Nebraska. Nebraska Conservation Bulletin **24**: 1-146.

Ingles, L. G. 1933. Studies on the structure and life history of *Ostiolum oxyorchis* (Ingles) from the California red-legged frog *Rana aurora draytoni*. University of California Publications in Zoology **39**: 135-161.

Irwin, M. S. 1929. A new lung fluke from *Rana clamitans* Latreille. Transactions of the American Microscopical Society **48**: 74-79.

Kennedy, M. J. 1980. Geographical variation in some representatives of *Haematoloechus* Looss, 1899 (Trematoda: Haematoloechidae) from Canada and the United States. Canadian Journal of Zoology **58**: 1151-1167.

Kennedy, M. J. 1981. A revision of the genus *Haematoloechus* Looss, 1899 (Trematoda: Haematoloechidae) from Canada and the United States. Canadian Journal of Zoology **59**: 1836-1846.

Korschgen, L. J., and T. S. Baskett. 1963. Foods of impoundment and stream-dwelling bullfrogs in Missouri. Herpetologica **19**: 89-99.

Krull, W. H. 1930. The life history of two North American frog lung flukes. Journal of Parasitology **16**: 207-212.

Krull, W. H. 1931. Life history studies on two frog lung flukes, *Pneumonoeces medioplexus* and *Pneumobites parviplexus*. Transactions of the American Microscopical Society **50**: 215-277.

Krull, W. H. 1932. Studies on the life history of *Haematoloechus longiplexus* (Stafford). Zoologischer Anzeiger **99**: 231-239.

Krull, W. H. 1933. Studies on the life history of a frog lung fluke, *Haematoloechus complexus* (Seely, 1906) Krull, n. comb. Zeitschrift für Parasitenkunde **6**: 193-206.

Krull, W. H. 1934. Some additional notes on the life history of a frog lung fluke, *Haematoloechus complexus* (Seely, 1906) Krull. Transactions of the American Microscopical Society **53**: 196-199.

Kruse, K. C. 1978. Causal factors limiting the distribution of leopard frogs in eastern Nebraska. Unpublished Ph.D. Thesis. University of Nebraska-Lincoln, 95 p.

Linzey D. W. 1967. Food of the leopard frog, *Rana p. pipiens,* in Central New York. Herpetologica **23**: 11-17

León-Règagnon, V., and D. R. Brooks. 2003: Molecular phylogeny of *Haematoloechus* Looss, 1899 (Digenea: Plagiorchiidae), with emphasis on North American species. Journal of Parasitology **89**: 1206–1211.

Manter, H. W. 1938. A collection of trematodes from Florida amphibia. Transactions of the American Microscopical Society **57**: 26-37.

McAlpine, D. F., and M. D. B. Burt. 1998. Helminths of bullfrogs, *Rana catesbeiana*, green frogs, *R. clamitans*, and leopard frogs, *R. pipiens*, in New Burnswick. The Canadian Field Naturalist **112**: 50-68.

McVey, M. E. 1985. Rates of color maturation in relation to age, diet, and temperature in male *Erythemis simplicicollis* (Say) (Anisoptera: Libellulidae). Odonatologica **14**: 101-114.

Muzzall, P. M., and C. R. Peebles. 1991. Helminths of the wood frog, *Rana sylvatica*, and the spring peeper, *Pseudacris c. crucifer*, from Southern Michigan. Journal of the Helminthological Society of Washington **58**: 263-265.

Muzzall, P. M., M. G. Gillilland, III, C. S. Summer, and C. J. Mehne. 2001. Helminth communities of green frogs *Rana clamitans* Latreille, from southwestern Michigan**.** Journal of Parasitology **87**: 962–968.

Needham, J. G., M. J. Westfall, Jr., and M. L. May. 2000. Dragonflies of North America. Revised edition. Scientific publishers, Gainesville, Florida, 939 p.

Pritchard, M. H., and G. O. W. Kruse. 1982. The collection and preservation of animal parasites. University of Nebraska Press, Lincoln , Nebraska, 141 p.

Sokal, R. R., and J. F. Rohlf. 1981. Biometry. Second edition. W. H. Freeman and Company, New York, New York, 859 p.

Snyder, S. D. 1996. Host specificity among species of *Haematoloechus* (Digenea: Haematoloechidae). Unpublished Ph.D. Thesis. University of Nebraska-Lincoln, 112 p.

Snyder, S. D., and J. Janovy Jr. 1994. Second intermediate host-specificity of *Haematoloechus complexus* and *Haematoloechus medioplexus* (Digenea: Haematoloechidae). Journal of Parasitology **80**: 1052-1055.

Snyder, S. D., and J. Janovy Jr. 1996. Behavioral basis of second intermediate host specificity among four species of *Haematoloechus* (Digenea: Haematoloechidae). Journal of Parasitology **82**: 94-99.

Snyder, S. D., and V. V. Tkach. 2001. Phylogenetic and biogeographical relationships among some Holarctic frog lung flukes (Digenea: Haematoloechidae). Journal of Parasitology **87**: 1433-1440.

Stafford, J. 1902. On the representation of *Distomum variegatum*. Zoologische Jahrbuecher Abteilung fuer Systematik Oekologie und Geographie der Tiere **16**: 895-912.

Stewart, M. M., and P. Sandison. 1972. Comparative food habits of sympatric mink frogs, bullfrogs and green frogs. Journal of Herpetology **6**: 241-244.

Waitz, J. A. 1961. Parasitic helminthes as aids in studying the distribution of species of *Rana* in Idaho. Transactions of the Illinois State Academy of Sciences **54**: 152-156.

Westfall, M. J. Jr., and M. L. May. 1996. Damselflies of North America. Scientific Publishers, Gainesville, Florida, 649 p.

Wetzel, E. J. and G. W. Esch. 1996. Influence of odonate intermediate host ecology on the infection dynamics of *Halipegus* spp., *Haematoloechus longiplexus*, and *Haematoloechus complexus* (Trematoda: Digenea). Journal of the Helminthological Society of Washington **63**: 1-7.

Whitehouse, C. A. 2002. A study of the frog lung flukes *Haematoloechus* (Trematoda: Haematoloechidae) collected from areas of Kentucky and Indiana. Proceedings of the Indiana Academy of Science **1**: 67-76.

Yoder, H. R. 1998. Community ecology of helminth parasites infecting molluscan and amphibian hosts. Unpublished Ph.D. Thesis. University of Wisconsin Milwaukee, 138 p.

Yoder, H. R. and J. R. Coggins. 1996. Helminth communities in the Northern Spring Peeper, *Pseudacris c. crucifer* Wied, and the Wood Frog, *Rana sylvatica* Le Conte, from Southeastern Wisconsin. Journal of the Helminthological Society of Washington **63**: 211-214.

Yoder, H. R., J. R. Coggins, and J. C. Reinbold. 2001. Helminth parasites of the green frog (*Rana clamitans*) from southeastern Wisconsin, U.S.A. Comparative Parasitology **68**: 269-272

Figure 1. Prevalence and mean abundance of *Haematoloechus* metacercariae infecting larva and teneral odonates collected from Nickol Pond, Cass County, Nebraska. A. Prevalence of *Haematoloechus* metacercariae in larval and teneral *Ischnura verticalis*. N = 80 for larvae and 28 for tenerals. B. Mean abundance of *Haematoloechus* metacercariae in larval and teneral *Ischnura verticalis*. N = 80 for larvae and 28 for tenerals. C. Prevalence of *Haematoloechus* metacercariae in larval and teneral *Erythemis simplicicollis*. N = 75 for larvae and 74 for tenerals. D. Mean abundance of *Haematoloechus* metacercariae in larval and teneral *Erythemis simplicicollis.* N = 75 for larvae and 74 for tenerals.

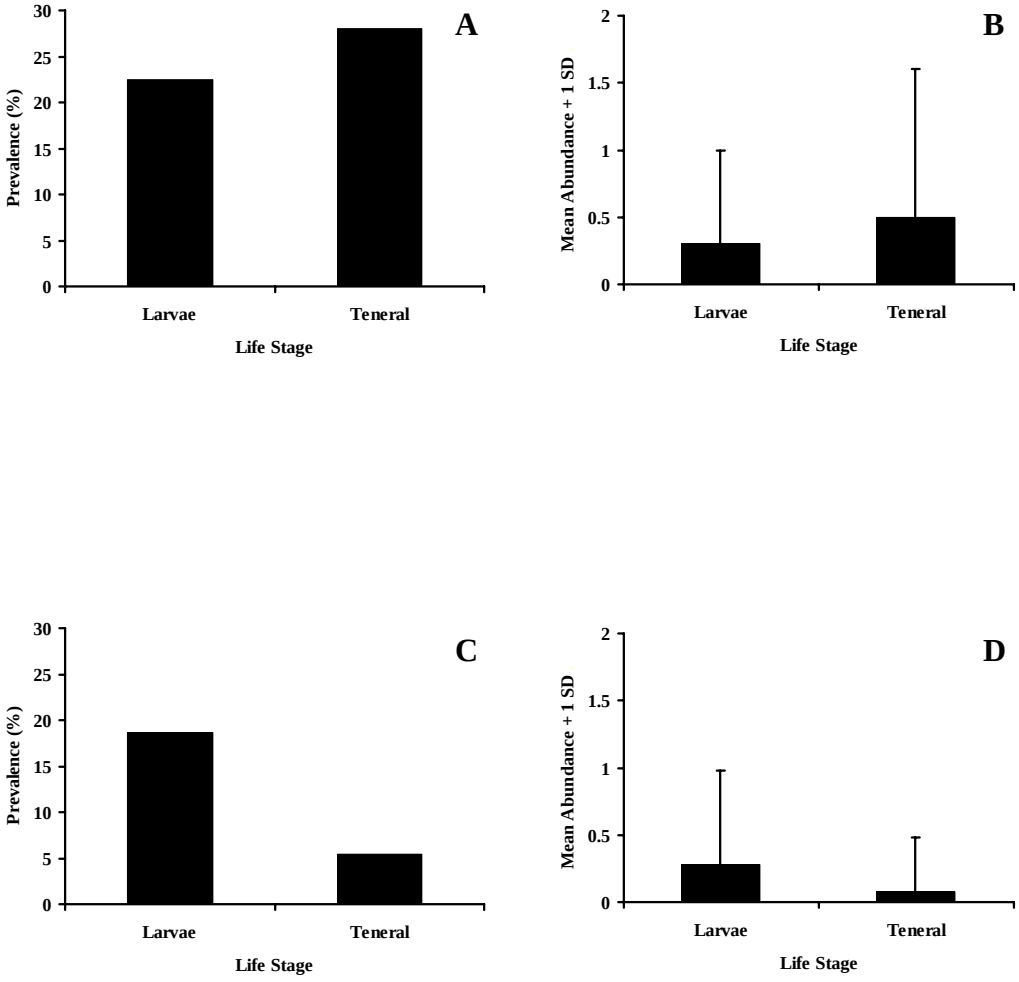

A
Prevalence (%)
30
25
20
15
10
5
0
Larvae
Teneral
Life Stage
B
Mean Abundance + 1 SD
2
1.5
1
0.5
0
Larvae
Teneral
Life Stage
C
Prevalence (%)
30
25
20
15
10
5
0
Larvae
Teneral
Life Stage
D
Mean Abundance + 1 SD
2
1.5
1
0.5
0
Larvae
Teneral
Life Stage

Figure 2. Prevalence and mean abundance of *Haematoloechus* metacercariae of laboratory metamorphosed odonates collected from Nickol Pond, Cass County, Nebraska. A. Prevalence of *Haematoloechus* metacercariae in larval and teneral *Ischnura verticalis*. N = 25 for each life stage. B. Mean abundance of *Haematoloechus* metacercariae in larval and teneral *Ischnura verticalis*. N = 25 for each life stage. C. Prevalence of *Haematoloechus* metacercariae in the branchial basket and thorax of larval and teneral *Erythemis simplicicollis*. N = 100 for each life stage. D. Mean abundance of *Haematoloechus* metacercariae in the branchial basket and thorax of larval and teneral *Erythemis simplicicollis*. N = 100 for each life stage.

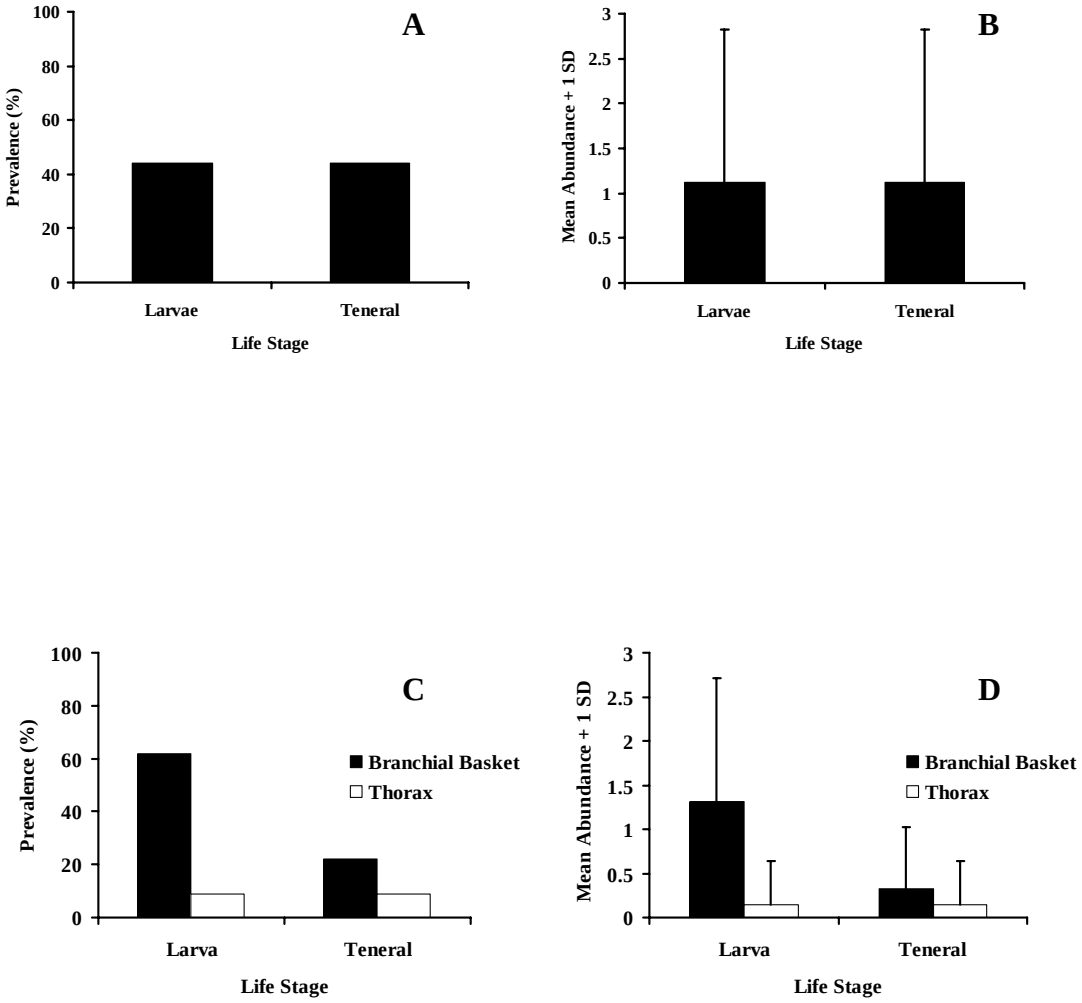

A
Prevalence (%)
100
80
60
40
20
0
Larvae
Teneral
Life Stage
B
Mean Abundance + 1 SD
3
2.5
2
1.5
1
0.5
0
Larvae
Teneral
Life Stage
C
Prevalence (%)
100
80
60
40
20
0
Branchial Basket
Thorax
Larva
Teneral
Life Stage
D
Mean Abundance + 1 SD
3
2.5
2
1.5
1
0.5
0
Branchial Basket
Thorax
Larva
Teneral
Life Stage

Figure 3. *Haematoloechus* metacercaria recovered from (A) the remnants of the branchial basket of an exuvia and (B) from the remnants of the branchial basket of a teneral *Erythemis simplicicollis*, naturally infected at Nickol Pond, Cass County, Nebraska. Scale bars = 40 μm. Note that the metacercaria from the exuvia is not encysted or encapsulated while the metacercariae from the teneral dragonfly is encysted and encapsulated.

