CONSEQUENCES OF VARIATION IN HOST SPECIES USED TO COMPLETE COMPLEX LIFE CYCLES OF HELMINTHS: AN EXPERIMENTAL STUDY ON LIFE CYCLES OF *HALIPEGUS* SPECIES (DIGENEA: HEMIURIDAE) FROM NORTH AMERICA

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"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"

—Dr. Wendell Krull (Ewing, 2001)

First of all, I need to acknowledge that this dissertation, and all of my accomplishments thus far, would not have been possible without the help of many colleagues, friends, and family. The course of my academic career has largely resulted from a series of serendipitous and fortunate encounters with several important people that have changed the trajectory of my life. Through these interactions, the quality of my life has become better, and for that I am forever grateful.

I begin this section of my dissertation with a quote from one of my scientific role models, Dr. Wendell Krull, who actually discovered the life cycles of many helminths including that of *Halipegus occidualis*. While I fully agree with Dr. Krull's statement that most biologists do not truly understand the amount of tedious and repetitious work required to complete complex life cycles of parasites in the laboratory, I consider myself extremely lucky to have fallen in love with one of the few people that can both understand and appreciate the dedication required to conduct this type of research. At this time, I thank my husband, Joshua Stigge, for continuously

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Abstract: Complex life cycles, in which discrete life history stages of a parasite are transmitted sequentially between different host species, are shared by many parasites and have evolved independently in several phylogenetically distinct parasitic groups including protozoans, acanthocephalans, cestodes, nematodes, and trematodes. The multiple origins of complex life cycles have resulted in an astounding diversity in patterns of host usage among complex life cycles of helminths. Unfortunately, parasitologists have made generalizations about the life cycles of groups of helminths from the described life cycles of only one or few parasite species from within that group; therefore, much of the variation that likely exists in life cycles of different helminth species is lost, and life cycles are depicted to be invariable within and among species of parasites. At every stage of a life cycle, the parasite has a chance to infect numerous potential host species, but not all potential hosts are equally suited for the development and transmission of the parasite. The objectives of this dissertation were to experimentally examine the extent to which the variation in host usage influences life history traits of trematodes. Specifically, I was interested in evaluating how host usage influences transmission dynamics of parasites and their ability to complete life cycles. In chapter II, I discuss the host specificity of *H. eccentricus* and *H. occidualis* in 4 microcrustacean second intermediate hosts. I also evaluated the development of metacercariae within each host and estimated their contribution to the completion of the life cycles. My primary objective for the study presented in Chapter III was to experimentally evaluate the effects of using a paratenic hosts on life history traits of parasites within their subsequent host. In chapter IV, I present data that suggests that site fidelity of *H. occidualis* is more variable than previously described, and that the site occupied by these worms is dependent on the host species infected. Data presented in this dissertation provide the groundwork for future hypothesis-driven studies on the evolution complex life cycles of parasites.

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CHAPTER I

OVERVIEW

"Life cycle investigation is somewhat simpler now than it was twenty years ago because enough cycles have been completed in many of the groups to indicate what a related unknown cycle may be like; 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."

—Dr. Wendell Krull (Ewing, 2001)

Complex life cycles, in which discrete life history stages of a parasite are transmitted sequentially between different host species, are shared by many parasites (Olsen, 1986) and have evolved independently in several phylogenetically distinct parasitic groups including protozoans, acanthocephalans, cestodes, nematodes, and trematodes (Mackiewicz, 1988; Rohde, 1994; Combes, 2001; Poulin, 2007). The multiple origins of complex life cycles have resulted in an astounding diversity in patterns of host usage among complex life cycles of helminths (Cribb et al., 2003). For example, in the case of digenetic trematodes, there are several examples of species that diverge from the most common type of life cycle involving 3 hosts by adding a fourth host to the life cycle, losing 1 of the hosts, or having a variable life cycle involving 1 to 3 hosts (Olsen, 1986; Shoop, 1988; Barger and Esch, 2000; Poulin and Cribb, 2002; Poulin, 2007; Bolek et al. 2009). Additionally, it has been suggested that hosts have been added and/or lost on

multiple occasions over the evolutionary histories of numerous parasite species (Combes, 2001). Therefore, the number and type of host used by a helminth to complete its life cycle is likely to be dynamic over time, and in the case of parasites with trophic transmission, life cycles should evolve to reflect trophic relationships between hosts. Accordingly, when food chains are stable, life cycles should remain stable; however, when the interactions between hosts change, life cycles could evolve by adjusting the number and types of hosts required for completion (Lafferty et al., 2008). Therefore, life cycles of parasites are dynamic over time, and hence, the invariable representations published in most studies and textbooks are unrealistic.

This portrayal of life cycles as fixed and invariable units is a major flaw because our knowledge on life histories of parasites is the foundation for concepts in parasite community and population ecology, life cycle evolution, and the epidemiology of diseases, and therefore, understanding the variability of host usage to complete life cycles is crucial. Despite the importance of this work, few parasitologists focus on life cycles of parasites as the center of their research. Furthermore, unfortunately, most parasitologists that have studied life cycles of parasites only did so until the life cycle could be completed, and once a solution of suitable host combinations were found, most investigators did not continue to search for other hosts through which the life cycle may be completed in nature. Furthermore, these published life cycles tend to be accepted as absolute truth, and their validity is rarely questioned (Bolek et al., 2009; Bolek et al., 2010).

Possibly even more problematic, parasitologists have made generalizations about the life cycles of groups of helminths from the described life cycles of only one or few parasite species from within that group; therefore, much of the variation that likely exists in life cycles of different helminth species is lost. As a result, the common perception of parasite life cycles is that of rigid iron wheels with defined parameters and little or no room for variability (Bolek et al., 2015). In this way, one can view a parasite life cycle as a puzzle with a definitive solution and only one

way to connect the pieces; in all cases, you start with an egg and end with an adult. However, life cycles of helminths are not as simple as they appear in typical textbook diagrams because in most cases the variability in life cycles, especially among closely related species, has been largely overlooked or simply ignored.

This is unfortunate because the variability in life cycles, including the host usage, is important for transmission dynamics and persistence of a parasite in the environment. Additionally, this variability certainly may play an important role in the evolution of life cycles and divergence of parasite species. When the parasite species infects a novel host species, it undoubtedly experiences different environmental conditions including new selective pressures and access to different resources. Therefore, the parasite could adapt to the new conditions in different hosts over evolutionary time. Theoretically, these adaptations could alter life histories of the parasite in subsequent hosts and lead to evolutionary divergence in a parasite species.

At every stage of a life cycle, the parasite has a chance to infect numerous potential host species, but not all potential hosts are equally suited for the development and transmission of the parasite. Obviously, some hosts will contribute to the completion of the life cycle frequently because they are consumed more often by subsequent hosts in the life cycle. However, these trophic interactions between host species do not necessarily ensure the completion of the life cycle. The parasite's development within those hosts is also important for its success. Variation in the development of worms among host species could cause differences in their ability to infect subsequent hosts and other life history traits within them. Previous studies have not thoroughly examined the extent to which the development of a parasite species differs in multiple host species and if those developmental differences influence the transmission and life history traits of parasites in subsequent hosts. The objectives of this dissertation were to experimentally examine the extent to which the variation in host usage influences life history traits of trematodes. Specifically, I was interested in evaluating how host usage influences transmission dynamics of

parasites and their ability to complete life cycles. Data presented in this dissertation provide the groundwork for future hypothesis-driven studies on the evolution complex life cycles of parasites.

Host-Parasite System

Trematodes are a good system to experimentally examine the extent to which host usage influences variation in development and transmission because trematode life cycles are complex, involving up to 4 hosts (Shoop, 1988), hence, they provide the opportunity to evaluate differences in parasite development within several distinct host species at multiple host levels within the life cycle. Furthermore, we can assess the extent to which small differences in development and transmission at each host level can collectively affect a parasite's overall ability to successfully complete its life cycle. Lastly, and possibly most importantly, the life cycles of some trematode species have been completed entirely in the laboratory, and therefore, the host usage by these species can be experimentally manipulated, and the consequences of these manipulations can be examined under controlled laboratory conditions.

Halipegus eccentricus* and *Halipegus occidualis

The genus *Halipegus* Looss, 1899 has a worldwide distribution and consists of 22 valid species that predominantly infect amphibian definitive hosts (Table I: Gibson and Bray, 1979; Prudhoe and Bray, 1982). Adults of *Halipegus* species reside in the stomach or various locations in the buccal cavity of their amphibian definitive host, including under the tongue or the eustachian tube cavities. Although most *Halipegus* species are morphologically indistinguishable, data suggest that species of *Halipegus* exhibit incredible site fidelity in their anuran hosts (Table I) which has been used previously for species identifications (Goater et al., 1989; Zelmer and Esch, 1999; Bolek et al., 2010). The life cycles of *Halipegus* species that have been described are typical of many trematodes in that they involve obligatory molluscan, arthropod, and vertebrate hosts. However, a number of *Halipegus* species add a fourth host to the life cycle (Fig. 1). Unfortunately, information on the complete life cycle is available for only 3 species, including 2

North American and 1 European species (Kechemir, 1978; Zelmer and Esch, 1998; Bolek et al., 2010); however, the life cycles of these *Halipegus* species demonstrate remarkable similarity. All 3 species use snails as first intermediate hosts, microcrustaceans as second intermediate hosts, amphibians as definitive hosts, and odonates as the forth host in the life cycle (Fig. 1).

The 3 known life cycles of *Halipegus* species begin as embryonated eggs that are swallowed by a definitive host and voided into the free environment within the host's feces. If deposited in water, snail first intermediate hosts become infected by ingesting eggs from the environment. After ingestion, the first larval stage hatches from the egg and penetrates the gut of the snail and migrates to its hepatopancreas and gonads where the trematode undergoes several generations of asexual reproduction and eventually produces hundreds of cercariae. Non-motile cystophorous cercariae are released from the snail host into the aquatic environment where they are ingested by various species of microcrustacean second intermediate hosts including copepods and ostracods (Krull, 1935; Thomas, 1939; Macy and DeMott, 1957; Macy et al., 1960; Zelmer and Esch, 1998; Bolek et al., 2010). Worms develop into the metacercaria stage within the microcrustacean host (Zelmer and Esch, 1998). Odonate hosts, which are optional hosts for the North American *Halipegus eccentricus* and *Halipegus occidualis* but are physiologically required for the European *Halipegus ovocaudatus*, become infected when they consume metacercariae within an infected microcrustacean. Lastly, amphibian definitive hosts can become infected when they consume an infected odonate host and/or directly from an infected microcrustacean. Within the amphibian host, metacercariae of *Halipegus* species remain in the stomach where they grow and develop for several weeks. After some time, the individuals of some species migrate to their appropriate location within the buccal cavity of the frog definitive host where they reach sexual maturity (Kechemir, 1978; Zelmer and Esch, 1998; Bolek et al., 2010).

For a number of reasons, *Halipegus* species are a unique system to examine the variation in life history traits that result from using different hosts to complete the life cycle. First, the life

cycles of the 2 North American *Halipegus* species can be complete and experimentally manipulated in the laboratory (Zelmer and Esch, 1998; Bolek et al., 2010). Secondly, previous work suggests that *Halipegus* species are capable of infecting multiple microcrustacean second intermediate hosts (Krull, 1935; Thomas, 1939; Macy and DeMott, 1957; Macy et al., 1960; Zelmer and Esch, 1998; Bolek et al., 2010) and amphibian definitive hosts (Bolek et al., 2010), and therefore, we can examine the development and transmission of worms from different host species at multiple levels within the life cycle. Furthermore, we can assess how each host contributes to the overall transmission of the parasites under controlled laboratory conditions to determine if all hosts should be considered equally suitable when describing host specificity. Third, *Halipegus* species are one of the few helminths that use paratenic hosts and have a domesticated life cycle that can be completed in the laboratory. Therefore, we can experimentally evaluate the changes in life histories that result from the addition of a host to a life cycle by comparing the life history traits of individuals of the same species that either use paratenic hosts or only intermediate hosts to infect subsequent hosts. Lastly, although *Halipegus* species were previously assumed to have strongly conserved site fidelity in their definitive hosts (Goater, 1989), it appears that the definitive host species may influence behaviors required for site selection by some *Halipegus* species. However, very few previous studies have examined the effect of host species on site-finding behaviors of parasites (Bolek et al., 2010).

The following 3 chapters in this dissertation discuss the extent to which multiple host species influence life history traits of *Halipegus* species. In chapter II, I discuss the host specificity of *H. eccentricus* and *H. occidualis* in 4 microcrustacean second intermediate hosts. I also evaluated the development of metacercariae within each host and estimated their contribution to the completion of the life cycles. In this chapter, I experimentally exposed 4 groups of microcrustaceans with 2 *Halipegus* species to test their host specificity at the second intermediate host level. Furthermore, I assessed the extent to which each of these 4 hosts contributed to

transmission by recording the rates at which each species consumed cercariae, how often exposed individuals became infected, and development of metacercariae within each host species. Then, I determined how often infected individuals of each species were eaten by the next host in the life cycle and the rates of establishment of metacercariae from each microcrustacean species in the odonates. This work suggests that there is variation in the suitability of the 4 intermediate hosts, but no significant differences in host usage between the 2 species of *Halipegus*.

My primary objective for the study presented in Chapter III was to experimentally evaluate the effects of using a paratenic hosts on life history traits of parasites within their subsequent host. Paratenic hosts have always been described as an optional host that is necessary for the transmission of the parasite but not for physiological development of the parasites. However, their role in the life cycle of parasites, other than bridging ecological or trophic gaps between obligate hosts, has largely has been ignored (Zelmer and Esch, 1998). Present-day paratenic and intermediate hosts are good comparative model systems to experimentally evaluate the extent to which a parasite's life history is altered from the addition of a new host to their life cycle. This is the first study to use experimental infections of both intermediate and paratenic hosts to evaluate the contribution of paratenic hosts to the life cycles of parasite. First, I use a comparative approach to determine any differences in the development of metacercariae of *H. eccentricus* within intermediate hosts and paratenic hosts. Next, I evaluate how life history traits of *H. eccentricus* within the definitive hosts differ between metacercariae of the same age that developed within intermediate or paratenic hosts. The major contribution of this study was that it is the first examination of the role of paratenic hosts in parasite life cycles using experimental infections and appropriate time-control groups to determine the extent to which the use of a paratenic hosts affects establishment, survival, and life history traits.

In chapter IV, I present data that suggests that site fidelity of *H. occidualis* is more variable than previously described, and that the site occupied by these worms is dependent on the

host species infected. Previous studies by Goater et al. (1989) indicate that *H. eccentricus* and *H. occidualis* always demonstrate strong site specificity in their definitive hosts. *Halipegus eccentricus* has been reported from only the eustachian tubes of both naturally (Brooks, 1976; Wetzel and Esch, 1996; Bolek and Coggins, 2001) and experimentally infected hosts (Bolek et al., 2010). In contrast, the site specificity of *H. occidualis* appears to be variable because it has been reported from under the tongue of green frogs and from the stomach of other anuran hosts including bullfrogs (Bouchard, 1951; Macy et al., 1960; Andrews et al, 1992; Wetzel and Esch, 1996; McAlpine and Burt, 1998; Schotthoefer et al., 2009; Mata-Lopez et al., 2010). However, these previous studies were field surveys, and none of them attempted to infect the different host species with *H. occidualis* to determine if host species influenced the site selected by the adult worms or if the worms in separate habitats actually were different *Halipegus* species. This was the first study to experimentally examine the site fidelity of *H. occidualis* in multiple anuran species.

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Table 1. The geographic distribution, habitat, and odonate host relationship of valid amphibian *Halipegus* species reported by Rankin (1944) or described thereafter (Gibson and Bray, 1979; Prudhoe and Bray, 1982). A ? represents information that is not known.

Species	Geographic Distribution	Site Fidelity Within Anuran	Odonate Relationship	Citation
H. africanus	Africa	?	?	Dollfus, 1950
H. alhaussani	Middle East	Stomach	?	Saoud and Roshdy, 1970
H.dubius	S. America	Under Tongue	?	Paraense, 1992
H. eccentricus	N. America	Eustachian Tubes	Paratenic	Thomas, 1939; Bolek et al. 2010
H. eschi	Central America	Esophagus	?	Zelmer and Brooks, 2000
H. genarchella	S. America	Buccal Cavity	?	Kohn and Fernandes, 1988
H. insularis	Madagascar	Under Tongue	?	Capron et al., 1961
H. japonicas	Asia	Under Tongue	?	Yamaguti, 1936
H. kessleri	India	Under Tongue	?	Grebnitzky, 1872
H. longispina	India	Under Tongue	?	Klein, 1905
H. mehransis	Asia	Stomach	Progenetic	Srivastava 1933; Nath and Pande, 1970
H. muradabadensis	India	Instestines	?	Chakrabarti, 2012
H. occidualis	N. America	UnderTongue/ Stomach	Paratenic	Krull, 1935; Goater, 1989; Zelmer and Esch, 1998
H. ovocaudata	Europe	Under Tongue	Intermediate	Kechemir, 1978
H. parva	S. America	Buccal Cavity	?	Kohn and Fernandes, 1988
H. psilonotae	Mexico	Under Tongue	?	Leon-Regagnon and Romero-Mayen, 2013
H. phrynobatrachi	Madagascar	Stomach	?	Maeder, 1969
H. rhodesiensis	United States	Stomach	?	Beverley-Burton, 1963
H. spindalis	India	?	?	Srivastava, 1933
H. tafonensis	Republic of Sudan	?	?	Pike, 1979
H. udairpurensis	India	?	?	Gupta and Agrawal, 1967
H. zweifeli	Papua New Guinea	Intestine	?	Moravec and Sey, 1989

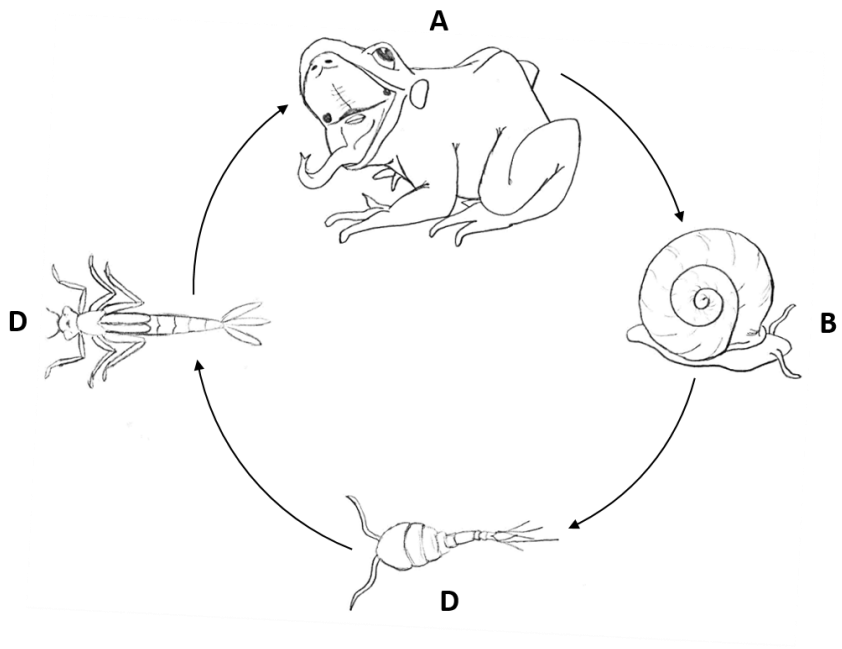

FIGURE 1. The life cycle for *Halipegus eccentricus* and *Halipegus occidualis*, as it is known to occur in nature. Adult worms occur either in the eustachian tubes or under the tongue of amphibian definitive hosts **(A)**, and eggs are released into the aquatic environment with the amphibian's feces. Snails first intermediate hosts **(B)** become infected when they consume *Halipegus* eggs. After asexual reproduction of the trematodes occurs within snail, the next infective stage, cercariae, is released into the environment. Then, microcrustacean second intermediate hosts **(C)** become infected when they consume a cercaria, and the trematodes develop into the metacercaria stage. Odonates larvae **(D)** serve as paratenic hosts for both of these *Halipegus* species. Odonates become infected when they consume infected microcrustacean. The life cycle is completed when an infected odonate is consumed by an anuran and metacercariae are transferred into it. However, in the laboratory, anurans can be directly infected by consuming infected microcrustaceans.

