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ECOLOGY AND RELATIONSHIPS OF *RHABDIAS* SPP. (NEMATODA:
RHABDIASIDAE) FROM NORTH AMERICAN AMPHIBIANS AND REPTILES

by

Gabriel J. Langford, Ph.D.

A DISSERTATION

Presented to the Faculty of
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Under the Supervision of Professor John Janovy, Jr.

Lincoln, Nebraska

May, 2010

ECOLOGY AND RELATIONSHIPS OF *RHABDIAS* SPP. (NEMATODA:
RHABDIASIDAE) FROM NORTH AMERICAN AMPHIBIANS AND REPTILES

Gabriel J. Langford, Ph.D.

University of Nebraska, 2010

Advisor: John Janovy, Jr.

Lungworms of the cosmopolitan genus *Rhabdias* (Nematoda: Rhabdiasidae) are among the most common parasites of amphibians and squamate reptiles. The life cycles, life histories, host specificities, and evolutionary relationships of *Rhabdias* spp. were studied through examination of their free-living and parasitic stages in amphibians and reptiles. This study found free-living development of anuran lungworms was primarily limited to heterogonic reproduction, whereas snake lungworms primarily reproduced homogonically. Infective anuran lungworms penetrated the skin of frogs and toads; in contrast, snake lungworms penetrated snake esophageal tissue during per os infections. Our molecular phylogeny strongly supported separate clades for anuran and snake lungworms, and supported our species identifications. Field studies and experimental infections indicated that snake lungworms were generalist parasites of snakes and maybe lizards, whereas lungworms from anurans ranged from strict host specificity to relative generalist. Host specificity in nature appeared to be limited by both ecological and physiological factors, which varied among worm species. Field studies found that the majority of lungworm infections appeared to occur within 2 m of wetland shorelines in Nebraska. Field and laboratory experiments suggested that the predominantly sandy soils

of Nebraska's Sandhills limit *R. joaquinensis* from infecting anurans in western Nebraska. This work is an important addition to the small number of previous studies that have used a multifaceted, comparative approach that includes field and laboratory experiments to investigate host-parasite relationships. Ultimately, this work provides much needed basic life history and ecological data for a fascinating group of parasites.

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INTRODUCTION

The nematode genus *Rhabdias* Stiles and Hassall, 1905, comprises approximately 60 species worldwide and at least 12 species in North America. Lungworms are among the most commonly encountered parasites of amphibians and reptiles, and often these worms are among the first parasites encountered by parasitology students. These worms infect a broad diversity of hosts that range from chameleons in Africa to caecilians in Asia. In North America, lungworms are known to primarily infect snakes and anurans, however they also have been found infecting salamanders (Kuzmin et al., 2003) and a lizard (Martínez-Salazar, 2006).

The life cycles of these nematodes alternate between parasitic and free-living generations. Parasitic individuals are protandrous hermaphrodites that feed on blood and produce eggs in the hosts' lungs. The eggs are swallowed and defecated into the soil, hatch, and begin a free living generation. The presence of homogonic, i.e. worms that forego sex, stages in the free-living generation has been controversial. Lungworms were initially thought to only undergo heterogonic reproduction, i.e., sexual reproduction (Mecznikow, 1865; Goodey, 1924); however, homogonic stages were later found in some species of lungworms (Railliet, 1899; Goodey, 1924; Walton, 1929; Chu, 1936). Currently, the mode of reproduction (heterogonic vs. homogonic) is unknown for most lungworm species. Next, infective juveniles enter host body cavities via skin penetration (Baker, 1979), orally (Chu, 1936), or potentially via a transport host (Baker, 1979), and eventually establish themselves as adults in the lungs.

Besides the initial works of Chu (1936) and Baker (1979), little experimental research has been conducted on the natural history and ecology of parasitic and free-

living generations in *Rhabdias* spp. Further research needs to be conducted on the biology of lungworms for several reasons: **(1)** Life cycles of several North American species remain unexplored, and some aspects of previously described life cycles are unsubstantiated; **(2)** Host specificity in lungworms is a controversial issue, primarily due to a lack of experimental research; **(3)** Evolutionary relationships within the genus are unknown, i.e., no phylogeny exists for these worms; **(4)** No studies exist on infective juvenile worms in nature. In addition, the increasing popularity of lungworms as ideal host–parasite models to test evolutionary principles and ecological interactions warrants a greater understanding of the worms’ life histories (see Langford and Janovy, 2009). Overall, we suggest lungworms are excellent model systems, yet our ability to interpret research results is limited by a lack of basic life history data.

The goal of this work is to elucidate and compare the life cycles, life histories, host specificities, evolutionary relationships, and free-living characteristics of several *Rhabdias* spp. that infect snakes and anurans in the United States and Canada. **Chapter 1** elucidates the life cycles and life histories of 6 species of lungworms, including the potential use of transport hosts in the life cycle. **Chapter 2** addresses host specificity in 7 species of lungworms, and produce a molecular phylogeny to elucidate evolutionary relationships and explore their role in host specificity. Lastly, **Chapter 3** reveals where anuran hosts encounter and become infected by the lungworm *Rhabdias joaquinensis* in Nebraska. This work utilizes the comparative method, which has been shown to be ideal for conducting host-parasite research (Poulin, 2007). In addition, this study combines field and laboratory experiments to fully elucidate host-parasite relationships and understand how these relationships function in nature.

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**CHAPTER ONE: COMPARATIVE LIFE CYCLES AND LIFE HISTORIES OF
NORTH AMERICAN *RHABDIAS* SPP. (NEMATODA: RHABDIASIDAE):
LUNGWORMS FROM SNAKES AND ANURANS**

Abstract: The present study used experimental infections to compare the life cycles and life histories of 6 *Rhabdias* spp. infecting snakes and anurans. Free-living development of anuran lungworms was primarily limited to heterogonic reproduction, and females utilized matricidal endotoky exclusively, whereas snake lungworms primarily reproduced homogonically and, when heterogonic reproduction occurred, females used a combination of releasing eggs and matricidal endotoky. Infective snake lungworms survived for longer periods in fresh water compared to anuran worms. Infective anuran lungworms penetrated into the skin of frogs and toads; few infections resulted from per os infections. In contrast, snake lungworms were unable to penetrate skin; instead, infective juveniles penetrated into snake esophageal tissue during per os infections. Despite separate points of entry, anuran and snake lungworms both migrated and developed in the fascia, eventually penetrating into the body cavity of the host. Worms molted to adulthood inside the body cavity and subsequently penetrated into the host's lungs, where they fed on blood while becoming gravid. Adult lungworm survival varied among lungworm species, but, in general, snake lungworms were longer lived than anuran worms. Anuran lungworms were poorly suited for transmission via transport hosts, whereas snake lungworms were consistently capable of establishing infections using transport hosts. Overall, these observations suggest that snake and anuran lungworms have discrepant life cycles and life history strategies.

INTRODUCTION

Comparative approaches in life cycle and life history research are useful in revealing life cycle variations between closely related parasites (Morand and Poulin, 2003; Lefebvre and Poulin, 2005; Poulin, 2007; Bolek et al., 2009). Using the comparative method, Snyder and Janovy (1994), Bolek and Janovy (2007a, 2007b; 2008), and Bolek et al. (2009) found that closely related parasites can and do use discrepant evolutionary avenues to complete their life cycles. These studies suggest life cycle variation is more common than previously assumed, at least in trematodes from anuran hosts. However, the pervasiveness of life cycle variation is unclear, because our understanding is limited to a few studies and parasites. Additional studies are needed to compare life cycles and life histories among closely related taxa infecting phylogenetically diverse and broadly distributed hosts. Species of *Rhabdias* fulfill these requirements and permit use of amphibian and reptilian hosts, which provide model host systems for studying life cycle evolution (see Bolek and Janovy, 2007a).

Species of the cosmopolitan nematode, *Rhabdias* Stiles and Hassall, 1905, are among the most common metazoan parasites of amphibians and reptiles. The life cycles of these nematodes alternate between parasitic and free-living generations. Parasitic individuals are protandrous hermaphrodites that produce spermatozoa in a “testis zone” prior to, and sometimes continuing through, egg development (Runey et al., 1978). Eggs are transported from the host’s lungs into the gastrointestinal tract, where they are defecated into the soil, hatch, and begin a freeliving generation. The free-living generation was initially thought to undergo only heterogonic reproduction, i.e., male and female sexual reproduction (Mecznikow, 1865; Goodey, 1924; Schaake, 1931). However,

in homogonic stages, i.e., juveniles that forego sex and molt to an infective stage, development also occurs (Railliet, 1899; Goodey, 1924; Walton, 1929; Chu, 1936; Baker, 1979); Chu (1936) found both homogonic and heterogonic development in some cultures of *Rhabdias fuscovenosa*. Regardless of developmental route, infective juveniles enter host body cavities via skin penetration (Baker, 1979), orally (Chu, 1936), or potentially via a transport host (Baker, 1979), and eventually establish themselves as adults in the lungs.

Since the work of Chu (1936) and Baker (1979), little experimental research has been conducted on the life cycles and life histories of parasitic and free-living generations in *Rhabdias* spp. (but see Spieler and Schierenberg, 1995). Life cycles of several North American species remain unexplored, and some aspects of previously described life cycles remain unsubstantiated. Given their lack of study, global distribution, and varied hosts, lungworms appear to be an ideal place to search for life cycle variation. Furthermore, the increasing popularity of lungworms as ideal host–parasite models to test evolutionary principles and ecological interactions (e.g., Goater, 1992, 1994; Goater and Ward, 1992; Christin et al., 2003; Gendron et al., 2003; Dare and Forbes, 2008a, 2008b) warrants a greater understanding of the worms' life histories. Our goal is to elucidate and compare the life cycles and life history characteristics of several *Rhabdias* spp. that infect snakes and anurans occurring in the United States and Canada.

MATERIALS AND METHODS

Amphibian and reptile field studies

Anurans and snakes were captured from May 2005 to June 2008 during both day and night using hand capture, pit-fall traps, funnel traps, drift fences, and by cruising roads

(Heyer et al., 1994). Animals were transported to either the parasitology laboratory at the University of Nebraska–Lincoln, Cedar Point Biological Station in western Nebraska, or the vertebrate museum at the University of South Alabama, where they were killed and measured for snout-vent length (SVL) and total length (TL), and all organs were examined for parasites within 48 hr of collection. *Rhabdias* spp. were removed and fixed in hot 70% ethyl alcohol; however, many adult hermaphrodites were set aside for use in life cycle experiments (see below). Representative specimens were cleared and temporarily mounted in glycerol for identification (Prichard and Kruse, 1982). All lungworms were identified according to Kuzmin et al. (2003). *Rhabdias americanus* Baker, 1978, were harvested from *Bufo terrestris* collected from the University of South Alabama campus, Mobile County, Alabama (30° 42' 7", -88° 11' 17"); *Rhabdias bakeri* Tkach, Kuzmin, Pulis, 2006, from *Rana sylvatica* collected in Jefferson County, Missouri (38° 21' 35", -90° 24' 0"); *Rhabdias joaquinensis* Ingles, 1935 from *Acris blanchardi* collected in Pawnee Lake, Lancaster County, Nebraska (40° 50' 35", -96° 31' 33"); *Rhabdias ranae* Walton, 1929, from *Rana sphenoccephala* collected in Baldwin County, Alabama (30° 27' 28", -87° 25' 59"); *Rhabdias eustreptos* (McCallum, 1921) from *Lampropeltis getulus* collected in the Grand Bay National Estuarine Research Reserve, Jackson County, Mississippi (30° 26' 10", -88° 25' 58"); and *Rhabdias fuscovenosa* (Railliet, 1899) from *Thamnophis sirtalis* collected in Keith County, Nebraska (41° 18' 19", -101° 55' 43"). Voucher specimens have been deposited in the H. W. Manter Parasitology Collection, University of Nebraska, Lincoln, Nebraska (accession numbers HWML 63512 *Rh. americanus*, 63513 *Rh. bakeri*, 63514 *Rh. eustreptos*, 63515 *Rh. fuscovenosa*, 63516 *Rh. joaquinensis*, and 63517 *Rh. ranae*).

Culturing lungworms in the laboratory

For this study, Chu (1936) was modified to provide an inexpensive, quick method for producing a large number of infective juveniles in a consistent manner. Culturing containers were constructed with a 10-cm diameter circular piece of unbleached paper towel, with a 2-cm diameter circle cut in the middle, thus forming a paper towel donut. This donut permitted viewing of lungworm development without disturbing culture conditions. Next, the donut was dampened with aged tap water and hand molded to the inside of a 5-cm Petri dish. The Petri dish was placed inside a larger (9 cm) Petri dish, and covered with a lid. Approximately 20 ml of aged tap water was added to the larger dish, thus forming a shallow water bath for the smaller container. Two grams of feces were added to the inner Petri dish to establish bacterial colonies for food. Finally, eggs were placed inside the small Petri dish adjacent to the feces. Cultures were kept at 26 C for all experiments. The development of free-living juveniles was easily observed. After a few days, infective juveniles migrated out of the small, inner Petri dish to reside in the outer Petri dish. Infective juveniles were easily maintained by removing the inner container following nematode migration. Water exchanges were necessary to prevent premature death of infective juveniles, due to stale water exposure (Chu, 1936). Petri dish lids also were removed for 5–10 min daily to provide gas exchange. Accidental inoculation of cultures with predators (mites and fungi) was avoided to a certain degree by centrifuging the feces and extracting the supernatant for bacteria (Spieler and Schierenberg, 1995). However, centrifuged cultures produced fewer infective juveniles than non-centrifuged ones; this technique was thus abandoned early in the research. Instead, cultures were inoculated with nematode-free feces and periodically checked for

predacious mites and fungi, which were promptly removed. The latter method was time consuming, but produced the greatest numbers of infective juveniles.

Acquiring uninfected hosts

Uninfected amphibians were obtained from Cedar Point Biological Station (CPBS) in western Nebraska (in .30 yr of constant sampling, no amphibians were infected with *Rhabdias* spp. [J. Janovy, Jr., pers. obs.]). Uninfected *Bufo woodhousii*, *Rana pipiens*, and *Rana blairi* were collected from CPBS. All other hosts used were obtained from areas where lungworms were potentially transmitted. These animals were placed individually in cages, and their feces were checked for juvenile *Rhabdias* spp. at 0 and 10–20 days. This time period permitted maturing of any infection the hosts may have obtained prior to capture, thus assuring only uninfected hosts were used in experiments. Other uninfected hosts were acquired by rearing uninfected tadpoles in the laboratory or isolating wild, gravid snakes in the laboratory and housing offspring in cages that prevented transmission.

Free-living experiments

Adult hermaphrodites were collected from lungs of captured wild hosts. Eggs released were pipetted into culture containers, with each receiving eggs from 6 to 7 adult worms. Cultures were checked every 2–3 hr to record developmental stage. Growth and timing of free-living juveniles were recorded by measuring total length, maximum width, esophagus length, and tail length of 10 specimens from each of the following stages: first stage juveniles, adult males and females, and third stage infective juveniles. The number of eggs per female was recorded for all measured specimens; however, only approximate egg counts were possible for *Rh. eustreptos* and *Rh. fuscovenosa* because both species

undergo partial matricidal endotoky, where females release some eggs prior to death (ca. 4–5), and remaining eggs hatch in utero and undergo typical matricidal endotoky. The remaining 4 species never released eggs; therefore, egg counts were exact. Numbers of infective juveniles produced by homogonic vs. heterogonic development were also recorded. Homogonic worms invariably, and rapidly, self separated, i.e., migrated into the outer water bath, prior to worms that developed heterogonically. Temporal variation in self separation allowed homogonic worms to be counted and placed in separate Petri dishes prior to emergence of heterogonic worms. After all infective worms migrated into the water bath, inner containers were removed from the culture container. Containers were considered viable if at least 1 infective juvenile remained alive.

Host experimental exposures

Infective lungworms used in snake and anuran host exposures were obtained using the modified culture chambers. Hosts were exposed to lungworms via skin penetration and orally to test the route(s) of infection used by each species. In skin exposures, infective worms were placed on a 9-cm diameter piece of moist, unbleached paper towel that was positioned on the bottom of a glass culture dish with a breathable glass lid. Hosts were placed individually in the infection chamber, where they remained for 12 hr; upon removal, hosts were rinsed thoroughly in aged tap water to remove any remaining nematodes. Host drinking was a concern in skin exposure experiments for snakes, but not anurans, since they do not acquire water orally (Pough et al., 2004). To prevent snakes from consuming nematodes orally, they were fitted with a 6-cm soft plastic collar around the neck. The collar allowed for normal movement and breathing, but kept the head of the snake dry. In the per os experiments, infective juveniles were

intubated into the esophagus along with 5 ml of aged tap water. Hosts were then rinsed in aged tap water to remove any juveniles that may have been pipetted onto the hosts' skin.

Depending on cannibalistic tendencies, hosts were housed individually or in small groups; they were provided access to fresh water and refugia. Snakes were fed frozen, thawed white fish (*Tilapia* sp.) and mice; anurans were fed a diet of commercial crickets (Top Hat Cricket Farm, Kalamazoo, Michigan) and cultured mealworms (Janovy Laboratory, University of Nebraska–Lincoln). Hosts were exposed to an appropriate temperature gradient and a 12-hr day/light cycle. Cages were lined with absorbent paper towels and cleaned 2–3 times per week to prevent development of infective juveniles in host feces and potential transmission of lungworms passed in feces from the per os experiment. All hosts were exposed to 30 infective juveniles. Host specificity problems were avoided by matching host species with appropriate *Rhabdias* spp. based on natural infections reported by Kuzmin et al. (2003) or found during this study. All experimental infection protocols used the following exposure combinations, *B. woodhousii* and *Rh. americanus*; *Ra. sylvatica* and *Rh. bakeri*; *A. blanchardi* and *Rh. joaquinensis*; *Ra. pipiens* and *Rh. ranae*; and *Thamnophis sauritus* and *Rh. eustreptos* or *Rh. fuscovenosa*.

Separate trials were conducted on host experimental exposures to determine the route and timing of development and morphological growth of the worms. In trial 1, hosts were exposed to lungworms using both per os and skin exposure and subsequently dissected. All organs, muscles, body cavities, and connective tissues were thoroughly searched for developing lungworms at regular intervals from 1 to 40 days post-infection (PI). Number and developmental stage of worms was recorded. In trial 1, 4 hosts (2 for per os, 2 for skin penetration) were dissected at each time period.

In trial 2, hosts were exposed to infective lungworms, using per os exposures for snakes and skin penetration exposures for anurans. Hosts were dissected and all tissues were thoroughly searched for developing worms at consistent intervals 1–30 days PI. Worms were categorized into the following developmental stages: juvenile (third stage), juvenile (fourth stage), adult (body cavity), adult (lungs), and gravid adult (lungs). Cuticular shedding was noted when possible. Ten worms per stage were selected at random and measured for total length, maximum width, esophagus length, and tail length. In the second trial, 2 hosts were dissected at each interval. Time-0 and time-T controls were used in both trials to ensure hosts did not develop spurious infections through the course of the experiments.

In trial 3, hosts were kept in clean, lungworm-free containers for extended periods of time to determine the duration of infection for each lungworm species. Containers and methods designed to prevent lungworm transmission (described above) were used throughout the experiment. In addition, host diets were supplemented with appropriate trace nutrients during long-term captivity. Anurans and snakes were not hibernated during the study. Hosts were each exposed to 30 infective lungworms (see trial 2), then placed in transmission-free containers. The following combinations of host species (No. of individuals) to parasite species were used in this experiment, *B. woodhousii* (12) and *Rh. americanus*; *Ra. sylvatica* (7) and *Rh. bakeri*; *Pseudacris triseriata* (12) and *Rh. joaquinensis*; *Ra. blairi* (12) and *Rh. ranae*; *T. sauritus* (3) and *Rh. eustreptos*; *T. sauritus* (3) and *Rh. fuscovenosa*. Fresh host feces were checked for juvenile lungworms twice a week in anuran lungworms and twice a month in snake lungworms. Duration of infection was measured in each host by recording the last date eggs, or juvenile worms, or both,

were collected from feces. Two negative fecal samples in succession were used as the stopping point for these experiments. Dissection was used to confirm hosts were uninfected.

Transport hosts

Infective juveniles of 4 lungworms species were obtained from feces and used to test the efficacy of potential transport hosts. Adult *Physa gyrina* were collected from Nevin's Pond, Keith County, Nebraska (41° 20' 18", -101° 41' 57") and exposed in groups of 10 snails to 150 infective worms 2–3 days post-capture. Snail exposures were conducted in a 9-cm diameter glass culture dish with an unbleached paper towel bottom and breathable glass lid in 1 cm of aged tap water. After 12 hr, snails were removed from the container, placed in clean aquaria, and allowed to void their gut for 12 hr. Four snails were then manually fed to individual hosts, which were exposed in various combinations, 7 *B. woodhousii* and *Rh. americanus*, 7 *A. blanchardi* and *Rh. joaquinensis*, 3 *T. sauritus* and *Rh. eustreptos*, and 3 *T. sauritus* and *Rh. fuscovenosa*. Anuran and snake hosts were dissected, and all tissues were searched for developing lungworms 8 days post-exposure. Twenty exposed snails were also dissected for all 4 species of lungworm to search for third stage juvenile worms. Snails were provided a diet of frozen spinach, unless otherwise noted. The earthworm *Eisenia foetida* and larval *Taenibro molitar* beetles from domestic stock were allowed to feed ad libitum on host feces containing numerous infective juveniles. After 12 hr earthworms and beetle larvae were placed in clean plastic containers and allowed to void their guts for 12 hr. Four beetles were fed to each host in the following combinations, 5 *B. woodhousii* and *Rh. americanus*, 6 *A. blanchardi* and *Rh. joaquinensis*, 3 *T. sauritus* and *Rh. eustreptos*, and 3 *T. sauritus* and *Rh. fuscovenosa*.

