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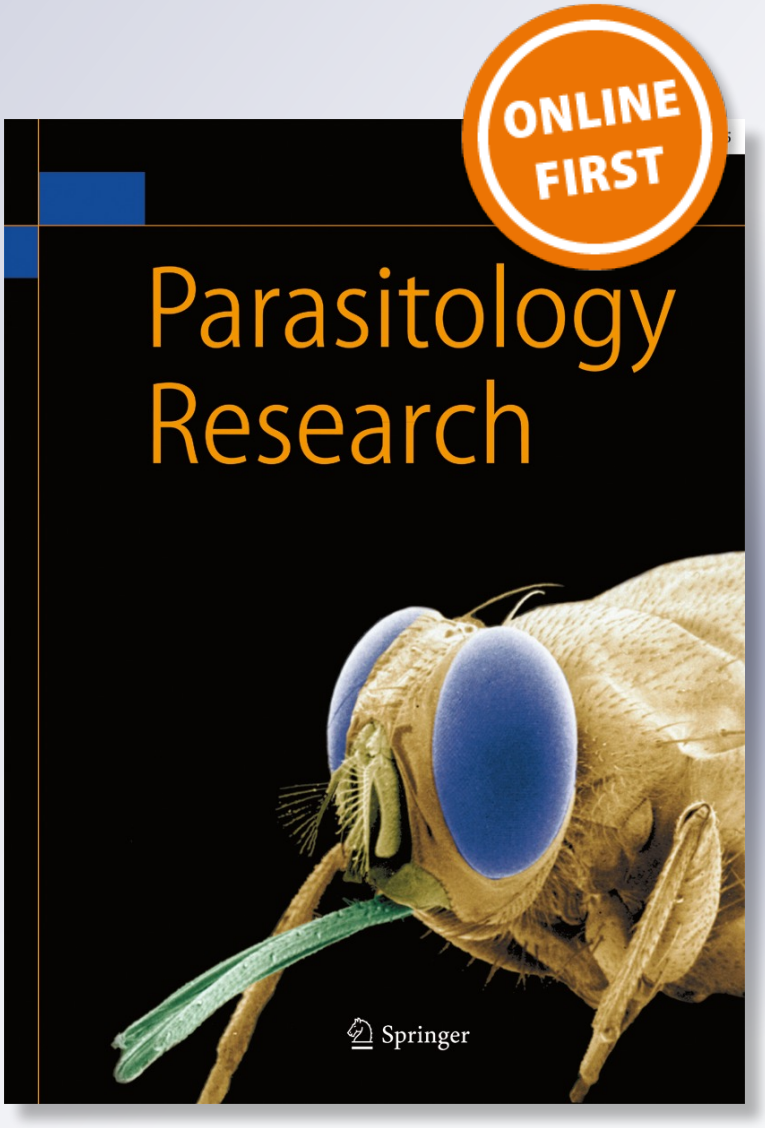

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ORIGINAL PAPER

Ecological factors responsible for the geographic distribution of *Rhabdias joaquinensis*: where do lungworms infect anurans in nature?

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Abstract The lungworm *Rhabdias joaquinensis* (Nematoda: Rhabdiasidae) is a common parasite of anurans in eastern Nebraska, yet absent from the same hosts in western Nebraska. This study investigated the ecology of the lungworm's free-living stages that reside in host feces and surrounding soils to establish the worm's free-living niche in eastern Nebraska. Using a comparative method, this study also investigated the absence of lungworms in western Nebraska's Sandhills. Soil composition, moisture, and temperature were experimentally varied in the laboratory to assess their effects on juvenile development and survival. Field mesocosm and host defecation experiments were used to determine where in nature lungworms survive and eventually infect frogs and toads and to discover if 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 and 35 °C, albeit at different rates, whereas only a limited number of non-infective worms developed at 40 °C. Field studies found that shoreline environments supported lungworm development, and the majority of lungworm infections appear to occur within a zone of infection adjacent to shorelines in eastern Nebraska. The prevalence in vegetation mesocosms was 100 %, and a significantly higher mean abundance was found in toads from containers with vegetation than without. Overall, these experiments suggest that the spatial distribution of *R. joaquinensis* in Nebraska is constrained by the worm's ability to survive and reproduce in a soil matrix.