CHAPTER II

EVALUATING THE BIOLOGICAL AND ECOLOGICAL FACTORS INFLUENCING TRANSMISSION OF LARVAL DIGENETIC TREMATODES: A TEST OF SECOND INTERMEDIATE HOST SPECIFICITY OF TWO *HALIPEGUS* SPECIES IN NORTH AMERICA

ABSTRACT: Host specificity of parasites is a basic principal in parasitology; however, it is not easily measured. Previously, host specificity was calculated as the number of species that a parasite infected, but this is not an accurate description of host usage because some species are capable of being infected but do not contribute to the completion of the life cycle. Instead, measures of host specificity should take into consideration interactions between a parasite and a potential host species as well as interactions between current and subsequent hosts in the life cycle. The objectives of this study were to track the development of 2 trematodes species, *Halipegus eccentricus* and *Halipegus occidualis*, in 3 phylogenetically distinct microcrustacean second intermediate hosts, and then, evaluate the extent to which each of these hosts contributed to transmission of each *Halipegus* species to the next odonate host in the life cycle. All 3 microcrustacean species exposed became infected with both species of *Halipegus*. The patterns of growth of *H. eccentricus* and *H. occidualis* were similar, but there were consistent differences in the rates of growth among the microcrustacean species in both *Halipegus* species. Regardless of host species infected, all individuals of both species were considered to be developmentally infective to the next host in the life cycle by 19 days post exposure (DPE) when they lost their

excretory bladder. Worms of varying sizes were capable of surviving without this structure suggesting that there is not a strong relationship between the rate of growth of the metacercariae and the development of their osmoregulatory system. Although *Halipegus* species were capable of living without an excretory bladder at 19 DPE, there were differences in the rates in which the 3 microcrustaceans contributed to transmission of the parasites to subsequent odonate hosts. Collectively, under controlled laboratory conditions, individuals that used the ostracod *Cypridopsis* sp. were more successful at completing their life cycle than those from either of the 2 copepod species. Therefore, despite all 3 microcrustacean species becoming infected, not all species were equally suited for transmission and completion of the life cycle.

INTRODUCTION

Host specificity or the extent to which a parasite can infect multiple host species is a fundamental concept of parasitology (Adamson and Caira, 1994; Poulin, 1998). The specificity that parasites demonstrate for their hosts not only varies greatly among parasite species, but host specificity can also drastically differ between different life history stages of a single parasite species (Poulin and Keeney, 2007). Despite such variation, parasitologists have traditionally assumed that most helminths are highly host specific because those parasites have been reported from only a single or few host species in nature. However, recent studies suggest that the host ranges of parasites are grossly underestimated based on field surveys alone (Poulin and Keeney, 2007). Unfortunately, relatively few studies have examined host specificity of helminths experimentally, and therefore, parasitologists have relied on these field surveys to draw conclusions about host ranges.

This is unfortunate because understanding host specificity of helminths is critical for evaluating transmission dynamics within natural systems (Bush and Kennedy, 1994; Poulin, 1998). Typically, host specificity has been reported as the number of host species infected (Lymbery, 1989); however, recently it has been stressed that host specificity is more complex

than this because not all host species have an equal probability of transmitting the parasite to the next host in the life cycle (Poulin and Mouillot, 2005). For example, some host species would not play a role in transmission if the parasite fails to develop to an infective stage in that host or if the parasite is not transmitted to subsequent host due to ecological or trophic gaps in the life cycle. Therefore, host specificity should not be based solely on whether a potential host species can be infected.

Instead, measures of host specificity should take into consideration the physiological and ecological interactions between the parasite and a potential host species as well as interactions between current and subsequent hosts in the life cycle. Fortunately, recent work has attempted to account for some of these interactions by weighting potential hosts by prevalence of the parasite within each host (Rohde 1980, 1993; Poulin and Mouillot, 2005), however, this does not take into consideration the trophic relationships or transmission of the parasite between host species. Although a high prevalence indicates that the parasite is capable of infecting a host species and that there must be considerable ecological overlap between it and the parasite, prevalence does not indicate whether parasites within that host species will successfully infect the next host in the life cycle. For example, some hosts could be infected frequently, hence having a high prevalence, but that species should be considered a poor host if transmission fails due to ecological gaps between hosts or if the parasite fails to develop within that host. To be considered a suitable host, it needs to assist in the completion of the life cycle.

Put simply, there are a number of events that must occur for a host species to contribute to the completion of the life cycle. First, a potential host species must come into contact with the propagules of the parasite, and it must be able to be infected by the propagules. Second, the parasites must be able to develop to become infective to the next host in the life cycle. Finally, the potential host species and the next host in the life cycle must interact so that the parasite is transmitted and survives in the next host. Unfortunately, none of the previous studies have

evaluated the quality of a host in terms of the development of a parasite and the frequency in which those hosts transmit parasites to the next host in the life cycle.

The objectives of this study were to determine if 2 trematodes species, *Halipegus eccentricus* and *Halipegus occidualis*, were able to infect 3 phylogenetically distinct second intermediate hosts. Then, the development of both *Halipegus* species was documented over 61 days to determine how worms developed within each of these 3 hosts. Lastly, the extent to which each of these hosts contributed to transmission of each *Halipegus* species to the next odonate paratenic hosts was evaluated. A major contribution of this paper is that it approached host specificity from physiological and ecological aspects including factors that influence transmission between the 2 trematode species and their intermediate hosts as well as the interactions between each of the 3 intermediate hosts and the subsequent paratenic host. This study documented the growth and development of *Halipegus* species in their second intermediate hosts to determine if all host species are equally suitable for development. Most of the previous studies on trematode host specificity have focused almost exclusively on development in the molluscan first intermediate or definitive hosts. However, relatively little is known on the development and host specificity of trematode metacercariae in invertebrate second intermediate hosts *(*Snyder and Janovy, 1996; Bolek and Janovy 2007). Secondly, this is the first study to experimentally evaluate host specificity in terms of the contributions of possible host species to transmission of the parasite to subsequent hosts including how often the host is eaten and the rate of transmission of the parasite between hosts.

Trematodes in the genus *Halipegus* were chosen because the life cycle of both of these species have been maintained in the laboratory at Oklahoma State University (see Appendix A), and previous work suggests that each species of *Halipegus* are capable of infecting phylogenetically distinct microcrustacean second intermediate hosts, including ostracods and copepods (Krull, 1935; Thomas, 1939; Macy et al., 1960; Zelmer and Esch 1998; Bolek et al.,

2010). Secondly, these hosts differ in their behavior, ecology, and morphology that may result in differences in the rates that each host is ingested by the next host in the life cycle (Thorp and Covich, 2001). Collectively, this variation in types of second intermediate hosts used by *Halipegus* species make them ideal to investigate differences in development of metacercariae in different second intermediate hosts and to evaluate the extent to which these hosts contribute to transmission.

MATERIALS AND METHODS

Microcrustacean second intermediate host specificity studies

Cypridopsis sp., *Phyllognathus* sp., and *Thermocyclops* sp. were used to assess the extent to which the growth and development of metacercariae of both *Halipegus* species differed within phylogenetically distinct intermediate hosts. For each species, individuals that were similar in size were selected to minimize the variation in resources available among individuals of the same host species. All microcrustaceans were exposed to cercariae of 1 *Halipegus* species that were collected daily from laboratory-infected snails and pooled separately in 250 ml of aged tap water within a 300 ml stackable preparation dish. Then, individual microcrustaceans of each species were exposed to 1 of the *Halipegus* species by placing a single cercaria, a microcrustacean, and approximately 0.25 ml of aged tap water into each well of a 96 cell culture plate. Each microcrustacean had 24 hrs to consume the cercaria; after this time, well plates were examined with a dissecting microscope to determine if all of the microcrustaceans consumed the cercariae. Individuals that had not consumed a cercaria were eliminated from the study.

After 2 days post exposure (DPE), each microcrustacean was pipetted onto a microscope slide with a drop of aged tap water and examined through their carapace for the presence of a metacercaria with a compound light microscope. If a metacercaria was not detected, microcrustaceans were dissected to confirm the lack of infection. The remaining infected

microcrustaceans were removed from the microscope slide and pooled in 300 ml stackable processing dishes with 250 ml of aged tap. Infected microcrustaceans were housed in these dishes for up to 61 days. During this time, approximately half of the water in each dish was replaced every other day, and 5-10 drops of pureed frozen romaine lettuce suspended in aged tap water was added to each well as food for the microcrustaceans.

For each microcrustacean species, a total of 210 individuals infected with *H. eccentricus* and 210 individuals infected with *H. occidualis* were examined for growth and development over 61 days. Approximately every 3 days, 10 infected individuals of *Cypridopsis* sp., *Phyllognathus* sp., and *Thermocyclops* sp. from the *H. eccentricus* and *H. occidualis* groups were dissected individually on a microscope slide. After a metacercaria was released from the host, it was transferred to a new microscope slide in a drop of tap water, and a coverslip was placed over the worm. Immediately following, 3 measures of growth and development were taken. First, to document metacercarial growth within each host species, metacercariae were measured for body length and ventral sucker diameter. Measurements were taken from live worms on wet-mounts while worms were relaxed and fully extended to their largest sizes. Second, the development of metacercariae was assessed by determining if worms were able to pinch off the excretory bladder from their posterior end, which has been suggested as an indicator that *Halipegus* species have developed to become infective to the next obligate host in the life cycle (Zelmer and Esch, 1998; Bolek et al., 2010). To accomplish this, each worm was observed on a wet mount while it was alive. Each worm was observed until the excretory bladder was detached or until the worm died. Individuals that had not developed to the point where they could pinch off their excretory bladder tend to live for a short period of time in water, typically less than 6 minutes. During this time, the inside of these worms would quickly become obscured as cells became opaque due to their inability to osmoregulate. When worms stopped moving, they were observed for an additional 5 minutes after the last movement to ensure that worms had died before they were discarded.

Odonate paratenic host infections

Unfortunately, laboratory stock cultures of odonate paratenic hosts could not be established, and therefore, damselflies of an *Ischnura* species used in experimental infections were field collected from San Borne Lake, Stillwater, Payne County, Oklahoma (36° 9' 17.8014", -97° 4' 40.4034") which is known to be free of both *Halipegus* species (Stigge, unpublished personal observations).

The extent that each of the 3 microcrustacean species contributed to transmission of each of the 2 *Halipegus* species was evaluated by documenting the rate that each microcrustacean species was consumed by the next odonate paratenic host in the natural life cycle. Then, the rates that metacercariae established infections in odonates from the 3 microcrustacean species was determined. To accomplish this, ultimate or pen ultimate instars of *Ischnura* sp. that were approximately the same size were collected from the field. Then, immediately after returning to the laboratory, the odonates were divided into 3 equal groups (N = 45) including time-0 controls, time-T controls, and those used for experimental infections with *H. eccentricus* or *H. occidualis*. Time-0 controls were dissected within 24 hrs after they were brought to the laboratory to determine if the field-collected odonates were naturally infected with any *Halipegus* species. Odonate larvae in the time-T and experimental groups were isolated in 266 ml plastic cups containing 250 ml aged tap water immediately after returning to the laboratory. Then, the time-T and experimental groups of *Ischnura* sp. were both separated into 3 subgroups (N=15) and randomly assigned 1 of 3 microcrustacean species that they would be given as prey. After 24 hrs of isolation, 20 uninfected laboratory-reared individuals of the assigned microcrustacean species were added to the plastic cup filled with 250 ml of aged tap water and a time-T control damselfly. Odonates in the experimental group were exposed to either *H. eccentricus* or *H. occidualis* by placing 20 infected individuals of the assigned microcrustacean species containing 19 day old metacercariae, into plastic cups filled with 250 ml of aged tap water and a single *Ischnura* sp. All

time-T and experimental *Ischnura* sp. fed on the infected microcrustaceans for 24 hrs. After this time, the number of individuals consumed for each of the 3 microcrustacean species was determined by counting the number of microcrustaceans that remained in the dish through a dissecting mircroscope. Time-T and experimental groups of odonates were dissected 2 days after they fed on microcrustaceans and the gut was examined for metacercariae of *Halipegus* species. Establishment was calculated as an average of the number of worms collected from an odonate larva divided by the total number of worms eaten because all individual microcrustaceans were infected with only a single metacercaria.

Statistical analyses

Prevalence, mean intensity, or mean abundance of *H. eccentricus* and *H. occidualis* in each species of microcrustacean and odonate were calculated according to Bush et al. (1997). The establishment rate of metacercariae in each of the 3 microcrustacean hosts was calculated by dividing the number of infected microcrustaceans by the number of microcrustaceans that ingested a cercaria. Additionally, the rate of establishment of metacercariae within odonate paratenic hosts was calculated by dividing the number of worms recovered from the odonate by the number of infected microcrustaceans (i.e. number of worm) eaten. Finally, the contribution of each host to the completion of the overall life cycle was calculated as: Transmission through each host = total cercariae used x % cercariae eaten x prevalence in microcrustacean x % of infected host eaten x % of worms that established in odonates.

The chi-square test for independence was calculated to compare differences in percent of cercaria consumed among different microcrustacean host species. The Kruskal–Wallis and Kolmogorov–Smirnov 2-sample post hoc tests were used to compare differences in the mean abundance of *H. eccentricus* or *H. occidualis* metacercariae among the 3 species of microcrustacean intermediate hosts, because variances were heteroscedastic. Additionally, a 2-way analysis of variance (ANOVA) and Scheffé post hoc tests were used to compare differences

in the percentage of each microcrustacean species consumed and to evaluate differences between the percentage of infected and uninfected individuals of each microcrustacean species eaten by odonates. Lastly, a 1-way analysis of variance (ANOVA) and Scheffé post hoc tests were used to determine if the differences in the percentage of worms transferred from the intermediate to the paratenic host were significant between microcrustacean species. All values are reported as a mean ± 1 SD (range), and 95% confidence intervals are reported for prevalence and percent of cercariae consumed (Sokal and Rohlf, 1981).

RESULTS

Microcrustacean infections via consumption of cercariae

Halipegus eccentricus cercariae were eaten by all 3 species of microcrustaceans; however, significant differences existed in the number of individuals of each microcrustacean species which consumed a cercaria ($\chi^2 = 229.309$, $P < 0.0001$). *Cypridopsis* sp. consumed cercariae most often (92.3%), followed by *Phyllognathus* sp. (75.2%), and *Thermocyclops* sp. consumed cercariae least often (64.1%). However, not all ingested cercariae were infective to microcrustaceans (Table I). Based on dissections, metacercariae of *H. eccentricus* developed in all 3 species of microcrustacean hosts although not all exposed individuals became infected. Prevalence and mean abundance was highest for *Cypridopsis* sp. but were similar for the 2 copepods (Table I). The mean abundance of *H. eccentricus* among microcrustacean species significantly differed (*H* corrected = 69.688, $P < 0.0001$). *Cypridopsis* sp. became infected with *H. eccentricus* at significantly higher mean abundance than either of the copepod species ($\chi^2 = 50.989$, $P < 0.0001$ for *Phyllognathus* sp.; $\chi^2 = 29.868$, $P < 0.0001$ for *Thermocyclops* sp.), but there was no significant difference in the mean abundance of *H. eccentricus* between *Thermocyclops* sp. and *Phyllognathus* sp. ($\chi^2 = 1.687$, $P = 0.86$).

The cercariae of *H. occidualis* were also eaten by all 3 species of microcrustaceans.

Similar to *H. eccentricus*, significant differences existed in the number of individuals of each microcrustacean species which consumed a cercaria of *H. occidualis* ($\chi^2 = 190.863$, $P < 0.0001$). *Cypridopsis* sp. consumed cercariae most often (90.0%), followed by *Phyllognathus* sp. (72.0%), and lastly *Thermocyclops* sp. consumed cercariae the least often (64.0%). Like *H. eccentricus*, metacercariae of *H. occidualis* developed in all 3 species of microcrustacean hosts, but not all individuals that ingested a cercaria became infected (Table I). Prevalence and mean abundance were highest for *Cypridopsis* sp. and lowest for *Thermocyclops* sp. (Table I). Significant differences in mean abundance of *H. occidualis* existed among microcrustacean species (*H* corrected = 157.441, $P < 0.0001$). *Cypridopsis* sp. became infected with *H. occidualis* at a significantly higher mean abundance than either of the copepod species ($\chi^2 = 77.440$, $P < 0.0001$ for *Phyllognathus* sp.; $\chi^2 = 100.409$, $P < 0.0001$ for *Thermocyclops* sp.), but there was no significant difference in the mean abundance of *H. eccentricus* among *Thermocyclops* sp. and *Phyllognathus* sp. ($\chi^2 = 2.068$, $P = 0.711$).

Growth and development in microcrustaceans

Metacercariae of both *Halipegus* species did not grow at the same rate in the 3 microcrustacean species. Additionally, growth was not continuous and metacercariae reached a point of development in which they appeared to stop growing within each of the 3 microcrustacean hosts (Fig. 1). For both *Halipegus* species, metacercariae grew faster within the ostracod host than within either of the 2 species of copepod hosts. Additionally, metacercariae of both *Halipegus* species reached an average maximum size sooner within the ostracod; whereas, the growth in both species of copepods was slower but comparable to each other (Fig. 1). A similar pattern of growth was observed for average metacercaria ventral sucker diameter (Fig. 2). The ventral suckers of metacercariae from *Cypridopsis* sp. grew faster than those from *Phyllognathus* sp. or *Thermocyclops* sp. However, the ventral sucker diameter of worms from both copepod species eventually converged on the average ventral sucker diameter of worms from *Cypridopsis* sp.

Halipegus eccentricus and *H. occidualis* took approximately the same time to develop to the point in which they could pinch off their excretory bladders (Fig. 3). All individuals that were able to pinch off their excretory bladder survived for over an hour of observation on a wet mount; however, all worms that were not developed to the point of pinching off this structure died within 6 minutes of being removed from the host and placed in tap water. Metacercariae of *H. occidualis* began pinching off their excretory bladders at 11 DPE in all 3 of the microcrustacean hosts; whereas, the first time *H. eccentricus* pinched off its excretory bladder was at 14 DPE. However, all individuals of both *Halipegus* species pinched off their excretory bladders by 19 DPE (Fig. 3).

Odonate infections

There was a significant main effect of microcrustacean species on the number of individuals eaten within the *H. eccentricus* group ($F_{2, 84} = 62.3$, $P < 0.0001$) and *H. occidualis* group ($F_{2, 84} = 40.7$, $P < 0.0001$) by odonates. There also was a significant main effect of infections status for both *Halipegus* species on the percent of individuals from each host group that were consumed by an odonate ($F_{1, 84} = 12.3$, $P < 0.0007$ for *H. eccentricus*, and $F_{1, 84} = 21.4$, $P < 0.0001$ for *H. occidualis*). Additionally, for *H. eccentricus* there was a statistically significant interaction between the effects of microcrustacean species and infection status on odonate ingestion rate ($F_{2, 84} = 3.3823$, $P = 0.039$). However, this interaction was not significant for *H. occidualis* ($F_{2, 84} = 1.88$, $P = 0.31$; Fig. 4).