Three *T. sauritus* were each fed 4 earthworms exposed to *Rh. eustreptos*, and 3 *T. sauritus* were each fed 4 earthworms exposed to *Rh. fuscovenosa*. Anuran and snake hosts were dissected and all tissues were searched for developing lungworms 8 days after being fed earthworms or beetle larvae. Twenty earthworms and 10 beetle larvae were also dissected for all 4 species of lungworm, after earthworms and beetles voided their guts, to search for third stage juvenile worms. Specimens of *Ra. blairi* were collected from Pawnee Lake, Lancaster County, Nebraska, and exposed to infective stages of *Rh. eustreptos* or *Rh. fuscovenosa*. Six frogs were exposed to *Rh. eustreptos* and 6 to *Rh. fuscovenosa* via passive exposure in culture dishes. Following the 12-hr exposure, frogs were rinsed and housed individually in plastic containers with access to fresh water and crickets. Frog transport hosts were housed for 7 days prior to being fed to final snake hosts. Six lab-reared *T. sauritus* were each fed 2 frogs from the same lungworm exposure, kept in the laboratory for 8 days, and dissected to determine the presence of developing lungworms. In addition, 5 frogs exposed to each lungworm species (10 frogs total) were dissected and searched for developing lungworms following parasite exposure and a 7 day resting period. For all transport host experiments, time-0 and time-T controls were used to ensure normal definitive and transport hosts were not previously infected and did not develop spurious infections during the experiment.

RESULTS

Free-living development

Lungworms of snakes: Lungworms (*Rh. eustreptos*, *Rh. fuscovenosa*) collected from snakes displayed similar development patterns, except timing of development (Table I). Approximately 50% of the eggs collected from the lungs of snakes were

embryonated; however, 100% of eggs removed from the lower intestine were embryonated. Lungworm eggs hatched on exposure to air, or fresh water, or both. On the average, *Rh. eustreptos* developed into third stage homogonic juveniles (65.8 hr) almost twice as fast as *Rh. fuscovenosa* (120 hr). Both species displayed homogonic development; heterogonic development occurred in 56% (10 of 18) of *Rh. eustreptos* cultures and 40% (8 of 20) of *Rh. fuscovenosa* cultures. In all snake lungworm cultures 2% of infective worms developed heterogonically. Infective juveniles acquired from heterogonic development in snakes were morphologically identical to those produced during homogonic development but were unable to infect snakes, whereas hosts exposed to homogonic juveniles developed infections consistently.

Infective lungworms often survived for long periods (97 days in 1 culture) as third stage juveniles in Petri dishes with regular water exchange. Infective juveniles in Petri dishes maintained a quiescent state until disturbed. When disturbed by vibration, juvenile worms undulated vigorously for approximately 15 min, and then returned to an inactive state. Desiccation killed free living juveniles and adults. Male and female worms were distinctly rhabditiform and easily distinguished from infective juveniles (see Baker, 1979). Mating was only observed on 8 occasions, i.e., the male moved alongside the female and grasped her with his tail. Copulation lasted 2 min; multiple mating was not observed under normal culture conditions, whereas multiple mating was observed when worm population densities were increased. Free living *Rh. eustreptos* females produced approximately 8 eggs, and *Rh. fuscovenosa* produced 10 eggs. Males died soon after mating. Eggs released prior to matricidal endotoky hatched quickly in the culture chamber and immediately began feeding on bacteria. In general, juveniles from released

eggs developed at a similar rate as those that developed via matricidal endotoky, i.e., outer dish migration times overlapped. *Rhabdias eustreptos* and *Rh. fuscovenosa* developed at different rates, yet were of similar size when measured at different developmental stages (Tables II, III).

Lungworms of anurans: Eggs collected from both the lungs and colons of anurans were fully embryonated and hatched upon exposure to air, or fresh water, or both. Lungworms collected from anurans, i.e., *Rh. americanus*, *Rh. bakeri*, *Rh. joaquinensis*, and *Rh. ranae*, displayed almost complete heterogonic development (see Table I). The notable exception was heterogonic development by *Rh. joaquinensis*; of 23 cultures, 3 produced 8, 12, and 17 homogonic juveniles. Infective juveniles acquired from homogonic development in *Rh. joaquinensis* were unable to infect *Ra. blairi* during skin exposure experiments. Mating was observed on numerous occasions in all species, except *Rh. bakeri*. Mating occurred when males aligned themselves parallel to females and grasped the females near the vulva with their tails. Copulation lasted 1–8 min; both males and females mated multiple times, usually with multiple partners. Males, however, died soon after mating.

Free-living females of all 4 species produced 2–3 eggs that developed exclusively through matricidal endotoky. Infective juvenile worms varied in maximum survival times from 16 days in *Rh. bakeri* to 37 days in *Rh. joaquinensis*. Infective juveniles in Petri dishes maintained a quiescent state until disturbed by agitating the Petri dish. When disturbed, juvenile worms behaved similarly to snake lungworms; likewise, worms of these 4 species were unable to survive desiccation. Adult females vary in average total length from 688 mm in *Rh. joaquinensis* to 778 mm in *Rh. ranae* (Tables IV–VII).

Despite interspecific differences in female size, infective juveniles reached similar total lengths.

Parasite development

Lungworms of snakes: Infective worms remained quiescent in culture chambers, until the Petri dish was disturbed by vibrations or the worms were prodded with forceps. On disturbance, worms responded similarly to anuran species (see below). During skin exposure experiments, infective juveniles were unable to penetrate the skin of *T. sauritus*. Infective worms continuously undulated over the surface of the snake, but worms established infections only when they entered the host's oral cavity. Infections were always established in per os infections. Following oral intubation, snake lungworms penetrated (or were in the process of) the esophagus and resided in the connective tissue of the esophagus. Penetration occurred throughout the entire esophagus, but primarily in the striated muscle of the anterior half. Next, worms molted to fourth stage juveniles, with no sheath. Both third and fourth stage juveniles were recovered from connective tissue that surrounded the esophagus, trachea, gastrointestinal tract, liver, and urinary bladder. Then, worms penetrated into the body cavity and primarily resided near the lower lung as adults. Eventually adults penetrated into the lungs, where they fed on blood to become gravid. Adults in the body cavity were never gravid, nor contained fully developed eggs. Timing of parasite development is provided in Figure I. *Rhabdias eustreptos* juveniles developed quicker and reached greater size than *Rh. fuscovenosa* (see Tables II–III). *Rhabdias eustreptos* released eggs in *T. sauritus* feces for 5, 9, and 9 mo; dead adults were shed in host feces starting 4 mo PI. *Rhabdias fuscovenosa* released eggs in *T. sauritus* feces for 7, 11, and 13 mo, the longest duration of any lungworm

species; dead adult worms were recovered from host feces starting 5 mo PI. For the first time, a non-gravid adult *Rhabdias* sp. was observed penetrating the lung of a host. Upon dissection of a snake infected in the laboratory with *Rh. eustreptos*, a worm initiated penetration and completely entered into a semi-inflated lung. Penetration took place on the ventral, posterior section of the vascularized lung over a period of 3 min; most of this time was dedicated to creating a small hole in the lung, through which the worm easily entered. Despite watching the worm enter through the hole, we struggled to locate the entry wound and noted few signs of trauma to the surrounding lung tissue. Overall, it appeared penetration by a lungworm caused little immediate damage to lung tissue.

Lungworms of anurans: During skin exposure experiments, infective juveniles penetrated the skin of anurans to gain entry. Nematodes immediately reacted to the presence of a toad and, within minutes of exposure, were seen crawling on anuran skin. Per os infections were only established in *Rh. joaquinensis*, i.e., 3 of 3 frogs established infections with 1, 1, and 2 worms collected. Infective juveniles were found both unharmed and viable in anuran feces from all 4 lungworm species. When not promptly removed, juveniles in these feces produced skin-penetrating infections, which could be easily mistaken for per os infections. Following skin penetration, all worms were found subcutaneously. Worms then migrated into the visceral fascia in the thoracic cavity, although some worms also remained subcutaneously in the pelvic region. Next, juvenile lungworms molted to fourth stage juveniles and penetrated into the body cavity where they developed into adults. Adults in the body cavity eventually penetrated into the lungs to feed on blood and become gravid. Adults in the body cavity were never gravid, nor did they contain fully developed eggs. Timing of parasite development is provided in Figure

I. During skin exposures, *Rh. joaquinensis* developed faster than any other species in this study, whereas *Rh. americanus* juveniles developed the slowest, but reached the largest adult length of any species in this study (see Tables IV–VII). The mean total length of *Rh. bakeri* gravid adults increased 3.02 mm in 13 days, which represented the greatest growth spurt of any lungworm species.

Of 12 *B. woodhousii* maintained in the laboratory with *Rh. americanus* infections, 8 survived to provide data on parasite longevity; worms from these toads released eggs for 13.4 ± 1.7 wk (range, 12–17 wk). Dead lungworms were found in feces starting at 10 wk PI. Four of 7 *Ra. sylvatica* maintained in the laboratory survived to provide data on parasite longevity. *Rhabdias bakeri* eggs were released in *Ra. sylvatica* feces for 10–16 wk. Dead adults were occasionally passed in host feces starting at 8 wk PI. *Rhabdias joaquinensis* from *A. blanchardi* released eggs for the shortest time period of any lungworm species, 34.5 ± 5.7 days (range, 24–45 days). Dead adults were occasionally passed in host feces starting 24 days PI. Finally, *Rh. ranae* from *Ra. blairi* released eggs in host feces for 15 ± 4.3 wk (range, 12–20 wk). Dead adult worms were found in host feces starting 8 wk PI.

Transport hosts

Lungworms of snakes: Physid snails when exposed to both species of snake lungworms, *Rh. eustreptos* (prevalence = 63%, mean intensity = 2.1 ± 1.1 , n = 7) and *Rh. fuscovenosa* (prevalence = 57%, mean intensity = 3.4 ± 2.6 , n = 7), produced infective juveniles. Worms were recovered from tissues of the snail's foot; they appeared as healthy third stage juveniles and, when fed to snakes, normal infections resulted. With *Rh. eustreptos*, 2 of 3 snakes became infected with 2 and 5 lungworms when fed transport

host snails. For *Rh. fuscovenosa*, 3 of 3 snakes became infected with 1, 3, and 4 lungworms following snail consumption. The earthworm *Eisenia foetida* and frog *Ra. blairi* also hosted dormant third stage juveniles of both snake lungworm species and served as suitable transport hosts. Earthworms harbored *Rh. eustreptos* (prevalence = 63%, mean intensity = 5.2 ± 3.2 , n = 7) and *Rh. fuscovenosa* (prevalence = 33%, mean intensity = 3.4 ± 3.2 , n = 7); juvenile worms were collected from the coelom and intestine. In *Rh. eustreptos*, 3 of 3 snakes became infected with 1, 2, and 7 lungworms when fed transport host earthworms, whereas 2 of 3 snakes exposed to earthworms that harbored *Rh. fuscovenosa* each became infected with 2 lungworms. Infective juveniles were found subcutaneously in the frog *Ra. blairi*, primarily in the hind legs. Frogs acted as transport hosts for *Rh. eustreptos* (prevalence = 44%, mean intensity = 6.4 ± 4.5 , n = 7) and *Rh. fuscovenosa* (prevalence = 73%, mean intensity = 4.5 ± 1.3 , n = 7). Three of 3 snakes fed frogs serving as transport hosts for *Rh. eustreptos* became infected with 2, 3, and 6 lungworms. Of 3 snakes exposed to frogs serving as transport hosts for *Rh. fuscovenosa*, 100% became infected with 1, 3, and 3 lungworms. Juvenile lungworms did not penetrate *Tenebrio molitor*, nor did infections result when snakes were fed beetles exposed to either lungworm species. None of the time-0 or time-T control snakes, snails, frogs, earthworms, or beetles were infected.

Lungworms of anurans: This study was able to establish infections in anurans that resulted from using snails as transport hosts for *Rh. americanus* in *B. woodhousii* (prevalence = 43%, mean intensity = 2.3 ± 2.3 , n = 7) and *Rh. joaquinensis* in *A. blanchardi* (prevalence = 50%, mean intensity = 2.6 ± 2.1 , n = 6). *Rhabdias bakeri* and *Rh. ranae* were not tested during transport host experiments due to a lack of appropriate

anuran hosts. Infections were not established in anurans fed earthworms or beetles. In terms of lungworm penetration into potential transport hosts, *Rh. joaquinensis* (prevalence = 73%, mean intensity = 4.8 ± 2.2 , n = 7) and *Rh. americanus* (prevalence = 81%, mean intensity = 6.7 ± 2.5 , n = 7) penetrated into the foot of *P. gyrina*. The study also recovered *Rh. joaquinensis* (prevalence = 16%, mean intensity = 2.9 ± 1.2 , n = 7) and *Rh. americanus* (prevalence = 10%, mean intensity = 2.3 ± 0.5 , n = 7) third stage juveniles from the coelom of the earthworm *E. foetida*. Worms recovered from earthworms and snails appeared unchanged and viable. Anuran lungworms were unable to penetrate *T. molitor*. No time-0 or time-T control anurans, snails, earthworms, or beetles were infected.

DISCUSSION

Initial commentary

This study is the first to establish several life history characteristics for anuran and snake lungworms, including data on fecundity and developmental rates. These results should provide valuable data for future ecological and evolutionary studies on lungworms and other nematodes. However, the primary contribution of this comparative study is the establishment of different life cycles and life history strategies between snake and anuran lungworms, adding species of *Rhabdias* to the list of studies that have identified life cycle variation (see Introduction). These results strengthen suggestions that life cycle variation within closely related parasites is more common than initially thought (e.g., Morand and Poulin, 2003; Bolek and Janovy, 2007a, 2007b; Poulin, 2007; Bolek et al., 2009). It is clear that more studies are needed to determine how widespread life cycle variation is within, and among, parasite taxa, and to understand the role life cycle variation plays in

parasite evolution. For example, our understanding of life cycle evolution in lungworms would benefit from a phylogenetic analysis to determine whether variation in life cycle and life history data are congruent with *Rhabdias* evolution, i.e., snake and anuran lungworms form separate clades.

Free-living experiments

In the present study, eggs released from adult worms in the lungs of anuran hosts were fully embryonated, whereas lungworm eggs from snakes were not fully embryonated until they reached the snake's colon. The difference in lung embryonation rates of anuran and snake species may be attributable to gastrointestinal length and differences in defecation patterns between host groups. Anurans tend to have short intestines and in nature defecate at least once every 24 hr (G. Langford, pers. obs.), whereas snakes have longer gastrointestinal tracts and inconsistent feeding patterns; as a consequence, many snakes only defecate 1–4 times per month (Secor and Diamond, 1995; Overgaard et al., 2002). Thus, it appears that lungworms of anurans are continuously released into the external environment compared to the lungworms infecting snakes. Under these conditions, our observations suggest anuran lungworms require rapid embryonation to ensure eggs hatch upon exposure to the external environment. In contrast, selective pressures on embryonation may be relaxed in snake lungworm species because most eggs accumulate in the snakes' colon for several days prior to being evacuated into the external environment. Baker (1979) suggested that rapid hatching of free-living juveniles upon exposure to the external environment is vital for lungworm survival given that eggs lack resistance to environmental fluctuations. In agreement, we

found that unhatched eggs of all lungworms are susceptible to desiccation and predation in culture chambers, likely making environmental embryonation a risky venture in nature.

Free-living female lungworms produce varying numbers of eggs based on host affinities. Lungworms from snakes produce 8–10 eggs per individual female worm, whereas worms from anurans only produce 2–3 eggs. The disparity in egg production between worms infecting snakes and anurans is perplexing, especially considering free-living females from all species are similar in size, developmental period, and habitat, i.e., feces and soil. Given these similarities, we would expect free-living lungworms to produce similar numbers of offspring, according to maximum fecundity studies conducted by Morand (1996). However, Chu (1936) was unable to infect snake hosts with infective juveniles produced during heterogonic reproduction, leading Baker (1979) to suggest that indirect development may be vestigial in snake lungworms. The present study found a small proportion (< 2%) of juveniles in snake lungworms unable to develop heterogonically, or to infect snakes, an observation that supports Baker's assertion that heterogonic reproduction is vestigial in snake lungworms. If heterogonic development is vestigial, then few evolutionary forces are expected to constrain egg production in snake lungworms. Therefore, comparing egg production between snake and anuran lungworms may have little evolutionary significance.

Homogonic, or direct, development of free-living juveniles was first described in snake lungworms by Railliet (1899), and later confirmed by Goodey (1924), Chu (1936), and Baker (1979). Our studies on the free-living stages of North American lungworms indicate that homogonic and heterogonic development can both, but do not always, occur in species infecting anurans and snakes, with a single dominant form of development in

each species. Homogonic development predominated in lungworms that infect snakes, whereas lungworms of anurans develop entirely heterogonically, except for a limited number of *Rh. joaquinensis*. Williams (1960) also found homogonic development in another anuran lungworm, *Rhabdias sphaerocephala*. Again, the evolutionary importance of the non-dominant form of development is unclear, because we were unable to establish infections with nondominant infective juveniles of any species. Baker's (1979) suggestion that non-dominant forms are vestigial may explain why these juveniles are uninfected; alternatively, it is possible that non-dominant forms can establish infections under conditions not reproducible in the laboratory.

Host experimental exposures

The route of transmission, i.e., oral versus skin penetration, prior to the present study was an unsettled issue in *Rhabdias* spp. biology. Previous researchers agreed that lungworms infecting amphibians primarily gain entry through pores in amphibians' moist, glandular skin (Fulleborn, 1928; Schaake, 1931; Williams, 1960; Baker, 1979), but the status of oral exposure as a viable route of infection in amphibian lungworms was unknown (Baker, 1979). In snake lungworms, Chu (1936) was only able to infect snakes orally, and he suggested that infective juveniles were unable to penetrate the thick, dry skin of snakes. However, when Chu (1936) injected infective juveniles subcutaneously into snakes, normal infections occurred, thus supporting his assertion that the dry, scaly property of snakeskin is the constraining factor preventing cutaneous penetration in snake lungworms. The present study confirms that snake lungworms are unable to penetrate reptilian skin, and suggests that oral inoculation is the primary, and likely only, route used by snake lungworms. Thus, upon per os inoculation, snake lungworms immediately

began penetrating into esophageal tissue, which is mechanically amenable to nematode penetration. Our study found esophageal penetration by snake lungworms was similar to skin penetration by those infecting anuran, including preferred penetration at, or near, a mucus gland (as described below). Subsequent to esophageal penetration, an active fascia and body cavity migration occurs in the snake host (Goodey, 1924; Chu, 1936; this study). In agreement with Goodey (1924) and Chu (1936), our study observed 2 molts during the migration of snake lungworms through the body cavity. The first molt was shed by fourth stage juveniles, while the second molt was retained by the mature adult as a sheath. Chu (1936) found an identical molting pattern in *Rh. fuscovenosa*, whereas Goodey (1924) observed both molts being retained as sheaths.

When anurans were exposed to 4 homologous species of lungworms by pipetting infective juveniles into their esophagus, only *Rh. joaquinensis* established infections; however, all species became infected when juveniles were pipetted into the mouth. These observations suggest anurans can become infected by ingesting juveniles in the soil or present externally on a prey item, which would result in a skin-penetrating infection near the margin of the mouth. Infective worms that bypass the oral cavity, i.e., worms inside of a transport host, appear to be less likely to establish infections, except in *Rh. joaquinensis*, which consistently established low intensity infections in our experiments. However, the present study was unable to completely rule out skin penetration by *Rh. joaquinensis*, since infective worms may have migrated out of the esophagus or cloaca, resulting in skin-penetrating infections.

The present study observed skin-penetrating ability by all 4 species of anuran lungworms. When worms were placed in the vicinity of a host they become markedly

more active and immediately began to search for the host. Infective juveniles appeared to use a combination of physical stimuli, i.e., host vibrations and chemotaxis, to locate hosts. Upon physically encountering a host, worms generally undulated over host skin for 1–2 min, near the lower limbs or underbelly of the host prior to initiating penetration, except in *Rh. americanus* infecting adult toads, where penetration almost always occurred on, or near, the seed patch. Our observations suggest that the thick cuticle of an adult toad's lower appendages is a poor site for worm penetration. Schaake (1931) suggested worms gained host entry through the numerous pores associated with amphibian mucous glands. In our experience, worms almost always penetrated at a mucous gland, which appears to facilitate dermal penetration. Generally, penetration and entry lasted approximately 2–3 min, although some worms entered extremely rapidly, without much apparent resistance.

Internal migration by amphibian lungworms was first proposed by Fulleborn (1928) and Schaake (1931). Both authors mistakenly thought juveniles were dispersed throughout the body via the circulatory system, and eventually reached the lungs and matured to adults. Subsequent studies have conclusively shown that infective worms undergo a body migration that does not involve the circulatory system (Williams, 1960; Baker, 1979; this study). Following penetration, we found worms residing in the fascia, where they undergo an incomplete molt by retaining the sheath. Next, worms penetrated the fascia and entered into the body cavity, which was followed by a complete molt to adulthood. In agreement with Baker (1979), our study found that both the third and fourth stage cuticles are shed simultaneously. Finally, worms penetrated the lungs and fed on blood to become gravid adults.