Keywords *Rhabdias* · Lungworm · Ecology · Free-living · Soil · Nematode · Frog · Toad · Transmission · Anuran · Environment

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 (Anderson 2000; Coyne & Smith 1994; O'Connor et al. 2006; Vlassoff et al. 2001), and soil transmission patterns of several entomopathogenic nematodes also are well known (Gaugler 2002; Kaya & Gaugler 1993). Unfortunately, few detailed studies have been conducted on the ecology of free-living stages of nematodes that infect wildlife, which limits our understanding of their transmission in nature, but see *Trichostrongylus tenuis* (Saunders et al. 1999; Saunders et al. 2000). Despite the recent popularity of using *Rhabdias* spp. that infect amphibians as model systems for the study of parasite ecology and evolution (Barton 1998; Dare et al. 2008; Goater & Ward 1992; Langford & Janovy 2009; Langford & Janovy 2013; Langford et al. 2013; Pizzatto & Shine 2011), no studies have addressed the ecology of the free-living stages of skin-penetrating nematodes that infect amphibians in nature. Previous studies have ignored the importance of the free-living stages of the worm's lifecycle to govern or constrain the spatiotemporal patterns of transmission.

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Soils are complex ecosystems that dictate the survival and infectivity of soil-transmitted nematodes (Kaya & Gaugler 1993; Levine 1980; Stromberg 1997). Free-living stages of parasitic nematodes may use the soil environment to deposit eggs, undergo juvenile development, sexually reproduce and lay eggs, and locate and infect an intermediate or final host (Anderson 2000). In addition to adaptations for a parasitic lifestyle, nematodes with free-living stages must maintain adaptations to survive abiotic conditions in the soil, such as temperature, rainfall, moisture content, pH, composition and chemistry, sunlight, wind, and biotic pressures from fungi, plants, prokaryotes, and numerous predaceous invertebrates (Kates 1950; Kaya & Gaugler 1993; Smith 1990; Stromberg 1997). In general, soil temperature, moisture, and composition are the primary abiotic factors that constrain distribution and abundance of the soil stages of parasitic nematodes (Bakonyi et al. 2007; Kaya & Gaugler 1993; Levine 1980; Payne 1923; Smith 1990; Stromberg 1997). Indeed, ecological interactions between free-living worms and the soil matrix may be one of the primary drivers of a parasite's distribution.

The skin-penetrating nematode *Rhabdias joaquinensis* Stiles & Hassall (1905) (Nematoda: Rhabdiasidae) provides an ideal candidate for studying the effects of soil microenvironments on the survival and geographic distribution of a nematode that parasitizes wildlife. The worms are heterogonic, i.e., they must sexually reproduce in the soil to produce an infective generation (Fig. 1); when compared to most other soil transmitted nematodes, *Rhabdias* spp. spend a large proportion of their life cycle in the soil. These worms also serve as a model organism for ecological study because the life cycle (Langford & Janovy 2009) and host specificity (Langford & Janovy 2013) of *R. joaquinensis* are fully elucidated in Nebraska. This lungworm is a generalist; our previous studies show that young-of-the-year (YOY) toads (*Anaxyrus woodhousii*) and juvenile and adult cricket frogs (*Acris blanchardi*) are always infected when exposed to infective *R. joaquinensis* juveniles in laboratory conditions and are commonly found infecting wild anurans in eastern Nebraska. Further, the lungworm's life cycle, from free-living stages to penetrating the lungs, can be completed quickly, i.e., in 7 days. Thus, this model system provides a quick and reliable assay of lungworm infectivity in the laboratory or field, a method that is not available for most parasites of amphibians or wildlife in general. In addition, lungworms from Nebraska are interesting candidates for study because, despite similar amphibian communities in the two regions, *R. joaquinensis* commonly infects anurans in eastern Nebraska, but is not known to infect anurans in western Nebraska (Langford & Janovy 2009; Langford & Janovy 2013). We hypothesize that *R. joaquinensis* is limited to the loam soils of eastern Nebraska by the inability of free-living stages to survive in the arid soils of western Nebraska. This hypothesis is based on previous lungworm studies that suggest (1) soil moisture may limit *Rhabdias pseudosphaerocephala* infection opportunities in its Australian host *Rhinella marina* (Barton 1998), (2) YOY toads are susceptible to infection by *Rhabdias bufonis* in Europe because they reside in wet soils (young toads are