Although all 3 microcrustacean species infected with *H. eccentricus* or *H. occidualis* were consumed by odonates at a higher frequency than uninfected controls, significant differences only occurred between infected and uninfected *Thermocyclops* sp. ($P < 0.001$ for *H. eccentricus*; $P < 0.001$ for *H. occidualis*). Additionally, independent of infection status *Phyllognathus* sp. and *Cypridopsis* sp. were consumed by odonates at a significantly higher rate than uninfected *Thermocyclop*s sp. ($P < 0.001$ for all comparisons; see Fig. 4).

There were significant differences in the percent of worms that established in the

odonates from the 3 microcrustacean species infected with *H. eccentricus* ($F_{2,42} = 16.1$, $P < 0.0001$) and *H. occidualis* ($F_{2,42} = 4.3$, $P < 0.0001$; Table II). Metacercariae of *H. eccentricus* from *Cypridopsis* sp. established in odonate paratenic hosts at a significantly higher rate than from either of the copepod species ($P < 0.0001$). However, there was no significant difference in the establishment rate of metacercariae of *H. eccentricus* from the 2 copepod species ($P > 0.05$). In contrast, the only significant difference for metacercaria establishment rate of *H. occidualis* in odonate hosts occurred for *Cypridopsis* sp. and *Thermocyclops* sp. ($P = 0.032$). There was no significant difference in the establishment rates of metacercariae from any of the other microcrustacean groups ($P > 0.05$; Table II).

Rate of transmission from cercariae to metacercariae in odonate

The pattern in transmission from cercariae to odonate through the 3 microcrustacean species was similar for both *Halipegus* species (Table III). Infections through *Cypridopsis* sp. yielded the highest percent of worms that established in odonates followed by *Phyllognathus* sp., and lastly, *Thermocyclops* sp. There was an approximately 2 fold difference in the average percent of worms that established in odonates from *Cypridopsis* sp. than from *Phyllognathus* sp., and the difference was nearly 3 fold between *Cypridopsis* sp. and *Thermocyclops* sp. (Table III).

DISCUSSION

A major contribution of this study is that it examines host specificity from physiological and ecological aspects. I evaluated the development of both trematode species in multiple species of microcrustacean hosts and then examined subsequent trophic relationships of those hosts with the next host in the life cycle. By doing so, the suitability of each host was evaluated in terms of parasite development and the frequency in which those hosts transmitted both trematode species to the next host in the life cycle.

Under controlled laboratory conditions, both *Halipegus* species were more successful in

establishing in *Cypridopsis* sp. than in the 2 other microcrustacean species. First, more individuals of *Cypridopsis* sp. consumed a cercaria, second ingested cercariae established infections more frequently within *Cypridopsis* sp., and more worms established within odonates when odonates consumed infected ostracods. In contrast, *Thermocyclops* sp. was the least suitable host of the 3 microcrustacean species. Fewer individuals of *Thermocyclops* sp. ingested a cercaria, they had the lowest prevalence, infected individuals were consumed by odonates significantly less often, and worms that developed in *Thermocyclops* sp. had a low establishment rate within odonates. Lastly, worms that developed in, *Phyllognathus* sp. had a higher success than *Thermocyclops* sp., but not as high as *Cypridopsis* sp. Therefore, the probability of a cercaria being transmitted through 2 successive hosts in the life cycle differed among the 3 microcrustacean species as did their role in the overall life cycle of both *Halipegus* species.

However, the contribution of each of the 3 microcrustacean host species to the transmission of each *Halipegus* species should only be considered as an estimate. Clearly, the 3 species of microcrustaceans used in this study represent only a few of the many microcrustacean species that may be available for infections in nature. Additionally, the experimental conditions of this study do not reflect the complex environment that affects transmission dynamics under natural conditions. However, these results demonstrate that when all conditions are equal all microcrustacean hosts are not equally suitable for the transmission of *Halipegus* species, and therefore, they should not be weighted equally when evaluating host ranges.

The observed differences in the transmission rates of both *Halipegus* species from microcrustacean to the odonate host may be related to differences in the development rates of these trematodes in the 3 microcrustacean species. Although the growth patterns of *H. eccentricus* and *H. occidualis* among the 3 microcrustacean species were similar, there were obvious differences in the development of both *Halipegus* species within the 3 microcrustacean hosts. The growth in body length and ventral sucker diameter of both *Halipegus* species reached a plateau

within the 3 microcrustacean species, and the maximum sizes of both trematode species were approximately the same in the 3 host species. However, there were differences in the growth rate of metacercariae among species of hosts. For both *Halipegus* species, the initial rate of growth, based on average body length, was faster within *Cypridopsis* sp. than in either of the 2 copepod species. Additionally, the average maximum size of the ventral sucker was reached fastest in *Cypridopsis* sp. than in the 2 species of copepods. Despite differences in growth, the developmental time required for metacercariae to begin pinching off their excretory bladders was similar across the 3 microcrustacean host species. *Halipegus occidualis* began losing this structure at 11 DPE in all 3 species of microcrustaceans, and by 19 DPE 100% of the metacercariae pinched off their excretory bladders. The metacercariae of *H. eccentricus* did not begin to pinch off the excretory bladder until 14 DPE, however as with *H. occidualis*, by 19 DPE all metacercariae of *H. eccentricus* pinched off their excretory bladders.

Importantly, the loss of the excretory bladder in worms was not dependent on worm size since worms that differed in average body length in the 3 microcrustacean hosts pinched off their excretory bladders at the same time. This suggests that there was not a strong relationship between the growth of the metacercariae and the development of their osmoregulatory system, which has been implicated as an important factor for infecting the next host in the life cycle (Zelmer and Esch, 1998). However, my study suggests that metacercariae size had an apparent effect on the infectivity of these parasites in the odonate host, as larger worms from *Cypridopsis* sp. established at higher rates in odonates than smaller worms from the 2 species of copepods.

Interestingly, previous comparisons of the size of *H. occidualis* metacercariae from laboratory infected microcrustacean hosts and field collected odonate hosts indicated that metacercariae from odonates are larger and develop much bigger suckers than worms from microcrustaceans (Zelmer and Esch, 1998). However, Zelmer and Esch (1998) concluded that the growth of metacercariae within odonates did not offer any benefit to metacercariae when

establishing within the frog definitive hosts. After exposing a single green frog with infected ostracods, Zelmer and Esch (1998) concluded that worms from ostracods, which are smaller than those from odonates, were 100% infective because 13 worms were recovered from their green frog, and this was slightly higher than expected based on the mean abundance of metacercariae from the infected ostracods that they dissected (1.6 ± 0.19 SE; the frog was exposed with 7 infected ostracods). Based on these results, they suggested that it was doubtful that the infectivity of metacercariae from ostracods could be enhanced by the growth of metacercariae that occurs in the odonate host. These observations are in contrast to the present study where larger metacercariae from microcrustaceans had a higher success rate of infecting odonate hosts. Furthermore, previous studies have shown that the ability of larval helminthes to acquire resources from their intermediate hosts can affect their success in definitive hosts (Rosen and Dick 1983; Lafferty and Kuris, 2002; Steinauer and Nickol, 2003; Fredensborg and Poulin, 2005; Benesh and Hafer, 2012). Clearly, future studies are needed to determine if difference in growth of metacercariae within microcrustacean and odonate hosts will affect the transmission of worms through the amphibian definitive host.

Finally, 1 interesting observation made during this study suggests that odonates consume infected microcrustaceans of some species significantly more frequently than uninfected individuals. This suggests that *Halipegus* species have a negative effect on their microcrustacean hosts and/or they manipulate the behavior of infected microcrustaceans to increase their transmission. However, future studies are needed to determine if odonates consume microcrustaceans infected with *Halipegus* species more frequently when both infected and uninfected microcrustaceans are available.

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Table I. Prevalence and mean abundance of *Halipegus eccentricus* and *Halipegus occidualis* metacercariae recovered from 3 species of microcrustacean species as determined through dissections. Lower case letters represent significant differences in mean abundance among host combinations ($P < 0.0001$ for all significant differences).

	Phyllognathus sp.	*Thermocyclops* sp.	*Cypridopsis* sp.
Halipegus eccentricus			
Prevalence ± 95% CI	62.5 ± 3.5%	66.0 ± 3.7%	80.0 ± 2.6%
(No. infected/No. exposed)	(470/752)	(423/641)	(739/923)
Mean abundance ± 1 SD	0.47 ± 0.50	0.43 ± 0.50	0.74 ± 0.50
Range	0-1[a]	0-1[a]	0-1[b]
Halipegus occidualis			
Prevalence ± 95% CI	65.0 ± 3.5%	61.0 ± 3.8%	87.0 ± 2.2%
(No. infected/No. exposed)	(468/720)	(391/640)	(783/900)
Mean abundance ± 1 SD	0.47 ± 0.50	0.40 ± 0.49	0.78 ± 0.50
Range	0-1[a]	0-1[a]	0-1[b]

Table II. Mean intensity and mean percent establishment of metacercariae in a larva of *Ischnura* sp. from 3 microcrustacean species infected with *Halipegus eccentricus* or *Halipegus occidualis*.

	Phyllognathus sp.	*Thermocyclops* sp.	*Cypridopsis* sp.
Halipegus eccentricus			
Mean Intensity ± 1 SD	7.9 ± 1.7	5.5 ± 1.5	9.3 ± 1.4
Range	5-10	4-9	7-11
Average Percent Established	62.9%	62.3%	73.7%
Range	50.0-75.0%	50.0-71.4%	63.6-84.6%
Halipegus occidualis			
Mean Intensity ± 1 SD	7.9 ± 1.7	5.6 ± 1.5	9.3 ± 1.1
Range	5-10	4-8	7-11
Average Percent Established	61.1%	57.6%	77.4%
Range	37.5-90.9%	33.3-90.0%	49.7-100%

Table III. Average percentage of *Halipegus eccentricus* and *Halipegus occidualis* transmitted from the cercariae stage into the 3 microcrustacean species and into odonate larvae.

	Phyllognathus sp.	*Thermocyclops* sp.	*Cypridopsis* sp.
Halipegus eccentricus			
Percent cercariae consumed ± 95% CI	75.2 ± 2.7%	64.1 ± 3.0%	92.3 ± 1.7%
Prevalence ± 95% CI	62.5 ± 3.5%	66.0 ± 3.7%	80.0 ± 2.6%
Infected microcrustacean consumed	63.0% (45.0-80.0%)	44.3 % (30.0-70.0%)	63.7% (45.0-80.0%)
Metacercariae established (Range)	62.9% (50.0-75.0%)	62.3% (50.0-71.4%)	73.7% (63.6-84.6)
Average overall transmission (Range)	**18.6% (9.6-30.8%)**	**11.7% (5.7-23.4%)**	**34.7% (20.1-52.5%)**
Halipegus occidualis			
Percent cercariae consumed ± 95% CI	72.0 ± 2.8%	64.0 ± 3.0%	90.0 ± 1.9%
Prevalence ± 95% CI	65.0 ± 3.5%	61.0 ± 3.8%	87.0 ± 2.2%
Infected microcrustacean consumed	67.0% (50.0-85.0%)	50.0% (40.0-55.0%)	63.7% (50-75%)
Metacercariae established (Range)	61.1% (37.5-90.9%)	57.6% (33.9-90.0%)	77.4% (49.7-100.0%)
Average overall transmission (Range)	**19.2% (8.0-39.6%)**	**11.2% (4.7-21.5%)**	**38.6% (18.6-61.5%)**

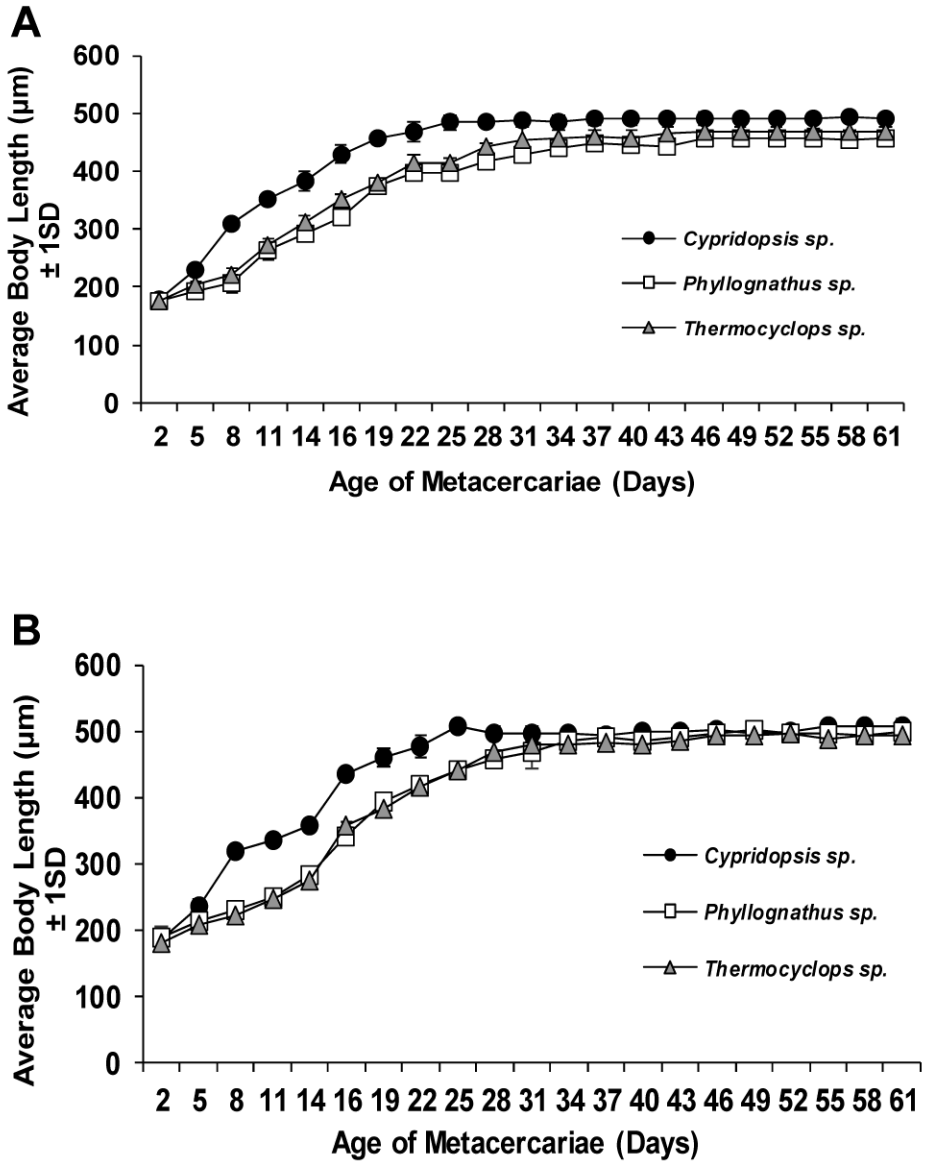

FIGURE 1. Average body length ± 1 SD of metacercariae of *Halipegus eccentricus* **(A)** and *Halipegus occidualis* **(B)** within 3 microcrustacean intermediate hosts over a 61 day development period.

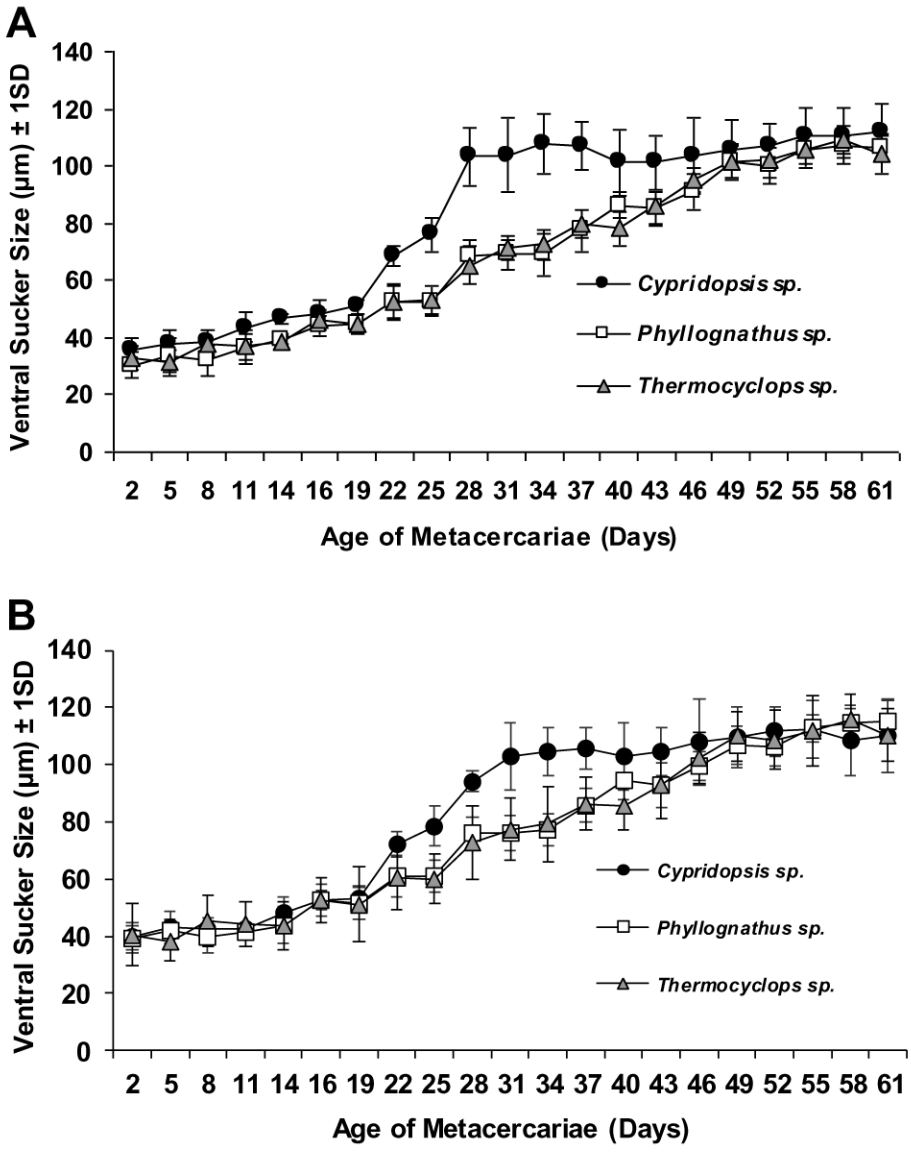

FIGURE 2. Average ventral sucker diameter ± 1 SD of metacercariae of *Halipegus eccentricus* **(A)** and *Halipegus occidualis* **(B)** within 3 microcrustacean intermediate hosts over a 61 day development period.