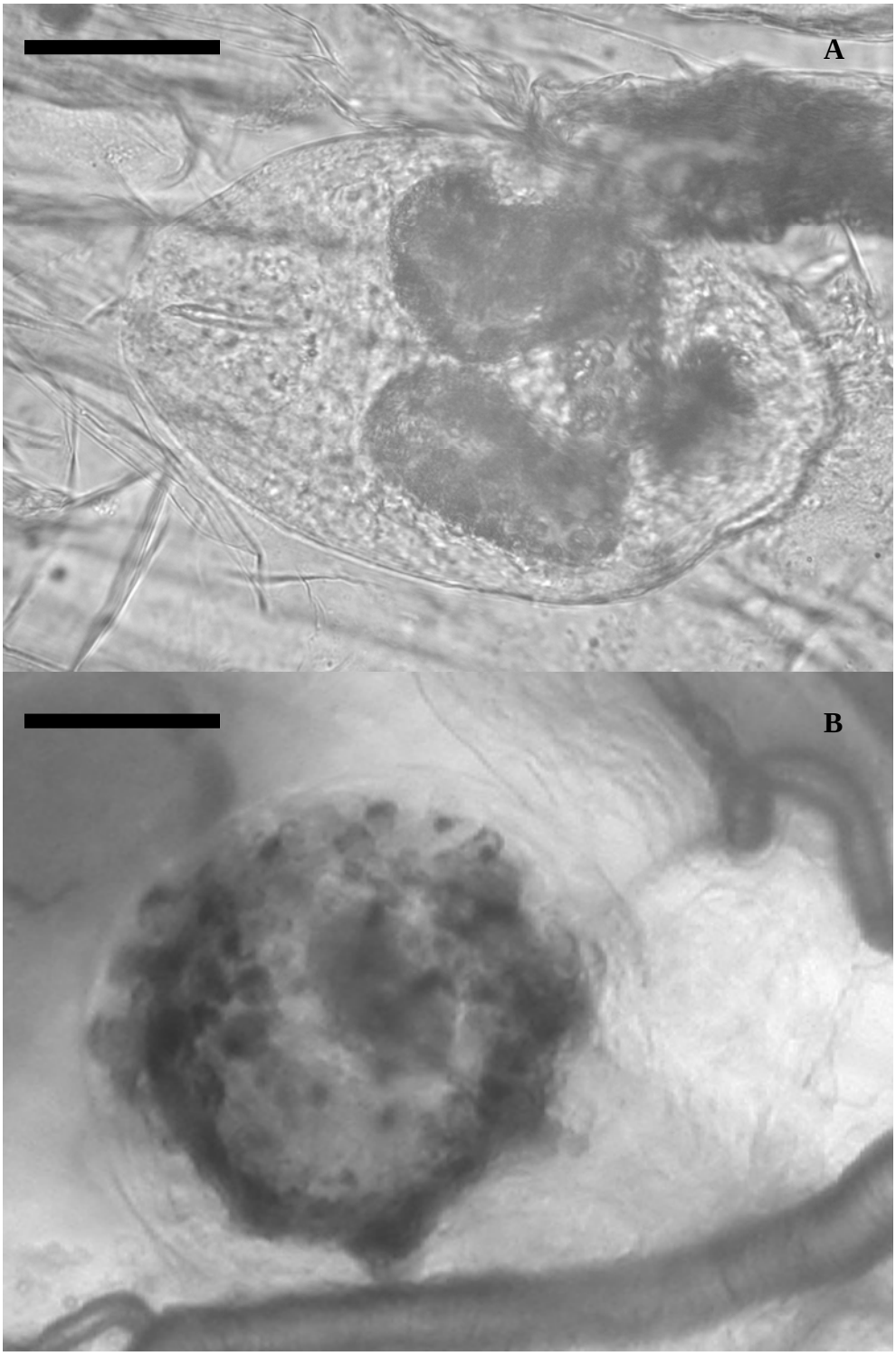
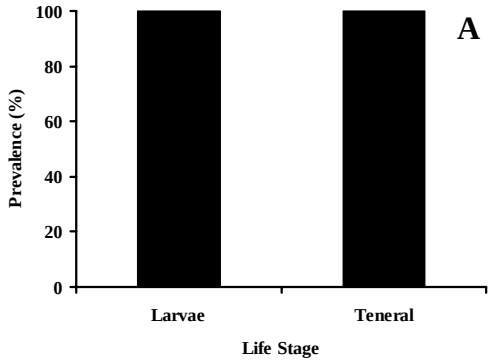
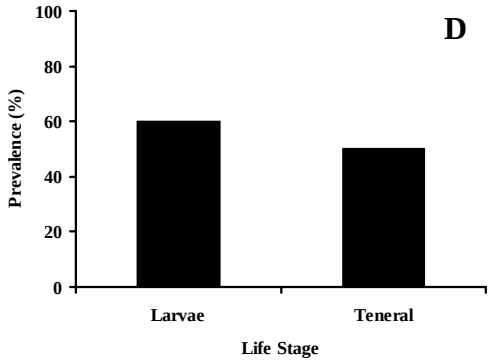
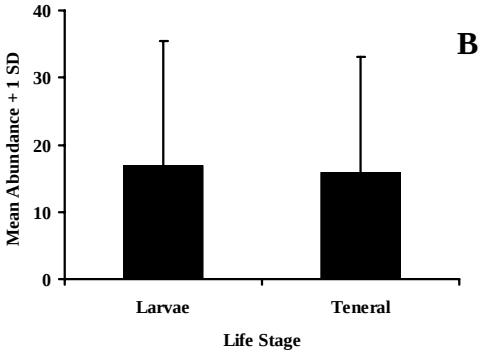
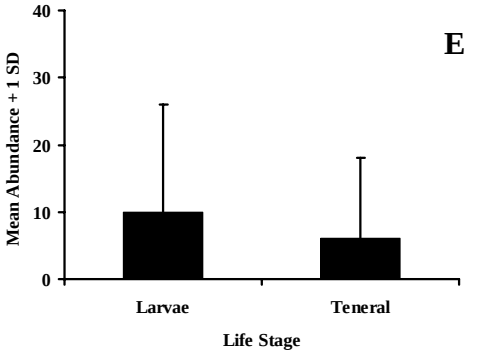

A
B

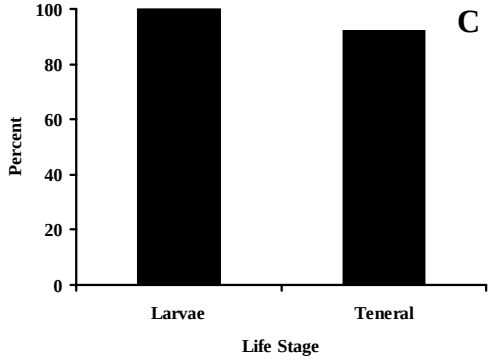
Figure 4. Prevalence, mean abundance, and percent of *Haematoloechus* metacercariae recovered from laboratory infected larval and teneral *Erythemis simplicicollis*. *Haematoloechus coloradensis* (N = 10; A, prevalence, B, mean abundance; C, percent metacercariae). *Haematoloechus complexus* (N = 10; D, prevalence; E, mean abundance; F, percent metacercariae).


A
Prevalence (%)
100
80
60
40
20
0
Larvae
Teneral
Life Stage


D
Prevalence (%)
100
80
60
40
20
0
Larvae
Teneral
Life Stage


B
Mean Abundance + 1 SD
40
30
20
10
0
Larvae
Teneral
Life Stage


E
Mean Abundance + 1 SD
40
30
20
10
0
Larvae
Teneral
Life Stage


C
Percent
100
80
60
40
20
0
Larvae
Teneral
Life Stage

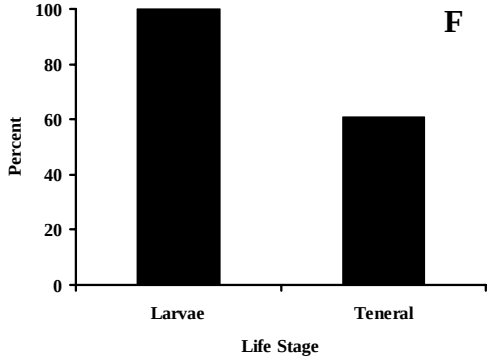
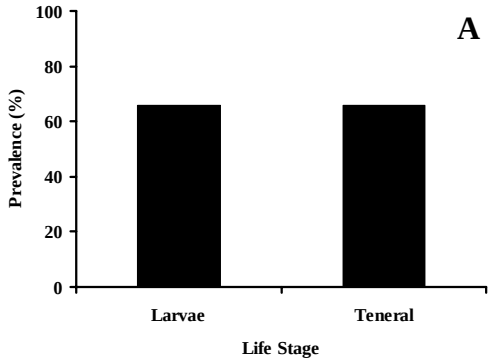
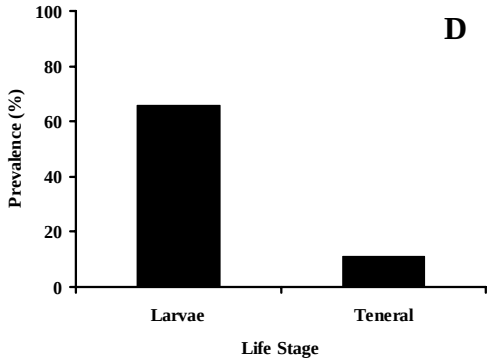
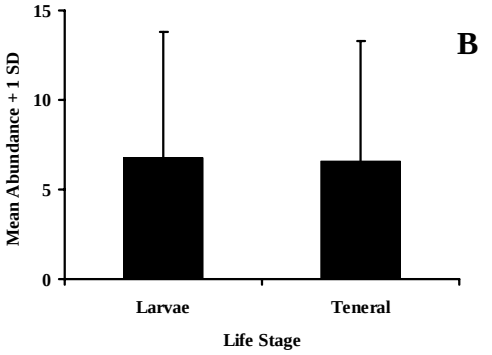
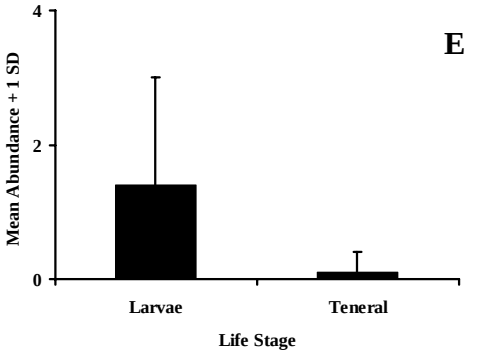

F
Percent
100
80
60
40
20
0
Larvae
Teneral
Life Stage

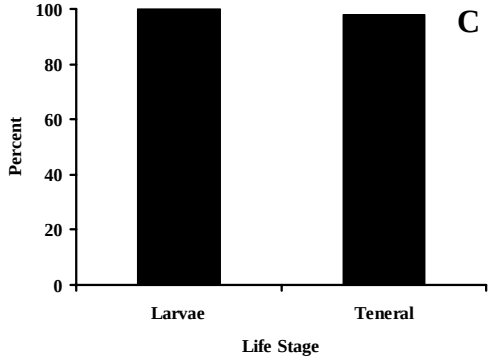
Figure 5. Prevalence, mean abundance, and percent of *Haematoloechus* metacercariae recovered from laboratory infected larval and teneral *Erythemis simplicicollis*. *Haematoloechus varioplexus* (N = 6; A, prevalence, B, mean abundance; C, percent metacercariae). *Haematoloechus longiplexus* (N = 9; D, prevalence; E, mean abundance; F, percent metacercariae).


A
Prevalence (%)
100
80
60
40
20
0
Larvae
Teneral
Life Stage


D
Prevalence (%)
100
80
60
40
20
0
Larvae
Teneral
Life Stage


B
Mean Abundance + 1 SD
15
10
5
0
Larvae
Teneral
Life Stage


E
Mean Abundance + 1 SD
4
2
0
Larvae
Teneral
Life Stage


C
Percent
100
80
60
40
20
0
Larvae
Teneral
Life Stage

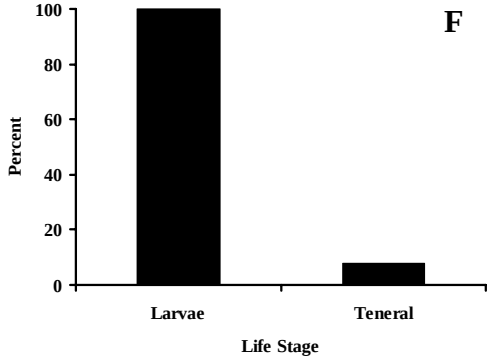

F
Percent
100
80
60
40
20
0
Larvae
Teneral
Life Stage

Figure 6. Line drawing of *Haematoloechus parviplexus* from a experimentally infected bullfrog, *Rana catesbeiana*, with a metacercaria collected from Nevens Pond, Keith, County, Nebraska. Scale bar = 1.5 mm.

Figure 7. Line drawing of *Haematoloechus varioplexus* from the northern leopard frog, *Rana pipiens,* collected from Nance County, Nebraska. Scale bar = 1.5 mm.

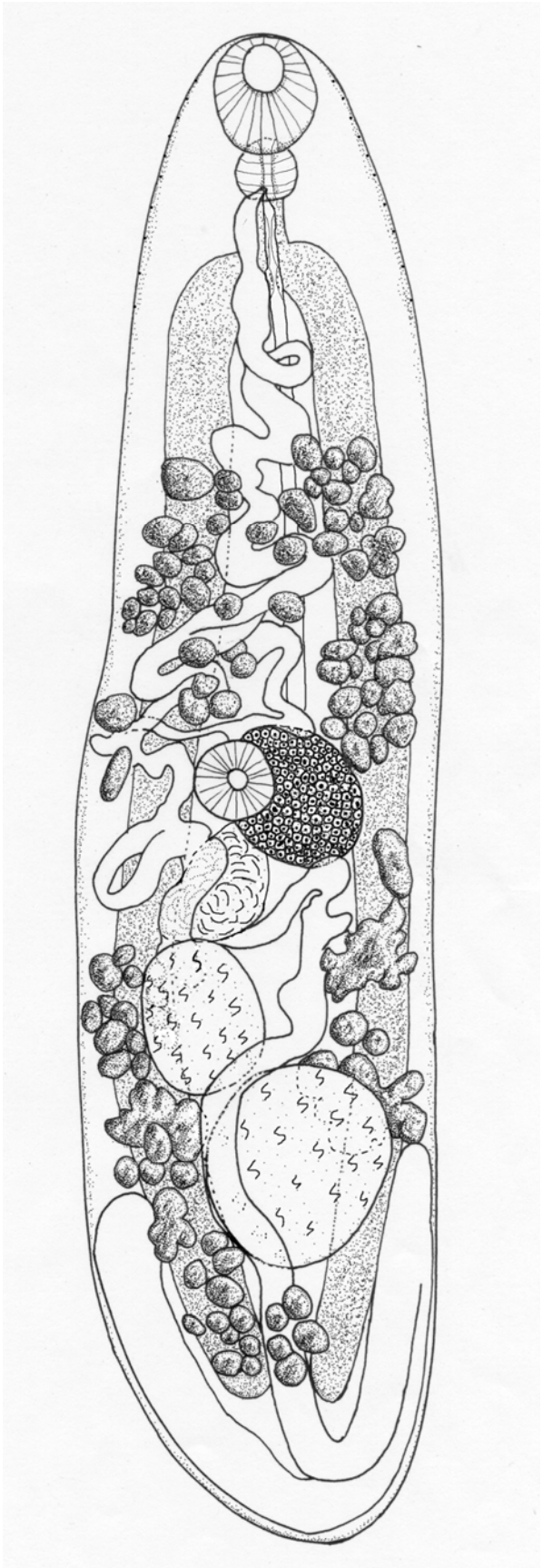


Table I. Population structure of *Haematoloechus complexus, Haematoloechus longiplexus*, and *Haematoloechus varioplexus* in six species of anurans collected from Pawnee Lake, Lancaster County, Nebraska during March 2001 to June 2005.

Anuran Species (n)	*Haematoloechus* species	Prevalence (%)	Mean Intensity ± 1 SD
Acris crepitans (50)			
	H. complexus	0	_
	H. longiplexus	0	_
	H. varioplexus	0	_
Bufo woodhousii (50)			
	H. complexus	0	_
	H. longiplexus	0	_
	H. varioplexus	0	_
Hyla chrysoscelis (36)			
	H. complexus	0	_
	H. longiplexus	0	_
	H. varioplexus	0	_
Pseudacris triseriata (93)			
	H. complexus	0	_
	H. longiplexus	0	_
	H. varioplexus	0	_
Rana blairi (70)			
	H. complexus	42.9	4.2 ± 4.3
	H. longiplexus	1.4	1
	H. varioplexus	0	_
Rana catesbeiana (100)			
	H. complexus	0	_
	H. longiplexus	24	5.7 ± 5.7
	H. varioplexus	19	5.1 ± 5.3

Table II. Population structure of *Haematoloechus* spp. metacercariae in 10 species of larval, teneral, and adult odonates collected from Nickol Pond, Cass County Nebraska during June-July 2001.

Odonate Species	Life stage (n)	Prevalence (%)	Mean Intensity ± 1 SD
Zygoptera			
Enallagma civile			
	Larva (15)	80	1.25 ± 0.6
	Teneral (4)	0	_
	Adult (5)	0	_
Ischnura verticalis			
	Larva (80)	22.5	1.3 ± 0.8
	Teneral (28)	28	1.9 ± 1.5
	Adult (46)	2.3	1
Total Zygoptera			
	Larva (95)	31.6	1.3 ± 0.8
	Teneral (32)	25	1.9 ± 1.5
	Adult (51)	2	1
Anisoptera			
Anax junius			
	Larva (24)	0	_
	Teneral (0)	_	_
	Adult (0)	_	_
Celithemis eponina			
	Larva (0)	_	_
	Teneral (0)	_	_
	Adult (2)	0	_
Erythemis simplicicollis			
	Larva (75)	18.7	1.5 ± 1.1
	Teneral (74)	5.4	2.2 ± 1.6
	Adult (5)	20	3

Table II continued.

Odonate Species	Life stage (n)	Prevalence (%)	Mean Intensity ± 1 SD
Anisoptera			
Libellula luctuosa			
	Larva (2)	0	_
	Teneral (9)	0	_
	Adult (3)	0	_
Pachydiplax longipennis			
	Larva (9)	33	1.3 ± 0.6
	Teneral (0)	_	_
	Adult (2)	0	_
Perithemis tenera			
	Larva (0)	_	_
	Teneral (0)	_	_
	Adult (1)	0	_
Sympetrum rubicundulum			
	Larva (0)	_	_
	Teneral (0)	_	_
	Adult (2)	0	_
Tramea lacerata			
	Larva (1)	0	_
	Teneral (0)	_	_
	Adult (0)	_	_
Total Anisoptera			
	Larva (111)	15.3	1.5 ± 1
	Teneral (83)	4.8	2.2 ± 1.6
	Adult (15)	6.6	3

Table III. Number infected and location of *Haematoloechus* spp. metacercariae in experimentally exposed *Erythemis simplicicollis* and *Ischnura verticalis* to cercariae of *Haematoloechus coloradensis, H. complexus*, *H. longiplexus,* and *H. varioplexus.*

	H. coloradensis		*H. complexus*		*H. longiplexus*		*H. varioplexus**	
Experimental Odonate	No. infected/no. exposed survivors	Location in host	No. infected/no. exposed survivors	Location in host	No. infected/no. exposed survivors	Location in host	No. infected/no. exposed survivors	Location in host
Odonata: Anisoptera								
Erythemis simplicicollis	10/10	Head, thorax, branchial basket	6/10	Head, thorax, branchial basket	6/9	Branchial basket	4/6	Branchial basket
Odonata: Zygoptera								
Ischnura verticalis	10/10	Head, thorax, abdomen	6/6	Head, thorax, abdomen	3/3	Head, thorax, abdomen	0/4	—

* From naturally infected snails.

Table IV. Experimental infections of laboratory reared *Rana catesbeiana*, *Rana pipiens* and field collected *Rana blairi* with metacercariae of *Haematoloechus varioplexus*.

Host	Prevalence (No. infected/no. exposed)	Mean Abundance ± 1 SD (range)	Number of worms recovered
Trial 1			
Rana pipiens	0 (0/4)	0	0
Rana catesbeiana	100 (4/4)	19.75 ± 15.5 (4-39)	79
Trial 2			
Rana blairi	0 (0/3)	0	0
Rana catesbeiana	100 (3/3)	5 ± 4.5 (1-5)	15

Table V. Morphological characteristics of adult *Haematoloechus varioplexus* and *Haematoloechus parviplexus*. Means and ranges (in parentheses) are given in µm unless otherwise noted.

	H. varioplexus n = 20 Mean (range)	*H. parviplexus* n = 20 Mean (range)	T-test	*P* /=/
Shape	Elongate	Elongate	__	__
Body Length (mm)	4.827 mm (3.200-7.830)	6.610 mm (2.320-9.150)	1.49	0.14
Body Width at Acetabulum (mm)	1.019 mm (0.650-1.620)	0.957 mm (0.480-1.420)	-0.52	0.60
Greatest Body Width (mm)	1.094 mm (0.760-1.630)	1.000 mm (0.400-1.500)	-0.98	0.16
Oral Sucker Length	342 (260-510)	230 (160-310)	-6.02	0.0001
Oral Sucker Width	303 (220-480)	247 (150-350)	-2.98	0.005
Pharnyx Length	167 (110-260)	150 (90-250)	-1.35	0.18
Pharanyx Width	187 (150-280)	174 (110-250)	-1.12	0.27
OS/PH Width Ratio	1.62 (1.37-2.00)	1.44 (1.15-1.92)	-2.98	0.005
OS/PH Length Ratio	2.07 (1.62-2.73)	1.56 (1.24-1.84)	-6.66	0.0001
Acetabulum Location from Anterior End	43% (32-52)	44 (37-53)	0.36	0.72

Table V continued.