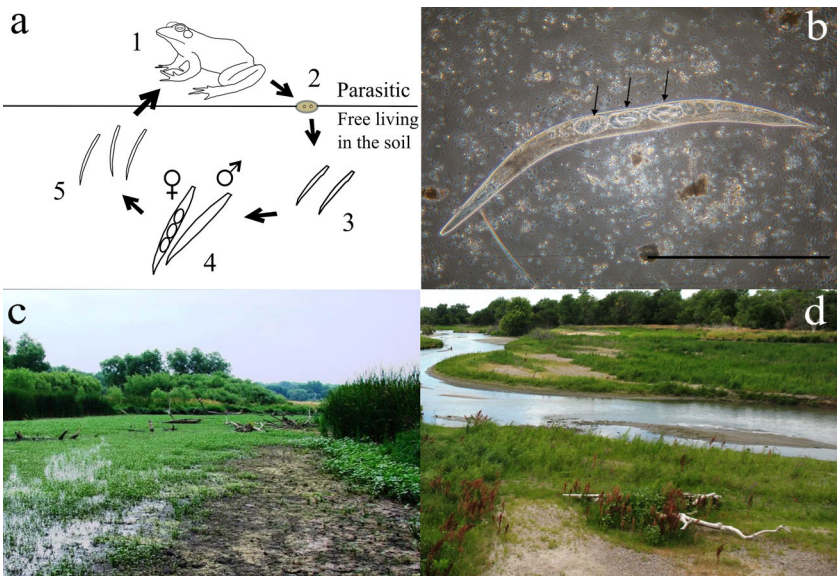

Fig. 1 **a** The life cycle of *Rhabdias joaquinensis*: *1* hermaphroditic adult worms feed on blood in the frog's lungs and produce eggs that are carried out of the lungs and into the gastrointestinal tract; *2* embryonated eggs are passed in the feces; *3* free-living juvenile worms feed and develop into males and females in the soil; *4* adult worms mate in the soil and females produce eggs that eventually rupture out of her cuticle (matricidal endotoky); *5* juvenile worms develop in the soil to the infective stage (J3) and subsequently penetrate the skin of a frog or toad, then worms undergo a developmental migration through the host's body cavity before penetrating into its lungs to mature and produce eggs. **b** Free-living gravid female lungworm; *arrows* indicate eggs. *Scale bar*=200 μm. **c** Loam soils at our shoreline collection site in eastern Nebraska. **d** Sandy soils along the Platte River in western Nebraska

susceptible to desiccation) that apparently are conducive to lungworm development (Goater & Ward 1992), and (3) the distribution of lungworm species in South America is geographically constrained because the free-living generation is directly influenced by the environment (Kloss 1974).

We are unaware of any studies attempting to determine how abiotic and biotic factors might affect amphibian lungworm development and distributions. Therefore, our main goals were to (1) determine the effects of soil temperature, moisture, and composition on survival of free-living worms in the laboratory; (2) discover where infected frogs defecate in nature; (3) establish where free-living lungworms develop in nature and infect anurans; and (4) determine if vegetation facilitates lungworm transmission. In combination, the experiments developed to achieve these goals enable us to elucidate the life history and ecology of a free-living lungworm and thus address the absence of *R. joaquinensis* in western Nebraska streams and wetlands.