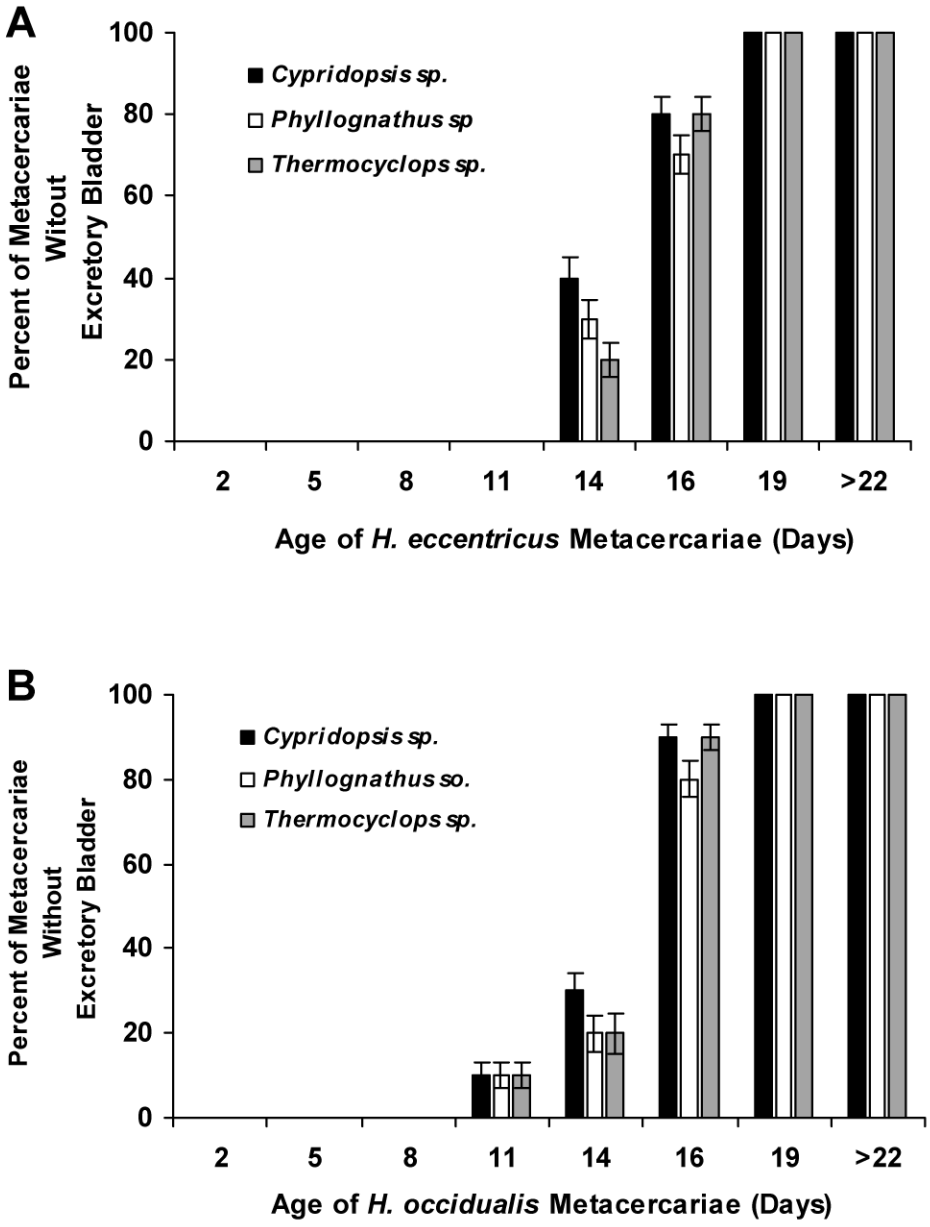

FIGURE 3. Percent ± 1 SD of *Halipegus eccentricus* **(A)** and *Halipegus occidaulis* **(B)** metacercariae that pinched off their excretory bladder over a 61 day development period.

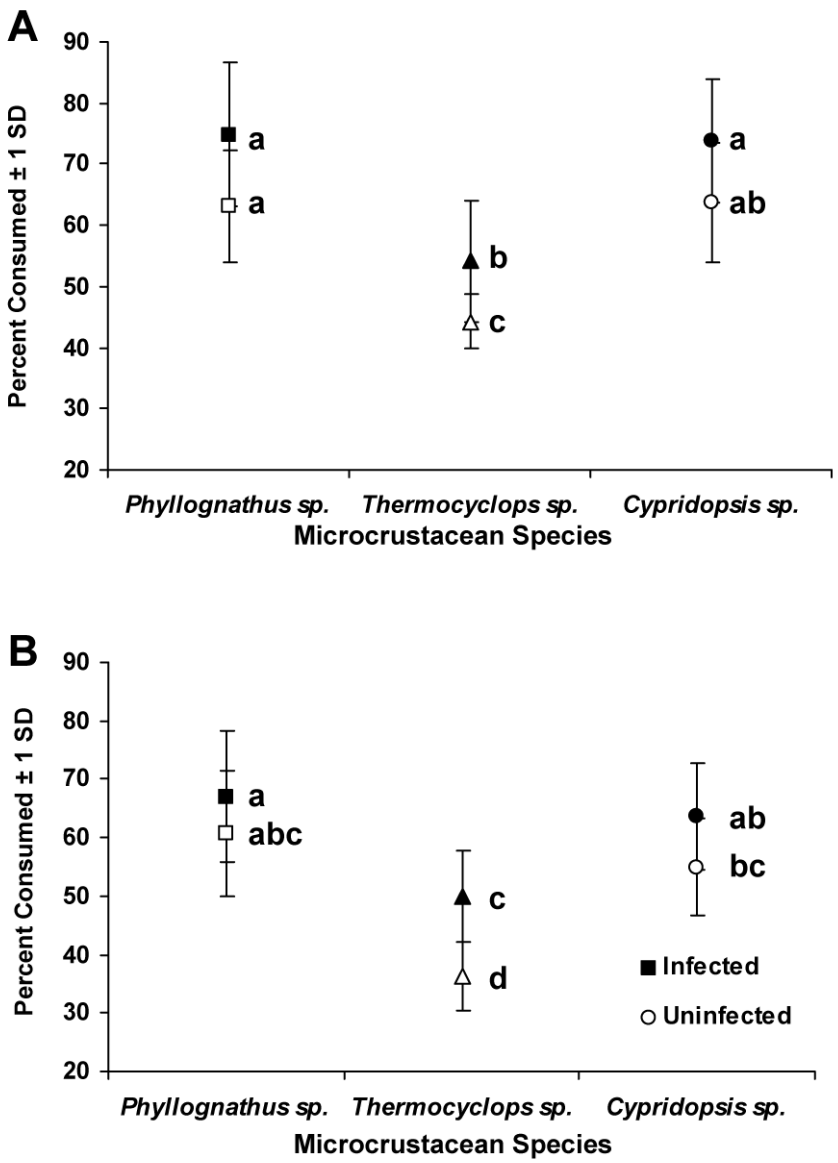

FIGURE 4. Average odonate consumption reported as an average percentage ± 1 SD of 3 microcrustacean species infected with *Halipegus eccentricus* **(A)** or *Halipegus occidualis* **(B)** and the consumption of each of the uninfected control groups. Lower case letters represent significant differences in mean percentages among microcrustacean species and infection statuses ($P < 0.04$ for all significant differences).

CHAPTER III

THE ALTERATION OF LIFE HISTORY TRAITS AND INCREASED SUCCESS OF *HALIPEGUS ECCENTRICUS* THROUGH THE USE OF A PARATENIC HOST: A COMPARATIVE STUDY

ABSTRACT: Complex life cycles are a hallmark characteristic of many parasites; however, little is known about the process by which life cycles become more complex through the addition of hosts. Paratenic hosts are present in the life cycles of several phylogenetically distinct groups of helminths suggesting that they may play a key role during this process. This study examined the development of metacercariae of *Halipegus eccentricus* within intermediate microcrustacean and odonate paratenic hosts. Then, a comparative approach was used to evaluate how life history traits of *H. eccentricus* within the anuran definitive hosts differ between metacercariae of the same age that developed within an intermediate ostracod host or a paratenic odonate host. The results of this study indicate that metacercariae of *H. eccentricus* do not grow at the same rate in different intermediate hosts and significant differences exist in growth within intermediate and paratenic hosts. Individuals from odonate paratenic hosts always had larger bodies and suckers than those of metacercariae of the same age that develop within microcrustacean intermediate hosts. Furthermore, metacercariae from odonates were more successful in establishing and migrating in definitive anuran hosts. Lastly, individuals from paratenic hosts began reproducing earlier within anuran definitive hosts than age-matched worms that develop within the

intermediate hosts. Collectively, these results suggest that the variation in body and sucker sizes within odonate and microcrustacean hosts may carry over to the definitive host, and in the case of *H. eccentricus*, using the paratenic host increases transmission and alters other life history traits within definitive hosts. These results indicate that using a paratenic host can affect the success of parasites in subsequent hosts, and therefore, these hosts may provide benefits other than just increasing transmission by bridging and ecological gap.

INTRODUCTION

Complex life cycles, in which discrete stages of a parasite are transmitted sequentially between different host species, have evolved independently in several phylogenetically distinct parasitic groups including protozoans, acanthocephalans, cestodes, nematodes, and trematodes (Olsen, 1974; Mackiewicz, 1988; Rohde, 1994; Poulin, 1998; Poulin, 2007). Current models indicate that complex life cycles were evolutionarily derived from simple life cycles by the addition of new hosts (Smith-Trail, 1980; Poulin, 1998; Gibson and Bray, 1994; Lafferty, 1999; Parker et al., 2003). Two primary hypotheses have been proposed to explain the evolution of complex life cycles of parasites. One hypothesis suggests that an intermediate host was the original host to the parasite, and all other types of hosts have been added over evolutionary time (Smith-Trail, 1980; Poulin, 1998; Parker et al., 2003). The second hypothesis suggests that the definitive host was the first hosts, and intermediate hosts have been added to the original life cycle (Smith-Trail, 1980; Gibson and Bray, 1994; Lafferty, 1999; Parker et al., 2003).

Despite these efforts, the evolution of complex life cycles has not been thoroughly addressed. The lack of a fossil record for most parasite species makes it difficult to identify the original host or the number of hosts that have been added or lost over evolutionary time. Additionally, incorporating or losing an obligate host is not likely to happen instantly, but instead, it probably occurs gradually as the parasite and host interact over a long evolutionary time span.

Therefore, in most cases, any changes in the number of hosts in a life cycle cannot be observed in their entirety by one investigator. As a result of these difficulties, little empirical and/or comparative data are available on the benefits of adding a host to a life cycle (Zelmer and Esch, 1998). However, present-day paratenic and intermediate hosts are good comparative model systems to experimentally evaluate the extent to which a parasite's life history is altered from the addition of a new host to their life cycle.

Paratenic hosts were first described as optional or temporary hosts (Joyeux and Baer, 1934; Baer, 1951), and subsequently, paratenic hosts have been associated with increasing transmission by bridging ecological gaps between obligate host species. Parasites do not significantly develop within paratenic hosts; hence, they are not required for the physiological completion of the life cycle but instead are necessary for transmission. Because paratenic hosts are critical for the persistence of some parasites, there is a close tie between those parasites and their paratenic hosts. As a result, the interactions between parasites and those hosts over their shared evolutionary history may have resulted in alterations of life history traits that benefit the parasite by increasing their success of completing the life cycle. As these benefits accumulate, the paratenic host may become required for the completion of the life cycle, in ways other than serving only as an ecological bridge for trophic gaps. Therefore, paratenic hosts may play a critical role in the evolution of complex life cycles by serving as the transition from a species that is not used as a host, because of either ecological or physiological reasons, to an obligate host.

Given the long time scale of such an evolutionary change and the difficulty of assessing these changes from historic and modern field data, the benefits of adding a host in a life cycle have not been thoroughly investigated (Zelmer and Esch, 1998). Fortunately, changes in life histories that result from the addition of a host can be examined experimentally by comparing the life history traits of individuals of the same parasite species that either use paratenic hosts or only intermediate hosts to infect subsequent hosts. Hemiurid trematodes in the genus *Halipegus* are an

example of a good system to experimentally examine the potential benefits associated with the addition of a paratenic host by using this comparative approach.

Currently, the life cycles of 2 North American species (*H. eccentricus* and *H. occidualis*) have been elucidated, and both incorporate paratenic hosts into their life cycles (Krull, 1935; Thomas, 1939; Zelmer and Esch, 1998; Bolek et al., 2010). Adults occur in the buccal cavity of anuran definitive hosts, and immature stages infect aquatic snails and microcrustaceans as the first and second intermediate hosts, respectively (Olsen, 1974). Although, amphibians can become directly infected with both *Halipegus* species when they consume infected microcrustaceans (Zelmer and Esch, 1998; Bolek et al., 2010), the adult frogs that serve as definitive hosts rarely consume microcrustaceans in nature (Werner et al., 1995; Hirai, 2004; Wu et al., 2005). To overcome this trophic gap in transmission, an odonate paratenic host has been added to the life cycles (Zelmer and Esch, 1998; Bolek et al., 2010).

All of the known life cycles of *Halipegus* species include an odonate host, and notably, a *Halipegus* species from India has been reported to mature progenetically within odonates suggesting that species of *Halipegus* have a long evolutionary history with odonate hosts (Nath and Pande, 1970; Goater, 1989). Additionally, it appears that some *Halipegus* species have adapted to make use of resources available within odonates. For example, the growth in body length and suckers of *H. occidualis* metacercariae continues within the odonate paratenic host (Zelmer and Esch, 1998). A larger body and suckers could be an asset for transmission of *Halipegus* species to amphibians because the size of the suckers might influence the ability of metacercariae to establish and remain attached to the active digestive tract of amphibian hosts. Metacercariae of *Halipegus* species attach in the stomach of frogs after being ingested where they continue to grow for a considerable amount of time, and eventually, they migrate up the gastrointestinal tract into the buccal cavity where they attach and mature. Therefore, a larger, and presumably stronger, ventral sucker may help these individuals attach to the stomach and prevent

being dislodged from the digestive tract as they develop and migrate. Thus, metacercariae from paratenic hosts could have a higher rate of establishment and successful migration than metacercariae from microcrustaceans that have smaller suckers. Additionally, worms that reach their final destination within the buccal cavity sooner may require less time to begin egg production if the habitat occupied by these worms influences their maturation.

There were 2 primary objectives for this study. First, I use a comparative approach to identify any differences in the development of metacercariae of *H. eccentricus* within intermediate hosts and paratenic hosts. For this, the body length and the ratio of oral-to-ventral sucker size of infective metacercariae recovered from 2 species of intermediate hosts were compared with that of individuals of the same age from a paratenic odonate host species. Lastly, I also used this approach to evaluate how life history traits of *H. eccentricus* within the definitive hosts differ between metacercariae of the same age that developed within an intermediate ostracod host or a paratenic odonate host. Specifically, I evaluated the potential benefits of using a paratenic host by infecting anurans with metacercariae of the same age from microcrustacean intermediate hosts and odonate paratenic hosts. I then compared (1) rate of initial establishment in the stomach, (2) time required to migrate to the buccal cavity, (3) establishment rates within the buccal cavity after migration, and (4) time to maturity. The major contribution of this study is the examination of the role of paratenic hosts in parasite life cycles using experimental infections and appropriate time-control groups to determine the extent to which the use of a paratenic hosts affects establishment, survival, and life history traits.

MATERIALS AND METHODS

A laboratory stock culture of *H. eccentricus* and all microcrustaceans used in this study was established according to Appendix A. All microcrustaceans were laboratory-raised; whereas, the odonates, *Ischnura* sp., were field-collected from James Creek Pond, Stillwater, Oklahoma

(36° 8' 50.661" N, -97° 4' 50.0412" W) which is known to be free of *H. eccentricus* (Stigge, unpublished personal observations). For this study, adult Woodhouse's toads, *Bufo woodhousii*, that were 4.8-5.2 cm in snout vent length were chosen as the definitive hosts because this species has never been reported as a host for *H. eccentricus* (see Bolek et al., 2010) ensuring that field-collected hosts are not unknowingly infected with individuals of *Halipegus* species. Furthermore, all toads used for infections were collected from Stillwater, Payne Co., OK (36° 7' 48.0174"N; (-97° 4' 25.3914"W), and in a previous study hundreds of toads were examined from this locality without a single toad being infected with any species of *Halipegus* (Vhora, 2012). Additionally, *B. woodhousii* was chosen as a model host for experimental infections because it is easy to maintain in the laboratory, and previous work indicates that there is no difference in the development, establishment and migration of *H. eccentricus* within *B. woodhousii* and the American bullfrog, *Rana catesbeiana*, the typical host of *H. eccentricus* in nature (Bolek et al., 2010). All toads used in experimental infections were housed individually in 37 L aquaria. Anurans were provided a 5 cm gravel substrate and a plastic water dish filled with aged tap water. Toads were maintained at 24 C and 24L:0D period and fed crickets and meal worms *ad libitum*. Water was changed every other day.

Intermediate host infections

Microcrustacean intermediate host infections were prepared daily by collecting cercariae from 29 laboratory infected *Physa gyrina* snails, and then pooling them in 250 ml of aged tap water in a stackable preparation dish. Within 24 hrs of collection, a single cercaria, an individual of 1 of 3 laboratory-reared microcrustacean species (*Phyllognathous* sp., *Thermocyclops* sp. and *Cypridopsis* sp.), and approximately 0.25 ml of aged tap water were pipetted into each well of a 96 cell culture plate. Because of their large body size, all individuals of *Bradlystrandesia* sp. were exposed to a single cercaria in approximately 1 ml of aged tap water in 1.5 ml wells of a 24 cell culture plate. All microcrustaceans fed on the cercariae for 24 hrs. After this time, the well plates

were examined using a dissecting microscope, and individuals that had not consumed the cercaria were eliminated from the study. The remaining microcrustaceans were maintained individually in 24-well culture plates containing 1.5 ml of aged tap water for 14 days. All exposed microcrustaceans were maintained under a 12:12 light-dark photoperiod at room temperature (24 ± 1 C). Approximately half of the water in each well was replaced and a drop of pureed frozen romaine lettuce suspended in aged tap water was added to each well every 2 days. After 14 DPE, the microcrustaceans were pipetted onto a microscope slide with a drop of aged tap water and examined through their carapace for the presence of metacercariae of *H. eccentricus* with a compound microscope. If a metacercaria was not detected, microcrustaceans were dissected to confirm the status of infection. Microcrustaceans that were identified as infected through external examination were carefully removed from the microscope slide and placed in groups based on species in a 300 ml stackable processing dish with 250 ml of aged tap water. All infected microcrustaceans were maintained in these dishes for an additional 14 days. Approximately half of the water in each dish was replaced every 2 days, and 5-10 drops of pureed frozen romaine lettuce suspended in aged tap water was added to each well as food for the microcrustaceans.

Paratenic host infections

For odonate infections, field-collected larval *Ischnura* sp. were divided into 3 equal groups (N=128), including time-0 controls, time-T controls, and experimental infections. Time-0 controls were dissected within 24 hours after damselflies were brought to the laboratory. Odonate larvae in the time-T and experimental groups were isolated in 266 ml plastic cups containing 250 ml aged tap water immediately after returning to the laboratory. All experimental odonates were exposed to *H. eccentricus* using infected *Cypridopsis* sp. because laboratory culture of this microcrustacean had the highest prevalence, and larval *Ischnura* sp. consumed *Cypridopsis* sp. the most frequently of the microcrustaceans available in the laboratory.

For odonate exposures, infected ostracods were divided into 2 groups (N = 221). The first group of infected *Cypridopsis* sp. was placed in 250 ml of aged tap water in a stackable processing dish for 15 days and served as a time-control for the experimentally infected odonates. The second group of infected *Cypridopsis* sp. was used to experimentally infect odonates with 14 day old metacercariae. Within 24 hrs of isolating *Ischnura* sp. within the experimental group, a single damselfly and an infected ostracod containing a 14 day old metacercaria were placed in a 266 ml plastic cups containing aged tap water. As a control, an uninfected laboratory-reared *Cypridosis* sp. was added to each cup containing the odonate time-T controls. Experimental and time-T odonates fed on the ostracods for 24 hrs; after which, each damselfly was transferred to a 355 ml glass mason jar containing 240 ml of aged tap water and a standard wood tongue depressor as a perch. The water within the glass jars was changed every 4-5 days, and at this time approximately 30-50 uninfected laboratory-raised ostracods from stock cultures were placed in the jars as food for odonates. After an additional 14 days, when metacercariae were 29 days old, the time-T control odonate, experimental odonate, and time-T ostracod groups were dissected for *H. eccentricus*.