	H. varioplexus n = 20 Mean (range)	*H. parviplexus* n = 20 Mean (range)	T-test	*P* /=/
Acetabulum Length	239 (180-380)	93 (50-150)	-11.88*	0.00001
Acetabulum Width	245 (180-410)	95 (50-130)	-10.95*	0.00001
OS/AC Width Ratio	1.25 (0.85-1.66)	2.68 (1.78-4.00)	9.56*	0.00001
Testes Length	566 (380-760)	915 (440-1800)	4.56*	0.0002
Testes Width	427 (240-550)	523 (150-880)	2.25	0.03
Ovary Length	405 (280-640)	777 (350-1180)	6.45*	0.0001
Ovary Width	271 (190-370)	350 (120-500)	3.42	0.002
Uterus	Longitudian extracecal uterine loops	Longitudianl extracecal uterine loops	—	—
Vitellaria Number	19.30 (15-23)	19.10 (13-23)	-0.30	0.77
Egg Length	36.40 (30.00-42.50)	24.60 (20.00-25.00)	-13.28*	0.00001
Egg Width	19.10 (15.00-22.50)	15.90 (12.50-18.00)	-4.18	0.0002

* Approximate t'_s -test.

CHAPTER THREE: PHYLOGENETIC AND LIFE HISTORY RELATIONSHIPS AMONG SOME HOLARCTIC FROG LUNG FLUKES (DIGENEA: HAEMATOLOECHIDAE)

> "…on the other hand one should not pin his hopes too closely on what is known, for cycles of closely related species may at times be quite different, and you certainly don't want to exclude any possibilities that are in the realm of procedure." Wendell Krull (Letter to Miriam Rothschild, 1953)

Abstract: A phylogenetic study of 23 specimens corresponding to 12 species of North American and European frog lung flukes belonging to the genus *Haematoloechus* was conducted using approximately 850 to 1,000 bases of the internal transcribed spacer region (ITS 1 – 5.8S – ITS 2). Molecular data from the present study support the separation of *H. parviplexus* from *H. varioplexus* and *H. coloradensis* from *H. complexus* into distinct species, and also confirm recent studies that *H. breviplexus* and *H. floedae* are distinct species. These results clearly indicate that the current synonymy of 16 different North American frog lung fluke species into six species was not warranted. Parsimony analysis of the data set revealed six most parsimonious trees 672 steps long, with a consistency index of 81%. All trees consisted of three distinct evolutionary lineages. Two of the three clades contained both European and North American species, supporting the hypothesis that the major lineages of *Haematoloechus* arose before the breakup of Laurasia and than radiated after Eurasia and North America split. This

analysis suggests that the longitudinal extracecal uterine loops are a poor character for assessing species relationships among haematoloechids, but supports use of the relative size of oral sucker to acetabulum for assessing species relationships. Mapping first and second intermediate host specificity, amphibian definitive host specificity, metacercaria morphology on the strict consensus tree revealed that within each of the three evolutionary lineages, members share similar patterns of arthropod host specificity and metacercaria morphology distinct from patterns found in the other lineages. These results suggest that second intermediate host specificity may be a trait that has been conserved through evolutionary time. The phylogenetic data presented in this study reveal the importance of second intermediate host specificity among the evolutionary lineages of frog lung flukes, because these second intermediate hosts serve as avenues for and constraints on the movement of these parasites to their respective definitive amphibian hosts.

INTRODUCTION

Morphological variability within species of *Haematoloechus,* the frog lung flukes, has caused controversy regarding their identification, taxonomy and phylogenetic relationships (Odening, 1960; Prokopic and Krivanec, 1974; Kennedy, 1980a, b, 1981). Based on morphological variability within species, Kennedy (1981) considered that only six of the 16 previously described *Haematoloechus* species from Canada and the United States were valid species. Kennedy's basis for his revision was based on morphological characters of eight species of North American lung flukes and life cycle studies on a single species, *H. buttensis*, in various anuran definitive hosts (Kennedy, 1980 a, b). He argued that the shape of the ovary and testes, arrangement of the uterine loops, presence or absence of spines on the body surface, sucker ratios, and egg size all exhibited intraspecific variation as a result of developmental stage and/or age of the parasite. Based on his life history studies of *H. buttensis*, he also indicated that the anuran species that the parasite infected or the amount of crowding effect affected these morphological characters. He concluded that these characters are not useful for differentiating species of *Haematoloechus*. However, my examination of some of the material used by Kennedy (1980b; 1981) in his revision of the North American *Haematoloechus*, and his drawings,, indicates that he based his conclusions on specimens of frog lung flukes which were misidentified, suggesting that some of his conclusions may not be warranted. Kennedy's data do, however, indicate that species identification can be controversial particularly when intraspecific morphological variability or when morphological characters are so conservative that there is little variation among species. Recently, researchers have argued that DNA sequence data can provide an independent source of

information for differentiating lineages such as frog lung flukes. These data can then be used to evaluate the usefulness of the morphological characters and life history characters that have been traditionally used in the taxonomy of parasitic groups such as frog lung flukes (Snyder and Tkach, 2001; León-Règagnon and Brooks, 2003).

A number of recent studies using DNA sequences have helped to resolve some of the taxonomical problems within the genus *Haematloechus* (see León-Règagnon et al., 1999; Snyder and Tkach, 2001; León-Règagnon and Brooks, 2003; León-Règagnon et al., 2005). However these studies have been controversial due to workers using different morphological characters to identify frog lung flukes or the lack of voucher specimens deposited in accredited museums for sequenced worms (León-Règagnon et al., 1999; Snyder and Tkach, 2001; León-Règagnon, 2003; León-Règagnon and Brooks, 2003). This situation, along with our limited knowledge of frog lung fluke life cycles, host specificity, and transmission dynamics has led to numerous controversies in the taxonomy, phylogenetic relationships and understanding of life cycle evolution within the genus *Haematoloechus*.

Frog lung flukes are good model organisms to examine life cycle strategies from a phylogenetic perspective for a number of reasons. First, frog lung flukes are common parasites of amphibians, cosmopolitan in distribution, and in all species that have been studied have complex life cycles all utilizing gastropods as first intermediate hosts, aquatic arthropods as second intermediate hosts, and amphibians as definitive hosts. This cosmopolitan distribution of the genus and its host species amphibians (anurans and caudatans) suggests a very ancient origin for this group. Second, life cycle studies on some African, European and North American frog lung flukes have shown distinct

patterns of molluscan, arthropod, and amphibian host specificity (Sinitzin, 1907; Krull, 1930, 1931, 1932, 1933; 1934; Grabda, 1960; Odening, 1960; Dobrovol'ski, 1965; Schell, 1965; Combes, 1968; Dronen, 1975, 1977, 1978; Underwood and Dronen, 1977; Bourgat and Kulo, 1979; Snyder and Janovy, 1994, 1996; Snyder, 1996; Chapter I; Chapter II). Recent molecular studies and these observations have led some workers to suggest that these different patterns of host specificity may have arisen among haematoloechids of Pangaean snails, and arthropods and were conserved over evolutionary time as lineages of parasites were isolated from one another by continental drift (Snyder and Tkach, 2001). Finally, recent DNA sequence data on North American freshwater gastropods and North American anurans that serve as hosts for frog lung flukes has given us a better understanding of phylogenetic relationships among the hosts of these parasites (Jorgensen et al., 2004; Hillis and Wilcox, 2005). Therefore this group of congeners provides an excellent opportunity to explore patterns of host use, host specificity and avenues for and constraints on the evolution of parasite life cycles within a single globally distributed genus of trematodes.

The present study has five main goals: (1.) to update and expand the nucleotide sequence data base for *Haematoloechus* species, using sequences of the variable regions of the internal transcriber spacer (ITS) region (ITS 1 – 5.8S – ITS 2) of the nuclear ribosomal DNA, (2.) re-evaluate previous identifications of voucher specimens of North American species of *Haematoloechus* for which the complete ITS region is available from GenBank and which were used in a previous phylogenetic study by Snyder and Tkach (2001) and León-Règagnon and Brooks (2003), (3.) to construct a phylogenetic hypothesis of species of some of the European and North American *Haematoloechus* (4.)

obtain additional data on anuran host specificity, and (5.) from a phylogenetic perspecitive examine some of the evolutionary and life history patterns of this subset of the genus.

MATERIALS AND METHODS

Specimen collection

Fourteen specimens of seven species of adult *Haematoloechus* were collected from multiple sites from Nebraska, North Dakota, South Dakota, Texas and Wisconsin U.S.A during March-September 2003-2004 to verify the intraspecific stability of the ITS DNA region. A single specimen of *Haematoloechus* (*Skrjabinoeces*) *similis* was collected from the Ukraine. Additionally, 10 sequences for seven *Haematoloechus* species and three plagiorchids species were obtained from GenBank from previous publications (Snyder and Tkach, 2001). Voucher specimens of all North American specimens previously sequenced and identified by Snyder and Tkach (2001), and used in this study, were compared to the original descriptions of *H. coloradensis*, *H. complexus*, *H. breviplexus*, *H. floedae*, Harwood, 1932, *H. parviplexus*, and *H. varioplexus* by Stafford (1902), Cort (1915), Irwin (1929), Harwood (1932) and Krull (1933) and type specimens of *H. floedae* borrowed from the U.S. National Parasite Collection, Beltsville Agricultural Research Center Beltsville, MD.

Living worms recovered from amphibians were allowed to release eggs in water, then thoroughly rinsed in water, identified and fixed in 95% ethanol before further processing. A 2 to 5 mm piece of the left or right side of each single alcohol preserved

worm was then cut to be used for DNA extraction. Once DNA was obtained the remaining worm was then stained and identified based on the original descriptions mentioned previously. Stained voucher specimens of all species are preserved. Species used in the analysis, hosts, collection locations, sequence lengths, the internal transcribed spacer (ITS) GenBank number, and the voucher accession numbers of species obtained from previous publications are listed in Table I.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from single adult worm pieces following the protocol of Snyder and Tkach (2001). With the exception of a few bases at the 5′ and 3′ ends, the entire ITS rDNA (ITS 1 + 5.8S + ITS 2) was amplified by polymerase chain reaction (PCR) using a forward primer in the 18S region, Br1 (5′-GTA GGT GAA CCT GCA GAA GG), and a digenean-specific reverse primer in the 28S region, DigL1 (5′-GTG ATA TGC TTA AGT TCA GC). Reactions were performed in a total volume of 100 µl and consisted of 30–50 ng of gDNA, 0.5 µM of each primer along with 10 µl of 10 × buffer with $MgCl_2$, 8 µl of dNTP mixture, and 2.5 units of *Taq* polymerase as provided in the Takara Ex Taq kit (Takara Biomedicals, Otsu, Japan). Reaction volume was brought to 100 µl with sterile deionized water. Reactions were run on a Biometra UNO under the following cycling conditions: 94 °C for 4 min followed by 40 cycles of 94 °C for 30 sec, 50–56 °C for 30 sec, and 72 °C for 2 min, followed by 1 cycle of 72 °C for 5 min for final elongation. Unincorporated PCR primers and nucleotides were removed from PCR products using High Pure PCR Purification Kit (Roche Diagnostics, Mannheim, Germany). Sequences were determined directly from PCR templates by

cycle sequencing using Big Dye fluorescent dye terminators and protocols and an ABI 377 automated sequencer (Perkin-Elmer, Foster City, California). Primers used for PCR amplification were also used in sequencing reactions.

Sequence analysis

Approximately 850 to 1,000 bases were determined from the complete ITS region of the rDNA for 23 specimens representing 12 *Haematoloechus* species. Sequences were assembled using Contig Express (v. 8.0, InforMax) and provisionally aligned using ClustalX using default settings (Thompson et al., 1997) followed by alignment by eye using the Bio Edit Sequence Alignment Editor (Hall, 1999) . The resultant sequence alignment was then edited by eye to remove ambiguous regions where I could not confidently identify positions of homology within these regions, yielding a 1,269 character alignment. Parsimony analyses were conducted in PAUP*4.0b10 (Swofford, 2001) using a heuristic search algorithm, and TBR branch-swapping option. Character states were treated as unordered and gaps were treated as missing data. Trees were rooted using *Plagiorchis koreanus* Ogata, 1938, *P. maculosus*, and *P. vespertilionis* (Mueller, 1780) as the outgroups. All outgroup taxa are Plagiorchiidae and have been demonstrated to be closely related to the Haematoloechidae within the Plagiorchioidea by Tkach et al., (2000; 2001). Sequence divergences among and within species of *Haematoloechus* were calculated using MEGA 3.1 (Kumar et al., 2004).

Haematoloechus coloradensis and *H. longiplexus* frog experimental infections

Anuran host specificity experiments were conducted in two separate trials. Three anuran species were used. In trial I, snails *Physa (Physella) gyrina* naturally infected with *H. coloradensis* were collected from Cedar Creek, Keith County, Nebraska (41.18080, -101.57973). A total of 318 *Physa (Physella) gyrina* snails were collected by dip-net and were individually isolated in 1.5 ml well plates filled with aged tap water. Of these 13 snails shed *Haematoloechus* cercaria. Larval damselflies *I. verticalis*, used in the second intermediate host infections came from Oak Lake, Lancaster County, Nebraska (40.83056, -96.70778) and were divided into three equal groups of experimental, time-0, and time-T controls, and exposed to *Haematoloechus* cercariae as previously described. Twelve newly metamorphosed green frogs, *R. clamitans*, collected from Eagle, Waukesha County, Wisconsin (42.5400, -88.29000) and nine newly metamorphosed Woodhouse's toads were collected from Beckius Pond, Keith County, Nebraska. Frogs and toads were divided into equal groups of experimental, time-0 and time-T controls. Experimental groups of frogs and toads were infected with metacercariae recovered from laboratory infected damselflies as previously described.

In trial II, larval damselflies (*Amphiagrion* sp.) naturally infected with metacercariae putatively identified as *H. longiplexus* were collected from Cedar Creek, Keith County, Nebraska. Twelve adult wood frogs, *R. sylvatica*, were collected from the University of Wisconsin-Milwaukee Field Station, Ozaukee County, Wisconsin (43.38875, -88.02208); and twelve juvenile Woodhouse's toads were collected from Beckius Pond, Keith County, Nebraska (41.20835, -101.61777). Frogs and toads were

divided into groups of four experimental, time-0 and time-T controls. Damselfly larvae were dissected in odonate saline, and all the recovered *Haematoloechus* metacercariae were thoroughly mixed, and experimental groups of frogs and toads were each given 20-45 metacercariae. A single experimental and time-T control wood frog and a single experimental and time-T control toad was necropsied two weeks after infections, while all other anurans were necropsied 30 day post infection. All experimental and time T-control groups of frogs and toads were maintained individually in plastic shoe boxes (35 cm X 25 cm X 15 cm), and fed crickets three times a week.

Data for evolution of *Haematoloechus* life history studies

Known life history data for species of *Haematoloechus* were obtained from chapter one and two, the current study and life history studies on *H. asper* Looss, 1899, by Sinitzin (1907), Odening (1960) and Dobrovol'ski (1965), *H. breviplexus* by Schell (1965), Dronen (1977) and Underwood and Dronen (1977), *H. coloradensis* by Dronen (1975; 1978), *H. complexus* by Krull (1933; 1934) and Snyder and Janovy (1994), *H. longiplexus* by Krull (1932), Snyder (1996), and Snyder and Janovy (1996), *H. medioplexus* and *H. parviplexus* by Krull (1930; 1931), Snyder (1996) and Snyder and Janovy (1994; 1996), *H. similis* by Grabda (1960) and *H. variegatus* (Rudolphi, 1819) by Sintzin (1907) and van Theil (1930). Known family of snail first intermediate hosts, ability to infect anisopteran, zygopteran, or other arthropod second intermediate hosts, North American amphibian definitive hosts specificity for each species of frog lung fluke, cercariae and metacercariae morphology, and some adult worm morphologies were

then mapped onto the phylogeny to obtain a better understanding of the avenues for and constraints on life cycle evolution in the genus *Haematoloechus*.

RESULTS

Voucher Identification from Snyder and Tkach (2001)

All but two voucher specimens deposited by Snyder and Tkach (2001) agreed with the original descriptions. The specimen identified as *H. varioplexus* by Snyder and Tkach (2001) and its sequence used under that name in the phylogeny by León-Règagnon and Brooks (2003) most closely resembled *H. parviplexus* described from the green frog by Irwin (1929) while the specimen identified as *H. breviplexus* most closely resembled *H. floedae* originally described from the bullfrog by Harwood (1932). Additionally, the specimen of *H. coloradensis* used in the molecular phylogeny by León-Règagnon and Brooks (2003) represents a previously undescribed species of *Haematoloechus* and should not be considered as the source of correct nucleotide sequence data for *H. coloradensis*.

Sequence analysis

Genetic divergence between the ingroup and the outgroup ranged from 14.1 to 15.6% and within the in-group, from 0.4 to 11.1% (Table II). Sequences of multiple specimens of five species were examined to determine intra specific sequence variation within the ITS 1, 5.8S, and ITS 2 rDNA. Two specimens of *H. parviplexus* collected from Lancaster County, Nebraska and Waukesha County, Wisconsin and compared to a previous sequence of *H. parviplexus*, previously identified as *H. varioplexus* by Snyder

and Tkach (2001) taken from Keith County, Nebraska, a distance of 383-1,095 km, were found to have no sequence variation across 843 bases. *Haematoloechus longiplexus* showed a similar pattern, two specimens taken from Lancaster and Keith Counties, Nebraska and compared to the previous sequence of *H. longiplexus* collected from Gage County, Nebraska by Snyder and Tkach (2001) (Table I), separated by a distance of 81-383 km, were found to have no sequence variation across 1,001 overlapping bases, while the two specimens of *H. longiplexus* collected from Lancaster and Keith Counties Nebraska, varied by a single base across their 1,025 overlapping bases. Similarly no sequence variation among 847 bases was found in two *H. coloradensis* collected from two different frog species in Keith County, Nebraska separated by a distance of approximately 30 km. Three *H. complexus* collected from a single population in Lancaster County, Nebraska compared to a single specimen collected from Gage County, Nebraska by Snyder and Tkach (2001) all from plains leopard frogs (*R. blairi*) had identical sequences but differed by two base pairs of 891 bases from a single specimen of *H. complexus* from green frogs (*R. clamitans*) collected in Waukesha County, Wisconsin a distance of 744 km. Finally, a single specimen of *H. varioplexus* collected from Kingsbury County, South Dakota differed by a single base of the 839 overlapping bases from two specimens collected in Grand Forks County, North Dakota, and Ozaukee County, Wisconsin, a distance of 250-895 km.

In the 1,269 character DNA sequence alignment 829 characters were constant, 179 variable characters were parsimony uninformative and 261 characters were parsimony informative. Parsimony analysis yielded six most parsimonious trees (tree length = 672; consistency index = 0.8110; homoplasy index = 0.1890; retention index =

0.8806). The trees differed only in the resolution of two clades. The clade comprised of *H. breviplexus*, *H. floedae*, *H. medioplexus* and *H. parviplexus* varied in that the clade comprised of *H. breviplexus* and *H. floedae* were basal to a clade comprised of *H. medioplexus* and *H. parviplexus* or they formed an unresolved polytomy. The clade comprising *H. varioplexus*WI, *H. varioplexus*ND and *H. varioplexus*SD varied in that *H. varioplexus*WI was basal to *H. varioplexus*ND and *H. varioplexus*SD, *H. varioplexus*ND was basal to *H. varioplexus*SD and *H. varioplexus*WI or they formed an unresolved polytomy. The strict consensus of the six most parsimonious trees obtained shows *Haematoloechus similis* as the sister species to the rest of the in-group. With the exception of the relationships among *H. breviplexus*, *H. floedae*, *H. medioplexus*, and *H. parviplexus* bootstrap support of nodes uniting all in-group taxa were strong across all clades (Figures 1 and 2).