Methods

Acquiring lungworms and uninfected hosts

Adult *A. blanchardi* were hand captured both day and night from a small pond adjacent to Pawnee Lake, Lancaster Co., NE (40.86, −96.89) from May to October 2008. Animals were transported to the parasitology laboratory at the University of Nebraska-Lincoln, euthanized, measured for snout-vent length (SVL), sexed, and all organs and tissues examined for *Rhabdias* spp. within 24 h 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 & Kruse 1982). All lungworms were identified according to Kuzmin et al. (2003) and a few were confirmed by molecular data (Langford & Janovy 2013); only *R. joaquinensis* were recovered in this study. Representative specimens were deposited in the H. W. Manter Parasitology Collection, University of Nebraska, Lincoln, NE (HWML 63516).

Uninfected YOY *A. woodhousii* were hand captured from the area surrounding Cedar Point Biological Station (CPBS), Keith County, NE (41.21, −101.62) and transported to the parasitology laboratory at the University of Nebraska-Lincoln for immediate use in mesocosm studies. Notably, *Rhabdias* spp. have never been collected from amphibians at CPBS or western Nebraska in over 30 years of parasitological explorations (JJJ pers. observ.).

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 & 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 each of the following temperatures: 5, 20, 30, 35, and 40 ° C. Fifty lungworm eggs or recently hatched juveniles were added to each Petri dish. 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. The experiment was repeated twice.

Soil moisture experiments

Fifty eggs or recently hatched juveniles were placed in 5 g of loam soil at 25 °C in a Petri dish and placed in the following soil moisture treatments: 30, 50, 70, 90, and 100 %, i.e., slightly beyond saturation. Aged tap water was added to containers to maintain moisture throughout the experiment. Outer Petri dishes were not used in these experiments because they altered soil moisture via wicking through the paper towel; 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. The experiment had five replicates and was repeated twice.

Soil composition experiments

Soil types used in this experiment are displayed in Table 1. 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. Aged tap water was added to containers to maintain moisture throughout the 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. The experiment had five replicates and was repeated twice.

Table 1 Soil characteristics used in free-living *Rhabdias joaquinensis* substrate experiments in the laboratory

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

Frog spatial defecation

Harmless fluorescent powder (Lightning Powder Company, Inc., Jacksonville, FL) was used to determine where *A. blanchardi* defecated in nature. A total of 80 frogs (some may have been recaptured) were collected from 100 m of creek shoreline that leads into Lake Pawnee, NE (40.86, −96.89) each morning from July 13 to 16, 2008. When a frog was captured, it was force fed ~1–2 g of moist fluorescent powder. After assuring the powder was swallowed, the frogs were immediately released within 5 min of capture. After sunset, from July 13 to 18, a handheld blacklight was used to illuminate frog feces within an area that extended 5 m from shoreline and was 50 m in length, thus including the frog's primary home range (Ferguson et al. 1967). The entire area was surveyed on foot to reveal illuminated feces. The following three parameters were measured for each fecal pellet discovered: distance to shoreline, soil temperature (which dropped slowly after sunset), 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 Lake Pawnee, where anurans often defecated, i.e., the apparent zone of potential infection. 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 20×20×10-cm box with no bottom. The open bottom was then pushed into the soil to prevent both soil disturbances by large animals and escape by toads placed in the mesocosms. Half of the mesocosms (n=8) were placed on naturally occurring loam soils, and the remaining mesocosms (n=8) were placed on sandy soils acquired from CPBS. Sandy mesocosms were created by excavating loam soils within the mesocosm to a depth of 5 cm, which were replaced with sandy soils. Vegetation displaced while digging plots was replanted, when possible.

On August 18, 2008, mesocosms were placed in the environment in groups of 16 at 10 distances from the shoreline of Lake Pawnee, NE. Fifty recently hatched juvenile *R. 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 *A. woodhousii* and permitted to develop for 5 days. Air temperature at the site ranged between 18 and 35 °C and humidity ranged between 31 and 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 and 4 pm. After 5 days, when the worms should have been able to develop into infective juveniles, one uninfected YOY toad, collected at CPBS, was added to each mesocosm for 12 h to assess the development and infectivity of free-living lungworms. Only one toad was added to each mesocosm to avoid pseudoreplication. Following exposure, toads were transported to the University of Nebraska-Lincoln parasitology laboratory and held for 7 days to allow infections to develop, with toad husbandry according to Langford & Janovy (2009), then euthanized, and all organs and tissues examined for *R. joaquinensis*. Importantly, several attempts were made to replicate this field mesocosm along the Platte River near CPBS in western Nebraska, where a variety of anurans were commonly encountered; however, no infections were established.