Development of metacercariae in intermediate and paratenic hosts

To determine if any differences existed in growth of metacercariae among intermediate and paratenic hosts, I compared the overall body size and sucker diameters for metacercariae of the same age recovered from 2 species of ostracod intermediate hosts and odonate paratenic hosts at 3 different time intervals. The 2 ostracod species (*Cypridopsis* sp. and *Bradlystrandensia* sp.) were chosen from the 4 microcrustacean species because both ostracod species varied in size and both species had large enough sample sizes to be used during both parts of the study, including the growth of metacercariae within intermediate and paratenic hosts and the effects of paratenic hosts on life history traits within the definitive host (see below). To accomplish this and control for differences in development time, 10 infected ostracods of each species, and 5 odonate larvae

exposed to 15 day old metacercariae were dissected at 30, 35, 43 DPE and 15, 20, and 28 DPE, respectively. Individual metacercariae were placed on a microscope slide in a drop of tap water containing a small sliver of a methanol crystal. Once each metacercaria relaxed and stopped moving, the methanol crystal was removed, and a coverslip was placed over the worm. The metacercariae were examined using a compound microscope. The total body length and the diameter of the oral and ventral suckers were measured from wet mount slides and ventral to oral sucker (v:o) ratios were calculated.

Effects of intermediate and paratenic hosts on establishment, migration and maturity of metacercariae in definitive hosts

To determine if using a paratenic host affected life history traits within the next host in the life cycle, I compared the establishment rate, migration time, and time until egg production in toad definitive hosts exposed to metacercariae of the same age recovered from microcrustacean intermediate or odonate paratenic hosts. Twenty-nine day old metacercariae recovered from 4 species of microcrustaceans intermediate hosts and 1 species of odonate paratenic hosts were pooled by host species in separate glass petri dishes (60 x 15mm) containing 25 ml of aged tap water. Within 5 minutes of collection, 10 metacercariae from each of the 5 host species were pipetted into the stomach of 5 groups (N = 6) of uninfected Woodhouse's toads, *B. woodhousii*. The exposed toads from each of the 5 groups were then divided into 2 additional groups. The first group (N = 3) was used to estimate the number of worms that initially establish within toads by examining the entire alimentary canal of each toad 2 DPE and counting the number of metacercariae present. The second group of toads was used to determine the amount of time it took for worms to migrate from the stomach into the buccal cavity, followed by the time required for worms to mature and produce eggs. The mouths of the 3 remaining exposed toads from each of 5 groups were monitored daily for the presence of worms, and once present, worms were removed daily from the mouth, placed on a wet mount, and examined for eggs using a compound

microscope. Once worms were examined for the presence of eggs, all worms were placed back into the mouth of the toad from which they were removed. Adult worms were considered gravid when at least 1 egg was observed. The total number of worms and number of gravid worms present within the buccal cavity and eustachian tubes was recorded daily. The 3 remaining toads from all 5 groups were dissected 90 DPE, and the entire alimentary canal was examined for the presence of worms to ensure that all worms were counted.

Statistical Analyses

Prevalence, mean intensity, or mean abundance of *H. eccentricus* in each species of microcrustacean, odonate and amphibian host was calculated according to Bush et al. (1997). The Kruskal–Wallis test and the Kolmogorov–Smirnov 2-sample post hoc tests were used to compare differences in the mean abundance of *H. eccentricus* metacercariae among the 4 species of microcrustacean intermediate hosts, because variances were heteroscedastic (Sokal and Rohlf, 1981). In contrast, a 1-way analysis of variance (ANOVA) and Scheffé post hoc tests were used to compare differences in mean body length and mean ventral to oral sucker ratio of metacercariae recovered from micro crustacean intermediate and odonate paratenic hosts. Additionally, a 1-way ANOVA and Scheffé post hoc tests were also used to compare differences in the mean number of worms that established 2 DPE, number of worms that migrated to the mouth, the average time worms migrated to the buccal cavity, and the average time for worms to produce eggs in toads exposed to metacercariae recovered from the 4 microcrustacean intermediate and 1 odonate paratenic hosts (Sokal and Rohlf, 1981). All values are reported as a mean ± 1 SD (range), and 95% confidence intervals are reported for prevalence (Sokal and Rohlf, 1981).

RESULTS

Intermediate and paratenic host infections

Halipegus eccentricus metacercariae developed in all 4 species of microcrustacean hosts, although not all exposed individuals became infected. Prevalence and mean abundance was highest for *Cypridopsis* sp. (93% and 0.9 ± 0.3) and lowest for *Bradlystrandesia* sp. (70% and 0.7 ± 0.5; Table I). The mean abundance significantly differed among microcrustacean species (*H* corrected = 79.72, $P < 0.0001$). There were significant differences in the mean abundance of *H. eccentricus* within *Cypridopsis* sp. and *Bradlystrandesia* sp. ($\chi^2 = 40.01$, $P < 0.0001$), *Cypridopsis* sp. and *Phyllognathous* sp. ($\chi^2 = 17.31$, $P = 0.0003$), and *Cypridopsis* sp. and *Thermocyclops* sp. ($\chi^2 = 16.97$, $P = 0.0004$; Table I).

Of the 114 *Ischnura* sp. exposed to *Cypridopsis* sp. infected with a single *H. eccentricus* metacercaria, 101 (88%) became infected with a mean abundance of 0.88 ± 0.5 (0-1). None of the time-0 or time-T control damselflies were infected with any hemiurid metacercariae.

Development of metacercariae in intermediate and paratenic hosts

There was no significant difference in mean body size or mean ventral:oral (v:o) sucker ratio for 15 day old metacercariae recovered from the 2 species of ostracod intermediate hosts or damselfly paratenic hosts ($F_{2, 27} = 2.917$, $P = 0.07$ for body size; $F_{2, 27} = 0.127$, $P = 0.88$ for v:o ratio; Fig. 1). However, significant differences existed in mean body size and mean v:o sucker ratio for 20 and 28 day old metacercariae among intermediate and paratenic host groups. The mean body length of 20 day old metacercariae was significantly longer in odonate paratenic hosts than in the 2 species of ostracod intermediate hosts ($F_{2, 27} = 778.30$, $P < 0.0001$, Scheffé $P < 0.0001$; Fig. 1). In contrasts, mean v:o sucker ratio of 20 day old metacercariae were significantly different among all groups of intermediate and paratenic hosts ($F_{2, 27} = 48.860$, $P < 0.0001$, Scheffé $P < 0.05$; Fig. 1). At 28 DPE, metacercariae were significantly longer in mean body length and had a significantly larger mean v:o sucker ratio in odonate paratenic hosts ($F_{2, 27} =$

25.352, $P < 0.0001$, Scheffé $P < 0.0001$; Fig. 1). However, there was no significant difference in mean body length or mean v:o sucker ratio of 28 day old metacercariae among the 2 species of ostracod intermediate hosts ($P = 0.91$; Fig. 1).

Effects of intermediate and paratenic hosts on metacercariae establishment in definitive hosts

All 15 (100%) toads examined 2 DPE became infected with a mean intensity of 7.1 ± 1.8 (range = 4-10). Of the original 150 metacercariae used to infect toads, 106 (71%) worms established in the stomach of toads. However, there were significant differences in establishment rates of metacercariae among the 5 toad groups ($F_{4, 10} = 6.971$, $P = 0.006$). The mean establishment rates 2 DPE significantly differed among toad groups infected with metacercariae recovered from *Phyllognathus* sp. and *Ischnura* sp. ($P = 0.001$) and *Thermocyclops* sp. and *Ischnura* sp. ($P = 0.002$). In contrast, there was no significant difference in mean establishment rate among metacercariae recovered from all other intermediate and paratenic host combinations ($P > 0.05$; Fig. 2).

Effects of intermediate and paratenic hosts on metacercariae migration and maturity in definitive hosts

All 15 (100%) toads which were exposed to 29 day old metacercariae, and allowed to live throughout the duration of the experiment, contained *H. eccentricus* in their buccal cavities with a mean intensity of 4.6 ± 2.4 (1-9). Of the original 150 metacercariae used to infect toads from all intermediate and paratenic hosts, only 69 (46%) worms migrated to the buccal cavity of toads. There were significant differences in the mean intensity of worms in the buccal cavity among the 5 toad groups ($F_{4, 10} = 17.094$, $P = 0.002$). Scheffé post hoc tests indicated that toads exposed to metacercariae recovered from odonate paratenic hosts had a significantly higher mean intensity of *H. eccentricus* in the buccal cavity than toads exposed to metacercariae recovered from all other microcrustacean intermediate hosts ($P < 0.003$; Fig. 3).

The average time for *H. eccentricus* to migrate to the buccal cavity of toads was 37.6 ± 6.9 (25-48) DPE. However, the average time worms took to migrate to the buccal cavity significantly differed among the 5 toad groups ($F_{4, 10} = 12.232$, $P = 0.0007$). Metacercariae recovered from the 4 microcrustacean intermediate hosts took a significantly longer time to migrate to the buccal cavity of toads than metacercariae used to infect toads from odonate paratenic hosts ($P < 0.006$; Fig. 3).

None of the worms contained eggs when the worms first appeared in the buccal cavity, and egg production only began after the adults reached the area within or surrounding the eustachian tubes of toads. All worms became gravid between 30 and 65 (49.5 ± 9.8) DPE. As with migration time, significant differences also existed in the average time to egg production among the 5 toad groups ($F_{4, 10} = 10.693$, $P < 0.001$). Metacercariae from the 4 microcrustacean intermediate hosts took a significantly longer time to become gravid than metacercariae used to infect toads from odonate paratenic hosts ($P < 0.02$; Fig. 3). None of the toads contained any additional worms in the stomach or buccal cavity when necropsied 90 DPE.

DISCUSSION

This is the first study to examine how using paratenic hosts influences life history traits of *H. eccentricus* in subsequent hosts with comparative laboratory infections of both paratenic and intermediate hosts. By using intermediate host infections as time controls, the age of the worms was a controlled factor that eliminated the possibility that the variation in life history traits between host groups was simply a result of developmental differences because of age. The results of this study indicate that metacercariae of *H. eccentricus* that are of the same age are bigger and have larger ventral-to-oral sucker ratios than metacercariae from intermediate hosts. The larger bodies and suckers of worms from paratenic hosts could be related to their higher establishment rate, earlier migration time, and earlier reproduction than metacercariae from intermediate hosts.

These results suggest that, in addition to increasing the probability of transmission across ecological gaps, paratenic hosts also can provide physiological benefits that give *H. eccentricus* advantages in transmission and development within subsequent hosts.

In the case of *H. eccentricus,* the body and ventral suckers of worms continue to grow to a larger extent within paratenic hosts than within either of the 2 species of ostracod intermediate hosts. This divergence in the growth rate occurred quickly. After only 5 days in the odonates, *H. eccentricus* were significantly larger on average than they were in both microcrustacean hosts, and this trend held through 28 DPE. Additionally, there were variations in the growth of the oral and ventral suckers between the 3 groups of hosts, and despite the sucker ratios being approximately equal at 15 DPE, the pattern of growth of oral and ventral suckers depended on the host species after that time. For example at 20 DPE, the ventral suckers grew at a slower rate than the oral suckers within *Bradlystradesia* sp.; however, the opposite occurred within *Cypridopsis* sp. Then, these growth patterns were reversed at 28 DPE. From 20 to 28 DPE, where the ventral suckers grew faster than the oral suckers within *Bradlystradesia*; however, the oral suckers of worms within *Cypridopsis* sp. grew faster than the ventral suckers. Perhaps most importantly, at both 20 and 28 DPE the ratio of suckers from metacercariae within the odonate always increased, and their suckers were always larger than those within either of the 2 species of ostracod intermediate hosts. It is currently unknown why metacercariae of *H. eccentricus* grow larger in odonatate paratenic hosts compared to metacercariae in microcrustacean intermediate hosts, but in this system odonate paratenic hosts may provide nutrients or other resources that allow metacercariae to become larger than individuals within intermediate hosts.

A study by Zelmer and Esch (1998) also found that metacercariae of the congener *H. occidualis* had larger bodies and larger ventral suckers within field collected odonate paratenic hosts than in laboratory infected ostracod intermediate hosts. They suggested that the growth within odonates was simply a continuation of the growth rate within ostracods that resulted from

the increase in space within the odonate (Zelmer and Esch, 1998). I attempted to address this hypothesis by using a relatively small ostracod, *Cypridopsis* sp. (approximately 0.3 mm in body length), and a much larger one, *Bradlystrandesia* sp. (approximately 4.2 mm in body length), to determine if this difference in body size would affect the growth of metacercariae (Thorp and Covich, 2010). However, despite the drastic difference in the size of these 2 species of ostracod hosts, there were no significant differences in the growth of body length of worms at 15, 20 and 28 DPE or growth of their suckers at 15 or 28 DPE. Additionally, there was a significant difference in body length and sucker ratios between metacercariae from odonates and both species of ostracods. This suggests that space was not likely to be the only limiting resource, and odonate paratenic hosts provide some other resource that affects growth and development of *H. eccentricus*.

The increased rate of growth and development of metacercariae within odonates appear to carry over to affect life history traits within the definitive hosts. On average, significantly more metacercariae established in toads 2 DPE from odonates than from the 2 copepod species, but there were not significant differences between the odonate and either ostracod groups at 2 DPE. In stark contrast, significantly more worms appeared in the buccal cavity of toads when infected with metacercariae from odonates than from the 4 microcrustacean species. This suggests that worms that use paratenic hosts are able to migrate from the stomach to the buccal cavity more successfully than worms from intermediate hosts. Additionally, worms from paratenic hosts arrived in the buccal cavity of toads and began producing eggs earlier than metacercariae that developed within any of the intermediate hosts. By reproducing early, *H. eccentricus* that develop in odonates could have a fitness advantage over those individuals that develop within microcrustacean hosts, assuming that the rates of egg production and longevity of adults that came from microcrustaceans and odonates are similar. Collectively, these results suggest that the variation in body sizes and sucker sizes within odonate paratenic hosts and microcrustacean

intermediate hosts may carry over to the definitive host, and in the case of *H. eccentricus*, using the paratenic host has a significant advantages in establishment, migration, and reproduction within the definitive hosts.

Currently, the exact mechanisms that cause worms from odonates to migrate faster, establish in the mouth more frequently, and begin egg production earlier is unknown, but their larger bodies and suckers may play an important role. It is possible that these metacercariae had greater success in migration and establishment because their large suckers allowed them to remain attached to the active digestive tract of the host, and the metacercariae could migrate faster because the larger body sizes created a greater working distance between suckers. Additionally, the metacercariae from microcrustraceans may take longer to appear in the mouths because they might require a longer period of time for development within the stomach of the definitive host before they migrate to the buccal cavity. In contrast, worms from odonates are larger and so they may require less time to develop in the stomach, and therefore, begin migration sooner. However, at this time, the mechanism that allows metacercercariae from odonates to complete the migration from the stomach to the buccal cavity earlier are still unknown, and should be examined in future studies.

Furthermore, future studies are needed to determine why worms from odonates mature faster. *Halipegus eccentricus* only began producing eggs once worms arrived in the eustachean tubes. Because worms from odonates arrive in the buccal cavity sooner than those from microcrustaceans, these worms could receive a cue or the resources necessary to trigger egg production earlier. Furthermore, metacercarie of *H. eccentricus* from odonates were larger than worms of the same age from microcrustaceans, and therefore, worms from odonates could be further developed to begin reproducing earlier. It seems likely that a combination of these factors may be occurring, however, future work is needed to determine the exact causes for the differences in life history traits between worms from paratenic and intermediate hosts. However,

perhaps more importantly, the results of the current study are important because they illustrate that in the laboratory *H. eccentricus* that use a paratenic host have several advantages in establishment and development over worms from an intermediate host species.

Previous studies have attempted to examine the role of odonates in the life cycles of *Halipegus* species (Kechemir 1978; Zelmer and Esch, 1998; Bolek et al., 2010). From this work, odonates have been identified as a paratenic hosts for *H. occidualis* (Zelmer and Esch, 1998) and *H. eccentricus* (Bolek et al, 2010); whereas, they are reported as an intermediate hosts for *H. ovocaudatus* (Kechemir, 1978). The results of the present study suggest that odonate hosts for *H. eccentricus* may be somewhere in between a paratenic host, that acts solely to bridge an ecological gap, and an intermediate host that is physiologically required for the completion of the life cycle.

The benefits of using a paratenic hosts by *H. occidualis* has also been evaluated, and Zelmer and Esch (1998) concluded that using a paratenic host did not affect the rate of development within the anuran definitive host. These results are in stark contrast to the present study. However, *H. eccentricus* and *H. occidualis* are distinct species and distinct evolutionary trajectories. It is likely that the benefits of using a paratenic host will vary across parasite species, and their host parasite evolutionary histories. Furthermore, interactions that have occurred between a parasite and it hosts over evolutionary time are unique to that system, and therefore, it is difficult to make general conclusions about host usage across species. Unfortunately, Zelmer and Esch (1998) evaluated the migration of metacercariae of *H. occidualis* into the buccal cavity of green frogs by comparing metacercariae of a known age recovered from laboratory infected ostracod intermediate hosts to previously studies by Krull (1935) and Macy et al. (1960) who exposed amphibians with metacercariae recovered from field collected odonate paratenic hosts. Because the metacercariae from field collected odonate hosts were of an unknown age, it is difficult to account for any developmental differences, due to age or environment, in

metacercariae of *H. occidualis* recovered from field-collected paratenic hosts by Krull (1935) and Macy et al. (1960) and of worms from experimentally infected intermediate hosts by Zelmer and Esch (1998). As a result, it is difficult to make any comparisons between the current study and those of Zelmer and Esch (1998).

Additionally, comparisons between this study and the studies by Zelmer and Esch (1998) and Kechemir (1978) should be made with caution because all 3 studies used different species of ostracod and copepod intermediate hosts. In this study, significant differences were observed in the development of metacercariae within 2 ostracod species, suggesting that differences in development of other *Halipegus* species in different microcrustacean hosts may confound any clear comparisons as to the role of odonates as paratenic or intermediate hosts. This is particularly important since nothing is known about microcrustacean host use for any species of *Halipegus* in nature. Previous work on various helminth groups indicates that helminth larval growth in the intermediate host can affect the survival and fitness of adult helminths within the definitive host. Therefore, the parasite's fitness is related to the worm's ability to obtain resources from the intermediate host (Rosen and Dick 1983; Lafferty and Kuris, 2002; Steinauer and Nickol, 2003; Benesh and Hafer, 2012). As a result, the differences in the role of odonates as either intermediate or paratenic hosts in previous studies on *Halipegus* species could be relate to the choice of intermediate microcrustacean host species used in those studies. For example, Kechemir (1978) determined that odonates must be intermediate hosts for *H. ovocaudatus* because the cyclopoid copepods used as intermediate hosts were not able to infect amphibians. However, in the current study, both species of copepods produced the fewest adult worms in experimentally infected toads suggesting that they were the least suitable of the 4 hosts for the establishment of *H. eccentricus* within amphibian definitive hosts. Hence, it is difficult to make any comparisons between these previous studies that used different species of intermediate hosts to accurately depict the role of paratenic hosts in life cycles of other species of *Halipegus*.

In conclusion, this is the first study to use experimental infections of both paratenic and intermediate hosts and the appropriate time controls to examine if life history traits of parasites can be altered by using paratenic hosts. These results suggest that *H. eccentricus* appears to benefit from using a paratenic host by establishing infections more frequently, migrating earlier to its final habitat within the definitive host, and reproducing earlier than worms that only use intermediate hosts. In this specific case, the odonate host appears to represent an evolutionary transition from a paratenic host to an intermediate host. For example, early in their evolutionary history paratenic hosts may benefit the parasite only by bridging trophic gaps between obligate hosts, however, over their long evolutionary history other interactions between the parasite and the host may take place resulting in other benefits from their use. If these benefits accumulate over the evolutionary time span, then the paratenic host could become physiologically necessary for the completion of the life cycle. Clearly, other experimental studies that use appropriate controls along with robust phylogenetic hypotheses for specific helminth groups are needed to fully understand the role of paratenic hosts in the evolution of complex life cycles in helminths.