Haematoloechus coloradensis and *H. longiplexus* frog experimental infections

In trial I of the three green frogs and three Woodhouse's toads exposed to *H. coloradensis* one toad died before necropsy. Of the surviving anurans none of the green frogs became infected while one out of two toads (50%) became infected with six *H. coloradensis*. None of the time-0, or time-T control green frogs or Woodhouse's toads were infected with any *Haematoloechus* species. In trial II the single experimental Woodhouse's toad examined two weeks after infection contained 39 immature *H. longiplexus* all containing toad blood in the ceca, while the single experimental wood frog contained a single inactive juvenile worm with no frog blood present in the worm's ceca. The three remaining experimental Woodhouse's toads necropsied 30 days post

infection all contained one to two adult *H. longiplexus*, while none of the three experimental wood frogs were infected. None of the time-0, or time-T control Woodhouse's toads or wood frogs were infected with any *Haematoloechus* species.

DISCUSSION

Sequences divergence

Molecular data from the present study supports the separation of *H. parviplexus* from *H. varioplexus* into two distinct species. These two taxa differed in 37 to 38 bases of the ITS rDNA across a gapped sequence length of 876 bases. This is a substantial sequence divergence considering that no intraspecific sequence variation was found in *H. parviplexus* while a single individual *H. varioplexus* only differed by one base from two other individuals. These data support my previous host specificity studies where *H. parviplexus* does not infect northern leopard and plains leopard frogs but infects bullfrogs (Chapter Two). Molecular data from the present study also indicate that *H. coloradensis* and *H. complexus* from Nebraska should be separated into two distinct species. These two taxa had no intraspecific sequence variation but differed in six base of the ITS rDNA across a gapped sequence length of 866 bases. Moreover, the North American *H. medioplexus* and *H. parviplexus* are sister species readily distinguished on morphological grounds but differ in their ITS by only four bases over a gapped sequence length of 851 bases. My morphological data on 20 *H. coloradensis* and 20 *H. complexus* from multiple frog and toad species indicate that although morphologically similar, these two species

are distinct, differing in their oral sucker to pharynx width and length ratios, uterine loop distribution, and placement of vitelline follicles (Chapter One).

My data also confirm previous studies by León-Règagnon (2003), León-Règagnon and Brooks (2003) and León-Règagnon et al. (2005) that *H. breviplexus* and *H. floedae* are distinct species. León-Règagnon and coworkers (2003; 2005) showed that these are distinct species based on morphology and two different molecular markers, the 28S of the rDNA and the mitochondrial COI. These worms differed in their testes shape, uterine loops and distribution of vitelline follicles and showed a 3.5% base composition difference of the 28S rDNA. My ITS data support their findings; these two species differed in 12 base (1.41%) of the ITS rDNA across a gapped sequence length of 852 bases. Additionally, the lung fluke *H. similis* is basal to all other members of the genus *Haematoloechus*. This species has been previously assigned to the genus *Skrjabinoeces* based on its distribution of vitelline glands and morphology of its cercaria and my molecular data indicate that the taxonomic status of this somewhat controversial genus may be valid. Finally, the phylogenetic analysis confirmed that two of the three clades of *Haematoloechus* were comprised of both European and North American species, indicating that the major lineages of *Haematoloechus* arose before the breakup of Laurasia and radiated after Eurasia and North America spilt (Snyder and Tkach, 2001; León-Règagnon and Brooks, 2003).

Life history characteristics

Examination of the literature suggests that the 12 European and North American frog lung fluke species examined in this study show distinct patterns of molluscan and

arthropod host specificity, and larval morphology (Figures 3-5). Studies on molluscan host specificity indicate that most species of frog lung flukes are host specific to a single species or two or three related species of gastropods (Krull, 1930; 1931; 1933; 1934; Snyder and Janovy, 1994, 1996). However, most *Haematoloechus* species including the basal European *H. similis* and *H. asper* and North American *H. longiplexus* species utilize snails in the family Planorbidae as the first intermediate hosts, (Figure 3) indicating that the parasitism of planorbids may be the ancestral condition within the genus.

Haematoloechus complexus and *H. coloradensis* two North American sister species are exceptions to this rule and utilize snails of the genus *Physa* in the family Physidae (see Dronen, 1975; Snyder and Janovy, 1996; Chapter One; Chapter Two). Interestingly, their close European relative *H. variegatus* utilizes a planorbid snail in the genus *Anisus* indicating that a host switch at the family level occurred. It is unclear if this family level host switch occurred after the break up of Laurasia because the life cycle of *H. varioplexus* the other North American close relative to *H. complexus* and *H. coloradensis* is not known.

The other exception to Planorbidae as the family of gastropods serving as first intermediate hosts for *Haematoloechus* is the North American *H. breviplexus* infecting bullfrogs in Texas. *Haematoloechus breviplexus* has been shown to utilize a species of *Ferrissia* in the family Ancylidae as the first intermediate host (Underwood and Dronen, 1977). This observation indicates that *H. breviplexus* also switched first intermediate hosts at the family level, since both *H. parviplexus* and *H. medioplexus*, its close

relatives, are known to utilize planorbid snails in the genus *Gyraulus*. However, recent morphological and molecular phylogenetic studies utilizing three different molecular markers indicate that Ancylidae and Planorbidae are paraphyletic and planorbids are nested within the ancylids (see Jorgensen, et al., 2004). It is unclear if this is a real family host switch because of the paraphyletic nature of the planorbidae and ancylidae and because another European species *Haematoloechus pyrenaicus* Combes, 1965 which was not included in this study uses the European freshwater limpet *Ancylus fluviatilis* as the first intermediate host indicating that some other Palearctic species of frog lung flukes utilize ancylids as first intermediate hosts (Combes, 1968).

Haematoloechus complexus, *H. coloradensis*, and *H. varioplexus* are parasites of North American anurans that form a well-supported clade with the European *H. abbreviatus* (Bychovskij, 1932) and *H. variegatus*. Snyder and Janovy (1994; 1996) and Bolek (Chapter One) recognized *H. complexus* and *H. coloradensis* as generalists within the second intermediate host because of the ability of the cercariae of these species to infect a wide variety of arthropods. The cercariae of both of these parasites are able to penetrate the exoskeleton of arthropods at any intersegmental membrane. Life cycle observations on the metacercariae of the European *H. variegatus* have been reported from zygopteran odonates (Sinitzin, 1907) and from nonodonate arthropods (van Theil, 1930), developing in the mosquito *Anopheles maculipennis*. Although experimental evidence is lacking for this species, these reports suggest a cercarial penetration ability similar to that of *H. complexus* and *H. coloradensis*. Unfortunately, the arthropod hosts of the European *H. abbreviatus* and the North American *H. varioplexus* are unknown, but the results of

the present phylogenetic analysis suggest that these parasites will be found to be second intermediate host generalists (Figure 4.)

Haematoloechus breviplexus, *H. floedae*, *H. medioplexus*, and *H. parviplexus* are all North American species. Although *H. medioplexus* differs greatly in its morphology from the other three species and has been considered by previous authors to represent a diverging lineage (Odening, 1960; Kennedy, 1981), the current study places them in a single clade with lower bootstrap support. The life cycle of *H. floedae* is currently unknown, whereas *H. breviplexus*, *H. medioplexus*, and *H. parviplexus* have cercariae that are considerably smaller than other North American *Haematoloechus* species and all are reported to infect anisopteran (dragonfly) odonates (Krull, 1931; 1932; Schell, 1965; Underwood and Dronen, 1977; Dronen, 1977; Snyder and Janovy, 1996). Importantly studies on arthropod second intermediate host specificity of *H. medioplexus* and *H. parviplexus* by Krull (1931; 1932), Snyder and Janovy (1994; 1996) and Bolek (Chapter Two) indicate that both of these species are second intermediate host specialists infecting the branchial basket of anisopteran odonates. The cercariae of these species infect dragonfly larvae by passive entry into the rectal gills where they form encysted metacercariae. Although, no efforts were made to infect other arthropods, Schell (1965) and Dronen (1977) reported encysted metacercariae of *H. breviplexus* from the branchial basket of experimentally infected anisopteran odonates. This site specificity and encysted metacercariae within an anisopteran host is consistent with the second intermediate host specialists *H. medioplexus* and *H. parviplexus* reported by Krull (1931; 1932), Snyder and Janovy (1994; 1996) and Bolek (Chapter Two), suggesting that this clade of four North American species is comprised of second intermediate host specialists

infecting the branchial basket of dragonflies (Figure 4) all having similar cercariae and metacercariae stages (Figure 5).

Examination of the literature also suggests the *H. similis*, *H. asper* and *H. longiplexus* share similar patterns of arthropod intermediate host specificity (Figure 4). The clustering of North American and European parasites in the same clade provides evidence of a second evolutionary lineage of *Haematoloechus* that was established before the breakup of Laurasia. Compared to other *Haematoloechus* species that have encysted metacercariae, all three of these species (*H. asper*, *H. longiplexus*, and *H. similis*) have been reported to have metacercariae that are unencysted or sometimes lightly encysted (*H. longiplexus*) in the second intermediate host (Krull, 1932; Grabda, 1960; Odening, 1960; Wetzel and Esch, 1996; Chapter Two; Figure 5.).

Both European species *H. asper* and *H. similis* have been reported to develop naturally in zygopteran (damselfly) odonates while the North American species *H. longiplexus* has been reported from zygopteran and anisopteran odonates (Grabda, 1960; Odening, 1960; Snyder and Janovy, 1996; Wetzel and Esch, 1996; Chapter Two). Unlike anisopterans, zygopteran odonates do not have a rectal gill that allows cercariae of *Haematoloechus* species passive access to the internal anatomy of dragonfly larvae. Both *H. longiplexus* and *H. similis* have been shown to actively penetrate the body of damselfly larvae whereas *H. asper* has never been reported from any other arthropods but zygoperan odonates (Grabda, 1960; Odening, 1960; Dobrovol'ski, 1965; Snyder and Janovy, 1996). It is unclear if *H. asper* and *H. similis* can infect anisopteran odonates as does *H. longiplexus* because both of these species have not been the subject of such

experiments. Observations indicate that metacercariae of *H. longiplexus* are unencysted or lightly encysted in the head and body cavity of zygopterans (Krull, 1932; Chapter Two). They have been reported as lightly encysted in the branchial basket of anisopteran odonates larvae and are lost after metamorphosis in these insects (Wetzel and Esch, 1996; Chapter Two). These life history observations suggest that the inability to form encysted metacercariae may preclude some *Haematoloechus* species such as *H. asper* and *H. similis* from infecting and/or surviving anisopteran metamorphosis. Experimentation with haematoloechid representatives found outside of North America is necessary to determine the limits of this kind of host specificity and ability to survive metamorphosis in anisopteran odonates. What ever the case may be the apparent pattern of damselfly host specificity and unencysted metacercaria in basal members of *Haematoloechus* (Figures 4 and 5) indicates that the parasitism of zygopteran odonates by unencysted metacercariae may be the ancestral condition within the genus. This is particularly true when considering the basal species *H. similis*, which infects damselflies with unencysted metacercariae. Additionally, *H. similis* has morphologically distinct cercariae from all other haematoloechid representatives (Figure 5). Although resembling the ornatae xiphidocercariae of haematoloechids (with a tail fin-fold) in size and body proportions cercariae of *H. similis* differ in having no tail fin-fold and a excretory system resembling cercariae of the armatae group of plagiorchids species that have been shown to be closely related to the Haematoloechidae and commonly used as outgroups in molecular studies (Tkach et al., 2000, 2001; Snyder and Tkach, 2001; León-Règagnon and Brooks, 2003). These observations give additional evidence that the use of damselflies and unencysted metacercariae may be the ancestral condition in the haematoleochids.

Recent studies have argued that no obvious coevolutionary patterns are revealed by Haematoloechidae amphibian host specificity (León-Règagnon and Brooks, 2003). However, most of the studies utilize field data reported in the literature to address issues of amphibian host specificity, which may include ecological host specificity. Data from the literature may not be reliable due to the common misidentification of these parasites unless voucher specimens have been examined or drawings of the parasites are provided in the publication. Finally no comprehensive studies have been made on phylogenetic relationships among the anuran definitive hosts from a single continent.

Recent phylogenetic studies of the new world *Rana* by Hillis and Wilcox (2005) have examined most of the described and undescribed species of true frogs from North, South and Central America and shed new light on the relationships of North American *Rana*. The Hillis and Wilcox (2005) study indicates that the new world *Rana* are comprised of six clades including the ones the authors call Amerana (*Rana boylii* group), *Rana sylvatica* (wood frog), Aquarana (*Rana catesbeiana* group), Ranula (*Rana palmipes* group), Torrentirana (*Rana tarahumarae* group), and Pantherana (*Rana pipiens* group). Hillis and Wilcox (2005) show that the yellow-legged and red-legged frogs of the western US are basal to all other new world *Rana*. More importantly they indicate that the wood frog, *R. sylvatica*, a small terrestrial frog of the north eastern US previously thought to be related to European *Rana* species (Farris et al., 1980), is most closely related to the bullfrog, *R. catesbeiana*, clade (Aquarana) of aquatic and semi-aquatic frogs comprised of seven species including the green frog, *R. clamitans*; all of which are distantly related to the semi-terrestrial Pantherana clade that include 30 species among them the northern leopard frog, *R. pipiens*, and plains leopard frog, *R. blairi*.

Mapping the definitive host specificity for the eight North American species of *Haematoloechus* examined in this study, using five *Rana* species that vary in their relatedness and a distantly related toad (Hay et al., 1995; Hillis and Wilcox, 2005), suggests a complex evolutionary history (Figure 6). The Woodhouse's toad, a distant relative of true frogs, has been shown to be infected with six of the eight species of frog lung flukes. No reports exist for *H. breviplexus* or *H. floedae* infecting this toad however its range does not overlap with the range of these parasites and laboratory infections will have to be conducted to demonstrate if Woodhouse's toad is susceptible. The two representatives of the Pantherana clade, the closely related northern leopard frog and plains leopard frog are infected with representatives of North American frog lung flukes from all three clades. However, my laboratory infections indicate that both are resistant to infection with *H. parviplexus*, a second intermediate host specialist, which infects frogs in the Aquarana clade. When considering the ecological host specificity data both of these frog species are predominantly infected with representatives of the second intermediate host generalist clade and rarely with *H. medioplexus* and *H. longiplexus*.

The two representatives of the Aquarana clade used in this study, the bullfrog and its sister species the green frog, show a different pattern from the two leopard frogs. Both of these species share three species of lung flukes from the second intermediate host specialist clade and *H. longiplexus*, which uses anisopteran and zygopteran as second intermediate hosts. Experimental studies on green frogs and field data on bullfrogs suggest that both species are resistant to infections with *H. medioplexus*, which predominantly infects members of the Panterorana. A study by Krull (1930) indicates that green frogs are resistant to infections with *H. medioplexus* but there are no such

studies of the susceptibility of bullfrogs to this parasite. However, of the 38 surveys of North American bullfrog parasites that included the lungs (Andrews et al., 1992; Snyder, 1996; McAlpine 1997; Goldberg et al., 1998; Bolek, Chapter One, Chapter Two) only one study reports a single *H. medioplexus* being found. However, because voucher specimens were not deposited and because frog lung flukes are commonly misidentified, it is doubtful if these investigators were dealing with *H. medioplexus*. Experimental infections need to be conducted to establish the validity of this single occurrence. The data suggest that these two sister species of frog lung flukes are restricted to two different clades of North American frogs *H. medioplexus* in the Panterorana and *H. parviplexus* in the Aquarana, with bufonids being infected with both of these species rarely in nature.

More interestingly, it appears that bullfrogs are resistant to most if not all the representatives of the generalist second intermediate host clade of North American frog lung flukes, whereas green frogs retained the ability to be infected with *H. complexus* but are resistant to infection with its sister species *H. coloradensis*. Finally wood frogs, which are more closely related to bullfrogs and green frogs than to the two leopard frog species, share two species (*H. complexus*, and *H. varioplexus*) from the generalist second intermediate host clade with leopard frogs and a single species with green frogs. Wood frogs are resistant to infections with *H. medioplexus*, which infects leopard frogs (Krull, 1931), and *H. longiplexus*, which infects all four other *Rana* species and a distantly related bufonid. This inability of certain clades of North American *Haematoloechus* to parasitize a single species of frog while other species within that clade have the ability to infect other closely related and unrelated ranids and bufonids indicates a complex evolutionary history among anuran definitive hosts. It indicates that for a frog lung fluke

to infect a certain species of frog that frog species must present the parasite with a series of physiological and immunological conditions that that parasite needs to survive and reproduce. Data indicate that those conditions vary greatly across different taxonomic levels of potentially susceptible anuran species.

Morphological data

Analysis of the phylogenetic history of *Haematoloechus* species has been hindered by the absence of any phylogenetic analyses based on a large suit of morphological traits. Currently, a small number of morphological characters are used to classify *Haematoloechus* species (see León-Règagnon and Brooks, 2003). Historically the arrangement of the uterine loops has been one of the most important taxonomical characteristics for classification of the groups. Numerous authors (Odening, 1960; Prudhoe and Bray, 1982) have considered that species without longitudinal extracecal uterine loops represent a different genus, *Ostiolum*. The current molecular data reject the evolutionary affinity of *Haematoloechus* species without longitudinal extracecal uterine loops as close relatives (Figure 7). This observation is in agreement with previous studies in which species without longitudinal extracecal uterine loops do not appear as closest relatives (León-Règagnon et al., 1999; Snyder and Tkach, 2001; León-Règagnon and Brooks, 2003). Importantly the two species of *Haematoloechus* (*H. coloradensis*, and *H. medioplexus*) without longitudinal extracecal uterine loops are predominantly parasites of leopard frogs. Two other species of frog lung flukes *H. complexus* without longitudinal extracecal uterine loops, and *H. varioplexus* with longitudinal extracecal uterine loops infect leopard frogs and green frogs and wood frogs respectively. Both of

these species are problematic in their identification because of the variation in their extracecal uterine loop morphology. *Haematoloechus complexus* in leopard frogs exhibits no longitudinal extracecal uterine loops, but can have small extracecal uterine loops in green frogs, while *H. varioplexus* exhibits well formed longitudinal extracecal uterine loops in wood frogs but can have reduced longitudinal extracecal uterine loops in leopard frogs. These observations suggest that leopard frogs may select for reduction of longitudinal extracecal uterine loops in their lung fluke parasites. Clearly laboratory life cycle studies among different anuran species will have to be conducted in order to test this hypothesis.

The other major taxonomical character for classification of frog lung flukes has been the relative size of the acetabulum to the oral sucker. In my analysis, species with acetabulum size of 50% or less of the size of the oral sucker are all included in the clade of North American second intermediate host specialists (Figure 7). These data are in contrast to previous studies by León-Règagnon et al., (2001) and León-Règagnon and Brooks (2003). The main reason for this discrepancy is that some of the species in their phylogenetic analysis were misidentified, particularly *H. varioplexus* of Snyder and Tkach (2001), which is actually *H. parviplexus* and their specimen of *H. coloradensis* which is an undescribed species and sequenced from an immature worm (León-Règagnon personal communication). Other species included in their analysis that were not available in the current study, particularly *Haematoloechus danbrooksi* León-Règagnon and Paredes-Calderón, 2002, a Mexican species and *Haematoloechus meridionalis* León-Règagnon, Brooks, and Zelmer, 2001, a Costa Rican species indicate that they are close relatives of *H. medioplexus*, a species which also has a small acetabulum compared to its

oral sucker. These data taken together suggest that this may be a valid taxonomic character for species relationships. Additional laboratory life cycle studies on the range of anuran hosts from other continents and sequence data for these species will be needed to determine if any host induced variation exists among some of these morphological characters.