Transport host experiments

The potential for transport hosts in the transmission of lungworms has been suspected since Fulleborn (1928) suggested that gastropods could serve as paratenic hosts for *Rh. americanus*. Previous studies have exposed snake and anuran lungworms to potential transport hosts to determine the ability of lungworms to penetrate into these hosts (Fulleborn, 1928; Chu, 1936; Baker, 1979). Chu (1936) reported that when *Rh. fuscovenosa* was fed to tadpoles, snails, copepods, and adult anurans, juvenile worms were passed unharmed in the feces and did not penetrate into the tissue of these animals. Fulleborn (1928) and Baker (1979) reported that anuran lungworms are capable of penetrating into snail tissue, and the latter author found a single instance of *Rh. fuscovenosa* penetrating into toads. However, no study has attempted to feed exposed transport hosts to homologous lungworm hosts. In the present study, both snake and anuran lungworms were capable of using transport hosts to establish infections in homologous host species in the laboratory. However, our results show lungworms vary in their ability to use transport hosts. Thus, anuran lungworms were able to use only snails as successful transport hosts, whereas snake lungworms were capable of using a variety of transport hosts, including frogs, earthworms, and snails. This study does not demonstrate infections of transport hosts in nature; however, snakes feed on a variety of prey items (Ernst and Ernst, 2003), including those used in this experiment. In addition, transport hosts used in this experiment are known to feed directly upon or near vertebrate feces (Campbell and Little, 1988; Bohlen and Edwards, 1995), which provide snake lungworms opportunities to penetrate potential transport hosts in nature. These observations suggest that snake lungworms may use transport hosts more frequently than

anuran lungworms to complete their life cycle. Snake lungworms rely on oral exposure to infect hosts (Chu, 1936; this study); therefore, the ability to penetrate host prey items should facilitate transmission to the final host, providing a viable evolutionary avenue for snake lungworms. Conversely, skin-penetrating anuran lungworms in our study appear to have little use for an oral transmission route because when infective juveniles enter a transport host their ability to encounter anuran epidermal tissue is drastically reduced, if not eliminated. Anuran lungworms in this study were poorly adapted to penetrating into the intestinal tract of anurans (see per os infections); transport host infections result when infective juveniles migrate out of the intestinal tract, resulting in skin penetrating infections near the margin of the mouth or cloaca. The large number of lungworms that pass (alive and dead) in feces following per os and transport host experiments support our assertion that anuran lungworms in this study are poorly adapted to oral routes of infection. The low prevalence and intensity of infections in these experiments make it unlikely transport hosts serve as an evolutionary avenue of infection for anuran lungworms. Nevertheless, other anuran lungworm species may use transport hosts successfully in nature if their anuran host(s) consume large numbers of semi-aquatic snails and/or display a greater propensity for intestinal penetration than found in this study.

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Figure I. Percentage of adult lungworms, (a) *Rhabdias joaquinensis*, (b) *Rhabdias ranae*, (c) *Rhabdias bakeri*, (d) *Rhabdias americanus*, (e) *Rhabdias eustreptos*, and (f) *Rhabdias fuscovenosa*, recovered during experimental infections of homologous anurans and snakes.

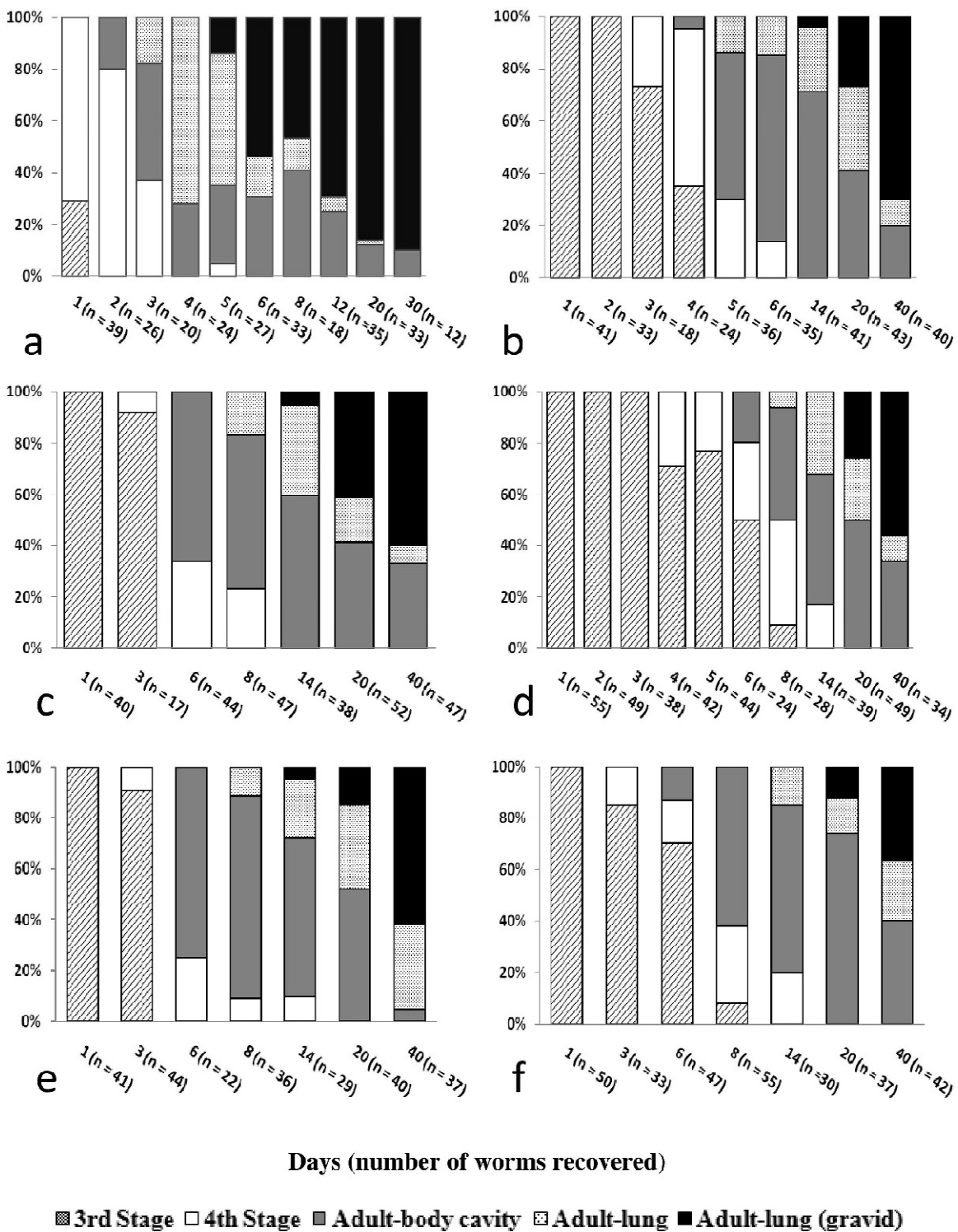


Table I. Characteristics of the free-living generation of *Rhabdias* spp. The species are divided into 2 categories, those that infect amphibians and those that infect reptiles. Mean, standard deviation, and range are reported as $\bar{x} \pm SD$ (range). Range only is provided for samples less than 5.

Species (# cultures)	Mean timing of development (hours)										Host (Location)
	Mating observed	Offspring hatch	Heterogonic 3 rd stage	Homogonic 3 rd stage	Days alive in petri dish	Number of eggs/female	Proportion heterogonic				
Amphibian											
<i>R. americanus</i> (n = 15)	22.6 ± 4.9 (15 - 31)	75.4 ± 31.3 (63 - 128)	144 ± 41 (90 - 195)	not observed	22	2-3	100%			<i>Bufo terrestris</i> (AL)	
<i>R. bakeri</i> (n = 6)	not observed	59.8 ± 5.8 (51 - 65)	92.7 ± 12.8 (78 - 114)	not observed	16	2-3	100%			<i>Rana sylvatica</i> (MO)	
<i>R. joaquiniensis</i> (n = 23)	8.2 ± 4.8 (3 - 14)	36.7 ± 14.5 (20 - 49)	73.2 ± 12.5 (66 - 95)	ca. 60	37	2-3	> 99%			<i>Acris crepitans</i> (NE)	
<i>R. ranae</i> (n = 8)	12.5 ± 7.1 (9 - 21)	52.2 ± 22.9 (45 - 68)	82.3 ± 25.2 (73 - 108)	not observed	18	2-3	100%			<i>Rana sphenoccephala</i> (AL)	
Reptile											
<i>R. eustreptos</i> (n = 18)	28 - 34	51 - 96	110 - 130	65.8 ± 23.5 (55 - 96)	97	ca. 8	< 2%			<i>Lampropeltus getulus</i> (MS)	
<i>R. fuscovenosa</i> (n = 20)	56 - 96	88 - 150	190 - 210	120 ± 15.2 (101 - 158)	90	ca. 10	< 1%			<i>Thamnophis sirtalis</i> (NE)	

Table II. Development of *Rhabdias eustreptos* experimentally exposed to *Thamnophis sauritus*. Free-living cultures established from wild *Thamnophis sirtalis*. Mean \pm SD (range) given in μ m.

	Free-living male	Free-living female	Third stage (infective)	Adult (lungs)	Adult gravid (lungs)	Adult gravid (lungs)
Days postinfection	-	-	-	10	18	30
No. measured	10	10	10	10	10	10
Total length	534 \pm 23 (500 - 583)	780 \pm 18 (714 - 836)	775 \pm 25 (743 - 840)	3.12 \pm 0.25 mm (2.67 - 3.78)	4.33 \pm 0.3 mm (3.74 - 4.7)	6.15 \pm 0.15 mm (5.67 - 6.28)
Maximum width	31 \pm 2 (27 - 35)	43 \pm 6 (35 - 59)	30 \pm 5 (26 - 44)	251 \pm 13 (237 - 268)	339 \pm 20 (298 - 373)	470 \pm 33 (401 - 494)
Oesophagus length	116 \pm 8 (109 - 138)	130 \pm 8 (120 - 152)	159 \pm 10 (140 - 177)	341 \pm 11 (322 - 360)	359 \pm 6 (337 - 382)	389 \pm 10 (371 - 401)
Tail length	29 \pm 4 (21 - 37)	50 \pm 5 (40 - 75)	57 \pm 6 (49 - 71)	213 \pm 21 (198 - 243)	250 \pm 8 (239 - 276)	290 \pm 6 (280 - 298)

Table III. Development of *Rhabdias fuscovenosa* experimentally exposed to *Thamnophis sauritus*. Free-living cultures established from wild *Lampropeltis getula*. Mean \pm SD (range) given in μ m.

	Free-living male	Free-living female	Third stage (infective)	Adult (lungs)	Adult gravid (lungs)	Adult gravid (lungs)
Days postinfection	-	-	-	14	20	30
No. measured	10	10	10	10	10	10
Total length	575 \pm 43 (514 - 629)	796 \pm 41 (705 - 850)	756 \pm 20 (740 - 810)	2.13 \pm 0.19 mm (2.01 - 2.45)	3.61 \pm 0.08 mm (3.54 - 3.78)	4.1 \pm 0.11 mm (3.89 - 4.21)
Maximum width	31 \pm 3 (25 - 37)	45 \pm 5 (33 - 55)	30 \pm 4 (23 - 39)	126 \pm 22 (116 - 141)	143 \pm 20 (133 - 160)	156 \pm 6 (149 - 166)
Oesophagus length	120 \pm 5 (111 - 127)	132 \pm 6 (122 - 148)	153 \pm 9 (141 - 178)	261 \pm 14 (242 - 288)	284 \pm 11 (259 - 301)	280 \pm 12 (272 - 300)
Tail length	30 \pm 5 (21 - 40)	52 \pm 6 (43 - 76)	55 \pm 7 (41 - 65)	123 \pm 14 (111 - 153)	168 \pm 18 (134 - 201)	180 \pm 11 (174 - 205)

Table IV. Development of *Rhabdias americanus* experimentally exposed to *Bufo woodhousii*. Free-living cultures established from wild *Bufo terrestris*. Mean \pm SD (range) given in μ m

	Free-living male	Free-living female	Third stage (infective)	Adult (lungs)	Adult gravid (lungs)	Adult gravid (lungs)
Days postinfection	-	-	-	9	18	30
No. measured	10	10	10	10	10	10
Total length	534 \pm 32 (489 - 598)	745 \pm 21 (700 - 761)	483 \pm 43 (414 - 554)	4.89 \pm 0.34 mm (3.8 - 5.6)	6.85 \pm 0.54 mm (5.2 - 7.21)	9.23 \pm 0.53 mm (8.76 - 10.03)
Maximum width	33 \pm 3 (29 - 35)	42 \pm 2 (39 - 45)	20 \pm 2 (17 - 25)	126 \pm 18 (103 - 158)	189 \pm 21 (156 - 201)	243 \pm 28 (227 - 266)
Oesophagus length	108 \pm 4 (100 - 111)	146 \pm 6 (138 - 159)	150 \pm 22 (120 - 182)	430 \pm 38 (411 - 454)	523 \pm 46 (501 - 559)	712 \pm 54 (640 - 742)
Tail length	32 \pm 3 (26 - 36)	49 \pm 5 (42 - 56)	42 \pm 7 (32 - 55)	156 \pm 24 (142 - 183)	247 \pm 29 (235 - 288)	344 \pm 41 (305 - 367)

Table V. Development of *Rhabdias bakeri* experimentally exposed to *Rana sylvatica*. Free-living cultures established from wild *Rana sylvatica*. Mean \pm SD (range) given in μm .

	Free-living male	Free-living female	Third stage (infective)	Adult (lungs)	Adult gravid (lungs)	Adult gravid (lungs)
Days postinfection	-	-	-	9	17	30
No. measured	10	10	10	10	8	10
Total length	501 \pm 33 (466 - 553)	748 \pm 24 (712 - 780)	490 \pm 30 (433 - 566)	3.03 \pm 0.22 mm (2.84 - 3.46)	4.22 \pm 0.26 mm (3.95 - 4.50)	7.24 \pm 0.09 mm (6.93 - 7.49)
Maximum width	33 \pm 2 (28 - 36)	49 \pm 5 (44 - 59)	20 \pm 2 (18 - 22)	94 \pm 9 (85 - 110)	127 \pm 6 (114 - 137)	134 \pm 4 (119 - 139)
Oesophagus length	112 \pm 4 (104 - 115)	146 \pm 9 (133 - 157)	140 \pm 27 (115 - 177)	334 \pm 9 (326 - 349)	353 \pm 11 (341 - 402)	401 \pm 11 (389 - 417)
Tail length	30 \pm 3 (28 - 37)	55 \pm 7 (48 - 74)	45 \pm 6 (35 - 59)	159 \pm 12 (145 - 177)	197 \pm 9 (177 - 211)	234 \pm 8 (221 - 245)

Table VI. Development of *Rhabdias joaquinensis* experimentally exposed to *Acris crepitans*. Free-living cultures established from wild *Acris crepitans*. Mean \pm SD (range) given in μ m.

	Free-living male	Free-living female	Third stage (infective)	Adult (lungs)	Adult gravid (lungs)	Adult gravid (lungs)
Days postinfection	-	-	-	4	6	20
No. measured	10	10	10	10	10	10
Total length	521 \pm 43 (478 - 585)	688 \pm 37 (642 - 720)	491 \pm 31 (448 - 530)	3.85 \pm 0.29 mm (3.3 - 4.0)	3.95 \pm 0.12 mm (3.7 - 4.25)	4.11 \pm 0.08 mm (3.81 - 4.18)
Maximum width	31 \pm 5 (28 - 34)	40 \pm 5 (37 - 46)	20 \pm 2 (17 - 21)	210 \pm 32 (186 - 254)	285 \pm 30 (274 - 333)	291 \pm 18 (279 - 329)
Oesophagus length	115 \pm 8 (108 - 119)	134 \pm 9 (128 - 139)	140 \pm 12 (136 - 158)	352 \pm 21 (342 - 372)	361 \pm 16 (349 - 381)	367 \pm 14 (357 - 375)
Tail length	29 \pm 3 (27 - 30)	53 \pm 4 (49 - 56)	42 \pm 4 (38 - 45)	143 \pm 24 (122 - 165)	160 \pm 18 (149 - 173)	160 \pm 10 (155 - 168)

Table VII. Development of *Rhabdias ranae* experimentally exposed to *Rana pipiens*. Free-living cultures established from wild *Rana sphenoccephala*. Mean \pm SD (range) given in μ m.

	Free-living male	Free-living female	Third stage (infective)	Adult (lungs)	Adult gravid (lungs)	Adult gravid (lungs)
Days postinfection	-	-	-	9	14	30
No. measured	10	10	10	8	10	10
Total length	474 \pm 34 (454 - 523)	778 \pm 37 (708 - 832)	519 \pm 21 (498 - 541)	3.18 \pm 0.39 mm (2.83 - 3.85)	3.87 \pm 0.22 mm (3.33 - 4.12)	6.3 \pm 0.12 mm (5.7 - 6.33)
Maximum width	26 \pm 4 (23 - 29)	58 \pm 5 (53 - 64)	20 \pm 2 (17 - 22)	109 \pm 12 (89 - 121)	120 \pm 8 (114 - 123)	123 \pm 4 (120 - 127)
Oesophagus length	109 \pm 12 (105 - 123)	144 \pm 6 (141 - 149)	151 \pm 7 (143 - 155)	348 \pm 18 (320 - 363)	371 \pm 17 (349 - 383)	401 \pm 11 (389 - 417)
Tail length	29 \pm 4 (27 - 31)	63 \pm 13 (52 - 81)	53 \pm 4 (47 - 59)	167 \pm 14 (135 - 181)	186 \pm 9 (177 - 214)	218 \pm 11 (211 - 223)

CHAPTER TWO: HOST SPECIFICITY OF NORTH AMERICAN *RHABDIAS* SPP. (NEMATODA: RHABDIASIDAE): COMBINING FIELD DATA AND EXPERIMENTAL INFECTIONS WITH A MOLECULAR PHYLOGENY

Abstract: Lungworms of the cosmopolitan genus *Rhabdias* are among the most common parasites of amphibians and squamate reptiles. The present study used experimental infections, field studies, and a molecular phylogeny to determine the host specificity of 6 *Rhabdias* spp. that infect snakes and anurans from North America. The molecular phylogeny suggests *Rh. ranae* from Nebraska and Mississippi may represent separate, cryptic species. In addition, the phylogeny strongly supports separate clades for anuran and snake lungworms. Field studies and experimental infections indicate that snake lungworms are generalist snake parasites; however, laboratory experiments also suggest lizards may be infected under some environmental conditions. Lungworms from anurans were unable to infect caudatans (i.e., salamanders) or reptiles in nature or in the laboratory, and anuran lungworm species ranged from strict host specificity (e.g., *Rh. ranae* from Nebraska) to relative generalist (e.g., *Rh. joaquinensis* from Nebraska). Host specificity in nature appears to be limited by both ecological and physiological factors, which vary between species and their hosts. In addition, lungworms appear to be tracking host resources instead of host phylogenies, an example of ecological fitting. Lastly, young anurans may be more susceptible to lungworm infection in some host-parasite combinations; in contrast, however, some lungworm species showed higher infection parameters in adult anurans.

INTRODUCTION

Host specificity is a universal and fundamental property of parasites and knowledge of host range is vital to understanding parasite evolution and ecology. In this study host range is defined as the number of host species a parasite can infect, thus an increase in host range corresponds to a decrease in host specificity. Host specificity provides insight into several intrinsic aspects of parasitism, such as parasite population dynamics, geographic limitations, taxonomic determinations, and past and future evolutionary avenues (Brooks and McLennan, 2002; Poulin, 2007). Yet despite its importance, little information is available on the limitations of host range for most parasite species (Perlman and Jaenike, 2003); and when available, specificity is often inferred from published records of parasite occurrence that may not reveal true host range (Brooks, 2003; Poulin, 2007). Preferably, experimental infections provide a comparative method to determine the limits of host range that avoids the constraints inherent to the sampling of parasite communities in nature (Snyder and Janovy, 1994; Krasnov et al., 2004; Bolek and Janovy, 2007; Poulin, 2007; Detwiler and Janovy, 2008; Dare et al., 2008).

In nature, host range can be restricted by phylogenetic, ecological, physiological, and immunological parameters (Combes, 2001; Poulin, 2007). The effects of each parameter on host specificity is difficult, if not impossible, to isolate using only parasite occurrence data. Alternatively, experimental infections can document host immune response and explain most ecological and some physiological barriers that may occur in nature to determine the probability of parasite establishment. These infections, when combined with phylogenetic data, can provide insight into the constraints placed on

parasite range by each of the 4 parameters. Recently studies by Bolek et al. (2009); Criscione et al. (2006); Dare et al. (2008); Detwiler and Janovy (2008); Edwards and Vidrine (2006); Little et al. (2006); and Munoz et al. (2007) have used this method to successfully elucidate host specificity, in the process distinguishing between physiological and ecological factors producing the specificity. These studies support the observations by Brooks (2003) and Poulin (2007) that establishing host specificity based on abundance records likely provides inaccurate results.

The increased availability of molecular tools to study host-parasite co-evolution has been a key component in recent host specificity research. Knowledge of a group's evolutionary history can provide support for the hypothesis of progressive specialization in host-parasite lineages (Brooks and McLennan, 2003). Furthermore, host specificity based on co-speciation events is only detectable using phylogenetic methods (Adamson and Caira, 1994), and often such specialization produces strict host specificity.

Alternatively, a lack of closely associated phylogenetic patterns suggests that current host range is not a result of common ancestry. If evolutionary history is not directly responsible for host range, then alternative factors, such as host-parasite ecological interactions, should be explored. In this case, the phylogeny provides evolutionary context for exploring alternative factors that may influence host range (Poulin, 2007). Phylogenies also are useful when working with morphologically cryptic species, which have the potential to artificially alter measurements of host range (Brooks and McLennan, 2003; Criscione et al., 2005; Emelianov, 2007; Jousson et al., 2000). Cryptic species are often impossible to distinguish morphologically, yet their life histories and

ecological interactions may vary widely; thus, leading to substantial differences in host specificity.