Vegetation mesocosms

In this experiment, laboratory mesocosms were established to determine if the presence of vegetation facilitates lungworm transmission to toads, similar to hookworm infections in humans (Smith 1990). A total of 50 mesocosms were established by placing 3 cm of soil, collected from Lake Pawnee, NE, into 30-cm (diameter) circular plastic trays. 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 and 90 %. Trays were exposed to a 12-h L/12-h D fluorescent photoperiod. White clover (*Trifolium repens*) was propagated in 20 trays and pruned to 50 % vegetative cover prior to beginning experiments. White clover was selected because it grows rapidly, even in poor soils, and the plant is common at many of our sampling locations. The second experimental group consisted of 20 trays with identical clay loam soils with no vegetation. For both experimental groups, 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 h. Nematodes were not added to 10 control trays (five bare soil, five vegetation). Following acclimation, one YOY *A. woodhousii*, collected at CPBS, was added to each mesocosm for 12 h to assess the transmission of infective lungworms. Following exposure, toads were held for 7 days to allow infections to develop, euthanized, and all organs and tissues examined for *R. joaquinensis*.

Statistical analysis

Prior to dissection, hosts were measured for SVL, and host sex was determined during dissection. Toads used in mesocosm

experiments were selected to not vary significantly in size (SVL=4.0±0.2 cm), and the prevalence and mean abundance did not vary between sexes, which was not surprising as they were YOY; thus, all data were combined. Prevalence (*P*) and mean abundance (MA) were calculated as measures of parasitism for anurans according to Bush et al. (1997). Values are reported as mean±1 SD. Analysis of variance (ANOVA) was used to compare the 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). Although female frogs were generally larger than males, no significant difference was found for sexual size dimorphism (female 2.8±0.3, n=50; male 2.7±0.4, n=88; t=0.81, P=0.242). Not surprisingly, 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 (Fig. 2).

A significant difference in the mean number of free-living worms that developed in Petri dishes was detected between temperature treatments (Fig. 3; 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 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 (Fig. 4; 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 types were significantly different in their ability to support lungworm development (Fig. 5; 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.

Fluorescent feces (n=49) of *A. blanchardi* were not discovered further than 1.7 m from shoreline and on average were

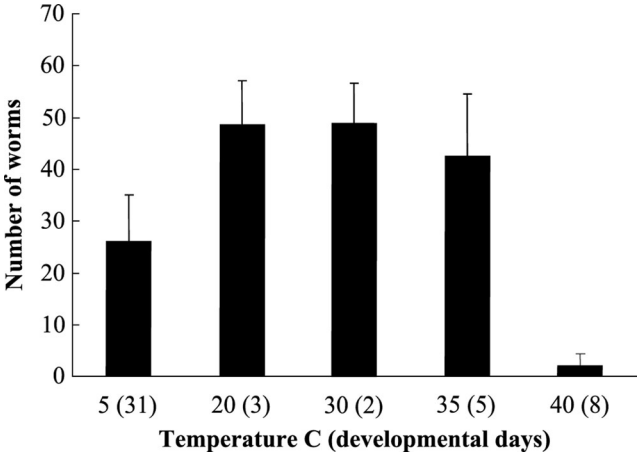

Fig. 3 Number of *Rhabdias joaquinensis* infective juveniles that developed at five temperatures in the laboratory. Worms reared at 45 °C were unable to infect toads