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Table I. Prevalence and mean abundance of *Halipegus eccentricus* metacercariae recovered from 4 species of intermediate hosts 29 days post exposure. Lower case letters represent significant differences in mean abundance among host combinations ($P < 0.0005$ for all significant differences).

	Phyllognathus sp.	*Thermocyclops* sp.	*Cypridopsis* sp.	*Bradlystrandesia* sp.
Measure of parasitism				
Prevalence ± 95% CI	74 ± 6.78%	74 ± 6.97%	93 ± 2.24%	70 ± 4.88%
(No. infected/No. exposed)	(119/161)	(112/152)	(462/498)	(238/339)
Mean abundance ± 1 SD	0.7 ± 0.4	0.7 ± 0.4	0.9 ± 0.3	0.7 ± 0.5
Range	0-1[a]	0-1[a]	0-1[b]	0-1[a]

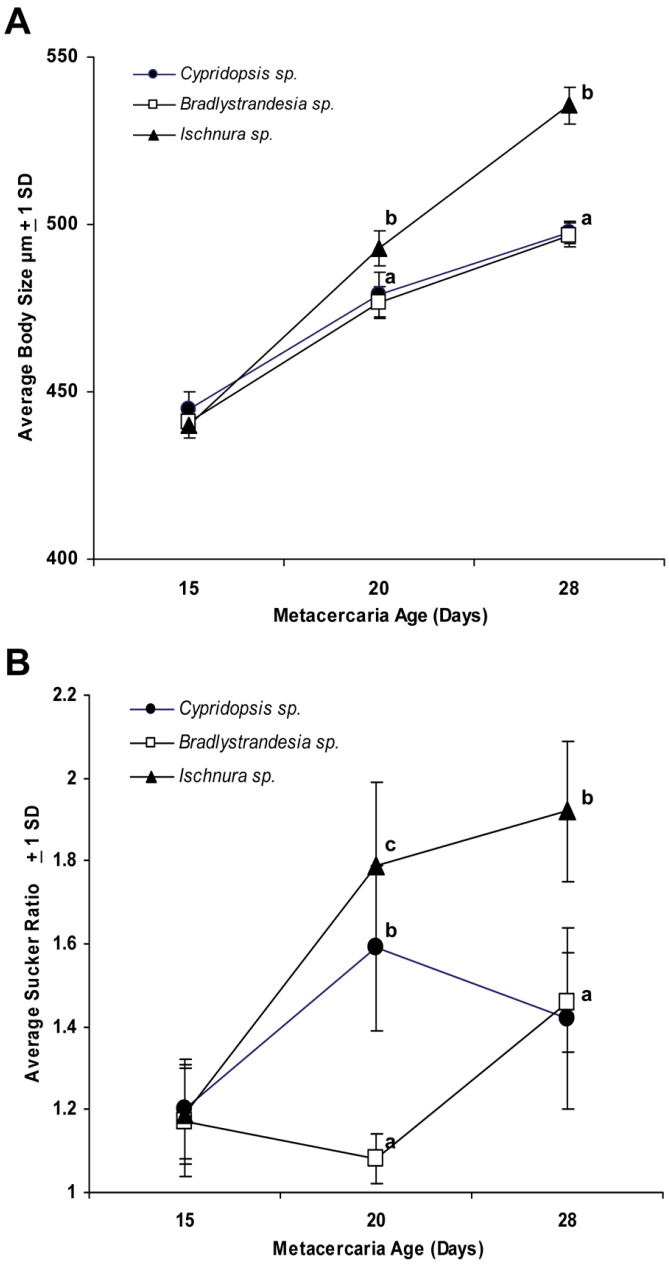

FIGURE 1. Mean body length (**A**) and mean ventral:oral sucker ratio (**B**) of 15, 20, and 28 day old metacercariae recovered from *Cypridopsis* sp. and *Bradlystrandesia* sp. intermediate hosts and *Ischnura* sp. paratenic hosts. Lower case letters represent significant differences in means among host combinations at the 3 developmental times ($P < 0.0001$ for all significant differences).

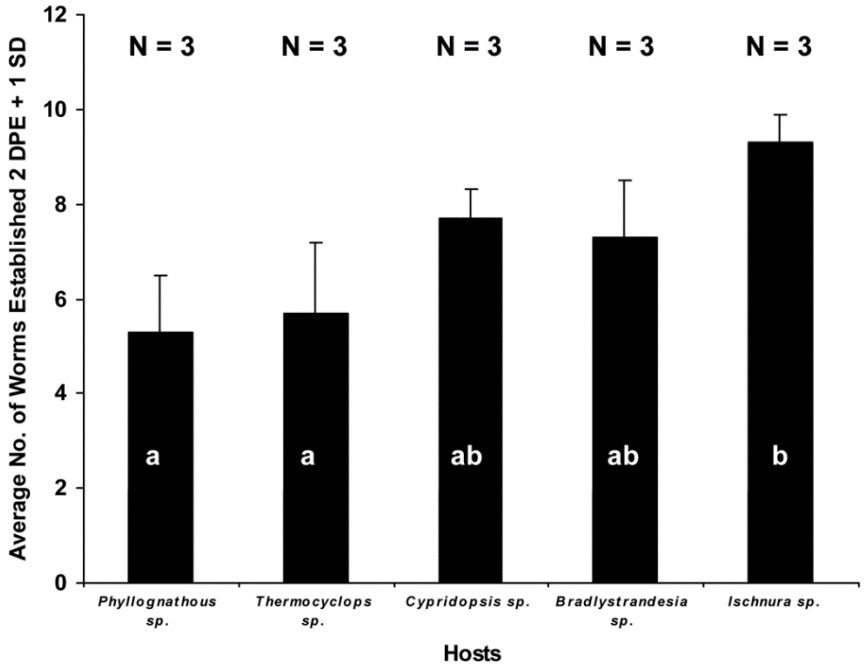

FIGURE 2. Average number of worms that established 2 days post exposure in the stomach of toads exposed to metacercariae recovered from 4 species of microcrustacean intermediate hosts and an odonate paratenic host species. N = number of toads in each group. Lower case letters represent significant differences in means among host combinations at the 3 developmental times ($P < 0.002$ for all significant differences).

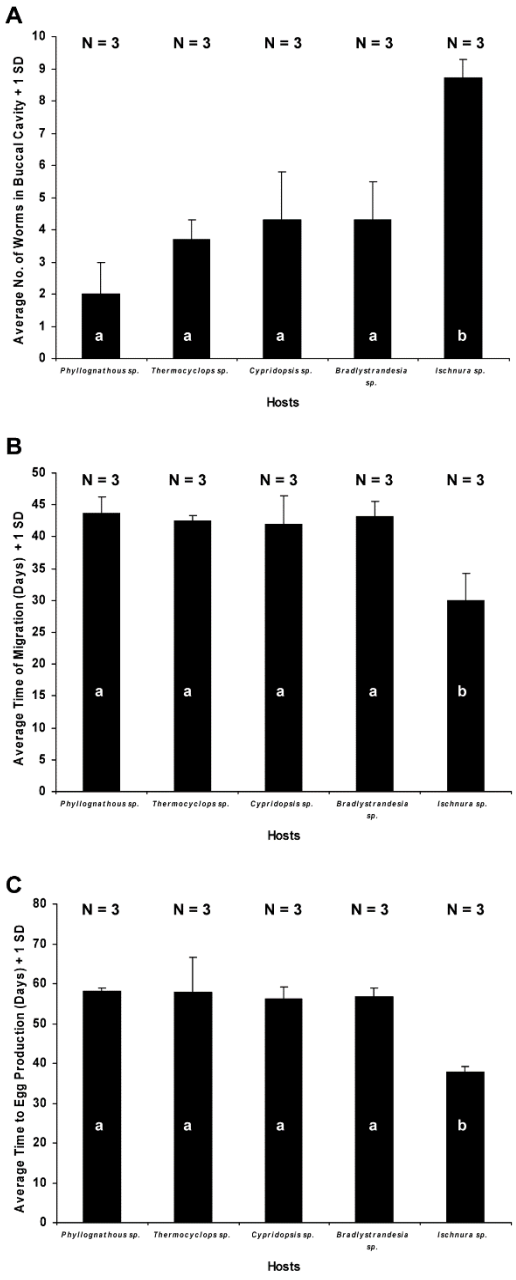

FIGURE 3. Average number of worms in the buccal cavity (**A**), average time of migration (**B**), and average time to egg production (**C**) in toads exposed to metacercariae recovered from 4 species of microcrustacean intermediate hosts and odonate paratenic hosts. N = number of toads in each group. Lower case letters represent significant differences in means among host combinations at 3 developmental times ($P < 0.02$ for all significant differences).

CHAPTER IV

ANURAN HOST SPECIES INFLUENCES SITE FIDELITY OF *HALIPEGUS OCCIDUALIS*

ABSTRACT: Helminths often demonstrate preferential site selection in which a parasite will only occur in one microhabitat or a restricted portion of its fundamental niche within its host. However, factors responsible for helminth site specificity are poorly understood and very little is known about how these factors vary among multiple host species. Some helminths, such as *Halipegus occidualis*, have been reported from different habitats (stomach or under the tongue) within multiple anuran host species suggesting that the site selected varies within anuran species. This study examined the site selection by *H. occidualis* in 7 definitive anuran host species using experimental infections. Then, the site fidelity of *H. occidualis* was further tested by transplanting worms from under the tongue to the stomach and vice versa in different anuran host combinations, and the movement of worms was documented. *Halipegus occidualis* occupied the habitat under the tongue in 6 of 7 anuran species. However, worms always occupied the stomach of bullfrogs and were never found under the tongue or in the mouth of these hosts. More importantly, all worms remained in the original habitat when transplanted from the stomach to the stomach or the buccal cavity to the buccal cavity within another individual of the same amphibian species. However, when worms were transplanted from the stomach to the buccal cavity or vice versa in the same host species, the worms always migrated back to the original habitat.

The main contribution of this study is that it experimentally documented the variability in the site fidelity of *H. occidualis* within multiple definitive host species and determined that site fidelity is not as strongly conserved in this genus as suggested previously. Additionally, this work suggests that the variation in site selection in different host species could lead to speciation of the parasites if host populations do not overlap.

INTRODUCTION

Many species of helminths occupy a restricted portion of their fundamental niche suggesting that the worms actively choose optimal microhabitats within their hosts. This preferential site selection by helminths within their hosts has been recognized for over a century (see Looss, 1905; Fűlleborn and Schilling-Torgau, 1911) and has been a well-documented phenomenon for various parasites (Crompton, 1973; Holmes, 1973; Sukhdeo and Bansemi, 1996). Despite decades of intensive research efforts, the factors that influence site selection by helminth species still remains one of the most poorly understood areas in parasitology (Goodchild, 1954, Sukhdeo and Bansemi, 1996).

Although these efforts did not lead to a clear identification of factors that enable site selection by helminths, collectively, this work has led to 2 general conclusions. First, many parasites are capable of choosing a site of attachment based on their perception of the environments within hosts, and these worms respond to stimuli that ultimately direct them toward a final habitat. Since a parasite's environment within a host is considered to be well defined and extremely predictable among individuals of the same host species, these stimuli should also be consistently present among those hosts (Holmes, 1973; Mettrick & Podesta, 1974; McVicar, 1979; Price, 1987; Sukhdeo, 1990). Hence, it not surprising that most parasites have evolved to consistently respond to their environmental conditions. Secondly, as a whole, previous work has demonstrated that the factors that control the process of site selection by parasites are different

among parasites species. Not all parasites species respond in the same way to host factors, and therefore, the process of site selection may be unique to each parasite-host system. Furthermore, a single helminth species also may not behave in the same way across multiple host species if there are differences in the quality of habitat that those hosts provide. However, very few previous studies have experimentally examined if site fidelity of helminths varies across multiple host species (Bolek et al., 2010).

Hemiurid trematodes in the genus *Halipegus* are a good model system for examining the site selection by helminths within different host species using an experimental comparative approach. There are currently 22 valid species of *Halipegus* reported from around the world, and all of these species are suggested to be highly site specific with each species occurring in only 1 habitat within their amphibian definitive hosts (Bolek et al., 2010; Zelmer and Brooks, 2000; León-Règagnon and Romero-Mayén, 2013). Additionally, the site specificity of *Halipegus* species is thought to be so conserved that the site of adult gravid worms has been used for species identification of the 2 North American species, *H. eccentricus* and *H. occidualis*, because the adults of these 2 species exhibit little morphological variation (Goater et al., 1990; Zelmer and Brooks, 2000; Bolek et al., 2010).

However, the site occupied by adult worms may not be a suitable characteristic for identification of all *Halipegus* species because the site fidelity of some of these species, e.g. *H. occidualis,* may be variable within different definitive hosts. *Halipegus occidualis* has been reported most frequently from under the tongue of green frogs, *Rana clamitans.* However, *H. occidualis* also has been reported from the stomach of other anuran species including American bullfrogs, *Rana catesbiana,* red-legged frogs, *Rana daytonii* and an unidentified species of leopard frog from the *Rana pipiens* complex from Mexico (Macy et al., 1960; Andrews et al, 1992; McAlpine and Burt, 1998; Mata-Lopez et al., 2010). More importantly, although *H. occidualis* was originally described from the margins of the mouth of American bullfrogs

(Stafford, 1905), it has not been reported from the buccal cavity of bullfrogs since its original description despite more than 30 parasitological surveys of bullfrogs throughout North America (Brooks, 1976; Andrews et al., 1992; Mata-Lopez et al., 2010; Bolek et al., 2010).

Reports of *H. occidualis* from 2 separate habitats in amphibian definitive hosts are conflicting with the previous conclusion that both species of North American *Halipegus* (*H. eccentricus* and *H. occidualis*) demonstrate strictly conserved site fidelity in different species of anuran definitive hosts. One explanation for these conflicting observations is that trematodes recovered from the stomach of bullfrogs, leopard frogs, and red-legged frogs were misidentified as *H. occidualis*. This may be likely given that the adults of most species of *Halipegus* are morphologically indistinguishable and site specificity of *Halipegus* species has always been described as being strictly conserved. Additionally, Bolek et al. (2010) found that the other North American species, *H. eccentricus,* occupied the same habitat regardless of the 3 definitive host species were infected in the laboratory. If the same is true for *H. occidualis,* adults of this species should establish only under the tongues of all definitive host species, and these worms should not be located in any other habitat. Under this scenario, gravid individuals of *Halipegus* sp. that occupy the stomach and those found under the tongue of different anuran species would be considered distinct species. However, if the worms from under the tongue and in the stomach of anurans were in fact *H. occidualis*, then the site fidelity of this species may not be as strongly conserved as has been suggested previously.

The first objective of this study was to experimentally examine the site occupied by gravid individuals of *H. occidualis* in 6 anuran species. Second, the strength of the specificity for the original habitat occupied by *H. occidualis* was tested by transplanting worms to other site (mouth or stomach) in different anuran species. The primary contribution of this study is to determine if the site fidelity of *H. occidualis* is variable in multiple definitive host species. If the site occupied by this species is variable, then the methods for identifying *Halipegus* species in

North America should be reevaluated. Additionally, this work is important since it suggests a mechanism that could promote speciation of *Halipegus* species because segregation of worms in different habitats within different species of amphibian hosts could result in speciation when amphibian host species are isolated.

MATERIALS AND METHODS:

Halipegus occidualis, *Planorbella trivolvis*, and *Cypridopsis* sp. used for this study were obtained from the laboratory stock cultures described in Appendix A. *Rana sphenocephala*, *Rana catesbeiana*, *Bufo woodhousii*, and *Bufo americanus* were collected from San Borne Lake, Stillwater, Payne County, Oklahoma (36° 9' 17.8014", -97° 4' 40.4034"); whereas, *Rana clamitans*, *Hyla versicolor*, and *Hyla cinerea* were collected from Clayton Lake, Pushmataha County, Oklahoma (34° 32' 27.6432", -95° 18' 34.38"). Both of these locations are known to be free of *Halipegus* species (Stigge, unpublished personal observations). Additionally, 2 adult *R. catesbiana*, were collected from James Creek Pond (36° 8' 50.661"N, -97° 4' 50.0412"W), where, at the time, the prevalence of *H. occidualis* in bullfrogs was 88.1% (89/101; Stigge, unpublished observation).

Site fidelity of *H. occidualis* within 6 amphibian species

Cercariae used in this study were shed by 4 laboratory infected *Planorbella trivolvis* that were all infected with eggs from a single adult *H. occidualis* removed from the stomach of a bullfrog. Each day, cercariae were pooled in 300 ml stackable processing dishes and then used to infect second intermediate ostracod hosts, *Cypridopsis* sp. To obtain ostracod infections, approximately 100 cercariae and 30 ostracods were placed in each well of a 6-well culture plate containing 15 ml of aged tap water. Ostracods fed on the cercariae for 24 hours, and then, all ostracods were pooled by exposure date and transferred to 300 ml stackable processing dishes containing 250 ml of aged tap. Approximately half of the water in each dish was removed and

replaced with aged tap water, and several drops of puréed romaine lettuce was added to each dish as a food source every 2 days. Exposed ostracods were maintained on this regimen for 20 days, at this time metacercariae are capable of losing their excretory bladder and infecting amphibian definitive hosts (see Appendix A).

At 20 DPE, anurans were exposed to *H. occidualis* by pipetting 20 exposed ostracods into the stomach of each individual of 6 amphibian species which included American bullfrogs *R. catesbeiana* (N=32), Woodhouse's toads, *B. woodhousii* (N=24), American toads, *B. americanus* (N=27), eastern gray tree frogs, *H. versicolor* (N=13), green tree frogs, *H. cinerea* (N=8), and southern leopard frogs, *R. sphenocephala* (N=12). All anurans used in experimental infections were housed in groups of 2-5 individuals based on species in 37 L aquaria. Anurans were provided a 5 cm gravel as a substrate and a plastic water dish filled with aged tap water. Anurans were maintained at 24 C and 24L:0D period and fed crickets and meal worms *ad libitum*. Water was changed every other day. After exposure, the buccal cavity of each anuran was checked daily for worms, and once *H. occidualis* were present they were individually removed from the mouth and checked for the presence of eggs by examining a wet mount of each worm with compound microscope. After worms were examined for the presence of eggs, all worms were placed back into the mouth of the anuran from which they were removed. Adult worms were considered gravid when at least 1 egg was observed in the uterus. All anurans were euthanized and dissected 95 DPE, and the entire alimentary canal was examined for the presence of worms to ensure that all worms were counted. Additionally, after dissections eggs from each worm were observed for the presence of a long abopercular filament that is a defining characteristic for *H. occidualis* (Krull, 1935). Finally, 2 naturally exposed adult bullfrogs, collected from James Creek Pond, an area with a high prevalence of *H. occidualis*, were maintained in the laboratory as previously described and their mouths were checked for worms daily for the first 95 days after collection. At this time 1 of the bullfrogs was dissected and examined for *Halipegus* species. The mouth of the

second naturally exposed bullfrog was examined for worms once a week between 95 and 365 days after collection. After 1 year, the bullfrog was dissected and examined for *Halipegus* species.