Amphibian and reptile parasites are good model hosts to study questions of host specificity. Numerous recent studies by Bolek and Coggins (2001; 2003), Gilliland and Muzzall (1999), Langford and Janovy (2009), McAlpine (1997), Schotthoefer et al. (2009), and Telford et al. (2001) have established basic life history data, such as life cycles, distribution, reproduction, and population and community structure in these hosts. These baseline data are necessary to conduct experimental host specificity research, yet few researchers have taken advantage of such studies to conduct host specificity research on these parasites (*Haematoloechus* spp. are the notable exception). Our previous study, Langford and Janovy (2009), established the life histories and life cycles of 6 *Rhabdias* spp. from North American snakes and anurans, however little is known about their host specificity. Of the more than 70 species of *Rhabdias* worldwide only 3 species have been systematically tested for host range experimentally. In 1936, Chu exposed *Rhabdias fuscovenosa* obtained from a snake to 2 species of frogs, a toad, and a turtle; he was unable to establish infections in these hosts and suggested *Rh. fuscovenosa* was restricted to snakes. In 1979, Baker conducted cross-transmission experiments on *Rhabdias americanus*, *Rhabdias ranae* (herein assumed to be *Rhabdias bakeri*, see Tkach et al., 2006), and *Rh. fuscovenosa*. In agreement with Chu, he was unable to establish non-snake infections using *Rh. fuscovenosa*. However, despite an apparent lack of cross-transmission in nature, Baker was able to establish infections of *Rh. americanus*, obtained from *Bufo americanus*, in *Rana blairi*. He also established *Rh. bakeri* infections, obtained from *Ra. blairi*, in *B. americanus*. Recently, Dare et al. (2008) successfully established

Rh. ranae, collected from *Ra. pipiens*, infections in sympatric *Ra. blairi* metamorphs, however molecular data suggested wild *Ra. blairi* were not infected with *Rh. ranae*. These studies suggest that additional evolutionary avenues are available to *Rhabdias* spp. in nature, but such infections likely remain undetected due to low host sample sizes and spatiotemporal limitations in sampling (see Poulin, 2007).

Currently, amphibian *Rhabdias* spp. are considered specialists, i.e., host specific, whereas lungworms infecting snakes are considered generalists (Dare et al., 2008; Kuzmin et al., 2001; 2003; Martı́nez-Salazar, 2007; Tkach et al., 2006). This conclusion is based on field surveys and/or molecular sequences, but not experimental infections. For example, Tkach et al. (2006) suggested that *Rh. ranae* only infects leopard frogs (i.e. host specific) and that *Rh. bakeri* only infects *Ra. blairi* (i.e. very host specific). However, their results are confusing because they found *Rh. bakeri* infecting metamorphic *Ra. ranae* during his field surveys, which they attributed to the altered immune status of the metamorphic frogs (see Carey et al. 1999). Their argument may be valid because age of amphibian hosts may alter some aspects of host-parasite relationships (Goater and Ward, 1992); however, it is unknown if these factors affect host specificity in lungworms, and without detailed experimental infections questions concerning lungworm host specificity cannot be answered. To address these questions of host specificity, the present study's goals are to (1) determine the host specificity of 6 North American lungworm species from native amphibians and reptiles using experimental infections, (2) determine if host age (estimated by size) affects host specificity, (3) develop a molecular phylogeny to elucidate relationships between lungworms, and reveal evolutionary and ecological avenues for and constraints on

lungworm transmission, and (4) use the phylogeny to address an unanswered question from Langford and Janovy (2009). Our previous study found anuran and snake lungworms had distinctly different life cycles, and suggested these differences could be reflected in the evolutionary history of these lungworms. If snake and anuran lungworms form separate clades, then the suggestions of Langford and Janovy (2009) would be supported.

MATERIALS AND METHODS

Amphibian and reptile field studies

Amphibians and reptiles were captured both day and night using hand capture, pit-fall traps, funnel traps, drift fences, and by cruising roads for dead and live animals (Heyer et al. 1994). Animals were transported to either the parasitology laboratory at the University of Nebraska-Lincoln's Cedar Point Biological Station in western Nebraska, or the vertebrate museum at the University of South Alabama, euthanized, measured for snout-vent (SVL), and all organs and tissues were examined for *Rhabdias* spp. within 24 hours of collection. Lungworms were removed and fixed in hot 70% ethyl alcohol for identification; however, most adult hermaphrodites were set aside for use in host specificity experiments. Representative specimens were cleared and temporarily mounted in glycerol for identification (Prichard and Kruse, 1982). All lungworms were identified according to Kuzmin et al. (2003), supplemented by Tkach et al. (2006). Lungworm specimens were deposited in the H. W. Manter Parasitology Collection, University of Nebraska, Lincoln, Nebraska (accession numbers HWML 63512 – 63517).

During June – July 2006, the following anurans were collected from Lancaster Co., Nebraska (40.86, -96.89): *Acris blanchardi*, *Bufo (Anaxyrus) woodhousii*, *Hyla*

chrysozelis, *Pseudacris triseriata*, *Rana (Lithobates) blairi*, *Rana (Lithobates) catesbeiana*, and *Spea bombifrons*. During the same period, the following herpetofauna were collected from Keith County, Nebraska (41.30, -101.92): *Ambystoma tigrinum*, *Cnemidophorus sexlineatus*, *Sceloporus undulatus*, *Thamnophis sirtalis*, and *Thamnophis radix*; and the following herpetofauna were collected from Shawnee County, Kansas (39.03930, -95.57741): *A. blanchardi*, *B. woodhousii*, *H. chrysozelis*, and *Elaphe obsoleta*. In July 2007, the following anurans from Saunders Co., Nebraska (41.02, -96.29): *Bufo (Anaxyrus) americanus*, *B. woodhousii*, *H. chrysozelis*, *P. triseriata*, *R. blairi*, and *R. catesbeiana*. In August 2007, the following herpetofauna were collected from the Grand Bay National Estuarine Research Reserve, Jackson Co., Mississippi (30.45, -88.42): *Acris gryllus*, *Agkistrodon piscivorous*, *Bufo (Anaxyrus) fowleri*, *Bufo (Anaxyrus) quercicus*, *Bufo (Anaxyrus) terrestris*, *Coluber constrictor*, *Gastrophryne carolinensis*, *Hyla cineria*, *Hyla versicolor*, *Kinosternon subrubrum*, *Lampropeltis getula*, *Nerodia fasciata*, *Rana (Lithobates) clamitans*, *Rana (Lithobates) sphenoccephala*, *Scaphiophus hoolbrooki*, *Thamnophis sauritus*, and *Trachemys scripta*. Lastly, in April 2008, road-killed *P. triseriata*, *Ra. sphenoccephala*, and *Ra. sylvatica* were collected from Jefferson County, Missouri (38.29, -90.45). Number of hosts collected at each location is provided in Table I; salamanders (n = 6), turtles (n = 9), and lizards (n = 21) were not infected in nature.

Acquiring uninfected hosts

Hosts used for experimental infections were collected during field surveys (see above). Young of the year (YOY) amphibians were collected as eggs, tadpoles, metamorphs, or bred from captive adults or road-killed specimens, via artificial

insemination of eggs, for experimental infections. Metamorphs were held for 2-3 weeks, dependent on species, to allow prepatent infections to develop; infected individuals were not used in experimental infections (see Langford and Janovy 2009). Given the young age of these uninfected metamorphs we assume they haven't been infected in their lifetime, based on lungworm life history data provided by Langford and Janovy (2009). Adult amphibians (according to length in Conant and Collins) also were held for 2-3 weeks to assure no prepatent infections existed.

Reptiles were not differentiated by size because they do not undergo metamorphosis and their immune system does not shift drastically during their lifetime, as seen in amphibians (Rollins-Smith, 1998). Captured reptiles were held for 3 weeks to allow prepatent infections to develop, thus assuring only uninfected individuals were used in experiments. Uninfected snakes also were acquired by isolating wild, gravid individuals in the laboratory and housing offspring in cages that prevented transmission. No reptiles were infected before 3 weeks of age. The infection history of wild captured hosts, both amphibian and reptile, is unknown, but it seems reasonable to assume that some adults were previously infected with at least one species of lungworm. It is not known if host immune response is altered by prior lungworm infection.

Lungworm experimental infections

Free-living lungworms were acquired directly from parasitic adults removed from host lungs to assure lungworm identification. Infective juveniles were raised in Petri dishes according to Langford and Janovy (2009), which produced numerous infective juveniles. Hosts were exposed to infective lungworms according to Langford and Janovy (2009). In brief, this method involved orally pipetting infective lungworms obtained from

snake hosts, which only infect hosts orally, into experimental hosts, whereas amphibian lungworms, which penetrate host dermal tissues, were placed in appropriately sized containers with experimental hosts. Hosts were each exposed to 30 infective juveniles, either per os or per container. Depending on cannibalistic tendencies, hosts were housed and cared for individually or in small groups. Cages were lined with absorbent paper towels and cleaned 2-3 times per week to prevent development of infective juveniles in host feces and potential transmission. Snakes were fed frozen, thawed white fish (*Tilapia* sp.) and/or mice; anurans were fed a diet of commercial crickets (Top Hat Cricket Farm, Kalamazoo, Michigan) and cultured mealworms (Janovy Laboratory, University of Nebraska–Lincoln). Aged tap water was provided ad lib. Hosts were given an appropriate temperature gradient via an under-the-tank heating pad and provided full spectrum lighting on a 12-hr day/light cycle.

Experimental infections of reptile and amphibian lungworms were conducted from July 2006 – October 2008. When groups of uninfected hosts became available they were exposed to sympatric lungworms. Trials of each lungworm species were conducted in succession to ensure host species were exposed to similar lungworms (i.e. worms collected from the same host species and location) to minimize issues of seasonality and local adaption. Every effort was made to standardize host-parasite exposures, and time-0 and time-T controls were used in all trials to ensure hosts did not develop spurious infections through the course of the experiments. Time-0 controls were dissected at the beginning of the experimental infections and time-T controls were maintained throughout the duration of the experiment and dissected along with the experimental group. The experimental groups were maintained until infections should have developed according to

Langford and Janovy (2009). At 30 days for *Rh. joaquinensis* and 40 days for the remaining 5 lungworm species, experimental groups were dissected and all organs, muscles, body cavities, and connective tissues were thoroughly searched for lungworms. Prior to dissection hosts were measured for snout-vent length (SVL), and host sex was determined during dissection.

Prevalence and/or mean abundance were calculated as measures of parasitism for amphibians and reptiles examined (Margolis et al., 1982). Values are reported as mean \pm 1 S.D. Host specificity was calculated using 2 measurements: host range (HR), the number of host species infected; S_{TD}^* , an index that combines phylogenetic and ecological data to assess host specificity, higher values indicate less host specificity. To determine S_{TD}^* , data on parasite prevalence and host taxonomy were used according to Poulin and Mouillot (2005), host taxonomy follows the recommendations of Pauly et al. (2009). S_{TD}^* has proven a useful calculation in comparative studies (e.g. Edwards and Veldrine, 2006), especially when parasite species are not collected from the same location and/or exposed to different sets of host species. Our goal was to determine the usefulness of these indices for lungworms. Student's 2-tailed *t*-test was used to compare mean abundance between young-of-the-year (YOY) and adult anurans from both experimental and natural infections with more than 30 individuals and only 1 species of lungworm. Host size (i.e. SVL) was used as a proxy to designate YOY vs. adult anurans. Anurans were considered YOY if SVL was < 50% of the adult size listed in Conant and Collins (1998).

Extraction, PCR, and sequence analysis

Genomic DNA was extracted, according to Tkach and Pawlowski (1999), using DNeasy tissue kits (Qiagen, Valencia, California), from single adult worms collected from the lungs. DNA fragments of approximately 2,200 base pairs and spanning the 3' end of 18S nuclear rDNA gene, internal transcribed spacer (ITS) region (ITS1 _ 5.8S _ ITS 2) and 5' end of the 28S (including variable domains D1–D3) were amplified by polymerase chain reaction (PCR) on an Eppendorf Master Gradient thermal cycler. PCR reactions were performed in a total volume of 51.5 ml (42ml H₂O, 5 ml Taq buffer, 1 ml dNTP at concentration 10 pM, 1 ml of each primer at concentration 10 pM, 0.25 ml of Taq, and 1–1.5 ml of template DNA solution, depending on the DNA concentration) by using Eppendorf Taq polymerase (concentration 5 units/1 ml). The Rhabdiasidae-specific forward primer ritf (59-GCGGCTTAATTTGACTCAACACGG-39) and the universal reverse primer 1500R (59-GCTATCCTGAGGGAACTTCG-39) were used for both amplification and sequencing. In addition, internal primers ITS5 (59-GGAAGTAAAAGTCGTAACAAGG-39), ITS4 (59-TCCTCCGCTTA TTGATATGC-39), 300R (59-CAACTTTCCTCACGGTACTTG-39) and ECD2 (59-CTTGGTCCGTGTTTCAAGACGGG-39) were used for sequencing. PCR products were purified directly using QIAGEN QIAquick columns (QIAGEN, Valenica, California). Sequences were determined directly from PCR templates by cycle sequencing using Big Dye fluorescent dye terminators and protocols and an ABI 377 automated sequencer (Perkin-Elmer, Foster City, California). Sequences for 6 lungworms species were assembled using Sequencher (version 4.1.4, GeneCodes Corporation, Ann Arbor, Michigan) and provisionally aligned using ClustalX with default settings (Thompson et al., 1997). The resultant sequence alignment was then edited by eye with Bio Edit

Sequence Alignment Editor (Hall, 1999) to remove ambiguous regions that appeared to lack homology, and the ends of each fragment were cut to match the shortest sequence in the alignment yielding an 2104 character alignment. Sequences were submitted to GenBank under accession numbers #–# (*Rh. americanus*), #–# (*Rh. bakeri*), #–# (*Rh. eustreptos*), #–# (*Rh. fuscovenosa*), #–# (*Rh. joaquinensis*), and #–#(*Rh. ranae*). Additional sequences acquired from GenBank, DQ264771.1 (*Rh. ranae*) and DQ264767.1 (*Rh. bakeri*), were included in the alignment.

Phylogenetic analysis

Using PAUP_ 4.0b10 (Swofford, 1998) maximum parsimony (MP) and maximum likelihood (ML) analyses were performed on the combined, ITS, and LSU datasets. MP was performed using a heuristic search with unweighted and unordered characters. All searches included 100 stepwise random addition replicates with tree bisection-reconnection branch (TBR) swapping and a maximum of 100 trees. A strict consensus tree was constructed from the MP analysis when more than 1 best tree was found. Using Modeltest v. 3.06 (Posada and Crandall, 1998) a general time reversible model (GTR+I+G; Rodriguez et al. 1990) of substitution was selected for use in ML analysis. All sequences were added to the tree using 100 random addition replicates with the TBR branch-swapping algorithm. To establish support for nodes, 1,000 bootstrap replicates were calculated using heuristic search criteria for both MP and ML analyses. Gaps were treated as missing data in both searches, and analyses were unrooted because no appropriate, alignable outgroup was available.

RESULTS

Amphibian and reptile field studies

Over a 2 year period more than 1,700 lungworms were recovered from the lungs of 615 amphibians and reptiles (Table I). *Rhabdias eustreptos* (HR = 2, S_{TD}^* = 3) and *Rh. fuscovenosa* (HR = 5, S_{TD}^* = 1.6) only infected snakes. Snake lungworms did not infect all available host species in nature, only infecting a subset of snake species collected at each location. Snake lungworm species both obtained 100% prevalence in at least 1 host, and mean abundance reached a high of 5.4 ± 4.4 for *Rh. eustreptos* and 6.2 ± 6.2 for *Rh. fuscovenosa*. The remaining 4 lungworm species only infected anurans (i.e. frogs and toads), however host range varied greatly among lungworm species: *Rh. americanus* (HR = 4, S_{TD}^* = 1) only infected species of Bufonidae, *Rh. bakeri* (HR = 2, S_{TD}^* = 3) infected 1 species of Bufonidae and 1 species of Ranidae, *Rh. joaquinensis* (HR = 12, S_{TD}^* = 2.34) infected 4 different host families, and *Rh. ranae* (HR = 4, S_{TD}^* = 1) only infected species of Ranidae. Except for *Rh. joaquinensis* (max. 90% prevalence), all anuran lungworms obtained 100% prevalence in at least one host species, and mean abundance reached a high of 12.1 ± 10.6 for *Rh. americanus*, 7 ± 3.6 for *Rh. bakeri*, 7.3 ± 10.1 for *Rh. joaquinensis*, and 4.7 ± 3.1 for *Rh. ranae*. The following 3 species of anurans were not infected at any location: *B. quercicus*, *G. carolinensis*, and *Sp. bombifrons*; the snake *C. constrictor* also was uninfected.

Phylogenetic analysis

Nucleotide frequencies for the combined rDNA data set were 0.242 (A), 0.202 (C), 0.270 (G), and 0.283 (T); frequencies were not significantly heterogeneous across taxa ($X^2 = 32.55$, $P = 0.67$). Of 2,104 bp, 1901 were invariant, 16 variable characters were parsimony uninformative, and 187 were parsimony informative (149 from ITS dataset). Maximum parsimony analysis yielded 2 trees with a consistency index (C.I.) =

0.95 and length of 226 steps. Both the MP and ML trees supported distinct clades for anuran and snake lungworms (Figures 1-2). The methods also agreed upon species designations, including 1 possible cryptic species, *Rh. ranae* collected in Mississippi. In the ML analysis, *Rh. ranae* from Mississippi were placed as a sister species to *Rh. joaquinensis*, instead of grouping with *Rh. ranae* collected from Nebraska and North Dakota. To avoid potential complications due to cryptic species, host specificity experiments in this study regard *Rh. ranae* from Mississippi as a distinct species. The sequence identity matrix supports this designation (Table II).

The LSU dataset (935bp) provided poor phylogenetic signal compared to the ITS dataset (see below). Forty sites were variable among all lungworm species, however only 11 variable sites occurred among lungworm species that infect anurans. A strict consensus of 14 MP trees and ML tree yielded polytomys for anuran lungworms (Figure III). However, despite poor phylogenetic signal, both MP and ML analyses supported distinct clades for anuran and snake lungworms.

The ITS dataset provided good phylogenetic signal. For ITS-1 (249 bp), 84 sites were variable, but only 29 sites varied among all lungworm species collected from anurans. The 200 bp ITS-2 data contained 70 variable sites among all lungworms, but only 17 among worms that infect anurans. Maximum parsimony analysis of the ITS dataset yielded 1 tree with a C.I. = 0.96 and length of 184 steps (Figure IV). The topologies of the ML and MP analyses from the ITS dataset agreed. The tree supports a sister species relationship between *Rh. ranae* from Mississippi and *Rh. joaquinensis* found during the combined analysis, but disagrees with the combined analysis by placing *Rh. ranae* as a recent common ancestor to *Rh. ranae* from Mississippi and *Rh.*

joaquinensis. The ITS analysis places *Rh. bakeri* basal to all other anuran lungworms, which disagree with the sister species relationship found in the combined analysis. The ITS dataset analysis agrees with both the combined and LSU datasets that anuran and snake lungworms form distinct clades.

Experimental infections

Infective juveniles of the 4 anuran lungworms species were unable to establish infections in salamanders or snakes, and varied in their ability to infect anurans (Table III). *Rhabdias americanus* (HR = 3, $S_{TD}^* = 1.78$) infected all *Bufo* spp. it was exposed to with a high prevalence of 100% and mean abundance of 28.1 ± 1.6 in *B. terrestris*. These lungworms also infected *Ra. sylvatica* with a prevalence of 57% and mean abundance of 12 ± 12.7 . *Rhabdias bakeri* (HR = 2, $S_{TD}^* = 3$) infected *Ra. sylvatica* with a prevalence of 100% and mean abundance of 12 ± 11.7 , but was unable to establish infections in *Ra. sphenoccephala*. However, the toad *B. americanus* was infected by *Rh. bakeri* with a prevalence of 71% and mean abundance of 11.2 ± 9.3 . *Rhabdias joaquinensis* (HR = 8, $S_{TD}^* = 2.7$) infected all anurans it was exposed to, except *G. carolinensis*, which was resistant to all lungworm exposures. Prevalence reached a high of 100% in 3 host species, and mean abundance reached a high of 28.1 ± 2.4 in *Ra. blairi*. *Rhabdias ranae* from Nebraska (HR = 2, $S_{TD}^* = 1$) only infected leopard frogs, *Ra. blairi* and *Ra. pipiens*, reaching 100% prevalence in both species and a maximum mean abundance of 28.5 ± 4.0 in *Ra. blairi*. *Rhabdias ranae* from Mississippi (HR = 4, $S_{TD}^* = 2.3$) displayed reduced host specificity when compared to worms from Nebraska. The worms infected 2 *Rana* spp. and 2 *Bufo* spp. with a high prevalence of 100% and mean abundance of 24.1 ± 5.4 in *Ra. sphenoccephala*; but were unable to infect the genus *Acris* and *Hyla*.

Experimental exposures of snake lungworms confirmed that *Rh. eustreptos* and *Rh. fuscovenosa* were unable to infect amphibians or turtles (Table IV). However, *Rh. fuscovenosa* was able to establish an infection in a lizard, with 1 of 5 *Sc. undulatus* infected with 15 gravid worms. Both snake lungworm species infected all snakes they were exposed to with a high prevalence of 100% in several host species, and mean abundance reached a high of 28 ± 2 for *Rh. eustreptos* in *L. getula* and 26.6 ± 4.2 for *Rh. fuscovenosa* in *C. constrictor*. None of the time-0 or time-T controls were infected with any lungworm species for any experiment.