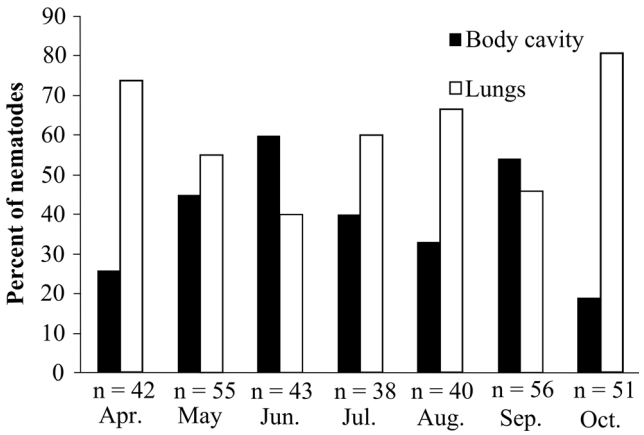

Fig. 2 Percent of *Rhabdias joaquinensis* recovered from the lungs and body cavity of *Acris blanchardi* collected at Lake Pawnee, NE, in 2008

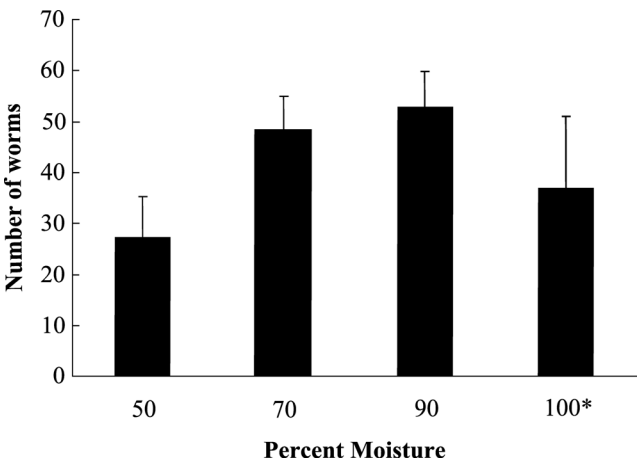

Fig. 4 Number of *Rhabdias joaquinensis* infective juveniles that developed at four soil moistures in the laboratory. Lungworms reared at 10 and 30 % did not produce infective juvenile worms, not displayed. *Asterisk* indicates slightly beyond soil saturation

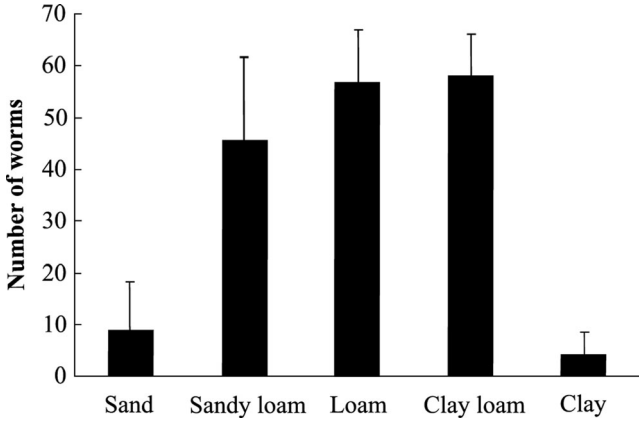

Fig. 5 Number of *Rhabdias joaquinensis* infective juveniles that developed in five soil compositions in the laboratory

0.8±0.5 m from shoreline. Frogs defecated in areas with an average soil temperature of 28±4 °C and soil moisture of 72 ±12 %. Fluorescent frog feces were not distributed randomly but tended to be clumped in small groups with unmarked feces. It is not clear if these feces aggregations were deposited by a single frog or if contributions from conspecifics were present.

Mesocosm experiments found infective juveniles reached the highest prevalence and mean abundance at 50–150 cm from shoreline (Table 2). In addition to infecting toads near shoreline, lungworms established infections when the mesocosm was placed on top of leaf litter. Lungworms were unable to infect toads 10 cm from shoreline in saturated soils; worms also failed to infect toads in dry soils (moisture 27–39 %). In mesocosms designed to test the influence of vegetation of lungworm transmission, the prevalence was higher in vegetated (100 %) than bare soil (65 %) containers. Similarly, a significantly higher mean abundance was found in toads from containers with vegetation ($\bar{x} = 10.25 \pm 7.4$) than without ($\bar{x} = 5.3 \pm 3.4$; $t = 4.19$, $P = 0.001$). Of note, infective lungworms ascended vegetation and often gathered in small groups on the vegetation, whereas nematodes in soil treatments did not obviously aggregate (but were harder to monitor to observe behaviors). Control containers produced no infected toads.