The present study demonstrates the advantages of molecular data in resolving issues of taxonomic confusion within the Haematoloechidae. More importantly it argues for additional studies on life cycles and transmission strategies of frog lung flukes from all continents. The phylogenetic data presented in this study reveals the importance of second intermediate host specificity among the evolutionary lineages of frog lung flukes, because these second intermediate hosts serve as avenues for and constraints on the movement of these parasites to their respective definitive amphibian hosts.

ACKNOWLEDGMENTS

The author greatly acknowledges Dr. Scott Snyder, University of Nebraska at Omaha for help with the molecular aspect of this study and Dr. Vasyl Tkach, University of North Dakota for providing specimens or sequences of *H. varioplexus* and *H. similis* and Dr. Norm Dronen Jr. Texas A&M University for providing specimens of *H. breviplexus*. I also thank, Dr. Eric Hoberg, U.S. National Parasite Collection, Beltsville Agricultural Research Center Beltsville, MD and Dr. Agustín Jiménez-Ruiz, Harold. W. Manter Laboratory of Parasitology, University of Nebraska State Museum, for providing

voucher specimens of *H. breviplexus* and *H. floedae*. Additionally, I thank my wife Melissa Bolek for help in collecting frogs, Randy Peterson, Dwaine Dunwoody, Dr. James Coggins and Dr. Gretchen A. Meyer, University of Wisconsin-Milwaukee, Dr. Susan Lewis, Carroll College, and Dr. Brent Nickol, University of Nebraska-Lincoln, for access to field sites, Dr. Brent Nickol for the use of his animal room, and Cedar Point Biological Station for providing facilities. This work was supported by grants from the Initiative for Ecology and Evolutionary Analysis, University of Nebraska-Lincoln, and The School of Biological Sciences, University of Nebraska-Lincoln.

LITERATURE CITED

Andrews, K. D., R. L. Lampley, M. A. Gillman, D. T. Corey, S. R. Ballard, M. J. Blasczyk, and W. G. Dyer. 1992. Helminths of *Rana catesbeiana* in southeastern Illinois with a checklist of helminths in bullfrogs of North America. Transactions of the Illinois State Academy of Science **85**: 147-172.

Bourgat, R., and S. D. Kulo. 1979. Cycle biologique d' *Haematoloechus johnsoni* Bourgat, 1977, (Trèmatode) parasite pulmonaire de *Dicroglossus occipitalis* (Günther, 1858) (Amphibien, Anoure) au Togo. Revue Suisse de Zoologie **86**: 467-472.

Combes, C. 1968. Biologie, écologie des cycles et biogeography de digénes et monogénes d'amphibiens dans l'est des Pyrénéés. Mémoires du Muséum National d'Histoire Naturelle **51**: 1-195

Cort, W. W. 1915. North American frog lung flukes. Transactions of the American Microscopical Society **34**: 203-243.

Dobrovol'ski, A. A. 1965. Life cycle of *Pneumonoeces asper* Looss, 1899 (Plagiorchiidae, Pneumonocinae). Materily k Nouchnoj Konferentsii Vesoyuznogo Obschestva Gel'mintologov. T. 4., Moscow, Nauka, p. 59-65. [In Russian].

Dronen, N. O. Jr. 1975. The life cycle of *Haematoloechus coloradensis* Cort 1915 (Digenea: Plagiorchiidae), with emphasis on host susceptibility to infection. Journal of Parasitology **61**: 657-660.

Dronen, N. O. Jr. 1977. Studies on the population structure of two species of *Haematoloechus* Looss, 1899 (Digenea: Plagiorchiidae) in raniid frogs in New Mexico. Proceedings of the Helminthological Society of Washington **44**: 68-72.

Dronen, N. O. Jr. 1978. Host-parasite population dynamics of *Haematoloechus coloradensis* Cort, 1915 (Digenea: Plagiorchiidae). American Midland Naturalist **99**: 330-349.

Farris, J. S., A. G. Kluge, and M. F. Mickevich. 1980. Paraphyly of the *Rana boylii* group. Systematic Zoology 28: 627–634.

Grabda, B. 1960. Life cycle of *Haematoloechus similis* (Looss, 1899) (Trematoda: Plagiorchiidae). Acta Parasitologica Polonica **8**: 357-367.

Goldberg, S. R., C. R. Bursey and H. Cheam. 1998. Helminths of two native frog species (*Rana chiricahuensis*, *Rana yavapaiensis*) and one introduced frog species (*Rana catesbeiana*) (Ranidae) from Arizona. Journal of Parasitology **84**: 175-177.

Hall, T. A. 1999. Bio Edit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symposium Series **41**: 95–98.

Harwood, P. D. 1932. The helminths parasitic in the amphibia and reptilia in Houston, Texas and vicinity. Proceedings of the U.S. National Museum **81:** 1–71.

Hay, J. M., I. Ruvinsky, S. B. Hedges, and L. R. Maxson. 1995. Phylogenetic relationships of amphibian families inferred from DNA sequences of mitochondrial 12S and 16S ribosomal RNA genes. Molecular Biology and Evolution **12**: 928-937.

Hillis, D. M., and T. P. Wilcox. 2005. Phylogeny of the new world true frogs (*Rana*). Molecular Phylogenetics and Evolution **34**: 299-314.

Irwin, M. S. 1929. A new lung fluke from *Rana clamitans* Latreille. Transactions of the American Microscopical Society **48**: 74-79.

Jorgensen, A., T. K. Kristensen, and J. R. Stothard. 2004. An investigation of the "Ancyloplanorbidae" (Gastropoda, Pulmonata, Hygrophila): preliminary evidence from DNA sequence data. Molecular Phylogenetics and Evolution **32**: 778-787.

Kennedy, M. J. 1980a. Host-induced variation in *Haematoloechus buttensis* (Trematoda: Haematoloechidae). Canadian Journal of Zoology **58**: 427-442.

Kennedy, M. J. 1980b. Geographical variation in some representatives of *Haematoloechus* Looss, 1899 (Trematoda: Haematoloechidae) from Canada and the United States. Canadian Journal of Zoology **58**: 1151-1167.

Kennedy, M. J. 1981. A revision of the genus *Haematoloechus* Looss, 1899 (Trematoda: Haematoloechidae) from Canada and the United States. Canadian Journal of Zoology **59**: 1836-1846.

Krull, W. H. 1930. The life history of two North American frog lung flukes. Journal of Parasitology **16**: 207-212.

Krull, W. H. 1931. Life history studies on two frog lung flukes, *Pneumonoeces medioplexus* and *Pneumobites parviplexus*. Transactions of the American Microscopical Society **50**: 215-277.

Krull, W. H. 1932. Studies on the life history of *Haematoloechus longiplexus* (Stafford). Zoologischer Anzeiger **99**: 231-239.

Krull, W. H. 1933. Studies on the life history of a frog lung fluke, *Haematoloechus complexus* (Seely, 1906) Krull, n. comb. Zeitschrift für Parasitenkunde **6**: 193-206.

Krull, W. H. 1934. Some additional notes on the life history of a frog lung fluke, *Haematoloechus complexus* (Seely, 1906) Krull. Transactions of the American Microscopical Society **53**: 196-199.

Kumar, S., Tamura, K. & Nei, M. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings in Bioinformatics **5**: 150–163.

León-Règagnon, V. 2003. Incorporating morphological and molecular data in biodiversity inventories: Parasites of leopard frogs. Journal of Parasitology: **89:** S141-148.

León-Règagnon, V., and D. R. Brooks. 2003: Molecular phylogeny of *Haematoloechus* Looss, 1899 (Digenea: Plagiorchiidae), with emphasis on North American species. Journal of Parasitology **89**: 1206–1211.

León-Règagnon, V., S. Guillèn-Hernandez, and M. A.Arizmendi-Espinosa. 2005. Intraspecific variation of *Haematoloechus floedae* Harwood, 1932 (Digenea: Plagiorchiidae), from *Rana* spp. in North and Central America. Journal of Parasitology **91**: 915-921.

León-Règagnon, V., D. R. Brooks, and G. Pèrez-Ponce De León. 1999. Differentiation of Mexican species of Haematoloechus Loss, 1899 (Digenea: Plagiorchiformes): Molecular and morphological evidence. Journal of Parasitology **85**: 935-946.

McAlpine, D. F. 1997. Helminth communities in bullfrogs (*Rana catesbeiana*), green frogs (*Rana clamitans*), and leopard frogs (*Rana pipiens*) from New Brunswick, Canada. Canadian Journal of Zoology **75**: 1883-1890.

Odening, K. 1960. Plagiorchiidae III. (Haematoloechinae) und Omphalometridae. Das Tierreich **75**: 1-75.

Prokopič, J., and Křivanec. 1974. Trematodes of the genus *Haematoloechus* Looss and their variability. Helminthologica **15**: 779-801.

Prudhoe, O. B. E, and Bray, R. A. 1982. Platyhelminth parasites of the Amphibia. Oxford University Press, Oxford, U. K., 217pp.

Sinitzin, D. T. 1907. Observations sur les mètamorphoses des trèmatodes. Zoologie Expèrimentale et Gènèrale **7**: 21-37.

Schell, S. C. 1965. The life history of *Haematoloechus breviplexus* Stafford, 1902, (Trematoda: Haplometridae McMullen, 1937), with emphasis on the development of the sporocysts. Journal of Parasitology **51**: 587-593.

Snyder, S. D. 1996. Host specificity among species of *Haematoloechus* (Digenea: Haematoloechidae). Unpublished Ph.D. Thesis. University of Nebraska-Lincoln, 112 p.

Snyder, S. D., and J. Janovy Jr. 1994. Second intermediate host-specificity of *Haematoloechus complexus* and *Haematoloechus medioplexus* (Digenea: Haematoloechidae). Journal of Parasitology **80**: 1052-1055.

Snyder, S. D., and J. Janovy Jr. 1996. Behavioral basis of second intermediate host specificity among four species of *Haematoloechus* (Digenea: Haematoloechidae). Journal of Parasitology **82**: 94-99.

Snyder, S. D., and V. V. Tkach. 2001. Phylogenetic and biogeographical relationships among some Holarctic frog lung flukes (Digenea: Haematoloechidae). Journal of Parasitology **87**: 1433-1440.

Swofford DL (2001) PAUP*: Phylogenetic analysis using prarsimony (*and other methods), version 4.0b10. Sinauer Associates, Sunderland, MA.

Stafford, J. 1902. On the representation of *Distomum variegatum*. Zoologische Jahrbuecher Abteilung fuer Systematik Oekologie und Geographie der Tiere **16**: 895-912.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. 1997. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research **25**: 4876-4882.

Tkach, V., J. Pawlowski, and J. Mariaux. 2000. Phylogenetic analysis of the suborder Plagiorchiata (Platyhelminthes: Digenea) based on partial lsrDNA sequences. International Journal for Parasitology **30:** 83– 93.

Tkach, V., J. Pawlowski, J. Mariaux, and Z. Swiderski. 2001. Molecular phylogeny of the suborder Plagiorchiata and its position in the system of Digenea. *In* Interrelationships of the Platyhelminthes, D. T. J. Littlewood and R. A. Bray (eds.). Taylor and Francis, London, U.K., p. 186–193

Underwood H. T., and N. O. Dronen. 1977. The molluscan intermediate hosts for species of *Haematoloechus* Looss 1899 (Digenea: Plagiorchiidae) from rannid frogs of Texas. Journal of Parasitology **63**: 122.

van Theil, P. H. 1930. Die Entwicklung von *Agamodistomum anophelis* zum *Pneumonoecues variegates* Rud. Zentralblatt für Backteriologie, Parasitenkunde, und Infektionskrankheiten **117**: 103-112.

Wetzel, E. J., and G. W. Esch. 1996. Influence of odonate intermediate host ecology on the infection dynamics of *Halipegus* spp., *Haematoloechus longiplexus*, and *Haematoloechus complexus* (Trematoda: Digenea). Journal of the Helminthological Society of Washington **63**: 1-7.

Figure 1. Strict consensus of the eight most parsimonious trees for the rDNA internal transcribed spacer data analyzed with maximum parsimony analysis. Names correspond to those assigned in Table I. Numbers above each node show bootstrap support from parsimony analysis.

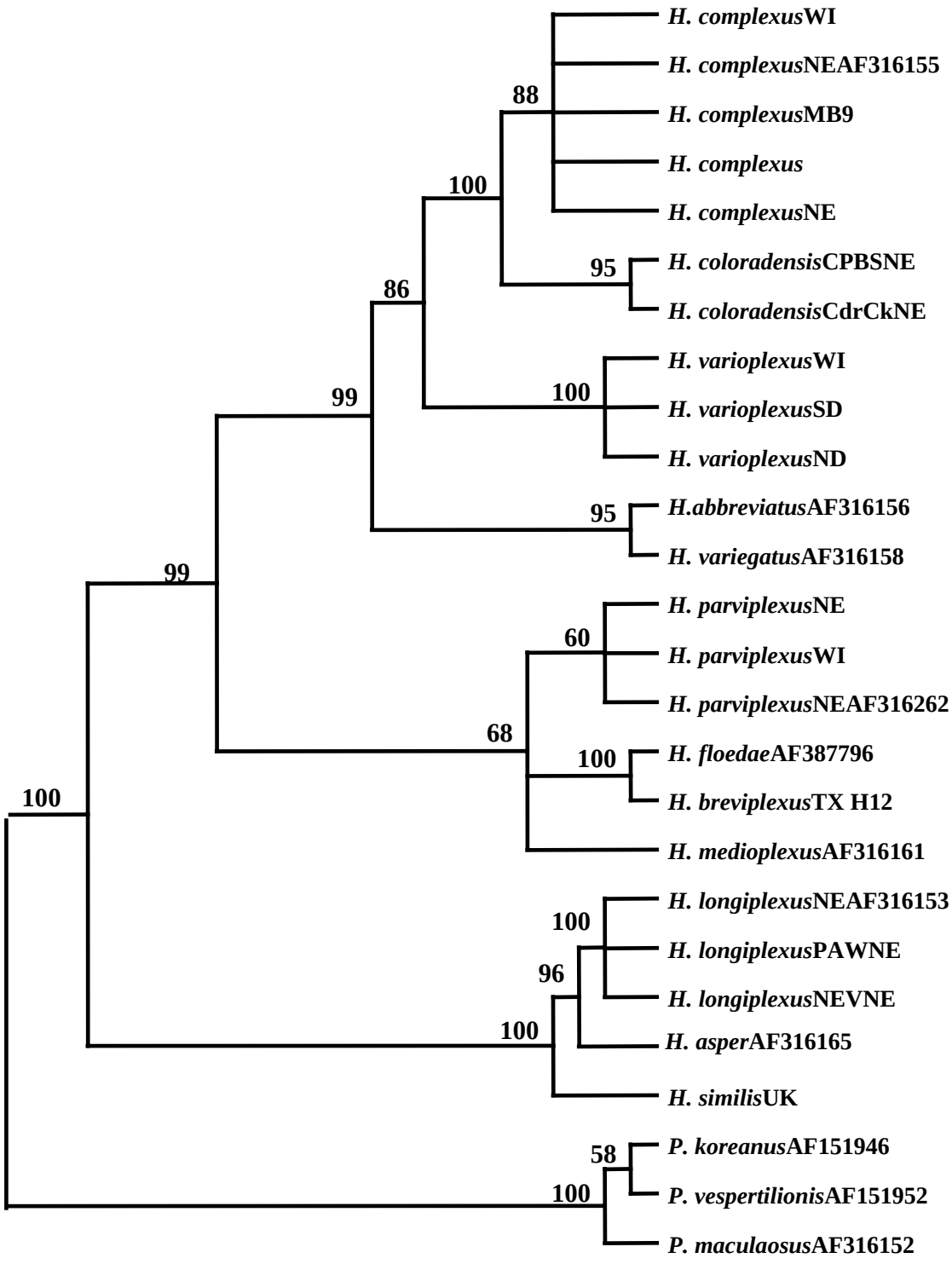

H. complexusWI
H. complexusNEAF316155
H. complexusMB9
H. complexus
H. complexusNE
H. coloradensisCPBSNE
H. coloradensisCdrCkNE
H. varioplexusWI
H. varioplexusSD
H. varioplexusND
H.abbreviatusAF316156
H. variegatusAF316158
H. parviplexusNE
H. parviplexusWI
H. parviplexusNEAF316262
H. floedaeAF387796
H. breviplexusTX H12
H. medioplexusAF316161
H. longiplexusNEAF316153
H. longiplexusPAWNE
H. longiplexusNEVNE
H. asperAF316165
H. similisUK
P. koreanusAF151946
P. vespertilionisAF151952
P. maculaosusAF316152
88
100
95
86
99
100
95
99
60
68
100
100
100
96
100
58
100

Figure 2. Phylogram of relationships among species of *Haematoloechus* of one of the six most parsimonious trees derived from the internal transcribed spacer data with maximum parsimony analysis. Names correspond to those assigned in Table I.