Comparisons of prevalence and mean abundance of lungworms infecting YOY vs. adult anurans are presented in Table V. Prevalence was higher in adult *A. gryllus*, *H. chrysoyelis*, and *P. triseriata* than in YOY anurans, whereas prevalence was higher in YOY *A. blanchardi*, *B. woodhousii*, *B. quercicus*, and *Ra. catesbiana* than in adult anurans. Statistically significant differences in mean abundance were observed for *Rh. joaquinensis* infecting *A. blanchardi*, *A. gryllus*, and *B. woodhousii*.

DISCUSSION

Our study combines field studies and experimental infections with a parasite phylogeny to comparatively establish host specificity in North American lungworms. Prior to this study, snake lungworm host specificity had not been formally addressed (but see Chu, 1936), and anuran lungworms were assumed to be strictly host specific based on field studies (Kuzmin et al., 2003 and references within). These assumptions likely over-estimated host specificity, i.e., assumed a decreased host range, due to parasite misidentification (see Kuzmin et al., 2003), low sample sizes, and limited geographic sampling (Poulin, 2007). Our study challenges universal strict host specificity in anuran

lungworms, and establishes lungworms collected from snakes as relative generalists. Furthermore, our research establishes *Rh. eustreptos* and *Rh. fuscovenosa* as parasites of squamate reptiles and *Rh. americanus*, *Rh. bakeri*, *Rh. joaquinensis*, and *Rh. ranae* as parasites of anurans. The study also suggests ecological fitting has played a role in lungworm host specificity.

The ITS region of rDNA has been shown to be a useful molecular region for investigating evolutionary patterns among nematodes (Powers et al., 1997). In this study the ITS region was highly variable and thus provides good phylogenetic signal, whereas the LSU dataset was shown to have a relatively poor phylogenetic signal (see results). These results suggest that our combined analysis may suffer from an over abundance of invariable sites and thus provide less than ideal results. Despite variations in phylogenetic signals all 3 datasets yielded separate clades for anuran and snake lungworms (see Figures 1-4). The genetic distance between snake and anuran lungworms suggests these 2 clades may need to be reclassified into separate genera, however data from other members of the Rhabdiasidae are needed before this designation can be confirmed. Separation of these 2 clades is supported by their different host specificities (this study) and life cycles (Langford and Janovy, 2009). The phylogeny also supports our species identifications and eliminates concerns about cryptic species (except among *Rh. ranae*, see below).

The combined dataset (see Figures 1-2) supports *Rh. americanus* and *Rh. bakeri* as sister taxa, however the ITS dataset places *Rh. bakeri* basal to all other lungworm species that infect anurans (see Figure IV). This close relationship between the 2 worm species may partially explain the ability of *Rh. bakeri* to infect toads (see below), and the

relationship suggests a host switch from frogs (Ranidae) to toads (Bufonidae) (or vice versa). The ITS and combined datasets agree on the distinct phylogenetic signal of *Rh. ranae* collected from Nebraska/North Dakota (herein referred to as *Rh. ranae* # 1) versus Mississippi (herein referred to as *Rh. ranae* # 2), which is supported by our sequence identity matrix (see Table II). The matrix shows that *Rh. ranae* # 1 and *Rh. ranae* # 2 have less similarity (0.988) than other established species (e.g., *Rh. americanus* vs. *Rh. bakeri*, 0.993). The ITS and combined datasets agree that *Rh. ranae* # 2 and *Rh. joaquinensis* are sister taxa, and according to the ITS dataset the 2 species share a common ancestor with *Rh. ranae* # 1. However, the combined dataset places *Rh. ranae* # 1 in a clade with *Rh. bakeri* and *Rh. americanus*. Given the morphological similarity between *Rh. ranae* # 1 and *Rh. ranae* # 2 it seems more likely that the relationship dictated by the ITS dataset will be confirmed with additional sequence data, specifically solid outgroups.

The host specificity of lungworms is a controversial issue (Tkach et al., 2006). A recent study by Tkach et al. (2006) concluded that *Rh. bakeri* displayed strict host specificity, only infecting *Ra. sylvatica*; however, earlier experimental infections conducted by Baker (1979) showed *Rh. bakeri* was able to infect *B. americanus*. Likewise, a study by Kuzmin et al. (2003) considered *Rh. americanus* to be a strict parasite of toads (*Bufo* spp.), despite previously successful cross-transmission studies that infected *R. sylvatica* (Baker, 1979). In both cases our results corroborate Baker's findings. Our experimental infections established *Rh. americanus* infections in both *Bufo* spp. and *R. sylvatica*, but only *Bufo* spp. were found infected in nature. Based on successful experimental infections, we consider *Ra. sylvatica* a viable host for *Rh.*

americanus, and suggest that future field studies will find the lungworm infecting wild *Ra. sylvatica* in habitats conducive to *Ra. sylvatica* and *Bufo* spp. overlap. Similarly, field studies in Missouri found *Rh. bakeri* infecting *Ra. sylvatica* (Ranidae) and the distantly related toad *B. americanus* (Bufonidae); these results were replicated in our experimental infections; therefore, we consider *B. americanus* a viable host for *Rh. bakeri*.

Interestingly, we were unable to infect *Ra. sphenocephala*, a close relative (both frogs are members of the subgenus *Aquarana*) of *Ra. sylvatica*. We found infective juvenile *Rh. bakeri* were able to penetrate *Ra. sphenocephala*, but worms never progressed further than the body cavity; whether the host immune system prevented development or worms became lost during migrations is unclear. The ability of *Rh. bakeri* to infect a distantly related host (i.e., separate families), but not *Ra. sphenocephala*, suggests that these worms are tracking host resources instead of host phylogenies, i.e., they are an example of ecological fitting (Brooks et al. 2006).

Our hypothesis is supported by the unique terrestrial behavior of *Ra. sylvatica*, which brings it into close contact with toads, but not other frogs. The majority of *Rana* spp. are aquatic or semi-aquatic, rarely spending extended time periods in terrestrial environments, however *Ra. sylvatica* has a distinctly terrestrial life style, inhabiting landscapes similar to most *Bufo* spp (see Conant and Collins, 1998). Inhabiting terrestrial habitats likely exposes *B. americanus* and *Ra. sylvatica* to each other's feces, where parasite transmission occurs, whereas *Ra. sylvatica* only shares habitat with other *Rana* spp. during the breeding season. Furthermore, *Ra. sylvatica* possesses physiological aspects, such as epidermal properties (e.g. desiccation times; Schmid, 1965), that are toad-like, which may facilitate parasite transmission between the 2 species. In other

words, the lungworm may not differentiate between the 2 species because they offer pseudo-interchangeable habitats, but the hosts must inhabit the same geographic space for cross-infection to occur. While the habitats provided by host species appear to be pseudo-interchangeable, i.e., amenable to lungworm establishment, they are likely not identical, based on greater prevalence and higher mean abundance found in *Rh. bakeri* infecting *Ra. sylvatica* when compared to *B. americanus*. These results suggest that a majority of *Rh. bakeri* in nature cycle through *Ra. sylvatica*, however the importance of infections in *B. americanus* should not be discounted. Infections in toads may represent an on-going or past host switch, and could provide the nematode with future evolutionary options. For example, in the event that *Ra. sylvatica* are extirpated in Missouri (they are imperiled in the state) *Rh. bakeri* may be able to subsist and adapt to exclusively infecting *Bufo* spp. Furthermore, spatiotemporal variations in host species relative abundances may result in a majority of lungworms cycling through *B. americanus* instead of *Ra. sylvatica* during different seasons and/or years. Such switches may be oscillatory, where parasite host range episodically alternates between hosts through time (see Janz and Nylin, 2007; Hoberg and Brooks, 2008). Finally, based on infections in both host species we can assume that *Ra. sylvatica* and *B. americanus* in Missouri share geographic space; but, these hosts may not sufficiently overlap to allow for cross-transmission in nature in southern Ontario, Canada (Baker, 1979). Further exploration of host habitat usage and additional field collections may shed light on these differences.

The lungworm *Rh. joaquinensis* has been previously collected from several *Rana* spp. and *B. americanus* (see Kuzmin et al., 2003). Our field studies and experimental infections found *Rh. joaquinensis* capable of infecting numerous species of anurans in

several families. We consider the lungworm to be a true generalist, infecting virtually all North American anurans it encounters (or that encounter it). The only exception we have found is the microhylid *G. carolinensis*, which was resistant to all lungworm experimental infections in this study, and natural lungworm infections have never been found. Upon exposure to infective juvenile lungworms *G. carolinensis* extrude a mucus substance that appears to impede lungworm skin penetration (G.J. Langford, pers. obs.). It also may be possible that the poison glands of *G. carolinensis* are activated by attempted lungworm penetration, which has previously been suggested as an anti-predatory mechanism (Garton and Mushinsky, 1979). The bullfrog, *Ra. catesbiana*, also appears to be a physiologically resistant host for all lungworm species tested in this study. While we did establish infections with low mean abundance with *Rh. joaquinensis* in young male bullfrogs from Nebraska, these worms produced few eggs and contained relatively few gravid eggs in comparison to control infections. In experimental infections the majority of worms successfully penetrated into bullfrogs, however upon dissection most lungworms were discovered dead in the body cavity. Our experimental infections and field surveys suggest that young bullfrogs may become infected with gravid lungworms in Nebraska, but not healthy adults. Furthermore, bullfrogs may represent evolutionary dead ends because they primarily defecate in water (G. J. Langford, pers. obs.); thus drowning all lungworm offspring, which are incapable of swimming.

Morphologically identical *Rh. ranae* from Mississippi and Nebraska displayed different host specificity tendencies in nature and in the laboratory. *Rhabdias ranae* # 1 from Nebraska are only capable of infecting leopard frogs (e.g. *Ra. blairi*, *Ra. pippens*, *Ra. sphenoccephala*), whereas *Rh. ranae* # 2 infect a variety of *Rana* spp. in nature and

have the potential to infect toads (*Bufo* spp.). The variation in host specificities is mirrored in our phylogeny (Figure I), which splits these worms into separate clades, suggestive of cryptic species; thus, we treat them as putatively separate species in this study. *Rhabdias ranae* # 1 displayed the strictest host specificity for all lungworm species in this study, which supports the Tkach et al. (2006) findings that wild *Rh. ranae* # 1 from North Dakota are strictly parasites of leopard frogs. It appears that *Rh. ranae* # 1 from the Upper Great Plains are leopard frog specialist. Conversely, we suggest *Rh. ranae* # 2, collected in Mississippi, are tracking a broader set of host resources, and thus can infect non-leopard frog *Rana* spp. and potentially toads. However, as mentioned above, toads and most *Rana* spp. (*Ra. sylvatica* is an exception), including the semi-aquatic *Ra. sphenocephala* and *Ra. clamitans*, do not share habitat for extended periods of time (sharing occurs during the breeding season), minimizing the likelihood of cross-transmission of *Rh. ranae* # 2 between toads and frogs.

Experimental infections with snake lungworms, *Rh. eustreptos* and *Rh. fuscovenosa*, indicate that they are each capable of infecting all North American snake species; whereas in nature our studies show that *Rh. eustreptos* only infected *L. getula* (Colubridae) and *A. piscivorous* (Viperidae), and *Rh. eustreptos* is a generalist infecting the Colubridae (Table III). The absence of infections in all wild hosts is likely ecologically mediated; unfortunately, because snake lungworms infect hosts orally, potentially via transport hosts (Langford and Janovy, 2009), deducing likely ecological constraints on lungworm infections is difficult. Despite these limitations, we noticed that neither *Rh. eustreptos* nor *Rh. fuscovenosa* infected field collected *C. constrictor*, yet laboratory exposures always established infections. The ecological tendencies of *C.*

constrictor tend to be more terrestrial, favoring high, dry habitats (Ernst and Ernst, 2003), which suggest that strictly terrestrial snake species are unlikely to be infected in nature. Further, strictly terrestrial snakes are less likely to consume potential transport hosts. For example, *C. constrictor* stomach contents in this study were primarily composed of terrestrial arthropods, which are not suitable transport hosts (see Langford and Janovy, 2009). The remaining snake species in this study often inhabit or visit aquatic sites (Ernst and Ernst, 2003), and many commonly consume potential transport hosts.

An unexpected result from our study was the ability of *Rh. fuscovenosa* to infect the lizard *Sc. undulatus*. Prior to this study snake lungworms had not been recovered from lizards, although several species of *Rhabdias* have been discovered infecting lizards outside of North America. The infection occurred when a malfunction of the under-the-tank heating system occurred during an experimental infection. We are unsure of the malfunction's duration, because the problem was not detected until the lizard had been dissected, when the lizard was noted to be cold to touch. The lizard appeared healthy at the start of the experiment. We hypothesize that the overhead UV lamp alone was not sufficient to warm the lizard to optimal temperatures and its decreased body temperature played a role in establishing a viable, i.e., capable of re-infecting snakes, lungworm infection. Previous studies have found temperature to alter immune function of ectotherms and thus mediate pathogen/parasitic infection (Kluger, 1979; 1991; Kluger et al. 1996; Carey et al. 1999). Specifically, behavioral fever, e.g. basking behavior, has been reported to occur and effectively increase host survivorship in response to parasite or pathogen infections in ectotherms that range from insects to reptiles (Kluger, 1979; 1991; Kluger et al. 1996; Moore, 2002). Thus, it seems possible that infections in lizards

may be physiologically mediated by host basking behavior. This possibility is supported when average and maximum basking temperatures for snakes and lizards are compared. Carpenter (1956) found the average temperature of 3 species of *Thamnophis* (a commonly infected genus of snakes) to range between 20-30° C, whereas 2 species of *Sceloporus* lizards had average temperature range between 31-35° C (Burns, 1970; Mayhew, 1963).

The affect of host age on host specificity is an unresolved topic in lungworm biology. Early experiments by Baker (1979) and Chu (1936) do not discern between adult and YOY hosts, and Tkach et al. (2006) suggest that when exposed to *Rh. bakeri* the immune system of young *Ra. pippens* is 'not efficient enough to resist infection by a nonspecific *Rhabdias* species.' Our results suggest that YOY may be more susceptible to lungworm infection in some host-parasite combinations; in contrast, however, some combinations showed higher infection parameters in adult anurans. Whether these results are due to host immune competency or a physiological, ecological, or behavioral factor is difficult to discern. Of the host-parasite combinations in Table IV, only the bullfrog's immune system appeared to kill juvenile worms, apparent by the presence of dead worms in adult bullfrog body cavities from experimental infections. In comparison, few, if any, dead worms were found in other host-parasite combinations in Table IV. Furthermore, only young, male bullfrogs contained gravid worms during experimental infections, lending some support to the assertion of Tkach et al. (2006) that young hosts may be more susceptible to lungworm infection. In contrast, reductions in adult prevalence and mean abundance in *B. woodhousii* exposed to *Rh. joaquinensis* are evidently due to a combination of tough skin in adult toads (see Langford and Janovy, 2009) and behavioral

avoidance of feces and/or penetrating nematodes displayed by adult, but not young toads. When placed in infection containers with infective lungworms and/or feces an adult toad will often raise their ventral side off of the ground in an apparent attempt to minimize ground contact points. In extreme cases we have witnessed toads wedge themselves off the surface of the container, virtually eliminating contact with infective juvenile worms. YOY toads do not display this behavior. Thus, while field survey's would suggest that adult toad immune systems are more effective at eliminating *Rh. joaquinensis* infections in Nebraska, our experimental infections found that reduced prevalence and mean abundance in adult toads are at least partially due to an ontogenetic shift in fecal and/or nematode avoidance behavior.

Overall, measurements of host specificity determined in this study, HR and S_{TD}^* , agree with our conclusion that host specificity varies greatly within the genus *Rhabdias* (HR range = 2 to 8, . S_{TD}^* range = 1 to 3). As we suspected, snake lungworms displayed little host specificity, whereas anuran lungworms ranged from strict host specificity (e.g. *Rh. ranae* from Nebraska; HR = 2, . S_{TD}^* = 1), to relative generalist (e.g., *Rh. joaquinensis*; HR = 8, . S_{TD}^* = 2.7). While these measures of host specificity are not perfect, e.g., we do not consider *Rh. bakeri* (S_{TD}^* = 3.0) to have less host specificity than *Rh. joaquinensis* (S_{TD}^* = 2.7), they do provide a generally accurate depiction of lungworm host specificity, and incongruence's between HR and S_{TD}^* point to interesting host-parasite relationships that need further study. For example, the snake lungworm *Rh. eustreptos* (HR = 2, S_{TD}^* = 3) has a relatively low HR and high S_{TD}^* in comparison to *Rh. fuscovenosa* (HR = 5, S_{TD}^* = 1.6). In the laboratory both lungworm species infected all snakes they were exposed to, thus it is confusing that *Rh. eustreptos* only infects 2

hosts in nature in Mississippi. Similarly, it seems odd that *Rh. fuscovenosa* infects several host species in nature, but only within the Colubridae and mostly natricines. Why does *Rh. fuscovenosa* not infect cottonmouths (*A. piscivorus*) in the wild?

In addition to this work's contribution to lungworm biology, our study is an important addition to the small number of studies that have used a multifaceted approach that includes phylogenetics and experimental infections to accurately investigate host specificity. We encourage other researchers to adopt this methodology and explore issues of host specificity, so we can better understand all the avenues for and constraints on parasite transmission.

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Figure I. Lungworm maximum likelihood tree (unrooted) based on entire rDNA dataset. Branch lengths equal substitutions/site. Bootstrap values are given as percentages near the individual nodes, with 1,000 bootstrap replicates using heuristic search criteria. Lungworm branches end in host species names and collection locations to provide information about host specificity. Lungworm species are designated to the right of host species. KS = Kansas, MO = Missouri, MS = Mississippi, NE = Nebraska, ND = North Dakota.

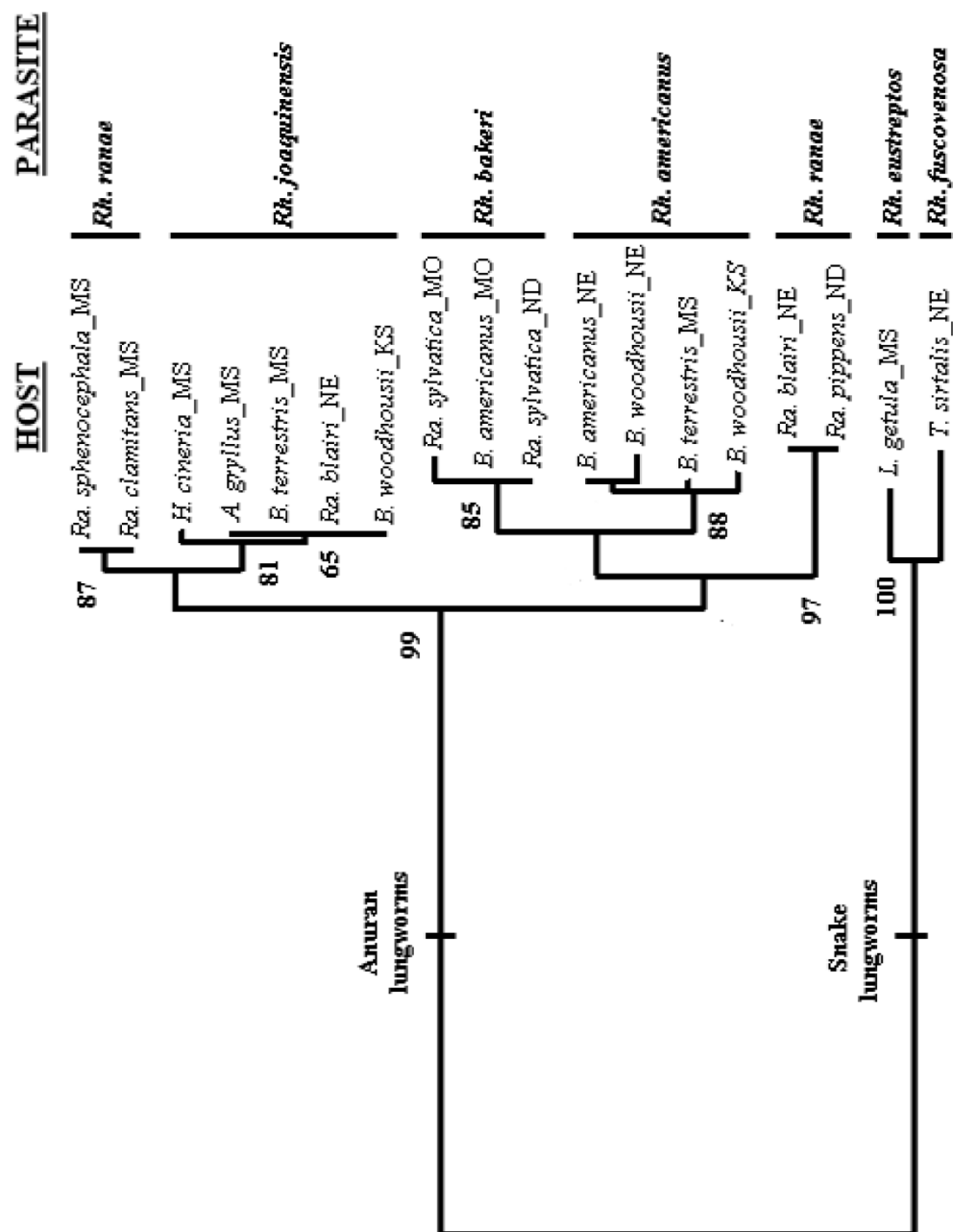


Figure II. Two unrooted maximum parsimony trees based on entire rDNA dataset. Bootstrap values are near the node, values <65 not shown. Branches end in parasite species names and collection locations. Trees agree on all relationships, except for the designation of *Rhabdias ranae* collected in Nebraska and North Dakota.