Transplant experiments

Transplant experiments were conducted in 3 species of anurans to determine if adult *H. occidualis* would demonstrate the same specificity for their original habitats when worms were transplanted from under tongue to the stomach or vice versa in different anuran species. First, 30 gravid adults of *H.* occidualis were removed from under the tongue of 3 gray tree frogs and 2 *H. occidualis* individuals were transplanted to the area under the tongue in each of 5 uninfected gray tree frogs which served as positive controls, and 5 uninfected green frogs and 5 uninfected bullfrogs, both of which are natural definitive hosts for *H. occidualis* in nature. Second, reciprocal transplant experiments were conducted by removing 8 adult worms from the stomach of an experimentally infected bullfrog (see above) to test the specificity of these worms after relocation. In this case, groups of 2 worms each were pipetted into the stomach of 1 uninfected gray tree frog and the stomach of an uninfected bullfrog. The remaining 4 worms were divided into groups of 2 and transplanted from the stomach of the bullfrog to under the tongues of an uninfected gray tree frog and an uninfected bullfrog.

The 5 bullfrogs in the first set of transplant experiments were dissected 2 weeks after worms were transplant to determine if worms that did not remain under the tongue were lost or if they had migrated to the stomach. All remaining anurans were dissected 58 days after worms were transplanted.

Statistical Analyses

Prevalence and mean abundance of *H. occidualis* in each species of amphibian host was calculated according to Bush et al. (1997). A 1-way analysis of variance (ANOVA) and Scheffé post hoc tests were used to compare differences in mean abundance of worms in the mouth, and

the average time worms migrated to the buccal cavity in different amphibian species (Sokal and Rohlf, 1981). All values are reported as a mean ± 1 SD (range), and 95% confidence intervals are reported for prevalence (Sokal and Rohlf, 1981).

RESULTS

Site selection of *H. occidualis* within 6 amphibian species

All 6 anuran species became infected with gravid adults of *H. occidualis*, however, not all individuals of each anuran species were infected. Prevalence was highest in American toads (93%) and lowest in southern leopard frogs (67%); whereas mean abundance was highest in Woodhouse's toads (8.1 ± 2.4) and lowest in southern leopard frogs (5.5 ± 1.4; Table I). All gravid worms contained eggs with long abopercular fillaments characteristic for *H. occidualis*, however, gravid worms did not occupy the same habitats in some of the anuran species (Fig. 1). Within 60 DPE, *H. occidualis* appeared in the buccal cavities of all of anuran species except bullfrogs (Table I). Worms that infected bullfrogs were never observed in the mouth during the 95 days that anuran mouths were examined. However, when the 6 anuran species were dissected 95 DPE, gravid worms were found in the stomach of only bullfrogs, and all other worms were located under the tongue of all infected individuals of the 5 other amphibian species (Fig. 1).

There were not significant differences in mean abundance of *H. occidualis* among the 5 anuran species that had worms in their buccal cavity ($F_{4,66} = 1.874$, $P = 0.125$). However, there were significant differences in the average time it took worms to migrate from the stomach to the buccal cavity in the 5 anuran species which had worms under their tongues ($F_{4,66} = 9.436$, $P < 0.0001$). Worms took a significantly longer time to migrate to the buccal cavity of southern leopard frogs ($P < 0.005$); whereas the average time of migration from the stomach to the buccal cavity in all other possible host species combinations were not significantly different from each other ($P > 0.05$; Table I).

Finally, observation on 2 naturally infected bullfrogs collected from James' Creek Pond, and maintained in the laboratory for up to a year, indicated that *H. occidualis* never migrated into the mouth of these 2 frogs. However, upon necropsy, 5 and 3 *H. occidualis* (identified based on egg morphology see Fig. 1) were recovered from the stomach of these bullfrogs 95 and 365 days after being collected from James' Creek Pond, respectively.

Transplant experiments

The 20 worms transplanted from under the tongue of gray tree frogs remained under the tongue of all 5 gray tree frogs and all 5 green frogs for the entire duration of the experiment (58 days). None of the worms in these 2 anuran species were observed migrating into different habitats during this time, and importantly all worms remained attached to the same lingual vein under the tongue where they originally attached after being transplanted. Additionally, no *H. occidualis* individuals were found in the digestive tract of any of these anurans when they were dissected 58 DPE. In contrast, all 10 worms that were transplanted from under the tongue of a gray tree frog to under the tongue of 5 bullfrogs moved from their site of transplant within the first 24 hrs. Over a period of 6 to 8 days, worms were observed in different locations within the mouths of bullfrogs, including being found on different lingual veins under the tongue as well as the margins and roof of the mouth. After being transplanted, worms remained in the mouth of bullfrogs for 6 to 8 days (6.8 ± 0.8), and at 7 days post transplantation some worms were observed migrating down the esophagus of bullfrogs (Fig. 2). Two weeks after being transplanted, necropsies revealed that 9 of 10 transplanted worms were recovered attached to the stomach of 5 bullfrogs and no other worms were found in any other location.

Additionally, 2 worms transplanted from the stomach of a bullfrog to under the tongue of a gray tree frog, remained under the tongue of the gray tree frog for 58 days. In contrast, the worms that were transplanted from the stomach of a bullfrog into the mouth of a second bullfrog migrated to the stomach within 6 days of being transplanted, and both worms were recovered in

the stomach 58 days after transplant. Finally, 2 worms that were transplanted from the stomach of a bullfrog into the stomach of a gray tree frog migrated in to the mouth of the tree frog and attached to the lingual veins under the tongue 5 and 7 days after being transplanted. In contrast, the 2 worms removed from the stomach of a bullfrog and transplanted into the stomach of a second bullfrog, never migrated from the stomach to the buccal cavity. Fifty-eight days after being transplanted and upon necropsy, 1 of the 2 *H. occidualis* was recovered attached to the stomach of the second bullfrog.

DISCUSSION

The primary contribution of this study is that it experimentally documents the variability in the site fidelity of *H. occidualis* in multiple definitive host species. All anuran infections during this study originated from the same laboratory culture of *H. occidualis* established using eggs collected from gravid individuals of *H. occidualis* that were taken from the stomach of naturally infected bullfrogs (see Appendix A). Even though worms from the stock culture originated from the stomach of bullfrogs, gravid worms established under the tongue of 5 of 6 anuran species experimentally infected with metacercariae. However, worms were never observed in the mouth of bullfrogs despite the numerous individuals recovered from the stomach of these frogs at the end of the study. Additionally, *H. occidualis* from naturally infected bullfrogs never appeared in the mouths of these hosts suggesting that adult worms from natural and experimental infections behave similarly in bullfrogs.

Halipegus occidualis was consistently attracted to the same site of the 2 habitats in each of the anuran species after the adult worms were transplanted. All worms remained in place when they were transplanted from the original habitat into the same habitat within another individual of the same species (i.e. stomach to stomach or buccal cavity to buccal cavity). However, when worms were transplanted from the stomach of one host to the buccal cavity of a second individual

of the same host species, or vice versa, the worms always migrated back to the original habitat. For example, *H. occidualis* transplanted from under the tongue of gray tree frogs remained under the tongue of a second group of gray tree frogs. Furthermore, the worms from under the tongue of gray tree frogs also remained under the tongues green frogs, which is the typical habitat and host species from which *H. occidualis* is reported in the eastern part of its range (Goater et al., 1989; Zelmer et al., 1999). Worms also remained under the tongue of the uninfected tree frog when transplanted there from the stomach of a bullfrog. However, when worms were transplanted from the stomach of a bullfrog to the stomach of an uninfected gray tree frog, the worms migrated into its buccal cavity within 5 to 7 days of being transplanted. Additionally, when worms were transplanted from either under the tongue of tree frogs or the stomach of bullfrogs to under the tongue of bullfrogs the worms always migrated out of the buccal cavity into the stomach of bullfrogs within a few days of being transplanted. However, when worms were transplanted into the stomach of bullfrogs the worms remained there for at least 58 days, when bullfrogs were necropsied.

Although this study did not test for the specific mechanisms that determine site selection by *H. occidualis* in different anuran species examined in this study, it suggests that there must be some fundamental difference in the stomach and/or mouth environment provided by bullfrogs and the other 6 anuran host species examined. However, at this time, we cannot conclude if the mouth or stomach environment in bullfrogs and the other 6 anuran species provided a less suitable environment for *H. occidualis*, respectively. Previous work on site selection by helminths suggests that other helminth species respond behaviorally to biochemical cues in the host (Sukhdeo and Mettrick, 1986). Therefore, it is possible that worms within bullfrogs receive a specific biochemical cue to stay in the stomach while that cue is not present in the other host species examined in this study. Additionally, the difference in site selection within bullfrogs could also result if worms are incapable of migrating out of bullfrog stomachs. However, this

scenario is highly unlikely since a congener, *H. eccentricus*, always migrates from the stomach to the buccal cavity of bullfrogs (Bolek et al., 2010). Clearly, future studies are needed to understand the factors responsible for site selection by *H. occidualis* in different anuran hosts.

More importantly, this work demonstrates that the site fidelity of *H. occidualis* is variable in different anuran species. Adult worms always occurred under the tongue in 6 of 7 anuran species that were experimentally examined, but were always found in the stomach of bullfrogs. From a phylogenetic perspective, it appears that anuran relationships did not affect the site selection of *H. occidualis* within their hosts, and worms occurred in the same habitat in distantly related species of anurans. For example, 6 species of anurans from 3 families including bufonids, hylids as well as ranids contained worms under their tongues. This observation is particularly interesting when considering that North American bufonids and hylids have never been reported as hosts for *H. occidualis* or any other *Halipegus* species in nature (Prudhoe and Bray, 1982). In contrast in this study the laboratory infections indicated that *H. occidualis* occurred in the stomach of bullfrogs but under the tongue of its sister species, the green frog (Austin et al., 2003; Lannoo, 2005). Notably, field surveys indicate that gravid *H. occidulais* have never been reported from under the tongue of bullfrogs or the stomach of green frogs despite numerous field surveys on both of these frog species (Brooks, 1976; Andrews et al., 1992; Zelmer et al., 1999; Bolek and Coggins, 2001; Bolek et al., 2010; Mata-Lopez et al., 2010). These observations suggest that bullfrogs maybe distinct from other anuran species in their influence on the site fidelity of *H. occiduals*. However, field surveys have also reported *H. occidualis* from the stomach of other amphibian species including various species of salamanders and red-legged frogs from the western United States and an unidentified species of leopard frog from Mexico (Prudhoe and Bray, 1982; McAlpine and Burt, 1998). Taken together these observations suggest that adult *H. occidualis* may reside in the stomachs of other amphibian species which have not been surveyed or experimentally infected in the laboratory.

These results bring into question if the site fidelity of *Halipegus* species within different species of amphibian hosts is a reliable character for species identification as has been suggested by others (Goater et al., 1989; McAlpine and Burt, 1998; McAlpine, 2006). The identification of *Halipegus* species based on the literature is arduous. The taxonomic status and nomenclature of most *Halipegus* species throughout the world is confusing and difficult to decipher because the majority of species were inadequately described and many species have been synonymized (McAlpine, 2006). Furthermore, species identifications of most *Halipegus* species are more difficult than many other helminth species because adults of most *Halipegus* species exhibit little morphological variation (Zelmer and Brooks, 2000). For example, in the case of the 2 valid North American species, *H. occidualis* and *H. eccentricus*, the names have changed repeatedly because the original descriptions are unclear (McAlpine and Burt, 1998; Zelmer and Esch 1999; McAlpine, 2006). As a result, some studies have relied on the morphology of non-adult stages, including eggs and cerciarae (Paraense, 1992), as well as the habitat of adult worms for species identification (Goater et. al., 1990; McAlpine and Burt, 1998; Zelmer and Esch 1999). However, descriptions of eggs and/or cercarial stages for most species of *Halipegus* are unknown and as a result, the habitat occupied by gravid worms has become the primary method for identifying *Halipegus* species, particularly those that occur in North America. Taken together, these observations suggest that because the site of infection appears to be a variable characteristic for at least some *Halipegus* species, species misidentifications can easily occur.

Understandably, the site occupied by *Halipegus* species is an exciting and a unique way to identify individuals, especially considering species identification using this method are quick and euthanizing hosts is not required for species that reside within the mouth (Goater et al., 1990; Zelmer et al., 1999). However, based on the results of our study, the site fidelity of *H. occidualis* within different amphibian species is more variable than previously described, and therefore, it alone is not a reliable characteristic for deciphering species. Importantly, 2 other species of

Halipegus from India have been reported from different habitats within different amphibian species suggesting that differences in site selection within multiple host species may occur in other species of *Halipegus* (Yamaguti, 1971). Therefore, investigators that rely on the site of establishment for species identifications in field studies risk misidentifying and/or missing individuals that may not occur in the type locations (Goater, 1989; Zelmer et al., 1999). Future identification of *Halipegus* species should be made with caution, and to do so accurately, investigators should use a combination of genetic data along with adult and non-adult morphological characteristics and habitat of adult worms as suggested previously by McAlpine and Burt (1998).

The discrepancy in the previously reported habitats of *H. occidualis* within bullfrogs, red-legged frogs, leopard frogs, and green frogs is likely result from differences within the anuran species that cause *H. occidualis* to select different habitats and not that the trematodes that reside in the mouth or stomach are distinct species of *Halipegus*. However, the segregation of worms of the same species in different species of hosts provides a mechanism that may play an important role in the diversification of species in this genus of trematodes that infects amphibians worldwide. For example, many of the reports of *H. occidualis* in the stomach of anurans have occurred in the western portions of the United States and Mexico (Prudhoe and Bray, 1982), while all of the reports of *H. occidualis* under the tongue have been from green frogs that do not occur in the western portion of the continent (Lannoo, 2005). Therefore, due to the differences in the site occupied within deferent amphibian species, adult worms occurring in the stomach of anurans in the western portion of North America are subjected to different evolutionary pressures than *H. occidualis* that occur under the tongue of green frogs in the eastern United States. In this case, cross fertilization between the adults in the stomach of some amphibian species, such as the red legged frog, and under the tongue of green frogs cannot occur due to the disjunctive ranges of these amphibian host species, and therefore, these populations of *H. occidualis* may be on separate evolutionary trajectories.

Finally, from an evolutionary perspective, the habitat switch of hemiurids from the stomach of marine fish to the buccal cavity of anurans, including hosts that are not commonly infected with these worms in nature such as true toads and tree frogs (Prudhoe and Bray, 1982), appears to be a relatively recent evolutionary shift in habitats that probably occurred after hemiurid invaded fresh water and eventually colonized semi-terrestrial anurans as hosts. Most hemiurids reside in the stomach of their marine fish definitive hosts (Gibson and Bray, 1979). However, hemiurid trematodes in the genus, *Deropegus*, have been reported from the stomach of freshwater salmonid fish and the stomach of frogs (McCauley and Pratt, 1961) suggesting that some hemiurids were able to make the evolutionary transition from fish to anuran (Gibson and Bray, 1979; Prudhoe and Bray, 1982). Once amphibian hosts were colonized, differences in the habitats provided by the different host species may have selected for some *Halipegus* species to occupy habitats other than the stomach, including the buccal cavity. Furthermore, Zelmer and Brooks (2000) suggested that the presence of some *Halipegus* species in the esophagus of anurans may represent this evolutionary transition from stomach to buccal cavity. Clearly, to test this hypothesis other life cycle studies, along with robust surveys of host will need to be conducted along with solid phylogenetic hypotheses will have to be constructed to get a better understanding of variation in site fidelity in the genus *Halipegus* and other hemiurids.

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Table I. Prevalence and mean abundance of *Halipegus occidualis* adults recovered from 6 species of anuran definitive hosts. If worms occurred in the mouth of the anuran species, the average days post exposure that worms first appear ± 1 SD is presented. Lower case letters represent significant differences between host groups.

	Bufo woodhousii	*Bufo americanus*	*Hyla versicolor*	*Hyla cinerea*	*Rana sphenocephala*	*Rana catesbiana*
Prevalence ± 95% CI	83 ± 15%	93 ± 9%	85 ± 19%	88 ± 23%	67 ± 8%	84 ± 13%
(No. infected/No. exposed)	20/24	25/27	11/13	7/8	8/12	27/32
Mean abundance in mouth ± 1 SD	8.1 ± 2.4[a]	7.4 ± 2.6[a]	7.2 ± 1.8[a]	7.4 ± 1.9[a]	5.5 ± 1.4[a]	0
Range	4-14	5-12	4-10	5-10	3-7	0
DPE arrived in mouth ± 1SD	40.7 ± 3.5[a]	41.8 ± 3.4[a]	42.1 ± 2.6[a]	42.1 ± 2.4[a]	50.1 ± 7.1[b]	N/A
Range	35-49	39-49	39-46	39-46	41-48	N/A
Mean abundance in stomach ± 1 SD	0	0	0	0	0	6.8 ± 2.0
Range	0	0	0	0	0	3-10

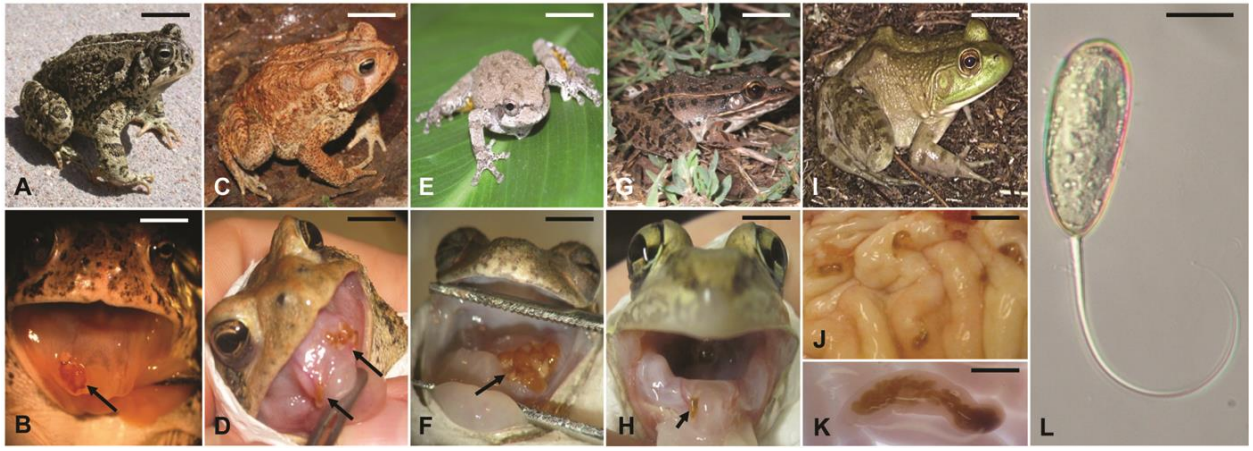

FIGURE 1. Anuran species and location of gravid *Halipegus occidualis* in experimentally infected anuran hosts examined 58 days post exposure. (**A-B**) *Bufo woodhousii* and 4 *H. occidualis* (black arrow) individuals attached to the lingual vein. Scale bars = 1 cm. (**C-D**) *Bufo americanus* and 7 *H. occidualis* (black arrows) individuals attached to the lingual veins. Scale bars = 1 cm. (**E-F**) *Hyla versicolor* and 10 *H. occidualis* (black arrow) individuals attached to the lingual vein. Scale bars = 2 cm. (**G-H**) *Rana sphenocephala* and 1 *H. occidualis* (black arrow) attached to the lingual vein. Scale bars = 1.5 cm. (**I-J**) *Rana catesbiana* and 5 individuals of *H. occidualis* attached to the stomach lining. Scale bars = 2 cm. (**K**) Higher magnification of a gravid *H. occidualis* attached to the stomach lining of an infected bullfrog. Scale bar = 3 mm. (**L**) Typical egg of *H. occidualis* recovered from a gravid worm removed from the stomach of a bullfrog. Note the long abopercualr filament. Scale bar = 20 µm.