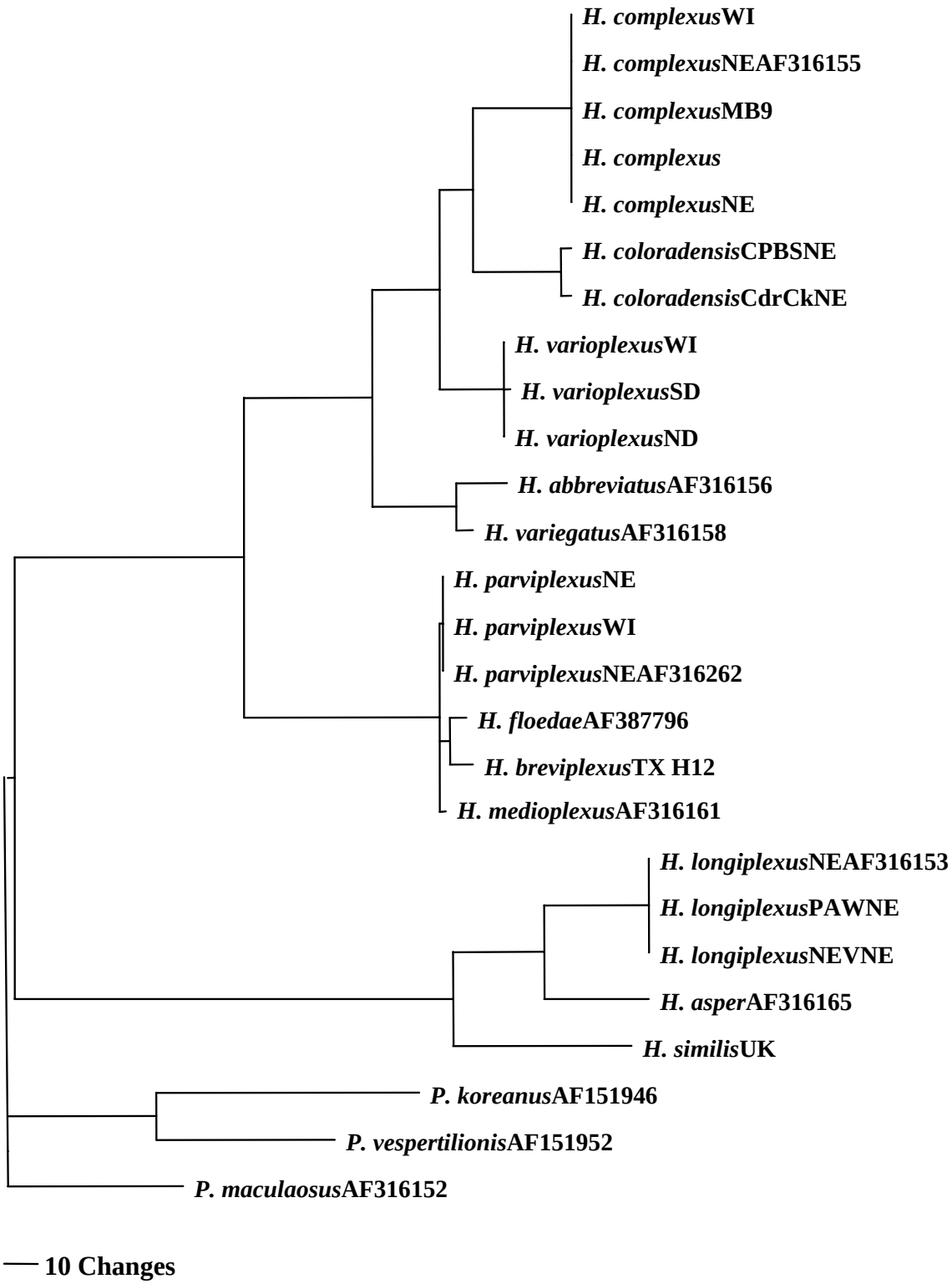

H. complexusWI
H. complexusNEAF316155
H. complexusMB9
H. complexus
H. complexusNE
H. coloradensisCPBSNE
H. coloradensisCdrCkNE
H. varioplexusWI
H. varioplexusSD
H. varioplexusND
H. abbreviatusAF316156
H. variegatusAF316158
H. parviplexusNE
H. parviplexusWI
H. parviplexusNEAF316262
H. floedaeAF387796
H. breviplexusTX H12
H. medioplexusAF316161
H. longiplexusNEAF316153
H. longiplexusPAWNE
H. longiplexusNEVNE
H. asperAF316165
H. similisUK
P. koreanusAF151946
P. vespertilionisAF151952
P. maculaosusAF316152
10 Changes

Figure 3. Molluscan host specificity at the family level and geographical distribution among species of *Haematoloechus* indicated on the tree derived from internal transcribed spacer rDNA data. Species in gray indicate that the life cycle is unknown. NA = North America. E = Europe.

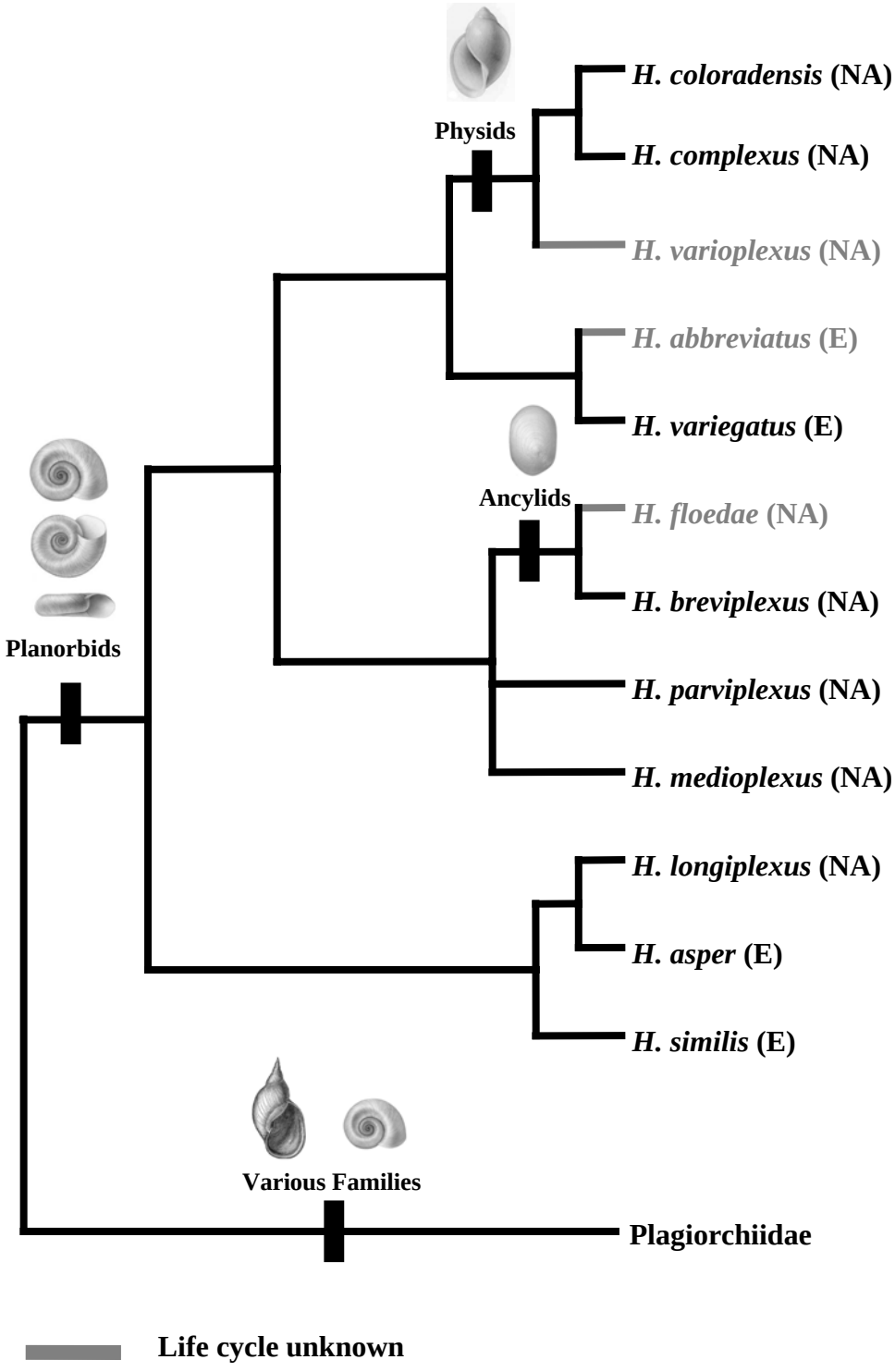

Physids
H. coloradensis (NA)
H. complexus (NA)
H. varioplexus (NA)
H. abbreviatus (E)
H. variegatus (E)
Ancylids
H. floedae (NA)
H. breviplexus (NA)
Planorbids
H. parviplexus (NA)
H. medioplexus (NA)
H. longiplexus (NA)
H. asper (E)
H. similis (E)
Various Families
Plagiorchiidae
Life cycle unknown

Figure 4. Arthropod host specificity and geographical distribution among species of *Haematoloechus* indicated on the tree derived from internal transcribed spacer rDNA data. Generalist parasites have the ability to form metacercariae in odonate and non-odonate arthropods. Species in gray indicate that the life cycle is unknown. NA = North America. E = Europe.

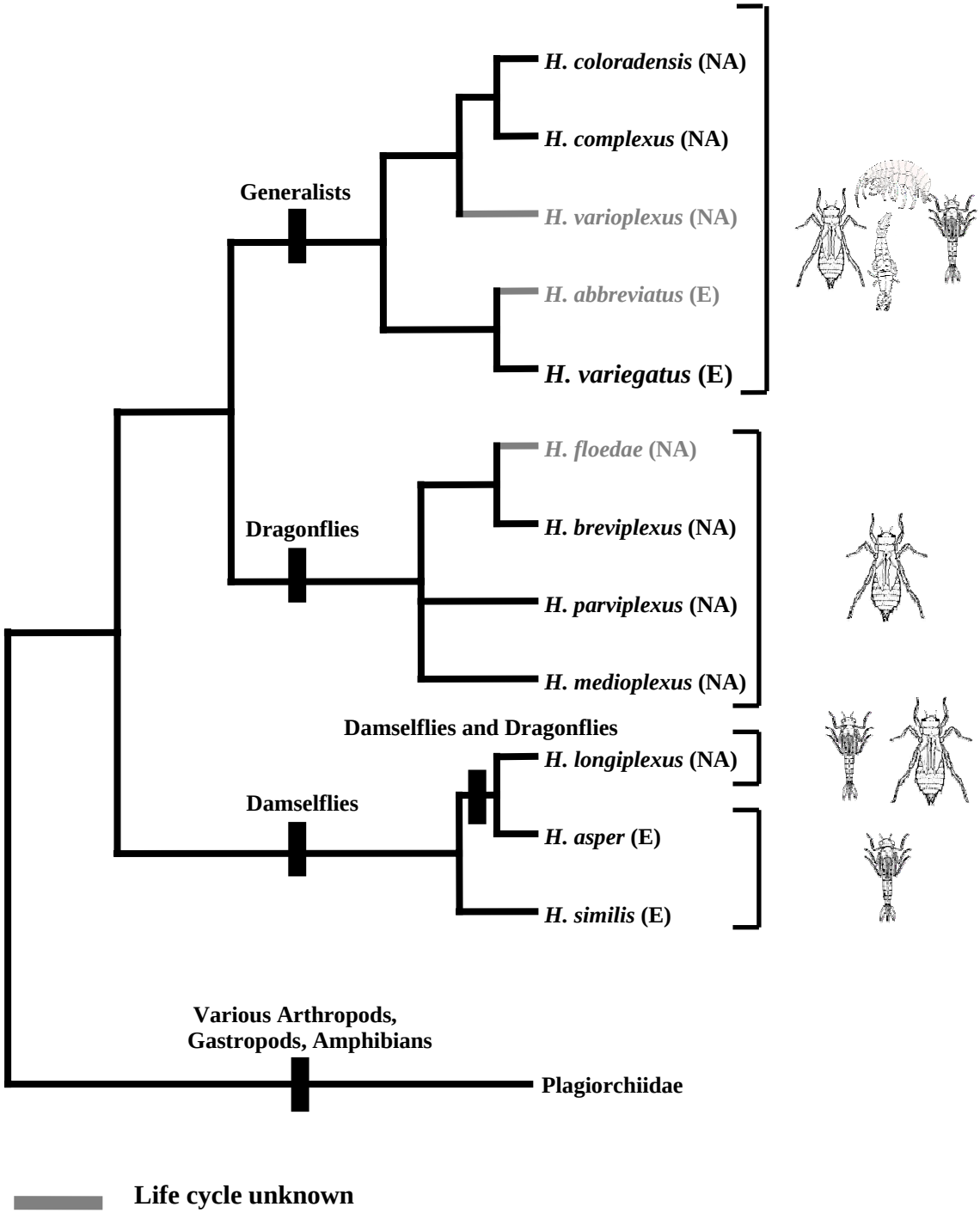

H. coloradensis (NA)
H. complexus (NA)
Generalists
H. varioplexus (NA)
H. abbreviatus (E)
H. variegatus (E)
H. floedae (NA)
Dragonflies
H. breviplexus (NA)
H. parviplexus (NA)
H. medioplexus (NA)
Damselflies and Dragonflies
H. longiplexus (NA)
Damselflies
H. asper (E)
H. similis (E)
Various Arthropods,
Gastropods, Amphibians
Plagiorchiidae
Life cycle unknown

Figure 5. Characteristics of cercaria and metacercaria morphology and geographical distribution among species of *Haematoloechus* indicated on the tree derived from internal transcribed spacer rDNA data. Species in gray indicate that the life cycle stage is unknown. NA = North America. E = Europe.

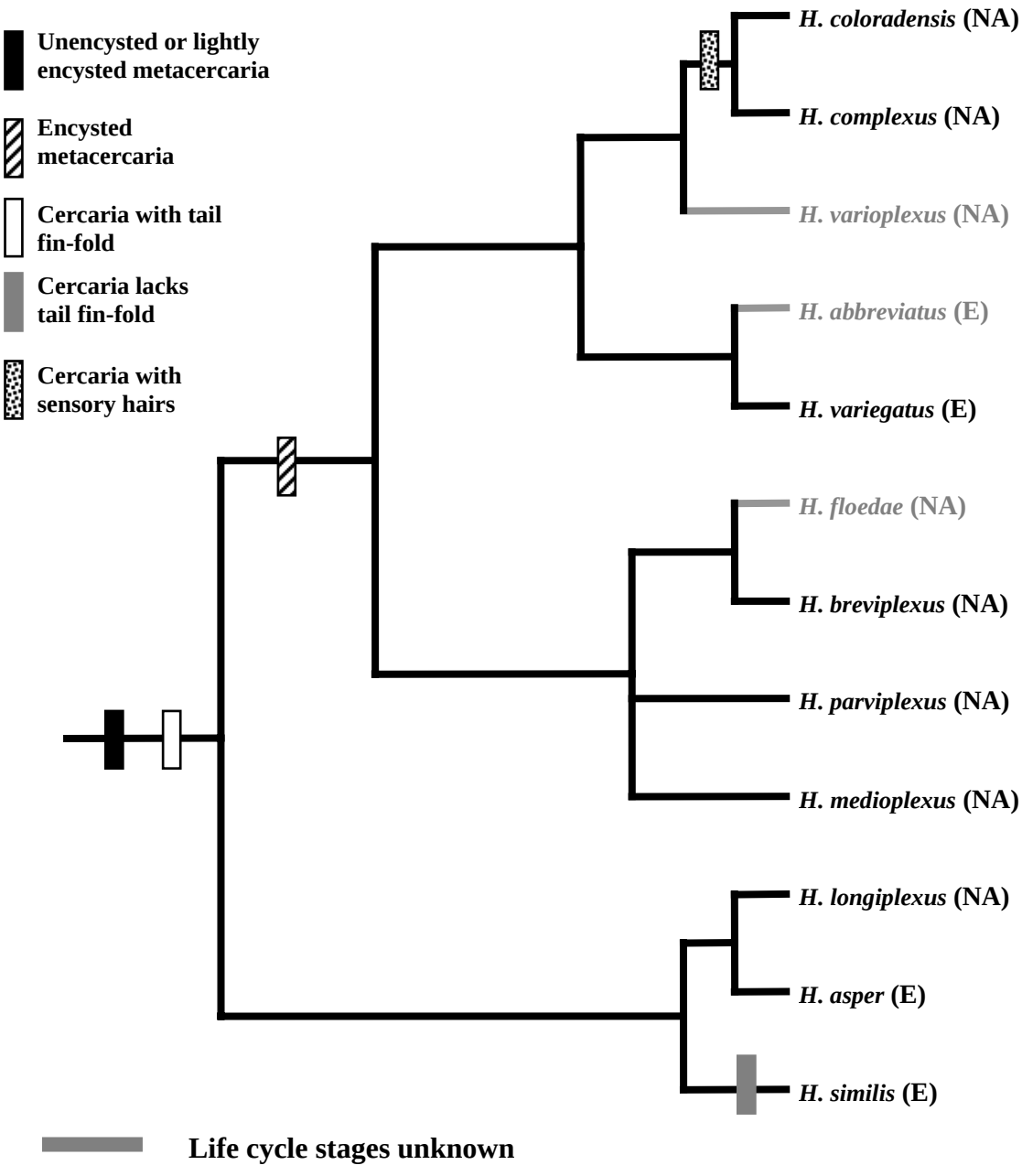

Unencysted or lightly encysted metacercaria
Encysted metacercaria
Cercaria with tail fin-fold
Cercaria lacks tail fin-fold
Cercaria with sensory hairs
H. coloradensis (NA)
H. complexus (NA)
H. varioplexus (NA)
H. abbreviatus (E)
H. variegatus (E)
H. floedae (NA)
H. breviplexus (NA)
H. parviplexus (NA)
H. medioplexus (NA)
H. longiplexus (NA)
H. asper (E)
H. similis (E)
Life cycle stages unknown

Figure 6. Anuran host specificity for five species of frogs and toads and geographical distribution among species of North American *Haematoloechus* indicated on the tree derived from internal transcribed spacer rDNA data. The X represents experimental infections that indicate that the particular anuran species is resistant to infection with a particular species of frog lung fluke, while a ? indicates that the susceptibility of the particular frog or toad species as a host for a lung fluke species is unknown. Rp = Northern leopard frog, *Rana pipiens*. Rb = Pains leopard frog, *Rana blairi*. Rcl = Green frog, *Rana clamitans*, Rc = Bullfrog, *Rana catesbeiana*. Rs = Wood frog, *Rana sylvatica*. Bw = Woodhouse's toad, *Bufo woodhousii*. NA = North America. E = Europe. Host specificity is only mapped for North American species of frog lung flukes.

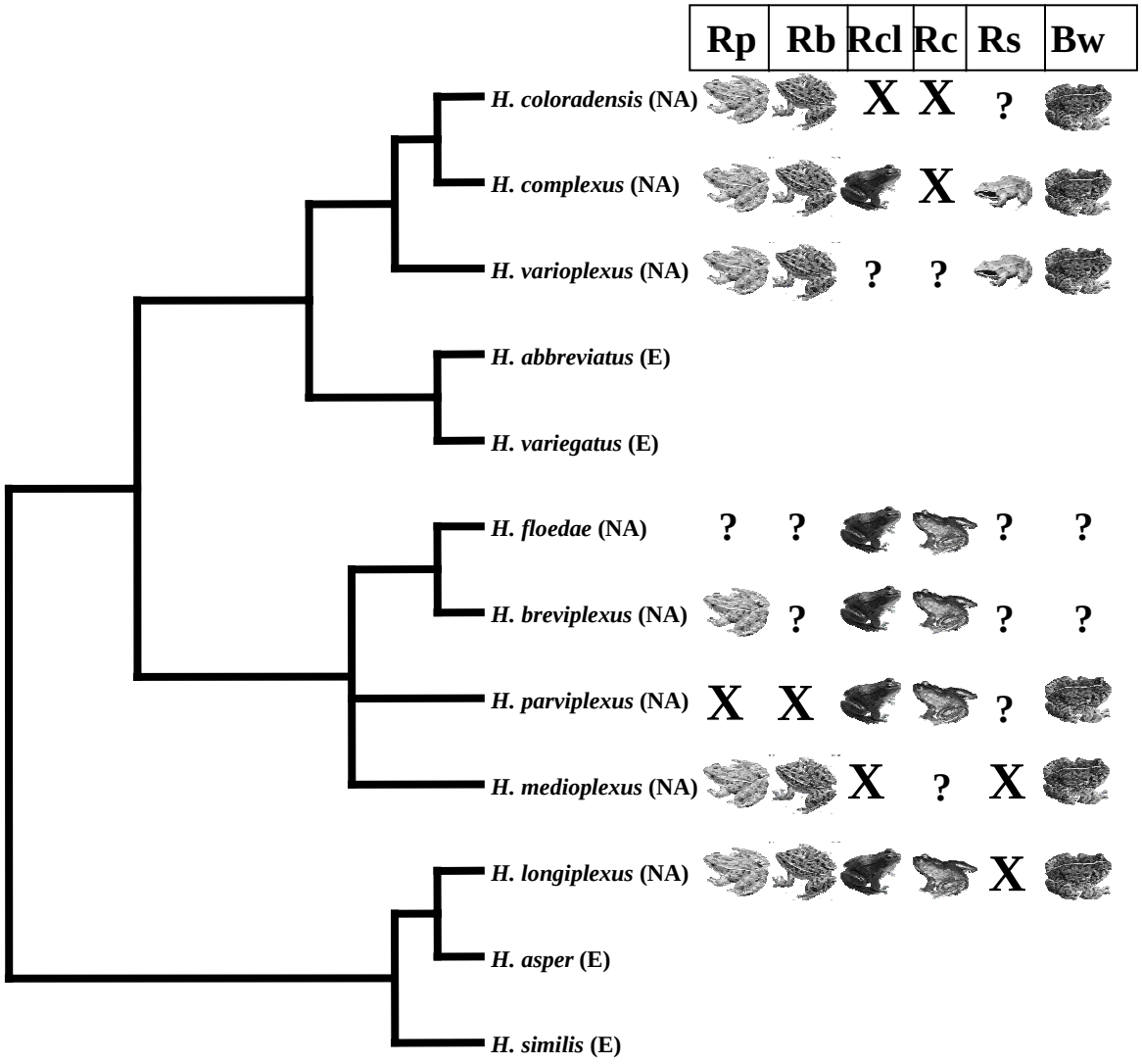

Rp
Rb
Rcl
Rc
Rs
Bw
H. coloradensis (NA)
H. complexus (NA)
H. varioplexus (NA)
H. abbreviatus (E)
H. variegatus (E)
H. floedae (NA)
H. breviplexus (NA)
H. parviplexus (NA)
H. medioplexus (NA)
H. longiplexus (NA)
H. asper (E)
H. similis (E)

Figure 7. Morphological characteristics of adult worms and geographical distribution among species of *Haematoloechus* indicated on the tree derived from internal transcribed spacer rDNA data. NA = North America. E = Europe.