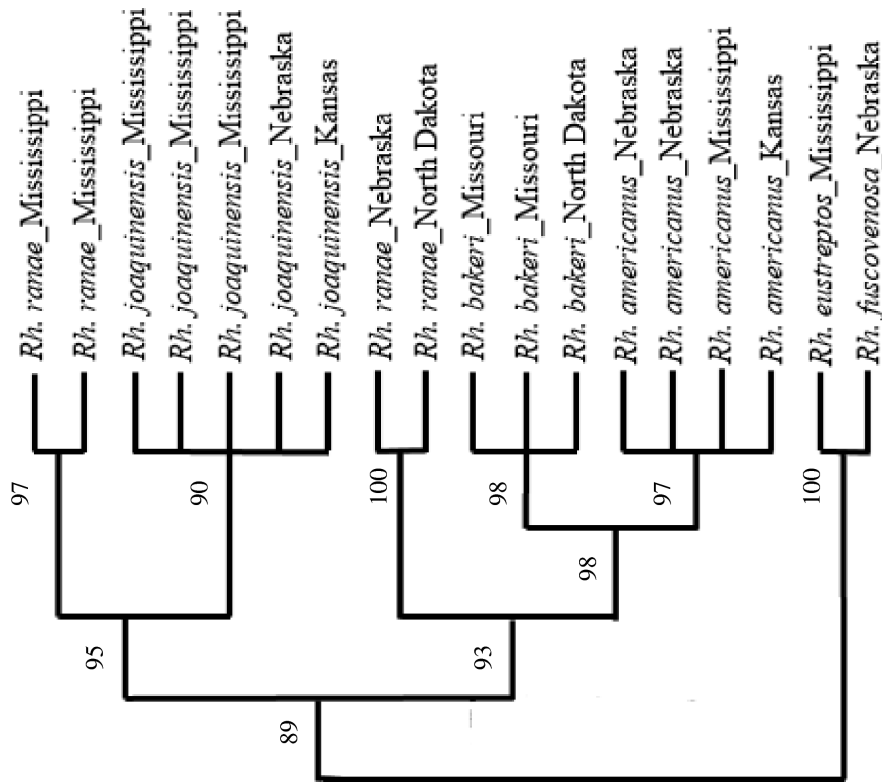
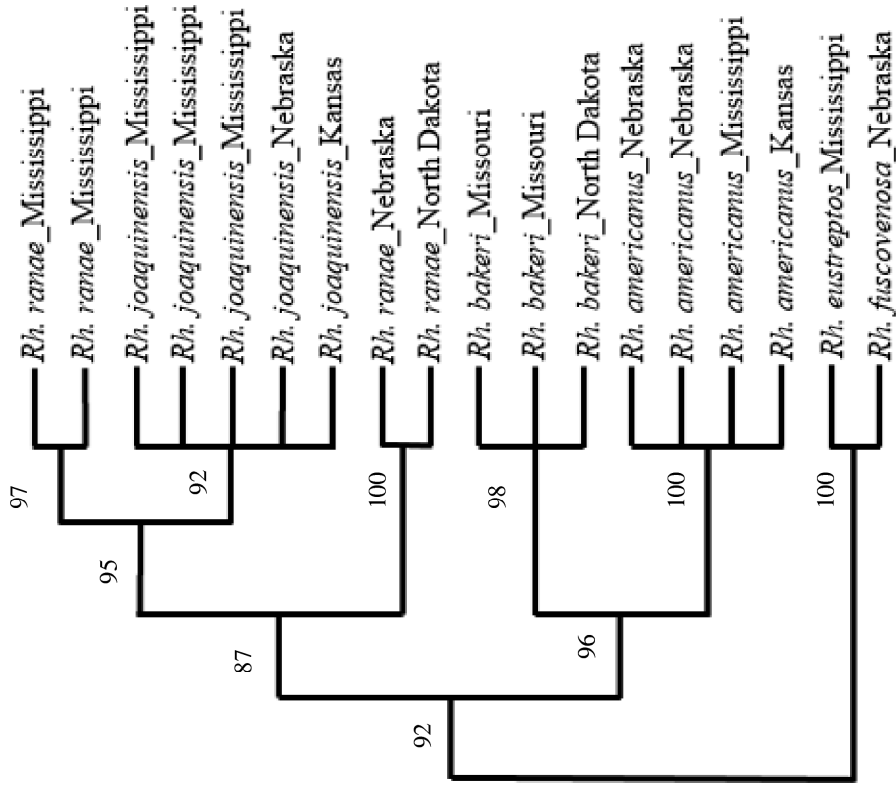


Figure III. Maximum parsimony (MP) strict consensus of 15 unrooted trees based on LSU dataset, maximum likelihood (ML) analysis agreed on tree topology. Bootstrap values are near the node, provided as MP/ML, values <65 not shown. Branches end in parasite species names and collection locations. The tree is unable to resolve relationships within anuran lungworms, however it is able to distinguish between anuran and snake lungworms.

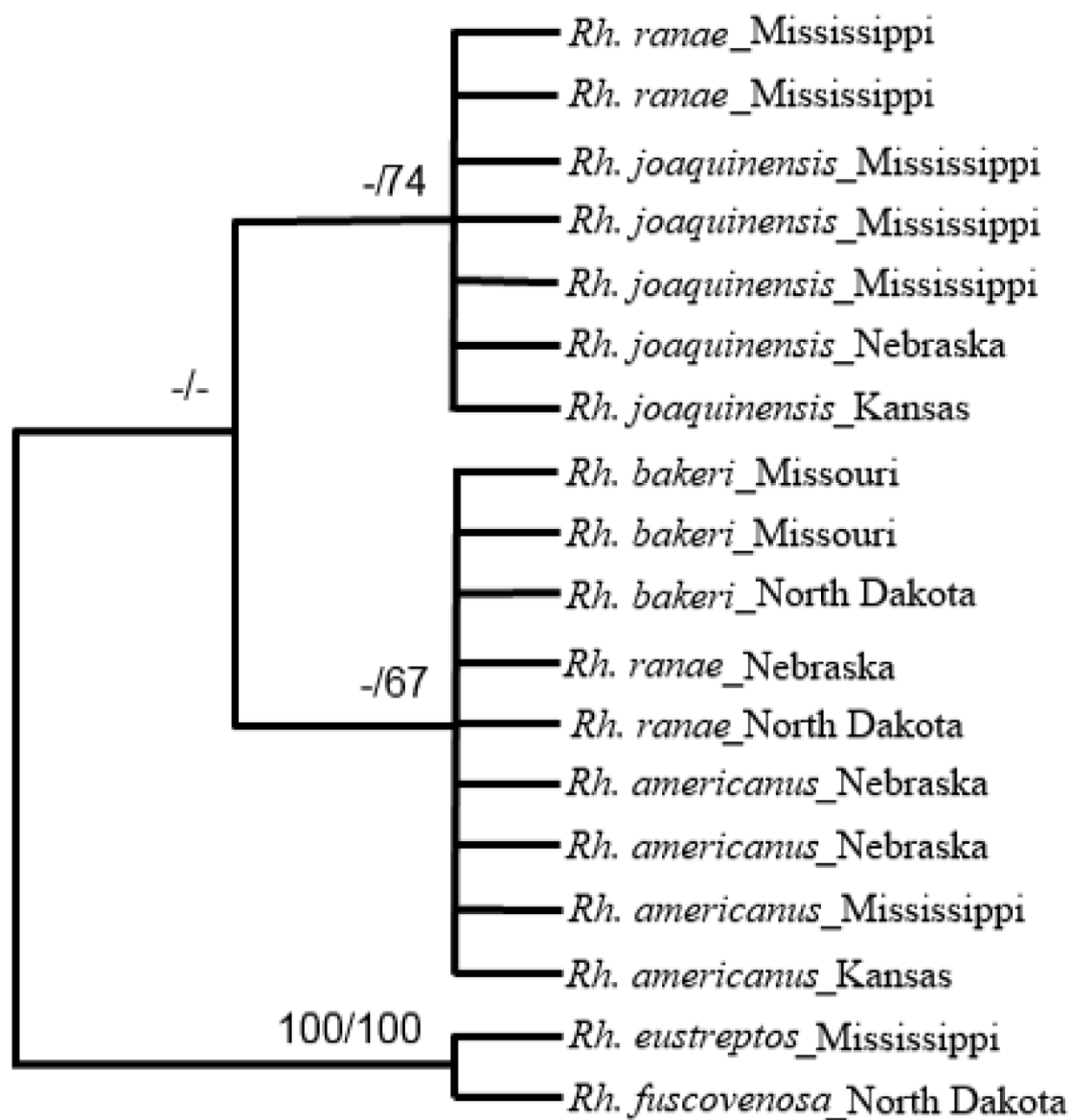


Figure IV. Maximum parsimony (MP) tree based on ITS dataset, maximum likelihood (ML) analysis agreed on tree topology. Bootstrap values are near the node, provided as MP/ML, values <65 not shown. Branches end in parasite species names and collection locations. The unrooted tree provides good resolution for all species, including the distinction of separate anuran and snake lungworm clades.

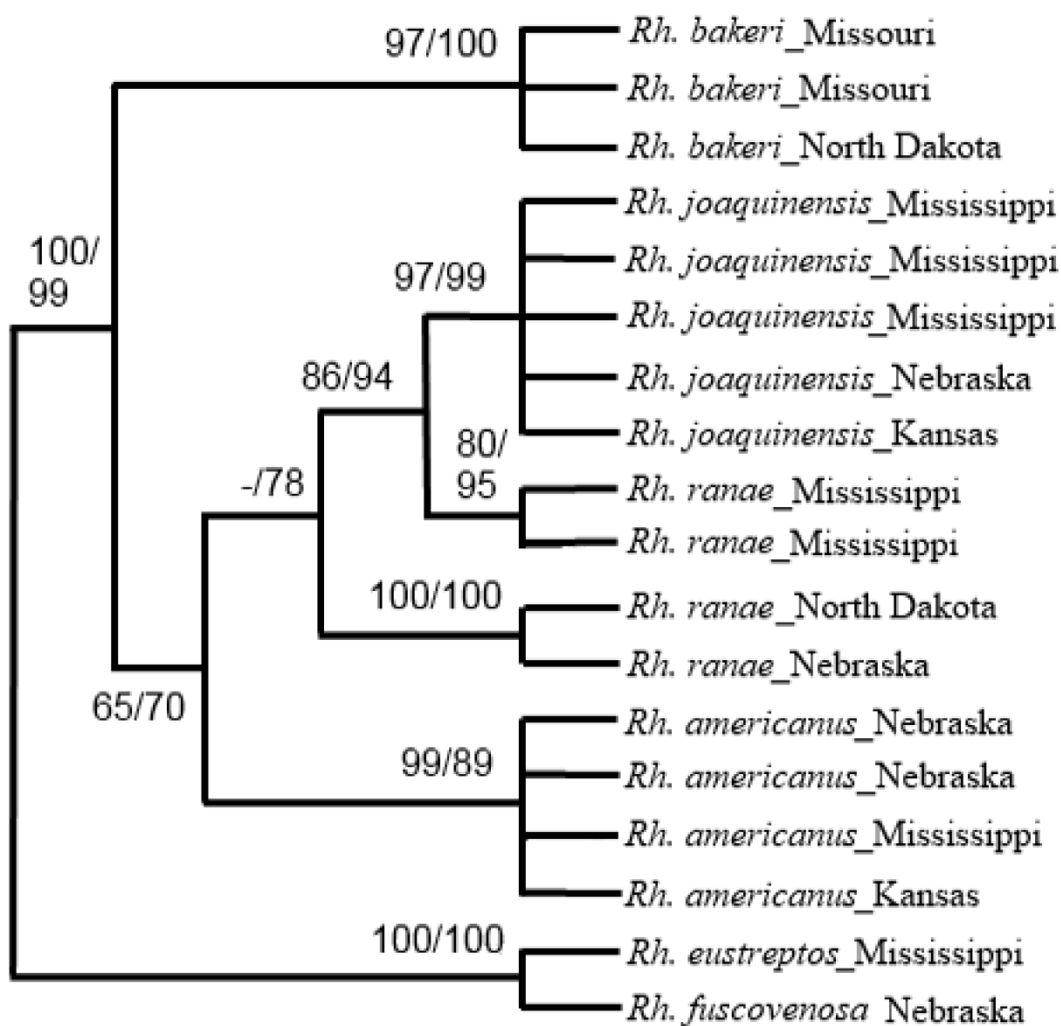


Table I. Prevalence (P) and Mean Abundance (MA) of *Rhabdias* spp. from anurans and snakes collected in Kansas (KS), Mississippi (MS), Missouri (MO), and Nebraska (NE).

Host species (location / n)	<i>Rh. americanus</i>		<i>Rh. bakeri</i>		<i>Rh. eustreptos</i>		<i>Rh. fuscovenosa</i>		<i>Rh. joaquiniensis</i>		<i>Rh. ranae</i>	
	P	MA ± SD	P	MA ± SD	P	MA ± SD	P	MA ± SD	P	MA ± SD	P	MA ± SD
Frogs and Toads												
<i>Acris blanchardi</i> (NE / 50)	0%	—	0%	—	0%	—	0%	—	86%	1.9 ± 2.7	0%	—
<i>Acris blanchardi</i> (KS / 50)	0%	—	0%	—	0%	—	0%	—	90%	0.9 ± 2.6	0%	—
<i>Acris gryllus</i> (MS / 33)	0%	—	0%	—	0%	—	0%	—	63%	3.8 ± 3.6	0%	—
<i>Bufo americanus</i> (MO / 6)	50%	4.2 ± 5.3	33%	3.3 ± 1.1	0%	—	0%	—	0%	—	0%	—
<i>Bufo fowleri</i> (MS / 8)	75%	4.6 ± 5.7	0%	—	0%	—	0%	—	25%	1.2 ± 2.1	0%	—
<i>Bufo quercicus</i> (MS / 15)	0%	—	0%	—	0%	—	0%	—	0%	—	0%	—
<i>Bufo terrestris</i> (MS / 14)	100%	12.1 ± 10.6	0%	—	0%	—	0%	—	21%	1.7 ± 3	0%	—
<i>Bufo woodhousii</i> (NE / 30)	0%	—	0%	—	0%	—	0%	—	27%	0.9 ± 2.7	0%	—
<i>Bufo woodhousii</i> (KS / 30)	53%	4.6 ± 7.9	0%	—	0%	—	0%	—	50%	1.6 ± 1.1	0%	—
<i>Gastrophryne carolinensis</i> (MS / 12)	0%	—	0%	—	0%	—	0%	—	0%	—	0%	—
<i>Hyla cinerea</i> (MS / 18)	0%	—	0%	—	0%	—	0%	—	0%	—	0%	—
<i>Hyla chrysoscelis</i> (NE / 20)	0%	—	0%	—	0%	—	0%	—	55%	2.1 ± 2.9	0%	—
<i>Hyla chrysoscelis</i> (KS / 20)	0%	—	0%	—	0%	—	0%	—	20%	7.3 ± 10.1	0%	—
<i>Pseudacris triseriata</i> (NE / 87)	0%	—	0%	—	0%	—	0%	—	45%	2.6 ± 2.5	0%	—
<i>Pseudacris triseriata</i> (MO / 5)	0%	—	0%	—	0%	—	0%	—	12%	0.3 ± 1.3	0%	—
<i>Rana blairi</i> (NE / 21)	0%	—	0%	—	0%	—	0%	—	0%	—	0%	—
									71%	6.7 ± 3.8	19%	0.9 ± 1.2

Table II. Sequence identity (%) matrix based on 2,104-bp-long sequences of ribosomal DNA (partial 18S, complete ITS I, complete 5.8S, complete ITS2, partial 28S) of 7 (1 putative) *Rhabdias* species.

Lungworm species	Sequence identity						
	<i>Rh. americanus</i>	<i>Rh. bakeri</i>	<i>Rh. joaquinensis</i>	<i>Rh. ranae_1</i>	<i>Rh. ranae_2</i>	<i>Rh. eustreptos</i>	<i>Rh. fuscovenosa</i>
<i>Rh. americanus</i>	—	0.993	0.987	0.989	0.989	0.916	0.913
<i>Rh. bakeri</i>	0.993	—	0.986	0.988	0.989	0.917	0.914
<i>Rh. joaquinensis</i>	0.987	0.986	—	0.987	0.996	0.916	0.914
<i>Rh. ranae_1</i>	0.989	0.988	0.987	—	0.988	0.912	0.91
<i>Rh. ranae_2</i>	0.989	0.989	0.996	0.988	—	0.917	0.915
<i>Rh. eustreptos</i>	0.916	0.917	0.916	0.912	0.917	—	0.994
<i>Rh. fuscovenosa</i>	0.913	0.914	0.914	0.91	0.915	0.994	—

Table III. Prevalence (P) and Mean Abundance (MA) from 5 amphibian *Rhabdias* spp. experimentally exposed to sympatric amphibians and reptiles. Hosts were exposed to 30 infective lungworms for 12 hours. NE = Nebraska, MO = Missouri, MS = Mississippi. **R. americanus* from MO *B. americanus*. ^*R. joaquinensis* from MS *A. gryllus*.

Experimental species (location)	<i>Rh. americanus</i> MS		<i>Rh. bakeri</i> MO		<i>Rh. joaquinensis</i> NE		<i>Rh. ranae</i> NE		<i>Rh. ranae</i> MS	
	P (n)	MA ± SD	P (n)	MA ± SD	P (n)	MA ± SD	P (n)	MA ± SD	P (n)	MA ± SD
Frogs and Toads										
<i>Acris blanchardi</i> (NE)										
<i>Acris gryllus</i> (MS)	0% (7)	—			100% (34)	26.7 ± 7.5			0% (5)	—
<i>Bufo americanus</i> (NE)							0% (18)	—		
<i>Bufo americanus</i> (MO)			71% (14)	11.2 ± 9.3						
<i>Bufo fowleri</i> (MS)	75% (12)	18.4 ± 7.1							50% (15)	3.9 ± 13.2
<i>Bufo quercicus</i> (MS)	94% (34)	24.5 ± 7.8								
<i>Bufo terrestris</i> (MS)	100% (33)	28.1 ± 1.6							64% (14)	6.9 ± 6.8
<i>Bufo woodhousii</i> (NE)					95% (58)	25.9 ± 5.2				
<i>Gastroph. carolinensis</i> (MS)	0% (5)	—			0% (7)^	—			0% (4)	—
<i>Hyla cineria</i> (MS)	0% (7)	—							0% (5)	—
<i>Hyla chrysocelis</i> (NE)					93% (30)	20.3 ± 10.2				
<i>Psuedacris triseriata</i> (NE)					45% (22)	8.1 ± 12.7			0% (20)	—
<i>Psuedacris triseriata</i> (MO)										
<i>Rana blairi</i> (NE)			0% (10)	—	100% (26)	28.1 ± 2.4	100% (16)	28.5 ± 4.0		

Table III cont.

<i>Rana catesbiana</i> (MS)	0% (10)	—	—	10% (30)	2.7 ± 8.2	0% (20)	—	0% (11)	—	
<i>Rana catesbiana</i> (NE)	0% (12)	—	—	100% (8)	24.6 ± 6.6	100% (21)	26.4 ± 3.5	75% (4)	11 ± 10	
<i>Rana clamitans</i> (MS)	0% (12)	—	—	0% (11)	—	—	—	100% (35)	24.1 ± 5.4	
<i>Rana pipiens</i> (NE)	0% (12)	—	—	57% (7)*	12 ± 11.7	100% (32)	26.4 ± 4.2	—	—	
<i>Rana sphenoccephala</i> (MS)	—	—	—	—	—	—	—	—	—	
<i>Rana sphenoccephala</i> (MO)	—	—	—	—	—	—	—	—	—	
<i>Rana sylvatica</i> (MO)	—	—	—	—	—	—	—	—	—	
<i>Spea bombifrons</i> (NE)	—	—	—	83% (6)	17.3 ± 9.8	0% (5)	—	—	—	
Salamanders										
<i>Ambystoma tigrinum</i> (NE)	—	—	—	0% (7)	—	0% (5)	—	0% (3)	—	
<i>Ambystoma tigrinum</i> (MS)	0% (4)	—	—	—	—	—	—	—	—	
Snakes										
<i>Agkistrodon piscivorus</i> (MS)	0% (2)	—	—	—	—	—	—	0% (2)	—	
<i>Elaphe obsoleta</i> (NE)	—	—	—	0% (4)	—	0% (2)	—	—	—	
<i>Thamnophis sauritus</i> (MS)	0% (6)	—	—	—	—	—	—	0% (3)	—	
<i>Thamnophis sirtalis</i> (NE)	—	—	—	0% (6)	—	0% (3)	—	—	—	

Table IV. Prevalence (P) and Mean Abundance (MA) from 2 snake *Rhabdias* spp. experimentally exposed to sympatric amphibians and reptiles. Hosts were given 30 infective lungworms. NE = Nebraska, MS = Mississippi. **R. fuscovenosa* from MS *N. fasciata*.