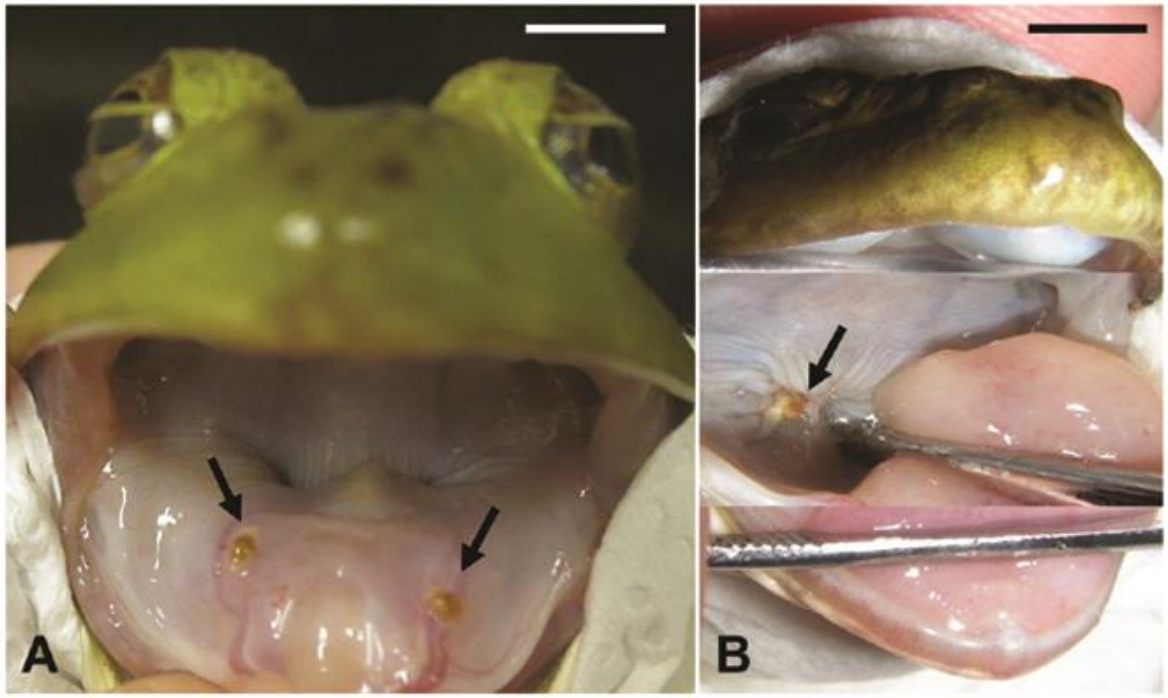

FIGURE 2. Locations of *Halipegus occidualis* in the buccal cavity of a bullfrog within seven days of being transplanted under the tongue. (**A**) Two individuals of *H. occidualis* (black arrows) attached to the lingual veins under the tongue of a bullfrog 12 hours after being transplanted from under the tongue of a gray tree frog. Scale bar = 1.0 cm. (**B**) Seven days after being transplanted, *H. occidualis* (black arrow) in the process of migrating down the esophagus of a bullfrog. Scale bar = 1.0 cm.

CHAPTER V

CONCLUSIONS

Nearly every free-living organism serves as a host to at least one, and in most cases several, parasite species suggesting that parasitism may be the most common and successful way of life (Price, 1980). Interestingly, among the inestimable number of parasite species, complex life cycles have been commonly selected for and have evolved independently in several parasitic groups (Mackiewicz, 1988; Rohde, 1994; Combes, 2001; Poulin, 2007). Additionally, the diversity in the number of hosts and the patterns in host usage is staggering (e.g. Olsen, 1986). However, the simplified life cycle diagrams that are presented in most textbooks underestimate their complexity because they do not represent all of the interactions that occur between parasites and the numerous prospective host species, especially when we consider that natural food webs can be dynamic and very complex (Anderson and Sukhdeo, 2011), and most of the free-living species that could serve as potential hosts for a parasite have not been examined for their parasites. Given the many opportunities for parasites to infect multiple host species and that many parasites may not be specialists at all host levels, it is important to understand how using different host species can affect life histories of parasites and influence their success in completing their life cycle. The objectives of this dissertation were to experimentally examine the extent to which variation in host usage influences life history traits and transmission of trematodes. The data presented in this dissertation are important because, in many cases, these were the first studies to experimentally examine how host use affects the development and transmission of parasites.

Therefore, these results provide the groundwork for future hypothesis-driven studies on the evolution of complex life cycles of parasites.

CHAPTER II: HOST-SPECIFICITY-NOT ALL HOSTS ARE AS SUITABLE FOR GROWTH AND TRANSMISSION

In chapter II, I examined the host specificity of *Halipegus eccentricus* and *Halipegus occidualis* in 3 microcrustacean second intermediate hosts. Host specificity of parasites is a basic principle in parasitology; however, it is not easily measured. Previously, host specificity was calculated as the number of species that a parasite infected (Lymbery, 1989), but this is not an accurate description of host usage because some species that are capable of being infected do not contribute to the completion of the life cycle due to physiological or ecological reasons. More recently, it has been suggested that prevalence should be included in host specificity indices (Rohde 1980 and 1993; Poulin and Mouillot, 2005). However, a high prevalence does not necessarily indicate that the species is a good host because they may not lead to transmission to subsequent hosts and therefore may represent a dead end host in the life cycle. To help resolve these problems, I suggest that measures of host specificity should take into consideration the physiological interactions between a parasite and a potential host species as well as ecological interactions between current and subsequent hosts in the life cycle.

In this study, I evaluated if both *Halipegus* species were capable of infecting 3 phylogenetically distinct second intermediate host species, and then, I examined the extent to which each of these hosts contributed to transmission of each *Halipegus* species to the next host. I found that both species of *Halipegus* infected all 3 microcrustaceans, and the 2 trematodes developed similarly within the same host species. All metacercariae we capable of developing to become infective within 19 DPE regardless of host species; however, there were significant differences in the growth of worms among the 3 second intermediate hosts and the size of worms

at the time of infectivity. Both species of *Halipegus* grew fastest in the ostracod species than either of the copepods. Therefore, metacercariae were largest within ostracods at the point in which they became infective (19 DPE) to the next host in the life cycle.

Additionally, in this study, I found that when all conditions are equal all microcrustacean hosts are not equally suitable for the transmission of *Halipegus* species, and therefore, they should not be weighted equally in host ranges. From the 3 microcrustaceans examined, the ostracods appear to be the most suitable host because they consumed cercariae most frequently, the prevalence of ostracods exposed was highest of the host examined, they were eaten frequently by odonates, and metacercariae were capable of establishing within odonates more often than from the 2 copepod species. Additionally, there were differences between the 2 copepod species. Cercariae that infected the ostracods were 3 times more likely to complete their life cycle through the odonate host than those from cyclopoid copepods, and metacercariae form ostracods were almost twice more likely to do so than those from the harpaticoid copepods. Therefore, the probability of a cercariae being transmitted to the odonate host is not the same in these 3 hosts, and their contributions to the life cycle are not equal.

The major contribution of this study is that it approached host specificity from physiological and ecological aspects including factors that influence transmission between the 2 trematode species and their intermediate hosts, as well as the interactions between each of the 3 intermediate hosts and the subsequent paratenic host. This study documented the growth and development of *Halipegus* species in their second intermediate hosts to determine if all host species are equally suitable for development. Majority of previous studies on trematode host specificity have focused almost exclusively on development in the molluscan first intermediate or definitive hosts. However, relatively little is known on the development and host specificity of trematode metacercariae in invertebrate second intermediate hosts *(e.g.* Snyder and Janovy, 1996). Secondly, this is the first paper to experimentally evaluate host specificity in terms of the

contributions of possible host species to transmission of the parasite to subsequent hosts including how often the host is eaten and the rate of transmission of the parasite between hosts.

CHAPTER III: THE ADDITION OF A PARATENIC HOST CAN AFFECT LIFE HISTORY TRAITS

My primary objective for the study presented in Chapter III was to experimentally evaluate the effects of using a paratenic hosts on life history traits of parasites within their subsequent host. Paratenic hosts have always been described as an optional host that is necessary for the transmission of the parasite, but not for its physiological development. However, their role in the life cycle of parasites, other than bridging ecological or trophic gaps between obligate hosts, has largely has been ignored (Zelmer and Esch, 1998). Present-day paratenic and intermediate hosts are good comparative model systems to experimentally evaluate the extent to which a parasite's life history is altered from the addition of a new host to their life cycle. The number of hosts that have been added or lost over evolutionary time is not easily deciphered, and any changes in the number of hosts in a life cycle cannot be observed in their entirety by one investigator. As a result of these difficulties, little empirical and/or comparative data are available on the benefits of adding a host to a life cycle (Zelmer and Esch, 1998). However, present-day paratenic and intermediate hosts are good comparative model systems to begin to address how hosts may be added to life cycles and become required for their completion.

In this study, I used a comparative approach to determine if *H. eccentricus* developed differently within intermediate hosts and paratenic hosts. For this, the body length and the ratio of oral-to-ventral sucker size of infective metacercariae recovered from 2 species of intermediate hosts were compared with that of individuals of the same age from a paratenic odonate host species. Next, I evaluate how life history traits of *H. eccentricus* within the definitive hosts differ between metacercariae of the same age that developed within intermediate or paratenic hosts.

Specifically, I compared their (1) rate of initial establishment in the stomach, (2) time required to migrate to the buccal cavity, (3) establishment rates within the buccal cavity after migration, and (4) time to maturity.

The results of this study indicate that metacercariae of *H. eccentricus* from the paratenic hosts are bigger and have larger ventral-to-oral sucker ratios than metacercariae that are the same age from intermediate hosts. Additionally, the metacercariae from odonate paratenic hosts had higher establishment rates, earlier migration time, and earlier reproduction with anuran hosts than metacercariae from intermediate hosts. The increased growth of metacercariae within odonates appear to carry over to affect life history traits within the definitive hosts, and the larger bodies and suckers of worms from paratenic hosts could be related to these differences. Furthermore, in chapter I, I found that growth rates of metacercariae varied between different intermediate host species, and by extension, these size differences might affect growth within paratenic hosts and its effects in anurans. In the future, it would be interesting to investigate if using different intermediate host to infect paratenic host would cause greater variation in life history traits within anuran hosts.

Collectively, these results suggest that, in addition to increasing the probability of transmission across ecological gaps, paratenic hosts also can provide physiological benefits that give *H. eccentricus* advantages in transmission and development within subsequent hosts. The major contribution of this study was that it is the first examination of the role of paratenic hosts in parasite life cycles using experimental infections and appropriate time-control groups to determine the extent to which the use of a paratenic hosts affects establishment, survival, and other life history traits. By using intermediate host infections as time controls, the age of the worms was a controlled factor eliminating the possibility that the variation in life history traits between host groups was simply a result of developmental differences because of age.

CHAPTER IV: HOST SPECIES CAN INFLUENCE SITE-SELECTION BEHAVIOR IN SOME *HALIPEGUS* SPECIES.

In chapter IV, I examined if site selection by *Halipegus* species is variable within multiple anuran host species. Helminths often demonstrate preferential site selection in which a parasite will only occur in one microhabitat or a restricted portion of its fundamental niche within its host. However, factors responsible for helminth site specificity are poorly understood and very little is known about how these factors vary among multiple host species. Furthermore, few previous studies have experimentally examined if site fidelity of helminths varies across multiple host species (Bolek et al., 2010).

In this study, I examined the site fidelity of *H. occidualis* in different anuran definitive host species to determine if host species was an important factor that influences site selection by these trematodes. Previous studies by Goater et al. (1989) indicate that *H. eccentricus* and *H. occidualis* always demonstrate strong site specificity in their definitive hosts. However, the site occupied by *H. occidualis* appears to be variable because it has been reported from under the tongue of green frogs and from the stomach of other anuran hosts including bullfrogs (Macy et al., 1960; Andrews et al, 1992; Wetzel and Esch, 1996; McAlpine and Burt, 1998; Schotthoefer et al., 2009; Mata-Lopez et al., 2010). Since adults of *Halipegus* species are known to occupy only 1 site within their definitive hosts, this could suggest that either the worms in the mouth and stomach are separate species or it indicates that the site fidelity of *H. occidualis* is not as strongly conserved as previously thought. However, the previous studies were field surveys, and none of them attempted to infect different species of definitive hosts with *H. occidualis* to determine if host species influenced the site selected by the adult worms of if the worms in separate habitats actually were different *Halipegus* species. This was the first study to experimentally examine the site fidelity of *H. occidualis* in multiple anuran species.

In this chapter, I presented data that suggests that site fidelity of *H. occidualis* is more

variable than previously described, and that the site occupied by these worms is dependent on the host species infected. Even though all worms from the stock culture originated from the stomach of bullfrogs, gravid worms established under the tongue of 5 of 6 anuran species experimentally infected with metacercariae. However, worms were never observed in the mouth of bullfrogs despite the numerous individuals recovered from the stomach of these frogs at the end of the study. Additionally, *H. occidualis* was consistently attracted to the same site of the 2 habitats in each of the anuran species after the adult worms were transplanted. All worms remained in place when they were transplanted from the original habitat into the same habitat within another individual of the same species (i.e. stomach to stomach or buccal cavity to buccal cavity). However, when worms were transplanted from the stomach of one host to the buccal cavity of a second individual of the same species, or vice versa, the worms always migrated back to the original habitat.

The main contribution of this study is that it brings into question if the site fidelity of *Halipegus* species within different species of amphibian hosts is a reliable character for species identification as has been suggested by others (Goater et al., 1989; McAlpine and Burt, 1998; McAlpine, 2006). Because these observations suggest that the site of infection appears to be a variable characteristic for at least some *Halipegus* species, species misidentifications can easily occur. Future identification of *Halipegus* species should be made with caution, and to do so accurately, investigators should use a combination of genetic data along with adult and non-adult morphological characteristics and habitat of adult worms as suggested previously by McAlpine and Burt (1998).

The discrepancy in the previously reported habitats of *H. occidualis* within bullfrogs, red-legged frogs, leopard frogs, and green frogs is likely result from differences within the anuran species that cause *H. occidualis* to select different habitats and not that the trematodes that reside in the mouth or stomach are distinct species of *Halipegus*. However, the segregation of worms of

the same species in different species of hosts provides a mechanism that may play an important role in the diversification of species in this genus of trematodes that infects amphibians worldwide.

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APPENDIX A

ESTABLISHING LABORATORY CULTURES OF *HALIPEGUS ECCENTRICUS* AND *HALIPEGUS OCCIDUALIS*

The life cycles of *H. eccentricus* and *H. occidualis* were established in the laboratory at Oklahoma State University. Briefly, laboratory stock cultures of *H. eccentricus* were established from 26 adult worms originally removed from the eustachian tubes of naturally infected American bullfrogs (*Rana catesbeiana*) collected in May 2011 from Neven's Pond, Keith County, Nebraska, U.S.A. (41°12.426'N, 101°24.510'W), and stock cultures of *H. occidualis* were started with 23 adult worms originally removed from stomachs of naturally infected American bullfrogs collected in May 2011 from James' Creek Pond, Stillwater, Payne County, Oklahoma, U.S.A. (36° 8' 50.661"N, -97° 4' 50.0412"W). To obtain eggs, gravid worms were removed from bullfrogs and placed separately in 70-mL plastic jars filled with aged tap water for 12 hrs. Eggs were then pooled in 1.5 ml vials filled with aged tap water, and all gravid worms were fixed in 70% ethanol. Worms were dehydrated in a graded ethanol series, cleared in xylene, and mounted in Canada balsam according to Pritchard and Kruze (1982). Worms were identified to species based on adult morphology, location of adults within amphibian hosts, and morphology of eggs according to Thomas (1939), Krull (1935), and Bolek et al. (2010).

To sustain a stock culture of *H. eccentricus* and *H. occidualis*, the life cycles of both species were maintained in the laboratory. Colonies of *Physa acuta* and *Planorbella trivolvis* snails that serve as first intermediate hosts for *H. eccentricus* and *H. occidualis*, respectively

were established in 37 L aquaria in the laboratory from field-collected individual from James' Creek Pond, Payne County, Oklahoma, U.S.A. in August 2010 according to Bolek and Janovy (2007). Snails were maintained on a diet of fresh and frozen iceberg lettuce, and Tetramin Tropical Fish Flakes (Tetra®). These snails were infected with the appropriate *Halipegus* species by placing a starved snail in an individual 5 ml cell of a well plate filled with aged tap water, and 10-20 eggs of the appropriate *Halipegus* species, and a small amount of crushed fish flakes. After 24 hrs exposure, all snails were removed and maintained in 3.78-L jars with aerated aged tap water at 24 C and 14L:10D period and fed iceberg lettuce *ad libitum*. Twenty-five days post exposure (DPE) all surviving snails were individually isolated in 5 ml well plates with aged tape water and observed daily for the presence of cercariae. Snails infected with *H. eccentricus* began shedding cercariae 27-34 DPE; whereas, snails infected with *H. occidualis* shed cercariae 59-71 DPE. All cercariae were collected and pooled by species each day.

Cultures of *Cypridopsis* sp., *Phyllognathous* sp., and *Thermocyclops* sp. micrucrustaceans were established using individuals that were brought into the laboratory with wild snails that were used to establish snail cultures. Additionally, a laboratory colony was established for a *Bradlystrandesia* sp. from dried sediment collected from a ditch 5 miles west of Chambers, Holt County, Nebraska (42 13'8.05, 98 53'55.21). Dried sediment was placed in the bottom of a 37.85 liter aquarium containing aerated tap water, and *Bradlystrandesia* sp. hatched from the sediments within 14 days. All microcrustaceans were maintained in 37.85 liter aquaria with gravel substrate that were filled with aerated aged tap water and all microcrustacean cultures were fed lettuce and crushed Tetramin Fish Flakes (Tetra®) at least once a week. Within 24 hrs of collecting the cercariae, microcrustaceans were infected by placing 2-5 cercariae, a single host, and 1 ml aged tap water in 1.5 ml well plates. Microcrustaceans fed on the cercariae for 24 hrs, and all microcrustaceans exposed on the same day were pooled by species of *Halipegus* and maintained in a 300 ml stackable processing dish filled with 250 ml of aged tap water. Every other day,

approximately half of the water was removed and replaced with fresh water, and several drops of pureed romaine lettuce were added to the dishes. After 16 days, the exposed microcrustaceans were used to infect field collected Woodhouse's toads (*Bufo woodhousii*).

Toads were infected with a single *Halipegus* species by pipetting 10-25 microcrustaceans that were exposed to 1 *Halipegus* species, into the mouth of each field-collected *B. woodhousii*. For this work, Woodhouse's toads were chosen as the definitive hosts because this species has never been reported to be infected with *Halipegus* species in nature (see Bolek et al., 2010). Additionally, *B. woodhousii* is easy to maintain in the laboratory, and previous work indicates that there is no difference in the development, establishment and migration of *H. eccentricus* within *B. woodhousii* and *R. catesbeiana*, the typical host of *H. eccentricus* in nature (Bolek et al., 2010). All toads used in experimental infections were housed individually in 37 L aquaria. Anurans were provided a 5 cm gravel substrate and a plastic water dish filled with aged tap water. Toads were maintained at 24 C and 24L:0D period and fed crickets and meal worms *ad libitum*. Water was changed every other day.

To maintain these life cycles in the laboratory, gravid worms, of each species, were removed from the buccal cavity of toads and allowed to release eggs, by placing individual worms in 70-mL plastic jars filled with aged tap water for 1-2 hrs. After worms released eggs they were returned to the buccal cavity of toads and the process was repeated periodically whenever eggs were needed for specific life cycle studies.

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Thesis: CONSEQUENCES OF VARIATION IN HOST SPECIES USED TO COMPLETE COMPLEX LIFE CYCLES OF HELMINTHS: AN EXPERIMENTAL STUDY ON LIFE CYCLES OF *HALIPEGUS* SPECIES (DIGENEA: HEMIURIDAE) FROM NORTH AMERICA

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