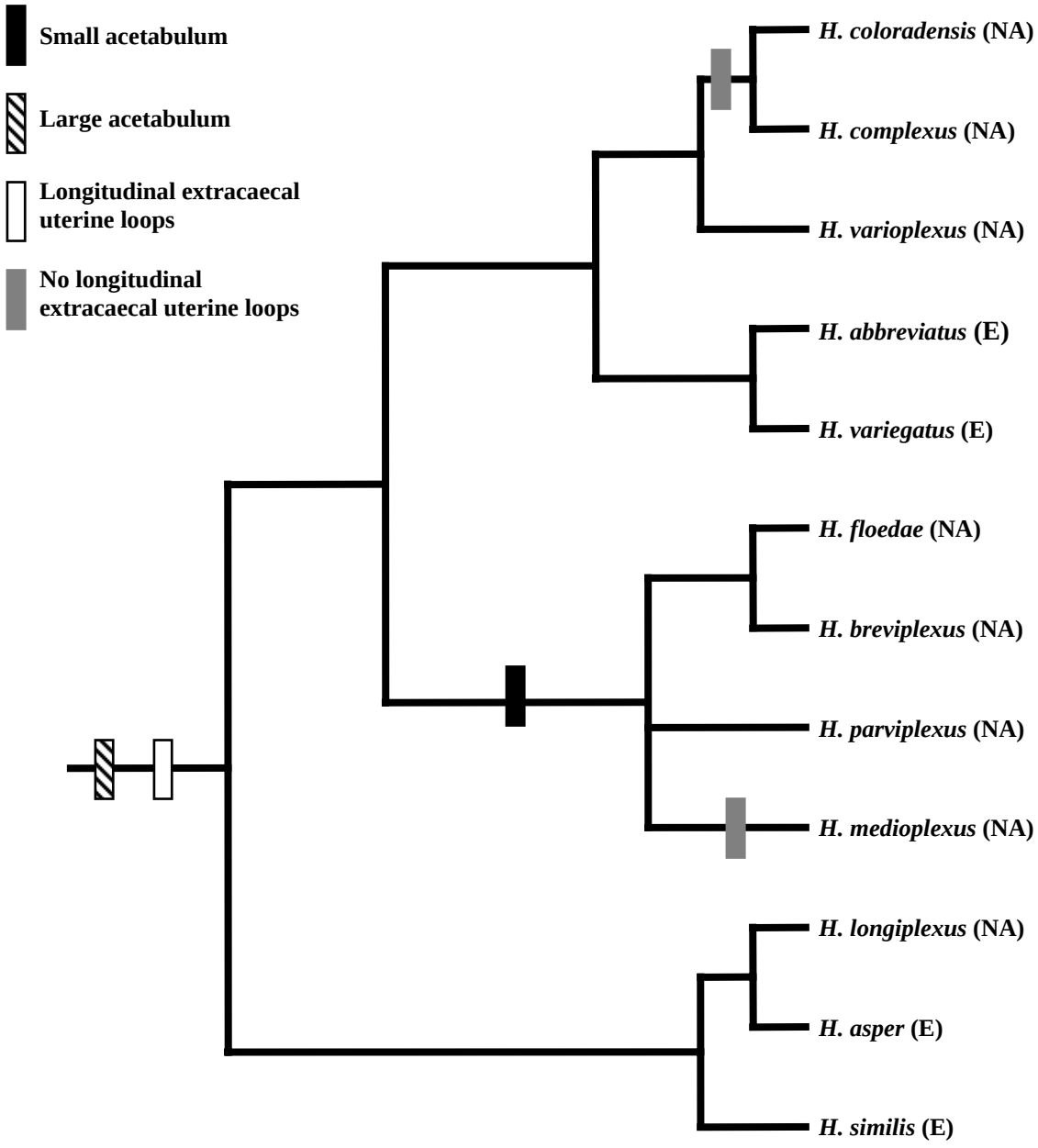

Small acetabulum
Large acetabulum
Longitudinal extracaecal uterine loops
No longitudinal extracaecal uterine loops
H. coloradensis (NA)
H. complexus (NA)
H. varioplexus (NA)
H. abbreviatus (E)
H. variegatus (E)
H. floedae (NA)
H. breviplexus (NA)
H. parviplexus (NA)
H. medioplexus (NA)
H. longiplexus (NA)
H. asper (E)
H. similis (E)

Table I. Digenean species used in this study, their hosts, geographical origin of specimens, GenBank accession number or name, sequence length, and accession numbers for vouchers of corresponding sequences of *Haematoloechus* species from previous studies.

Digenean taxa	Host species	Geographic origin	Name or ITS GenBank no. (Sequence length)	Vouchers
Haematoloechidae				
Haematoloechus abbreviatus	*Bombina variegata*	Khust District, Zakarpatska Region, Ukraine	AF316156 (845)	USNPC 091503.00
H. asper	*Rana ridibunda*	Vilkova, Kilia District, Odessa Region, Ukraine	AF316165 (1,009)	USNPC 091506.00
H. breviplexus	*R. catesbeiana*	Brazos County, Texas, U.S.A.	TXH12 (849)	__
H. coloradensis	*R. pipiens*	Keith County, Nebraska, U.S.A.	CdrCkNE (847)	__
H. coloradensis	*R. blairi*	Keith County, Nebraska, U.S.A.	CPBSNE (847)	__
H. complexus	*R. blairi*	Lancaster County, Nebraska, U.S.A.	__ (863)	__
H. complexus	*R. blairi*	Gage County, Nebraska, U.S.A.	AF316155 (845)	USNPC 091508.00
H. complexus	*R. blairi*	Lancaster County, Nebraska, U.S.A.	MB9 (862)	__
H. complexus	*R. blairi*	Lancaster County, Nebraska, U.S.A.	NE (860)	__
H. complexus	*R. clamitans*	Waukesha County, Wisconsin, U.S.A.	WI (881)	__
*H. floedae**	*R. catesbeiana*	Cachise County, Arizona, U.S.A.	AF387796 (851)	USNPC 091507.00
H. longiplexus	*R. catesbeiana*	Lancaster County, Nebraska, U.S.A.	PAWNE (1,025)	__
H. longiplexus	*R. catesbeiana*	Keith County, Nebraska, U.S.A.	NEVNE (1,024)	__

Table I continued.

Digenean taxa	Host species	Geographic origin	Name or ITS GenBank no. (Sequence length)	Vouchers
Haematoloechidae				
H. longiplexus	*R. catesbeiana*	Gage County, Nebraska, U.S.A.	AF316153 (1,001)	USNPC 091509.00
H. medioplexus	*R. pipiens*	Winnebago County, Wisconsin, U.S.A.	AF316161 (838)	USNPC 091511.00
H. parviplexus†	*R. catesbeiana*	Keith County, Nebraska, U.S.A.	AF316262 (850)	USNPC 091515.00
H. parviplexus	*R. catesbeiana*	Lancaster County, Nebraska, U.S.A.	NE (851)	—
H. parviplexus	*R. clamitans*	Waukesha County, Wisconsin, U.S.A.	WI (843)	—
H. similis	*R. ridibunda*	Vilkovo, Odessa Region, Ukraine	UK (859)	—
H. variegatus	*R. arvalis*	Rakhiv District, Zakarpat'ska Region, Ukraine	AF316158 (849)	—
H. varioplexus	*R. pipiens*	Grand Forks County, North Dakota, U.S.A.	ND (838)	—
H. varioplexus	*R. pipiens*	Kingsbury County, South Dakota, U.S.A.	SD (864)	—
H. varioplexus	*R. sylvatica*	Ozaukee County, Wisconsin, U.S.A.	WI (867)	—
Plagiorchiidae				
Plagiorchis koreanus	*Myotis daubentoni*	Sumy Region, Ukrain	AF151946 (1,163)	—
P. maculaosus	*Fringilla coelebs*	Kiev, Ukrain	AF316152 (1,100)	—
P. vespertilionis	*M. daubentoni*	Kiev, Ukrain	AF151952 (1,238)	—

* Originally identified as *H. breviplexus* by Snyder and Tkach, 2001, † originally identified as *H. varioplexus* by Snyder and Tkach, 2001.

Table II. Pairwise genetic distance between *ITS* genotypes. Uncorrected p-distances are expressed as percentages in the lower portion of the matrix. Standard error estimates are based on bootstrap analysis with 1,000 pseudoreplicates and are given in the upper portion of the matrix. H. col = *Haematoloechus coloradensis*, H. com = *H. complexus*, H. vario = *H. varioplexus*, H. abb = *H. abbreviatus*, H. var = *H. variegates*, H. par = *H. parviplexus*, H. med = *H. medioplexus*, H. flo = *H. floedae*, H. bre = *H. breviplexus*, H. lon = *H. longiplexus*, H. asp = *H. asper*, H. sim = *H. similis*, OutG = *Plagiorchis koeanus*, *P. maculaosus*, and *P. vespertilionis*.

	H. col	**H. com**	**H. vario**	**H. abb**	**H. var**	**H. par**	**H. med**	**H. flo**	**H. bre**	**H. lon**	**H. asp**	**H. sim**	**OutG**
H. col	-	0.002	0.007	0.008	0.007	0.009	0.009	0.009	0.009	0.011	0.012	0.011	0.012
H. com	0.4	-	0.007	0.008	0.008	0.009	0.009	0.009	0.009	0.011	0.012	0.011	0.012
H. vario	3.7	3.6	-	0.006	0.005	0.008	0.008	0.007	0.007	0.009	0.010	0.010	0.011
H. abb	5.4	5.5	3.0	-	0.005	0.008	0.008	0.008	0.008	0.009	0.010	0.010	0.012
H. var	4.6	4.8	2.6	2.4	-	0.008	0.008	0.008	0.008	0.009	0.010	0.010	0.012
H. par	6.3	6.2	4.4	5.8	5.0	-	0.002	0.003	0.004	0.009	0.010	0.010	0.011
H. med	6.4	6.6	4.6	6.0	5.2	0.4	-	0.003	0.004	0.009	0.010	0.010	0.011
H. flo	6.2	6.1	4.3	5.6	4.9	0.7	0.8	-	0.004	0.009	0.010	0.010	0.011
H. bre	6.4	6.4	4.0	5.6	4.9	1.1	1.2	0.9	-	0.009	0.010	0.010	0.011
H. lon	10.2	10.1	7.5	8.3	7.6	8.1	8.1	8.2	8.5	-	0.009	0.010	0.012
H. asp	11.1	11.0	8.8	9.4	9.0	8.9	9.0	9.0	8.7	5.2	-	0.010	0.012
H. sim	10.6	10.5	9.1	9.9	9.1	9.1	9.3	9.0	9.4	8.5	9.7	-	0.012
OutG	14.5	14.4	14.0	15.0	14.3	14.3	14.1	14.1	14.2	14.2	14.5	15.6	-

CONCLUSION

> "One must be able to identify the different kinds of organisms encountered and know their habits and habitats; the more one knows about the food and life of these animals…. the more success one is likely to have…..Exceptional physical endurance is an asset for one must follow the maxim…..Above all, one must have command of his or her time for twenty-four hours a day, seven days a week."
>
> Wendell Krull (Letter to Miriam Rothschild, 1953)

THE ROLE OF NON-ODONATE ARTHROPOD SECOND INTERMEDIATE HOSTS

This work indicates that the role of non-odonate arthropod second intermediate hosts is critically important in the recruitment of *H. coloradensis* and *H. complexus* to leopard frogs. The present study shows that non-odonate arthropods are a viable avenue for the transmission of these two species of frog lung flukes. These observations clearly suggest that *H. coloradensis* and *H. complexus* can colonize young of the year leopard frogs more commonly than can other *Haematoloechus* species (*H. longiplexus*, and *H. medioplexus*), which only use odonates as second intermediate hosts.

The main reason that *H. coloradensis* and *H. complexus* are so successful in colonizing leopard frogs are two fold: 1) the large number of species of second intermediate hosts utilized that are represented in the frogs' diet, and 2) the range of sizes

of second intermediate hosts utilized. Comparisons of population structure of adult flukes indicate that the generalist nature of *H. coloradensis* and *H. complexus* metacercariae leads to larger populations of adults of these species in both young of the year northern leopard frogs and adult plains leopard frogs (Chapter One; Chapter Two). Due to this second intermediate host specificity both of these species are more successful in leopard frogs than are their congeners, *H. longiplexus* and *H. medioplexus*.

A similar pattern has been reported for two European species: *H. variegatus* a second intermediate host generalist and its congener *H. abbreviatus* assumed to be a second intermediate host generalist. In a study of helminth parasites of amphibians of the Czech Republic Prokoič and Křivanec (1975) reported that *H. variegatus* was the most common frog lung fluke in the edible frog, *Rana esculenta*, infecting 81 of 305 (26.6%) frogs, whereas *H. similis* and *H. asper*, both species that use damselflies as intermediate hosts, infected 10 (3.3%) and four (1.3%) of these frogs respectively. In a similar study of ranid frogs in Poland Kuc and Sulgostowska (1988) reported *H. varigatus* as the most common lung fluke in *R. esculenta*, 72 of 202 (36 %) frogs, whereas *H. asper* and *H. similis* had a lower prevalence infecting 8 (4 %) and 30 (15 %) frogs respectively. Additionally, Prokoič and Křivanec (1975) showed that *H. abbreviatus* infected 38 of 146 (26%) yellow-bellied toads, *Bombina variegata,* whereas *H. asper* only infected two (1.4%) of these toads. Clearly the trait of adding additional intermediate hosts has led to avenues for greater reproductive success in some European and North American species of *Haematoloechus*.

THE ROLE OF ODONATE METAMORPHOSIS IN THE SURVIVAL OF THE METACERCARIAE

The work from the present study on metacercariae survival during dragonfly metamorphosis indicates that the fate of a metacercaria depends on its location in the odonate host and the ability of that metacercaria to encyst. Experimental infections of dragonflies showed that metacercariae of the two second intermediate host generalists, *H. coloradensis* and *H. complexus*, were located in the head, thorax and branchial basket of these hosts, whereas metacercariae of *H. longiplexus* and *H. parviplexus* were restricted to the branchial basket of dragonflies. Metacercariae of *H. coloradensis*, *H. complexus* and *H. longiplexus* infected the head, thorax and abdomen of damselflies but these insects were resistant to infection with *H. parviplexus*.

Subsequent studies on experimentally infected dragonflies indicated that most unencysted *H. longiplexus* metacercariae were lost from the branchial basket during metamorphosis whereas most encysted metacercariae of the other three species of lung flukes survived dragonfly metamorphosis. Although *H. medioplexus*, a parasite of leopard frogs that has been shown to survive dragonfly metamorphosis, was rarely found in leopard frogs during this study, it has been shown to infect adult northern and plains leopard frogs in Nebraska with prevalence reaching as high as 18% (Krull, 1930; 1931; Brooks, 1976; Snyder, 1996). Taken together, these observations suggest that the observed ecological host specificity of *H. longiplexus* in semi-terrestrial leopard frogs may be due to few metacercariae of *H. longiplexus* reaching these frogs in a terrestrial environment. Because the metacercariae of *H. longiplexus* are usually found free or only

lightly encysted in the branchial basket of dragonflies and are lost during metamorphosis it is unclear what evolutionary advantage if any *H. longiplexus* gains from these unencysted metacercariae. The inability of *H. longiplexus* metacercariae to survive metamorphosis in dragonflies precludes it from infecting semi-terrestrial frogs and therefore decreases this lung flukes' reproductive potential. However, molecular studies show that *H. longiplexus* is most closely related to *H. similis* and *H. asper*, two European species that also have unencysted metacercariae found in damselflies.

Studies on the unencysted metacercariae of *H. asper* and *H. similis* indicate that both of these species have metacercariae that are progenetic (see Odening, 1960; Grabda, 1960). Grabda (1960) found that metacercariae of *H. similis* show precocious sexual maturity in larval damselflies. Within a month of infecting damselflies the unencysted metacercaria had well developed testes, an ovary, a seminal receptacle and a uterus filled with round or oval dark brown bodies. These similar bodies are often found in the uterus of *Haematoloechus* species in frogs and their appearance usually precedes the production of eggs (Grabda, 1960). These observations suggest that the evolutionary history of *H. asper* and *H. similis* has enabled metacercariae of these species to reach adulthood in the second intermediate host as unencysted metacercariae.

Other amphibian plagiorchid trematodes closely related to the Haematoloechidae have been shown to either shorten their life cycles with gravid metacercariae in the second intermediate host or lose the metacercaria stage altogether (see Grabda-Kazubska, 1976). Studies on trematode life cycle evolution suggest that omitting hosts in complex life cycles by developing progenetic metacercariae should be advantageous if that life

cycle can be completed with fewer transmission steps, thus avoiding risks during transfer from one host to another. This omission of hosts can be advantageous if one host is temporarily very rare or if predation rate by the specific down-stream host during the trophic transmission is too low (Poulin and Cribb, 2002). Both *H. asper* and *H. similis* have been reported with low prevalence in European frogs, indicating rare predation rates on damselflies by these relatively small anurans (Prokoič and Křivanec, 1975; Juszczyk, 1987). These observations support the hypothesis that the low predation rate of these anurans may be selecting for the development of progenetic metacercariae in their arthropod intermediate hosts and suggest that the ancestral amphibian host(s) of these flukes may have had similar selective pressures in their evolutionary past.