Experimental species (location)	<i>Rh. eustreptos</i> MS		<i>Rh. fuscovenosa</i> NE	
	P (n)	MA \pm SD	P (n)	MA \pm SD
Snakes				
<i>Agkistrodon piscivorous</i> (MS)	100% (5)	21.2 \pm 5.2	100% (3)*	18 \pm 5.3
<i>Coluber constrictor</i> (NE)			100% (5)	26.6 \pm 4.2
<i>Coluber constrictor</i> (MS)	100% (6)	20 \pm 3.4		
<i>Elaphe obsoleta</i> (NE)			100% (4)	22.5 \pm 3.5
<i>Lampropeltis getula</i> (MS)	100% (4)	28 \pm 2		
<i>Nerodia fasciata</i> (MS)	75% (8)	13.9 \pm 10.7		
<i>Thamnophis sauritus</i> (MS)	50% (8)	8 \pm 7.1		
<i>Thamnophis sirtalis</i> (NE)			100% (8)	20 \pm 5.7
Lizards				
<i>Cnemidophorus sexlineatus</i> (NE)			0% (5)	—
<i>Sceloporus undulatus</i> (NE)			20% (5)	15
<i>Sceloporus undulatus</i> (MS)	0% (4)	—		
Turtles				
<i>Kinosternon subrubrum</i> (MS)	0% (3)	—		
<i>Trachemys scripta</i> (MS)	0% (4)	—		
Frogs and Toads				
<i>Bufo woodhousii</i> (NE)			0% (10)	—
<i>Rana blairi</i> (NE)			0% (7)	—
<i>Rana catesbiana</i> (MS)	0% (15)	—		
<i>Rana sphenoccephala</i> (MS)	0% (6)	—		
<i>Spea bombifrons</i> (NE)			0% (3)	—

Table V. Comparison of prevalence and Mean Abundance (MA) of *Rhabdias* spp. infecting Young of the Year (YOY) and adult anurans from experimental and natural infections with ≥ 30 hosts and only 1 lungworm species. Host size (SVL) was used as a proxy to designate YOY vs. Adult anurans. KS = Kansas, MS = Mississippi, MO = Missouri, NE = Nebraska.

Parasite (location)	Host (location)	Prevalence		MA \pm SD		t-test	P
		YOY (n)	Adult (n)	YOY	Adult		
Experimental infections							
<i>Rh. joaquinensis</i> (NE)	<i>A. blanchardi</i> (NE)	100% (20)	100% (14)	27.1 \pm 5.8	25 \pm 9.5	0.76	0.459
	<i>B. woodhousii</i> (NE)	100% (43)	80% (15)	27.8 \pm 3.1	22.7 \pm 8.9	3.28	0.005
	<i>H. chrysosecelis</i> (NE)	92% (13)	94% (17)	23.8 \pm 9.5	17.5 \pm 10.4	1.73	0.096
	<i>R. catesbiana</i> (NE)	15% (19)	0% (11)	4.2 \pm 10	0	—	—
<i>Rh. americanus</i> (MS)	<i>B. quercicus</i> (MS)	100% (19)	86% (15)	23.2 \pm 9.8	24 \pm 9.9	0.77	0.456
	<i>B. terrestris</i> (MS)	100% (16)	100% (17)	27.6 \pm 2.5	28.5 \pm 0.9	1.46	0.161
<i>Rh. ranae</i> (MS)	<i>R. sphenoccephala</i> (MS)	100% (16)	100% (19)	24.1 \pm 5.8	24.2 \pm 4.9	0.02	0.986
<i>Rh. bakeri</i> (MO)	<i>R. sylvatica</i> (MO)	100% (23)	100% (9)	25.9 \pm 4.6	27.7 \pm 3.2	1.24	0.229
Natural infections							
<i>Rh. joaquinensis</i> (NE)	<i>A. blanchardi</i> (NE)	100% (21)	79% (29)	2.1 \pm 3.5	2.1 \pm 3.9	0.02	0.986
	<i>B. woodhousii</i> (NE)	46% (15)	7% (15)	1.7 \pm 3.7	0.1 \pm 0.5	1.65	0.12
	<i>P. triseriata</i> (NE)	0% (21)	17% (66)	0	0.4 \pm 1.5	—	—
<i>Rh. joaquinensis</i> (KS)	<i>A. blanchardi</i> (KS)	23% (34)	12% (16)	1.3 \pm 4.9	0.2 \pm 0.5	2.07	0.046
<i>Rh. joaquinensis</i> (MS)	<i>A. gryllus</i> (MS)	31% (16)	94% (17)	0.8 \pm 1.4	6.6 \pm 2.7	7.91	0.001

CHAPTER THREE: ECOLOGY OF THE FREE-LIVING STAGES OF *RHABDIAS JOAQUINENSIS* IN NEBRASKA: WHERE DO ANURANS BECOME INFECTED IN NATURE?

Abstract: The lungworm *Rhabdias joaquinensis* is a common parasite of anurans in eastern Nebraska. This study investigated the ecology of the lungworm's free-living stages that reside in host feces and surrounding soils. The study also investigated the absence of lungworms in western Nebraska's Sandhills. Soil type, moisture, and temperature were experimentally varied in the laboratory to assess juvenile development and survival. Field mesocosm experiments were used to determine where in nature lungworms infect hosts. Laboratory mesocosms were established to determine if the presence of vegetation facilitates lungworm transmission to hosts. The results found loam soils were amenable to lungworm development, whereas soils with high clay or sand content produced few infective lungworms. Soil moisture < 50% did not support lungworm development. Infective juveniles successfully developed between 5 - 35° C. A limited number of worms developed at 40° C, but were unable to infect juvenile toads. Field studies found that shoreline environments supported lungworm development, and the majority of lungworm infections appear to occur within 2 m of wetland shorelines in Nebraska. Prevalence in vegetation mesocosms was 100%; however, a significantly higher mean abundance was found in toads from containers with vegetation than without. Field and laboratory experiments suggest that the predominantly sandy soils of Nebraska's Sandhills limit *R. joaquinensis* from infecting anurans in western Nebraska.

INTRODUCTION

A wealth of knowledge is available on the ecology of the free-living stages of skin penetrating nematodes of humans (Smith, 1990) and domestic animals (Coyne and Smith, 1994; Anderson, 2000; Vlassoff et al., 2001; O'Conner et al., 2006;), and soil transmission patterns of several entomopathogenic nematodes also are well known (Kaya and Gaugler, 1993; Gaugler, 2002). Yet few detailed studies have been conducted on the ecology of free-living stages of nematodes that infect wildlife (but see the *Trichostrongylus tenuis* papers by Saunders et al., 1999; 2000), a situation that limits our understanding of their transmission in nature. Nematodes of amphibians are no exception, and despite their recent popularity as model systems for the study of parasite ecology and evolution (Goater and Ward, 1992; Tkach et al., 2006; Dare et al., 2008; Langford and Janovy, 2009); no studies have addressed the ecology of the free-living stages of amphibian skin-penetrating nematodes in nature. Previous studies of amphibian nematodes have recorded development time in the laboratory and simply stated that skin-penetrating nematodes are acquired directly from the soil (see Anderson, 2000). Soils are complex ecosystems that dictate the survival and infectivity of soil transmitted nematodes, and detailed studies combining field and laboratory experiments are needed to determine how soils affect nematode distribution and abundance.

The skin-penetrating nematode *Rhabdias joaquinensis* Stiles and Hassall, 1905 (Nematoda: Rhabdiasidae) provides an ideal candidate for studying the free-living ecology of a nematode parasite of amphibians. The life cycle (Langford and Janovy, 2009) and host specificity (see Chapter 2) are fully elucidated in Nebraska. These studies show that young-of-the-year (YOY) toads (*Bufo woodhousii*) are always infected when

exposed to *R. joaquinensis* juveniles in experimental infections, and the lungworm's life cycle, including free-living stages, can be completed quickly in Nebraska, i.e., in as few as 8 days. Thus, this model system provides a quick and reliable method to assess lungworm infectivity in both the laboratory and field, a method that is not available for most parasites of amphibians. In addition, lungworms from Nebraska are interesting candidates for study because, despite similar amphibian communities in the 2 regions, lungworms commonly infect anurans in eastern, but never in western Nebraska (see Langford and Janovy, 2009). We suspect that *R. joaquinensis* is limited to eastern Nebraska by the inability of free-living stages to survive in the arid, sandy soils of western Nebraska. This hypothesis is based on studies of other skin-penetrating nematodes that show nematode distributions are often limited by environmental factors such as soil moisture, temperature, type, and chemistry, microhabitat humidity, and the presence of vegetation (reviewed in Gaugler, 2002; Smith, 1990; Vlassoff et al., 2001). Prior to this study it was unknown how these factors affect amphibian lungworms.

In this study, our main goals are to (1) determine the effect of soil temperature, soil moisture, and soil type on survival of free-living stages in the laboratory, (2) establish where in nature lungworms are able to develop and infect toads, and (3) determine if the presence of vegetation facilitates increased lungworm transmission.

METHODS

Acquiring lungworms and uninfected hosts

Adult *Acris blanchardi* were hand captured both day and night from a small pond adjacent to Pawnee Lake, Lancaster Co., Nebraska (40.86, -96.89) from May – October 2008. Animals were transported to the parasitology laboratory at the University of

Nebraska-Lincoln, euthanized, measured for snout-vent (SVL), sexed, and all organs and tissues were examined for *Rhabdias* spp. within 24 hours of collection. Lungworms were removed and fixed in hot 70% ethyl alcohol for identification; however, most adult hermaphrodites were set aside for use in experimental infections. Representative specimens were cleared and temporarily mounted in glycerol for identification (Prichard and Kruse, 1982). All lungworms were identified according to Kuzmin et al. (2003), only *R. joaquinensis* were recovered. Representative specimens were deposited in the H. W. Manter Parasitology Collection, University of Nebraska, Lincoln, Nebraska (HWML 63516).

Uninfected YOY *Bufo (Anaxyrus) woodhousii* were hand captured from the area surrounding Cedar Point Biological Station (CPBS), Keith County, Nebraska (41.21, -101.62) and transported to the parasitology laboratory at the University of Nebraska-Lincoln for immediate use in mesocosm studies.

Temperature experiments

For all studies free-living lungworms were acquired directly from parasitic adults removed from host lungs to assure lungworm identification. Infective juveniles were reared in Petri dishes according to Langford and Janovy (2009), except 5 g of loam soil with 80% moisture was added to the inner Petri dish over the paper towel. Soil was added to mimic conditions in nature. Five Petri dishes were incubated at the following temperatures: 5° C, 20° C, 30° C, 35° C and 40° C. Fifty lungworm eggs or recently hatched juveniles were added to each Petri dish. The experiment had 5 replicates and was repeated twice. Aged tap water was added to containers to maintain moisture throughout experiment. Containers were monitored for development and treatments were ended

when all infective juveniles migrated into the outer Petri dish. Infective juveniles were easily counted in the outer dish.

Soil moisture experiments

Fifty eggs or recently hatched juveniles were placed in 5 g of loam soil at room temperature (~25° C) per Petri dish, and placed in the following soil moisture treatments: 30%, 50%, 70%, 90%, and 100% (slightly beyond saturation). The experiment had 5 replicates and was repeated twice. Aged tap water was added to containers to maintain moisture throughout experiment. Outer Petri dishes were not used in these experiments because they altered soil moisture, thus when treatments were ended at 4 days all Petri dishes were thoroughly searched for live infective juveniles. Soil moisture and pH were measured using a Kelway® Soil pH and Moisture Meter.

Soil composition experiments

Soil types used in this experiment are displayed in Table I. Aged tap water was added to each soil type until 80% moisture was obtained. Next, 5 g of moist soil was added to each Petri dish, along with 50 eggs or recently hatched juvenile lungworms. The experiment had 5 replicates and was repeated twice. Aged tap water was added to containers to maintain moisture throughout experiment. Treatments were ended at 4 days, when all infective juveniles migrated into the outer Petri dish. Infective juveniles were easily counted in the outer dish.

Frog spatial defecation

Harmless florescent powder (Lightning Powder Company, Inc., Jacksonville, FL) was used to determine where *A. blanchardi* defecated in nature. A total of 80 (some may have been recaptured) frogs were collected from a creek shoreline that leads into Pawnee

Lake, Nebraska (40.86, -96.89) each morning from July 13 -16, 2008. When a frog was captured it was force fed a 1 g pellet of moist florescent powder. Frogs were immediately released within 5 minutes of capture. After sunset each day, a handheld blacklight was used to illuminate frog feces within an area that extended 5 m from shoreline and was 50 m in length. The following 3 parameters were measured for each fecal pellet discovered: distance to shoreline, soil temperature, and soil moisture. Soil type was consistent throughout the experimental area.

Toad infection mesocosms

Mesocosms were placed in the moist habitat near the shoreline of Pawnee Lake, where anurans often defecated (see RESULTS). In addition, mesocosms were placed in other representative habitats to determine their likelihood of transmitting infective lungworms. Soil mesocosm enclosures were created from 1 cm hardware cloth that was formed into a 20 x 20 x 10 cm box with no bottom. The open bottom was then pushed into the soil to prevent both soil disturbance by large animals and escape by toads placed in the mesocosms. Half of the mesocosms (n = 8) were placed on naturally occurring loam soils, remaining mesocosms (n = 8) were placed on sandy soils acquired from the CPBS collection location. Sandy mesocosms were created by excavating loam soils within the mesocosm to a depth of 5 cm, which were replaced with sandy soils. Vegetation within these plots was replaced, when possible. On August 18, 2008 mesocosms were placed in the environment in groups of 16 at 10 distances from the shoreline of Pawnee Lake, Nebraska. Fifty recently hatched juvenile *Rhabdias joaquinensis*, i.e. non-infective, obtained from the lungs of *A. blanchardi*, were placed in the center of each mesocosm with a 10 g fecal pellet from an uninfected *B. woodhousii*

and permitted to develop for 5 days. Air temperature at the site ranged between 18 - 35° C and humidity ranged between 31 – 90%, no measureable rainfall occurred during the experiment. Loam soils in the experimental area were uniform, with a pH of 6.4 - 6.7 and organic content of 4.2%. Sandy soils had a pH of 6.5 and organic content of 1.8%. Soil moisture was measured in loam soils at the beginning of the experiment, and all mesocosms at a given distance were maintained to match the highest recorded soil moisture at each distance. For example, all 16 mesocosms placed 50 cm from shoreline were maintained at 90% soil moisture. Maximum soil temperature did not exceed 37° C in any mesocosms, except those on bare ground (43° C). Mesocosm soil moisture and maximum surface soil temperature was checked daily between 1-4pm. After 5 days, 1 uninfected YOY toad, collected at CPBS, was added to each mesocosm for 12 hours to assess the development and infectivity of free-living lungworms. Following exposure, toads were transported to the University of Nebraska-Lincoln parasitology laboratory and held for 7 days to allow infections to develop (toad husbandry according to Langford and Janovy, 2009), then euthanized, and all organs and tissues examined for *R. joaquinensis*.

Vegetation mesocosms

During toad infection mesocosm experiments (above), we observed that infective juvenile worms ascended low-lying vegetation and waved their anterior ends, similar to the behavior seen in human hookworms (see Smith, 1990). In this experiment, laboratory mesocosms were established to determine if the presence of vegetation facilitates lungworm transmission to toads. On September 2, 2008 a total of 50 mesocosms were established by placing 3 cm of soil, collected from Pawnee Lake, Nebraska, into 20 cm (diameter) circular plastic buckets. Clay loam soils used in mesocosms were mixed for

uniformity, with a pH of 7.0 and organic content of 3.9%. Soil temperature and moisture were maintained at 24° C and 80%, and relative humidity ranged between 80-90%. Buckets were exposed to a 12L:12D florescent photoperiod. White clover (*Trifolium repens*) was propagated in 20 trays, and pruned to 50% vegetative cover prior to beginning experiments. On September 20, 100 infective juveniles, raised in optimal laboratory conditions, were placed in the center of each mesocosm with 5 g of toad feces, and permitted to acclimate for 12 hours. Nematodes were not added to 10 control buckets (5 bare soil, 5 planted). Following acclimation, 1 YOY *B. woodhousii*, collected at CPBS, was added to each mesocosm for 12 hours to assess the infectivity of free-living lungworms. Following exposure, toads were held for 7 days to allow infections to develop, then euthanized, and all organs and tissues examined for *R. joaquinensis*.

Statistical analysis

Prior to dissection hosts were measured for snout-vent length (SVL), and host sex was determined during dissection. Hosts used in mesocosm experiments were selected to not vary significantly in size (SVL = 4.0 ± 0.2 cm), and prevalence and mean abundance did not vary between sexes, thus all data were combined. Prevalence (P) and mean abundance (MA) were calculated as measures of parasitism for anurans according to Margolis et al. (1982). Values are reported as mean ± 1 S.D. Analysis of variance (ANOVA) was used to compare mean abundance between treatments in laboratory experiments, and Tukey's HSD test was used when significant differences were detected during ANOVA (Zar, 1999). A student's *t*-test was used to compare differences in mean abundances, and a Chi-square test of independence was used for differences in parasite

prevalence. Alpha was set at 0.05 for all statistical procedures. All statistics were performed using Minitab® 15 (Minitab Inc., State College, PA, 2007).

RESULTS

Lungworms infected 92 (66.7%) of 138 *A. blanchardi* captured April – October, 2008, which produced 325 lungworms (MA = 2.3 ± 2.7 ; range = 0 - 6). Frog sexual size dimorphism was not found (female: 2.8 ± 0.3 , n = 50; male: 2.7 ± 0.4 , n = 88; $t = 0.81$, $P = 0.242$), and no differences were noted in lungworm prevalence ($X^2 = 1.23$, $P = 0.287$) and mean abundance ($t = 0.58$, $P = 0.592$) between males ($P = 64.7\%$; MA = 2.4 ± 2.6 ; range = 0-6) and females ($P = 70\%$; MA = 2.1 ± 3.0 ; range = 0-6). During June – August lungworms were consistently recovered from both host body cavities and lungs, whereas lungworms primarily resided in the lungs during April and October (Figure I).

A significant difference in the mean number of free-living worms that developed in Petri dishes was detected between temperature treatments (Figure II; $F = 83.02$, $P = < 0.0005$). Worms at 30° C developed quickest and reached a high for mean abundance; however, a similar number of worms developed at 20° C and 35° C, although worms at these temperatures developed slower than those at 30° C. Normal infective juveniles, i.e., capable of infecting toads, developed slowly at cool temperatures (5° C), whereas worms that developed at high temperatures (40° C) were unable to infect toads. Free-living lungworm development was significantly different between soil moisture treatments (Figure III; $F = 23.07$, $P = < 0.0005$). Lungworms did not survive in 10% and 30% treatments, these results are not displayed. Lungworm survival peaked at 70% and 90% moisture, and decreased both above and below these soil moisture levels. Soil treatments were significantly different in their ability to support lungworm development (Figure IV;

$F = 95.09$, $P = < 0.0005$). Loam and clay-loam soils produced the greatest number of juveniles, whereas clay and sand treatments produced the fewest. Florescent feces ($n = 49$) of *A. blanchardi* were not discovered further than 1.7 m from shoreline, and on average were 0.8 ± 0.5 m from shoreline. Frogs defecated in areas with an average soil temperature of $28 \pm 4^\circ \text{C}$ and soil moisture of $72 \pm 12\%$.

Mesocosm experiments found infective juveniles reached highest prevalence and mean abundance at 50 – 150 cm from shoreline (Table II). In addition to infecting toads near shoreline, lungworms established infections in leaf litter. Lungworms were unable to infect toads 10 cm from shoreline in saturated soils; they were also unable to infect toads in dry soils (moisture: 27 -39%). Prevalence in vegetation mesocosms was 100%; however, a significantly higher mean abundance was found in toads from containers with vegetation ($\bar{x} = 10.25 \pm 7.4$) than without ($\bar{x} = 5.3 \pm 3.4$; $t = 4.19$, $P = 0.001$). Infective lungworms ascended vegetation and often gathered in small groups on the vegetation, whereas nematodes in soil treatments did not aggregate. Control containers produced no infected toads.

DISCUSSION

This is the first study to explore ecological factors that affect the development and transmission of the free-living stages of amphibian lungworms. Our laboratory studies suggest that free-living lungworms are constrained to soil habitats that have high moisture content (Figure II) and that lack exposure to high temperatures (Figure III), and lungworms developed poorly in soils with high proportions of either clay or sand (Figure IV). Field studies using florescent feces found that *A. blanchardi* often defecate in shoreline environments ($\bar{x} = 0.8 \pm 0.5$ m from shoreline) conducive to lungworm

development. The potential for shoreline environments to successfully support lungworm development and infection opportunities was confirmed during our field mesocosm experiments (see Table II). In combination, these studies suggest that the majority of lungworm infections at our site occur within 2 m of the shoreline. Baker (1979) and Bolek and Coggins (2000) have established a precedent for using seasonality data to establish transmission patterns in *Rhabdias* spp.; in this study, the consistent presence of worms in both the lungs and body cavity suggests constant transmission during warm months (see Figure I), especially when the relatively short lifespan (<45 days; Langford and Janovy, 2009) of this worm is considered. Consistent transmission in this population further shows that *R. joaquinensis* is an excellent model organism for ecological studies when compared to other lungworm species. Transmission in other known *Rhabdias* species is strongly seasonal and limited to 1-2 generations per season (Baker, 1979; Bolek and Coggins, 2000), which may limit their usefulness in some experimental systems.

Previous studies on the free-living stages of skin penetrating nematodes of humans and domestic animals have concluded that temperature and moisture are the primary limiting factors in nematode survival (Anderson et al., 1970; Coyne and Smith, 1994; Kung et al., 1970; Smith, 1990). In this study, temperatures >35° C limited lungworm development and produced un-infective juvenile worms in the laboratory (see Figure II). However, during our field experiments maximum soil temperatures in the primary zone of infection (i.e., within 2 m of shoreline) did not reach the upper limit of lungworm tolerance. Furthermore, frogs at our site defecated in soils with temperatures conducive to lungworm development (range 28-33.5° C; G.J. Langford, unpub. data).

These combined results suggest that temperature may not be a limiting factor for free-living juveniles deposited in the primary zone of infectivity, whereas lungworms deposited outside of this area may be exposed to greater maximum temperatures, thus limiting or preventing development.