In North America, the inability of *H. longiplexus* metacercariae to encyst and survive dragonfly host metamorphosis apparently has not precluded this parasite from colonizing its primary definitive host the bullfrog. Field studies indicate that in Nebraska *H. longiplexus* is the most common lung fluke in bullfrogs. Brooks (1976) and Snyder (1996) found over 40% of bullfrogs infected with this trematode, whereas field data from the current study indicates that depending on the bullfrog population *H. longiplexus* infects 24 % to 52 % of these frogs. Bullfrogs are large aquatic frogs that feed on large aquatic prey such as dragonfly larvae (Stewart and Sandison, 1972). These facts taken together suggest that the unencysted metacercaria of *H. longiplexus* may be a relic of selective pressure of the past but does not hinder this parasite from being successful at infecting its definitive bullfrog host. More importantly these data show the importance of understanding the evolutionary history of parasite life histories because these histories impose constraints on current life cycle evolution.

PHYLOGENETIC RELATIONSHIPS AMONG FROG LUNG FLUKES

The phylogenetic hypothesis constructed during this study demonstrated three distinctive evolutionary lineages within the Holarctic *Haematoloechus*. Two of the three clades were comprised of both European and North American species, supporting previous studies that lineages of *Haematoloechus* arose before the breakup of Laurasia and radiated after Eurasia and North America split (Snyder and Tkach, 2001; León-Règagnon and Brooks, 2003). Mapping the first and second intermediate host specificity and amphibian definitive host specificity on the phylogenetic hypothesis revealed that within each of the three evolutionary lineages, members share similar patterns of arthropod host specificity distinct from patterns found in the other lineages.

Results of the current and previous research do not allow for anything but speculative discussion of the evolutionary history of amphibian definitive host utilization because no European frogs were available for experimental infections. However, based on the information presented in this study a case can be made that the lineage represented by species of *Haematolochus* was ancestrally a parasite of amphibians. As both the amphibian lineage and the parasite lineage speciated, certain parasites, for reasons unrelated to phylogeny, were able to parasitize some amphibian species and unable to parasitize others. Although some frog lung fluke species can parasitize a range of related and unrelated anuran hosts, when considering ecological host specificity most individuals of a particular species are predominantly found in a single species of frog in a particular amphibian community, suggesting that such host species may have the biggest selection factor on lung fluke transmission evolution (see Brandt, 1936; Bolek and Coggins 2003).

When considering arthropod second intermediate host specificity, the apparent pattern of damselfly host specificity and unencysted metacercaria in basal members of *Haematoloechus* indicates that parasitism of zygopteran odonates by unencysted metacercariae may have been the ancestral condition within the genus. From those ancestors two other lineages of second intermediate host specificity arose among the Holarctic haematoloechids: a generalist clade composed of North American and European species and a specialist clade comprised of strictly North American species. These observations suggest that two different selective pressures scenarios might account for the two contrasting second intermediate host utilization patterns observed among these lineages.

Among the five European and North American lung fluke species representative of the generalist clade, all are known to infect small to medium size frogs that are gape limited "sit-and-wait" predators. The European species *H. varigatus* and *H. abbreviatus* are common parasites of *Rana esculenta* and *Bombina variegata* respectfully, whereas *H. coloradensis*, *H. complexus*, and *H. varioplexus* infect leopard frogs, leopard frogs and green frogs, and leopard frogs and wood frogs, respectfully. In Europe *Rana esculenta* is a medium size semi-terrestrial anuran with an average SVL of 7.6 cm (range 5.2-10.6) whereas *B. variegata* is a small semi-aquatic species averaging 4.6 cm in SVL (range 2.5-5.6). The diet of *B. variegata* is predominantly represented by small aquatic invertebrates including larval and adult dipterans and small crustaceans make up over 85% of this toads diet, whereas diet of *R. esulenta* has been reported to predominantly consist of small terrestrial invertebrates including dipterans, coleopterans, hymenopterans, annelids, and molluscs, all making up over 80% of this frogs' ingesta (Juszczyk, 1987). In North

American the anuran species that serve as the predominant definitive hosts for *H. coloradensis*, *H. complexus* and *H. varioplexus* range in SVL size from 3.8 cm (range 2.6-5.2) for wood frogs, to 6.2 cm (range 4.4-8.9) for northern leopard frogs, 7.1 cm (range 4.0-10.0) for plains leopard frogs and 7.3 cm (range 5.9-8.8) for green frogs (McAlpine and Diworth, 1989; personal observations). Diet studies on these frogs also show that they feed on predominantly terrestrial prey of appropriate size (Knowlton, 1944; Hamilton, 1948; Whitaker, 1961; Stewart and Sandison, 1972; Werner et al., 1995; Bolek, 1998).

Data on prey size selection by adult green frogs and adult northern leopard frogs indicate that both of these species feed on relatively small organisms. Studies by McAlpine and Dilworth (1989) showed that northern leopard frogs and green frogs selected prey much smaller than the length of adult dragonflies, namely, 13.2 ± 4.2 mm (range 3.0-55.0) in northern leopard frogs and 12.9 ± 5.1 mm (range 3.0-28.0) in green frogs (Needham et al., 2000). McAlpine and Dilworth (1989) indicated that this small prey size selection by these frogs was related to their relatively small gape size, with green frogs having an average head width of 26.5 ± 3.92 (range 20.0-32.1) whereas northern leopard frogs had an average head width of 21.6 ± 4.1 (range 14.7-32.2). Studies on the transmission dynamics of *H. coloradensis* presented in Chapter One clearly indicate that newly metamorphosed northern leopard frogs (3.8 ± 0.7 cm in SVL) recruit *H. coloradensis* predominantly by feeding on small (6.8 mm; range 0.5-18) non-odonate arthropod hosts. A sub-sample (N=30) of these frogs indicates that they had an average head width of 13.0 ± 2.0 mm (range 10.0-17.0). Although such ecological data are lacking for the North American *H. complexus* and *H. varioplexus* these two species

predominantly infect small or medium size frogs. Both adult plains leopard frogs and adult wood frogs that commonly serve as hosts for *H. complexus* and *H. varioplexus* respectfully, have relatively small head widths, namely, 15.5 $\pm$ 1.1 (range 14-17; N = 25) for wood frogs and 20.5 $\pm$ 4.1 mm (range 12-26; N = 25) for plains leopard frogs, indicating that they predominantly feed on invertebrates smaller than adult dragonflies (Figure 1). These observations suggest that small gape size of some frogs may have been an important selective pressure for the generalist pattern of small arthropods as second intermediate hosts.

Among the four strictly North American representatives of the dragonfly second intermediate host specialist clade all but one, *H. medioplexus,* predominantly infect bullfrogs. Because *H. medioplexus* and *H. parviplexus* are sister species readily distinguished on morphological grounds but differing in their ITS sequences by only four bases over a gapped sequence length of 851 bases, this observation suggests a recent evolutionary host switch from bullfrogs to leopard frogs. Bullfrogs differ from most other North American anuran species being strictly aquatic and also being the largest frog species in North America, commonly reaching a SVL of 180-200 mm (Werner, et al., 1995). These observations suggest that this clade of second intermediate host specialists arose in bullfrogs or their close recent ancestor.

Studies on the diet of bullfrogs indicate that unlike most other North American and European anurans, these frogs predominantly feed on large aquatic prey (McAlpine and Dilworth, 1989; Werner, et al., 1995). McAlpine and Dilworth (1989) showed that this feeding behavior was related to the bullfrogs' aquatic habitat and a large gape size.

Their data indicate that the head width of adult bullfrogs (40.0 $\pm$ 9.3, with a range of 16.6-52.3) was significantly larger than that of adult northern leopard frogs and adult green frogs. More importantly, McAlpine and Dilworth (1989) also indicated that as a result of a larger gape size, adult bullfrogs fed on significantly larger prey than adult green frogs or adult northern leopard frogs. In their study bullfrogs fed on organisms ranging in size from 3.0-102.2 mm with an average length of 29.3 $\pm$ 16.1 mm (Figure 1).

Dietary studies on bullfrogs indicate that they commonly prey on aquatic larval odonates (Stewart and Sandison, 1972; Dronen, 1977). Importantly dragonfly larvae in their ultimate instar range in body size from 18 to 42 mm, a size that falls within one standard deviation of the average prey size selected by bullfrogs (McAlpine and Dilworth, 1989; Bouchard, 2004). Studies on the life history of odonates indicate that dragonflies are most susceptible to mortality by predators during metamorphosis of this ultimate larva stage (Corbet, 1999). Stomach contents data from bullfrogs in central Missouri farm ponds indicates that odonate larvae during their period of emergence were the second most important food item in the diet of bullfrogs (Korschgen and Moyle, 1955). Preliminary data from Nevens Pond on 20 adult bullfrogs collected on a single night suggests that larval and teneral dragonflies and damselflies can make up 8.3 % of their diet on a given night with all dragonflies recovered being in the ultimate larval stage (personal observations) suggesting that bullfrogs commonly prey on odonate larvae as they emerge to transform. Taken together these observations suggest the gape size and an aquatic habitat may have played an important role as a selective pressure for the evolution of the specialist clade of strictly North American species of *Haematoloechus*. The specialist arthropod host utilization has arisen only in North America, probably after

the break up of Laurasia, although a more extensive sampling of Palearctic *Haematoloechus* may well reveal Eurasion members of this clade and must be done to test the hypothesis that gape size of large aquatic anurans may have selected for this second intermediate host specificity.

The present study is a unique comparative examination of life cycle transmission events among closely related parasite species. The results of this study reveal new information about transmission events and evolutionary relationships among a genus of trematodes. To more precisely determine the evolution of life cycle strategies and the evolutionary nature of host specificity within the family Haematoloechidae, specimens must be collected and experimental host specificity studies conducted on all continents. Such an effort may reveal much about the biogeographical patterns of both parasite and host and provide further insight into the origins of parasitism in amphibians.

LITERATURE CITED

Bolek, M. G. 1998. A seasonal and comparative study of helminth parasites in nine Wisconsin amphibians. M.S. Thesis, University of Wisconsin-Milwaukee, 134 p.

Bolek, M. G., and J. R. Coggins. 2003. Helminth community structure of sympatric eastern American toad, *Bufo a. americanus*, northern leopard frog, *Rana pipiens*, and blue-spotted salamander, *Ambystoma laterale*, from southeastern Wisconsin. Journal of Parasitology **89**: 673-680.

Bouchard, R. W., Jr. 2004. Guide to aquatic macroinvertebrates of the upper Midwest. Water Resources Center, University of Minnesota, St. Paul, Minnesota, 208 p.

Brandt, B. B. 1936. Parasites of certain North Carolina Salientia. Ecological Monographs **6**: 491-532.

Brooks, D. R. 1976. Parasites of amphibians of the Great Plains: Part 2. Platyhelminths of amphibians in Nebraska. Bulletin of the University of Nebraska State Museum **10**: 65-92.

Corbet, P. S. 1999. Dragonflies, behavior and ecology of odonata. Cornell University Press, Ithaca, New York, 829 p.

Dronen, N. O. Jr. 1977. Studies on the population structure of two species of *Haematoloechus* Looss, 1899 (Digenea: Plagiorchiidae) in raniid frogs in New Mexico. Proceedings of the Helminthological Society of Washington **44**: 68-72.

Grabda, B. 1960. Life cycle of *Haematoloechus similis* (Looss, 1899) (Trematoda: Plagiorchiidae). Acta Parasitologica Polonica **8**: 357-367.

Grabda-Kazubska, B. 1976. Abbreviation of the life cycles on plagiorchid trematodes. General remarks. Acta Parasitological Polonica **24**: 125-141.

Juszczyk, W. 1987. Płazy i gady krajowe część 2 płazy-amphibia, 2nd ed. PWN, Warszawa, 384 p.

Kennedy, M. J. 1981. A revision of the genus *Haematoloechus* Looss, 1899 (Trematoda: Haematoloechidae) from Canada and the United States. Canadian Journal of Zoology **59**: 1836-1846.

Knowlton, G. F. 1944. Some insect food of *Rana pipiens*. Copeia **1944**: 119.

Korschgen, L, J. and D. L. Moyle. 1955. Food habits of the bullfrog in central Missouri farm ponds. American Midland Naturalist **54**: 332-341.

Krull, W. H. 1930. The life history of two North American frog lung flukes. Journal of Parasitology **16**: 207-212.

Krull, W. H. 1931. Life history studies on two frog lung flukes, *Pneumonoeces medioplexus* and *Pneumobites parviplexus*. Transactions of the American Microscopical Society **50**: 215-277.

Kuc, I., and T. Sulgostowska. 1988. Helminth fauna of frogs in the forest of Kampinos near Warszawa. Acta Parasitologica Polonica **33**: 267-272.

León-Règagnon, V., and D. R. Brooks. 2003: Molecular phylogeny of *Haematoloechus* Looss, 1899 (Digenea: Plagiorchiidae), with emphasis on North American species. Journal of Parasitology **89**: 1206–1211.

McAlpine, D. F., and T. G. Dilworth. 1989. Microhabitat and prey size among three species of *Rana* (Anura: Ranidae) sympatric in eastern Canada. Canadian Journal of Zoology **67**: 2,244-2,252.

Needham, J. G., M. J. Westfall, Jr., and M. L. May. 2000. Dragonflies of North America. Revised edition. Scientific publishers, Gainesville, Florida, 939 p.

Odening, K. 1960. Plagiorchiidae III. (Haematoloechinae) und Omphalometridae. Das Tierreich **75**: 1-75.

Prokoič J,, and K. Křivanec. 1975. Helminths of amphibians, their interaction and host-parasite relationships. Acta Scientiarum Naturalium Academiae Scientiarum Bohemoslovacae Brno **9**: 1-48.

Poulin, R., and T. H. Cribb. 2002. Trematode life cycles: short is sweet? Trends in Parasitology **18**: 176-183.

Snyder, S. D. 1996. Host specificity among species of *Haematoloechus* (Digenea: Haematoloechidae). Unpublished Ph.D. Thesis. University of Nebraska-Lincoln, 112 p.

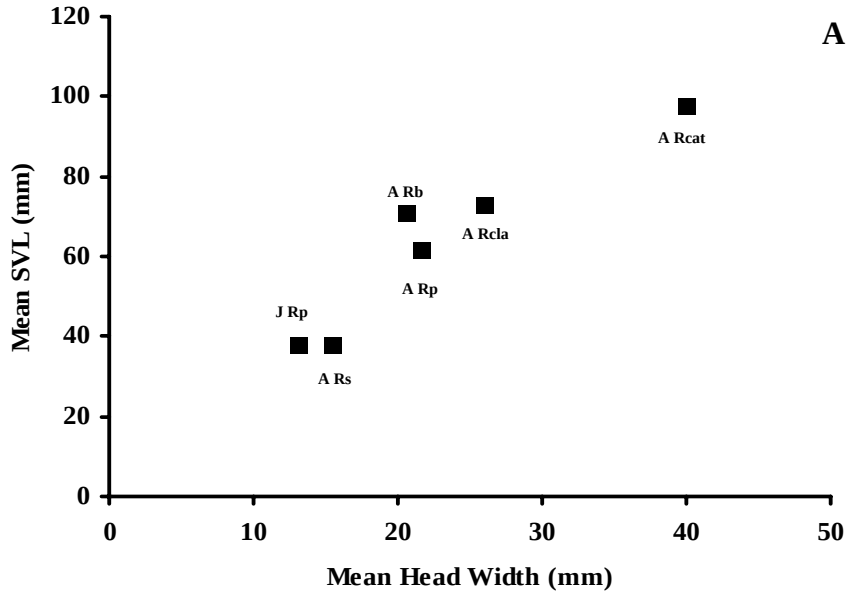
Snyder, S. D., and V. V. Tkach. 2001. Phylogenetic and biogeographical relationships among some Holarctic frog lung flukes (Digenea: Haematoloechidae). Journal of Parasitology **87**: 1433-1440.

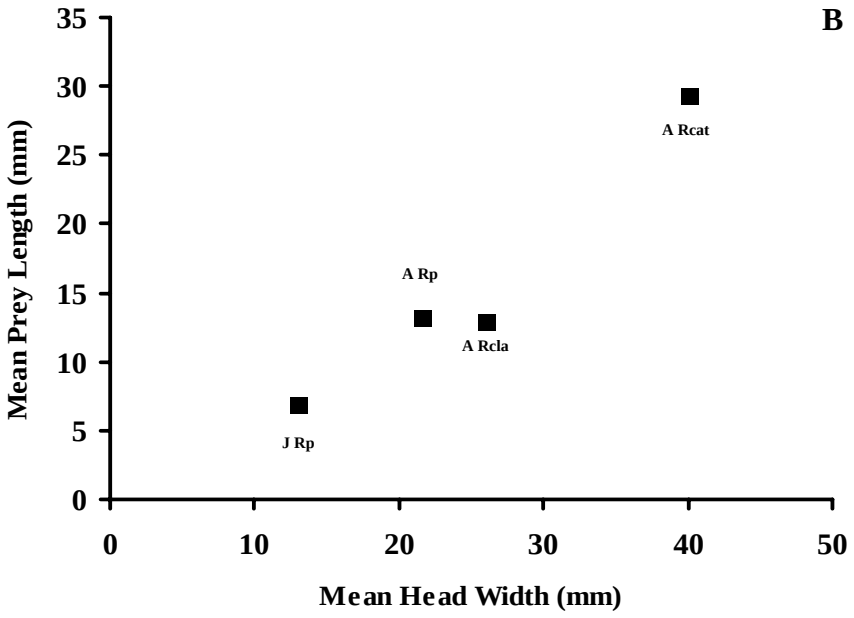
Stewart, M. M., and P. Sandison. 1972. Comparative food habits of sympatric mink frogs, bullfrogs and green frogs. Journal of Herpetology **6**: 241-244.

Werner, E. E., G. A. Wellborn, and M. A. McPeek. 1995. Diet composition in postmetamorphic bullfrogs and green frogs: implications for interspecific predation and competition. Journal of Herpetology **29**: 600-607.

Whitaker, J. O. Jr. 1961. Habitat and food of mousetrapped young *Rana pipiens* and *Rana clamitans*. Herpetologica **17**: 173-179.

Figure 1. Scatter plots of mean head width versus mean snout vent length (SVL) and mean prey length of five species of North American true frogs. A. Mean head width versus mean snout vent length of six populations of North American *Rana*, $r = 0.95$, df = 4, $P < 0.01$. B. Mean head width versus mean prey length of four populations of North American *Rana*, $r = 0.94$, df = 2, $P > 0.05$. A Rb = adult plains leopard frogs, *Rana blairi*, from Pawnee Lake, Lancaster County, Nebraska, A Rcat = adult bullfrogs, *Rana catesbeiana*, from New Burnswick, Canada, A Rcla = adult green frogs, *Rana clamitans*, from New Burnswick, Canada, A Rp = adult northern leopard frogs, *Rana pipiens*, from New Burnswick, Canada, A Rs = adult wood frogs, *Rana sylvatica*, from the University of Wisconsin-Milwaukee Field Station, Ozaukee County, Wisconsin, J Rp = juvenile northern leopard frogs, *Rana pipiens*, from Cedar Creek, Keith, County, Nebraska. Data for A Rcat, A Rcla and A Rp from McAlpine and Dilworth (1989).


A
Mean SVL (mm)
120
100
80
60
40
20
0
A Rcat
A Rb
A Rcla
A Rp
J Rp
A Rs
0
10
20
30
40
50
Mean Head Width (mm)


B
Mean Prey Length (mm)
35
30
25
20
15
10
5
0
A Rcat
A Rp
A Rcla
J Rp
0
10
20
30
40
50
Mean Head Width (mm)