The shoreline area also provided ideal soil moisture parameters for lungworm development and transmission (see Table II and Figure III). Moisture levels evidently define the zone of infectivity in our study, assuming temperature rarely reaches lungworm tolerances. Soil moisture is critical for nematode development because nematodes require a film of water to move through soils (Anderson, 2000). Yet too much moisture is problematic because soil moisture has an inverse relationship with oxygen availability and a reduction in oxygen levels is predicted to reduce nematode survivorship (Kung et al., 1990), explaining why most skin-penetrating nematodes thrive in soil moistures well below saturation. In contrast, this study showed that lungworms are able to survive beyond the soil saturation point under laboratory conditions; however, nematodes in our field mesocosms did not infect toads in saturated soils, but were able to establish infections at soil moistures of 90%. The lack of infections in saturated soils appears to be due to the mesocosm's proximity to the water's edge, wave action along the shoreline was noted to wash soil and presumably nematodes into the lake (G.J. Langford, pers. obs.).

The ability of lungworms to survive in very moist soils appears to be an adaption to frog defecation patterns. Our florescent powder study found that *A. blanchardi* primarily deposit juvenile lungworms in moist, shoreline habitats, which likely selects for the ability to thrive in near saturated soils. In contrast, nematodes infecting terrestrial

hosts (e.g. trichostrongylids) are most frequently deposited in soils with low to moderate soil moistures (e.g. pastures; Anderson et al., 1970), which would generate little selective pressure for survival in soils at or near saturation for extended time periods.

Previous studies on entomopathogenic nematodes show that soil type can drastically alter survivorship (Georgis and Gaugler, 1991). Finer textured soils, i.e., clay or silt, have decreased pore space between soil particles, which may constrict nematode movement (Gray and Lissmann, 1974) and/or create anoxic conditions (Kung et al. 1990). Finer soils also retain soil moisture longer than coarse, sandy soils; alternatively, larger particle soils, i.e., sand, provide sufficient pore size to facilitate movement, but dry quickly (Koppenhofer and Fuzy, 2006). Our study found that even under ideal laboratory conditions juvenile lungworms are unable to overcome constraints placed on their development by soils with high clay or sand content (see Figure IV). While a few juveniles developed in clay and sand treatments, the numbers were drastically lower than those produced in sandy loam, loam, and clay loam soils. In clay soils, worms mated successfully, however several female worms and their offspring were found dead. The females' deaths were expected because these worms undergo matricidal endotoky (Langford and Janovy, 2009), but the offspring normally survive the mother's death and emerge from her cuticle as infective juveniles. The offspring's death may be attributed to a lack of oxygen in the clay soils. Specifically, offspring inside of the maternal cuticle may have struggled to obtain oxygen across the mother's cuticle. The offspring would have been spatially restricted and unable to move to an area with relatively high oxygen.

Soil type is often the primary determinant of soil microbial communities, and sandy soils support fewer bacterial species with lower abundances when compared to

clay and loam soils (Paul, 2007). We suggest that food, i.e., bacteria, was limited in sand treatments, thus bacterial species or quantities necessary for lungworm growth and development were unavailable. Our hypothesis is supported by Chu (1936), who found that free-living juvenile lungworm growth and development was limited by their bacterial food source. His studies showed that several species of bacteria failed to support lungworm growth and such cultures did not produce infective juvenile worms. Alternatively, lungworms may encounter greater predation pressures (see Karagoz et al., 2007) and/or have reduced locomotor capabilities (see Hunt et al., 2001) on sandy soils compared to loam soils; however, neither was apparent during our experiments.

The evidence presented here rejects our initial hypothesis that western Nebraska anurans were uninfected due to the dry soils of the area. Instead our results suggest that sandy soils alone limit *R. joaquinensis* from infecting anurans in western Nebraska. *Rhabdias joaquinensis* primarily infects anurans in a zone around wetlands, where soil moisture is consistently high. Wetlands that provide a moist zone of soil are common around CPBS in western Nebraska and within the Platte River channel that connects eastern and western Nebraska (G.J. Langford, pers. obs.). Thus, the availability of moist soils does not appear to be a limiting factor for lungworm survival in western Nebraska. Instead, our mesocosm and laboratory experiments found that sandy soils (even when moist) are not conducive to lungworm development and transmission (see above for potential explanations). In the mesocosm experiments, lungworms placed on sand transplanted from CPBS were only able to establish infections in 2 of 80 (2.5%) exposed toads; in comparison, lungworms on natural loam soils generated infections in 26 of 80 (32%) toads. Therefore, based on these results we feel that lungworms would not survive

in the sandy soils of Nebraska's Sandhills, even in moist soils; however, we did not confirm these findings by conducting mesocosm studies at CPBS because of the minimal risk of introducing a novel parasite to the anurans of western Nebraska.

Soils offer a multitude of potential environments; therefore it may be possible that pockets of favorable habitat exist in western Nebraska. For example, 2 of 8 (25%) toads became infected with lungworms in sandy soil mesocosms placed underneath hardwood (primarily *Ulmus* spp.) leaf litter. This result was unexpected, yet we suspect leaf litter may supplement sandy soil nutrient levels and/or inoculate sandy soils with bacteria (from the original loam soil) suitable for lungworm growth and development (see Chu, 1936). Dense stands of hardwood trees are limited in western Nebraska, except along the Platte River. The Platte River is characterized as a sandy, braided stream, subject to seasonal flooding, and lined primarily by plains cottonwoods (*Populus deltoids*) in central and western Nebraska. Cottonwoods create a layer of moist leaf litter on the banks of the Platte River in the fall season that appears favorable for lungworm transmission, yet 5 years of collecting anurans in these sandy habitats (west of Kearney, Nebraska) has produced no infected hosts (G. J. Langford, unpub. data).

Another factor that may reduce the ability of free-living forms to survive in sandy soils along the Platte River is the disturbance of fecal pellets in the zone of infectivity. This study attempted to determine anuran defecation patterns along the Platte River in western Nebraska, but removal of feces was rapid compared to Pawnee Lake in eastern Nebraska. Feces in western Nebraska were often disturbed within 12 hours, whereas feces at Pawnee Lake in eastern Nebraska remained relatively undisturbed, sometimes for several days. The consumption and/or removal of feces by invertebrates would likely kill

nematodes in the fecal pellet and remove a source of bacteria for nematodes that migrated into nearby soils, forcing them to survive on sandy soils alone.

Many soil-inhabiting nematodes are transferred to hosts via ascending clumps of vegetation (see Anderson, 2000). This behavior appears to increase transmission opportunities (Holaseva et al., 1989; Saunders et al., 2001; Smith, 1990), and offer favorable microhabitats for nematodes (Boff et al., 2002). In this study, the presence of vegetation nearly doubled the mean abundance of lungworms when compared to bare soil. Although this result was not surprising, this study provides the first evidence of vegetation facilitating lungworm transmission. Our laboratory experiments did not suggest this behavior, likely because infective juveniles were only provided a simple container of water with glass sides that prevented climbing. Without field studies this climbing behavior would not have been observed, thus demonstrating the importance of combining laboratory and field studies while studying parasite transmission.

In conclusion, our combined field and laboratory experiments suggest that vegetated areas within a moist zone of infection near shorelines support lungworm transmission in eastern Nebraska. The experiments also suggest that sandy soils are the limiting factor preventing lungworm transmission in western Nebraska. Hopefully this study emphasizes the importance of the free-living stages to understanding lungworm transmission in nature. This study is an important addition to the small number of studies that have used a multifaceted approach to investigate the free-living ecology of skin-penetrating nematodes that infect wildlife, and to our knowledge this study is the first to address these factors in a nematode that parasitizes amphibians. We encourage other

studies on amphibian nematodes with free-living stages, such as *Cosmocercoides* spp., to provide a better understanding of this important, yet understudied life stage.

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Figure I. Percent of *Rhabdias joaquinensis* recovered from the lungs and body cavity of *Acris blanchardi* collected at Pawnee Lake, NE in 2008. n = number of lungworms.

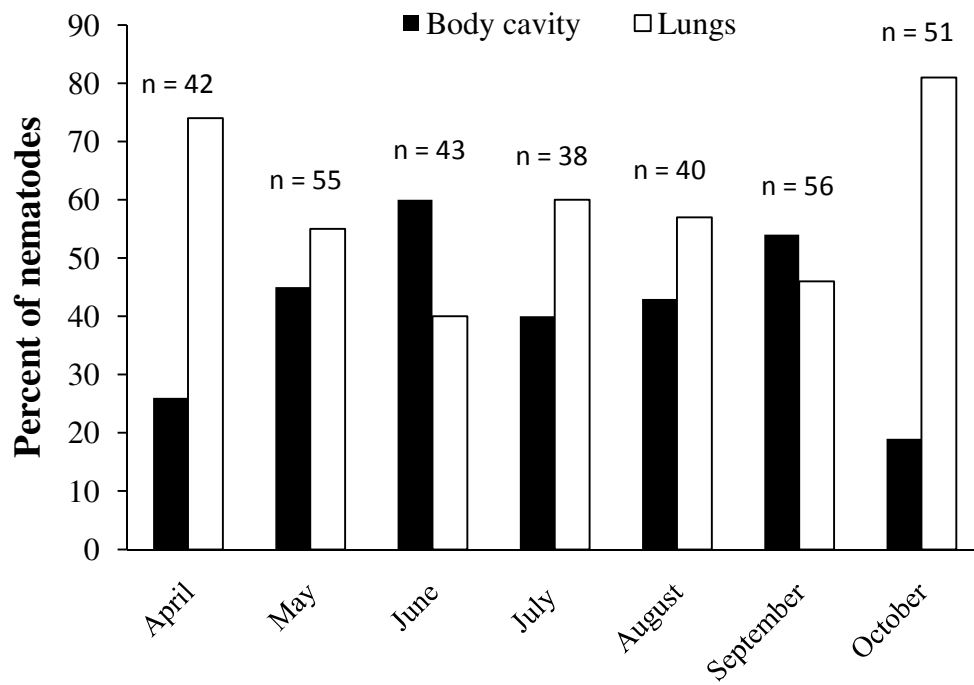


Figure II. Number of *Rhabdias joaquinensis* infective juveniles that developed at 5 different temperatures in the laboratory. Worms reared at 45° C were unable to infect toads. Different letters among treatments represent significant differences.

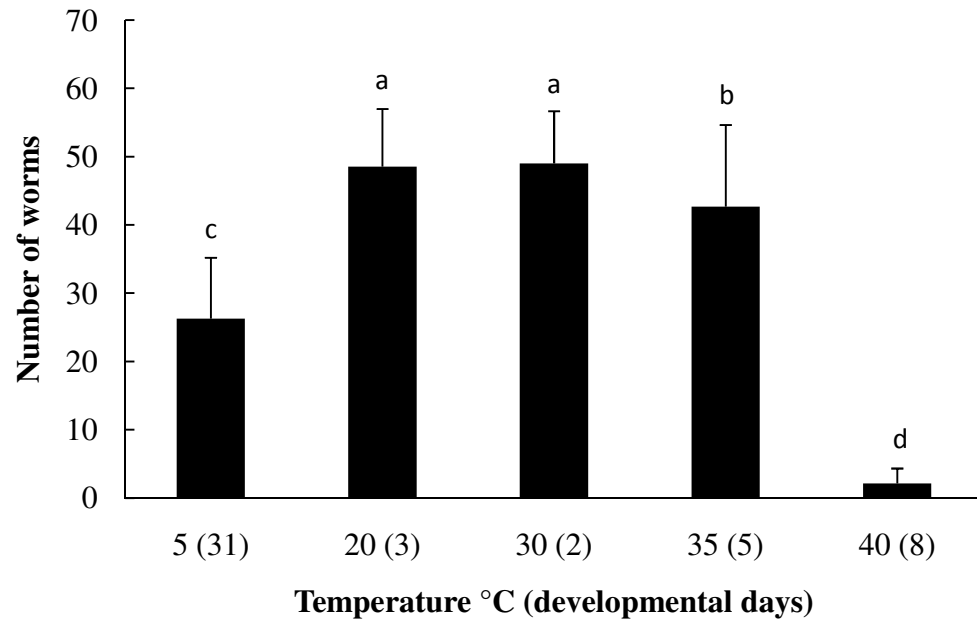


Figure III. Number of *Rhabdias joaquinensis* infective juveniles that developed at 4 different soil moistures in the laboratory. Lungworms reared at 10% and 30% did not produce infective juvenile worms, data not shown. Different letters among treatments represent significant differences.* = slightly beyond soil saturation

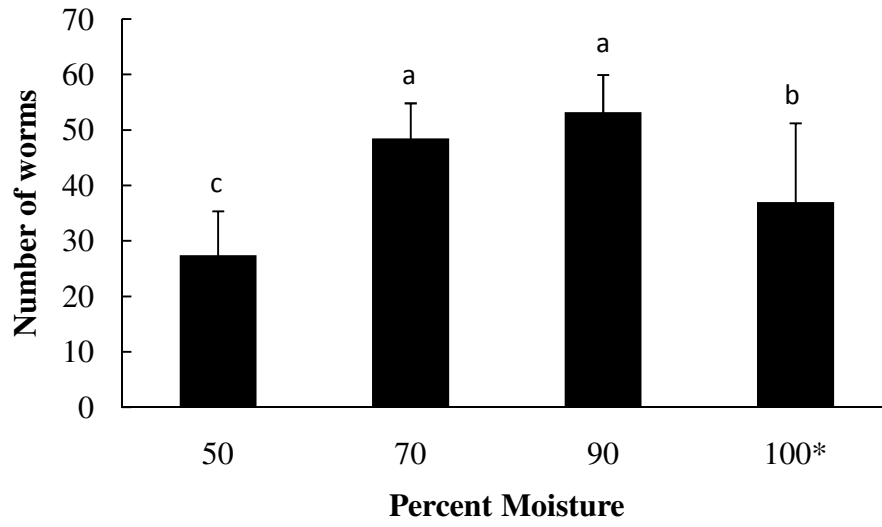


Figure IV. Number of *Rhabdias joaquinensis* infective juveniles that developed in 5 different soil types in the laboratory. Different letters among treatments represent significant differences.

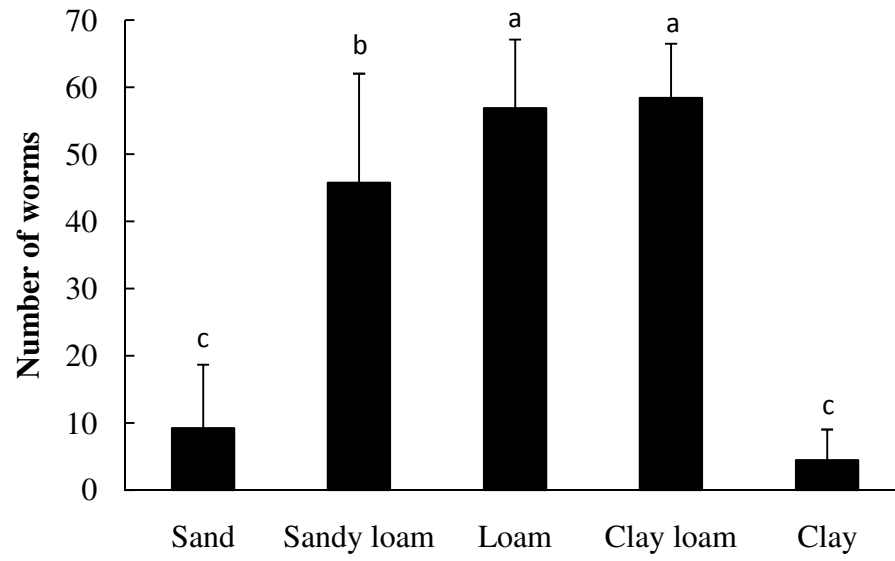


Table I. Soil characteristics used in substrate experiments.

Substrate type	Percent sand/silt/clay	pH	OM	Moisture
Sand	90/10/0	6.0	1.5	80%
Sandy loam	60/25/15	6.3	2.9	80%
Loam	37/44/19	6.3	3.5	80%
Clay loam	25/35/40	7.0	4.2	80%
Clay	0/10/90	7.0	3.8	80%

Table II. Prevalence (P) and Mean Abundance (MA) of *Rhabdias joaquinensis* recovered from young *Bufo woodhousii* in field mesocosms near Pawnee Lake, NE.

Distance from shoreline (cm)	Dominant Vegetation	Soil Moisture	<u>Loam Soil</u>		<u>Sandy Soil</u>	
			P	MA \pm S.D.	P	MA \pm S.D.
10	Shoreline plants	Saturated	0%	—	0%	—
50	Shoreline plants	90%	75%	4.5 \pm 3.5	0%	—
100	Shoreline plants	85%	100%	7.1 \pm 4.8	0%	—
150	Shoreline plants	71%	100%	6.8 \pm 4.2	0%	—
200	Shoreline plants	30%	0%	—	0%	—
400	Bare soil	27%	0%	—	0%	—
500	Shrubs	50%	13%	2.0 \pm 2.0	0%	—
550	Lawn grasses	39%	0%	—	0%	—
700	Tall grasses	47%	25%	1.0 \pm 1.5	0%	—
800	Elm leaf litter	55%	13%	1.2 \pm 1.9	25%	0.9 \pm 1.3

CONCLUSION

"Maybe you understand frogs, and maybe you don't understand 'em; and maybe you've had experience, and maybe you an't only a amateur."

Mark Twain (1865)

Elucidation of an organism's life cycle is vital to understanding all other aspects of its biology (Poulin, 2007; Roberts and Janovy, 2008). This work has established a solid understanding of the life cycles and life histories of several species of North American lungworms (Chapter 1). It was shown that anuran and snake lungworms have different lifecycles, and that snake worms have the potential to and likely do use transport hosts. Specifically, anuran lungworms penetrated the moist, glandular skin of amphibians; whereas, snake lungworms were unable to penetrate the dry scales of snakes. Snake lungworms only infected hosts upon entering the oral cavity, where worms penetrated esophageal tissue. This major difference in host entry (oral vs. dermal) separates snake and anuran worms into 2 separate categories of parasite transmission mechanisms: trophic transmission and direct penetration. Thus, future studies of anuran lungworm transmission should concentrate on direct host-parasite interactions (i.e., areas of physical contact), whereas snake lungworm transmission will require more detailed studies on host consumption (food, water, and incidental) of lungworms. The relatively long survival period of infective snake lungworms compared to anuran worms suggests that snake worms may be adapted to waiting long periods in the environment (or possibly inside a transport host) prior to infecting a definitive host.

Our molecular phylogeny suggests *Rh. ranae* from Nebraska and Mississippi represent separate, cryptic species (Chapter 2); however, additional sequences and an appropriate out-group are needed before a new species is described. Future research may demonstrate shifts among in-group relationships, especially when an appropriate out-group is established for *Rhabdias* spp. Issues concerning cryptic species do not appear to be a concern among the remaining lungworm species, which is convenient for those interested in using these lungworm species in ecological studies. The phylogeny also strongly supports separate clades for anuran and snake lungworms, although whether these clades represent separate genera is a question that still needs to be addressed. The molecular distance between the 2 clades supports separate genera, especially when the variation in life cycles and host specificities between the clades is considered. However, the morphological similarities between the clades suggest otherwise, and the addition of other genera (e.g. *Entomelas*) to the phylogenetic analysis may clarify the current classification.

Host specificity studies indicated that snake lungworms are capable of infecting a wide variety of snake species and perhaps lizards too (Chapter 2). Our preliminary studies (unpublished) suggest that increased environmental temperatures can prevent lungworm establishment in snakes and lizards, and to a lesser degree resolve established infections. Lungworm species collected from anurans ranged from those exhibiting strict host specificity (e.g., *Rh. ranae* from Nebraska) to relative generalists (e.g., *Rh. joaquinensis* from Nebraska), and provided examples of ecological fitting (Chapter 2). Host specificity studies found that most lungworm species are capable of infecting a greater number of hosts than they encounter (or that encounter them) at any one location

or time in nature. For example, our experimental exposures (Chapter 2; unpublished) have found that all lungworm species in this work are capable of establishing infections in allopatric populations and/or novel host species, and many worm species established infections in distantly related hosts (i.e. different families). These experimental infections suggest that phylogenetic relationships among hosts are not as important for lungworm establishment as the resources offered by the host (serving as examples of ecological fitting; see Brooks et al., 2006). Our understanding of ecological fitting in these worms may benefit from research on the spatial and temporal overlap among hosts (e.g. *Ra. sylvatica* and *B. americanus*), in conjunction with lungworm studies on survival of free-living stages in overlapping host environments. Another direction for future research on lungworm host specificity would be to address the immune response of bullfrogs or narrowmouth toads to lungworm exposure. These 2 host species offer an opportunity to study physiologically-mediated host specificity in lungworms.

This dissertation also found that vegetated areas within a moist zone of infection support lungworm transmission in eastern Nebraska (Chapter 3). The zone of infection at Pawnee Lake extended from the shoreline to approximately 2 m outwards. A similar zone of infection would likely be found in other environments similar to eastern Nebraska. However, landscapes comprised of extensive wetlands would likely contain an extended zone of infection, due to the pervasive moist habitats in these wetlands. This study also suggests that sandy soils are the limiting factor preventing lungworm transmission in western Nebraska. Yet, it remains unknown if sandy soils limit lungworm development outside of Nebraska's Sandhills; our preliminary studies (unpublished) in Mississippi suggest that *Bufo* spp. maintained on bare sand in nature are rarely infected by *Rh.*

joaquinensis or *Rh. americanus*. Perhaps bacterial growth in sandy soils from western Nebraska is not conducive to *Rh. joaquinensis* development, but this aspect of lungworm biology remains largely unstudied. Future studies on free-living stages may address the role of bacteria in juvenile growth and development. In addition, further study is needed to understand how vegetation facilitates *Rh. joaquinensis* transmission. Does vegetation increase juvenile survival in the soil and/or provide a more effective mode of transferring to the host? Lastly, we note that Chapter 3 was only conducted on *Rh. joaquinensis*, thus the response of other lungworm species to the free-living environment remains unknown.

Overall, it is our hope that this foundation of *Rhabdias* biology will encourage other biologists to use lungworms and their hosts as model host-parasite systems for ecological and evolutionary research.

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