Discussion

The heterogonic life cycle of amphibian lungworms makes them ideal candidates to study constraints on nematode development in free-living microhabitats, yet this is the first study to assess the ecological factors that affect the development and transmission of free-living lungworms. The experimental evidence we present establishes a "zone of infection" wherein ecological conditions are conducive for worm development and susceptible anurans may acquire infections. Based on the ecological conditions in the zone of infection, we must reject our initial hypothesis that anurans from western Nebraska were uninfected with *R. joaquinensis* because of the dry soils in the region. We found that moist soils and wetland habitats, similar to areas where transmission was common in eastern Nebraska, were pervasive in areas that supported amphibians in western Nebraska. Our results, however, suggest an alternative explanation for the spatial distribution of lungworms in Nebraska: that soil composition, i.e., sandy soil, was a limiting factor preventing *R. joaquinensis* from developing in the soil and infecting anurans in western Nebraska. Indeed, the ecological requirements of the free-living generation constrict the distribution of heterogonic lungworms in nature.

In favorable loam soils, we found that free-living lungworms develop better in soils with high moisture content and moderate temperatures and that transmission is enhanced when vegetation is present. In addition, we found that cricket frogs commonly defecate in shoreline environments that are conducive to lungworm development. Indeed, the potential

Table 2 Prevalence (*P*) and mean abundance (MA) of young-of-the-year toads (*Anaxyrus woodhousii*) exposed to *Rhabdias joaquinensis* in field mesocosms (loam vs. sandy soils) near Lake Pawnee, NE

Distance from shoreline (cm)	Dominant ground cover	Soil moisture	Loam soil		Sandy soil	
			P (%)	MA±SD	*P* (%)	MA±SD
10	Shoreline plants	Saturated	0	–	0	–
50	Shoreline plants	90 %	75	4.5±3.5	0	–
100	Shoreline plants	85 %	100	7.1±4.8	0	–
150	Shoreline plants	71 %	100	6.8±4.2	0	–
200	Shoreline plants	30 %	0	–	0	–
400	Bare soil	27 %	0	–	0	–
500	Shrubs	50 %	13	2.0±2.0	0	–
550	Lawn grasses	39 %	0	–	0	–
700	Tall grasses	47 %	25	1.0±1.5	0	–
800	Elm leaf litter	55 %	13	1.2±1.9	25	0.9±1.3

for shoreline environments to successfully support lungworm development and infection opportunities was confirmed during our field mesocosm experiments where toads were infected when confined to the zone of infection in nature. Thus, our results suggest that lungworms develop and infect frogs in eastern Nebraska stream and lake microenvironments within 2 m of the shoreline, i.e., the zone of infection. Lungworms deposited outside of this area may be exposed to greater maximum temperatures and reduced soil moisture, thus limiting or preventing development.

Moisture levels evidently define the zone of infectivity in our study, assuming loam soils are present and temperatures rarely reach 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 (Anderson 2000; Smith 1990). In contrast, our laboratory findings showed that lungworms are able to survive beyond the soil saturation point; however, nematodes in our field mesocosms did not infect toads in saturated soils. The lack of infections in saturated soils is attributable to the mesocosms proximity to the water's edge; wave action was noted to wash soil and presumably nematodes into the lake (GJL pers. obser.). Our results are a departure from previous studies on free-living forms of parasitic nematodes, which primarily find survivorship highest in moderate soil moisture (Anderson et al. 1970; Coyne & Smith 1994; O'Connor et al. 2006). These studies have concentrated on nematodes (e.g., trichostrongylids) that parasitize terrestrial hosts (e.g., sheep) that infrequently reside in or adjacent to water bodies, whereas frogs and toads may spend their entire life in these moist habitats. Thus, it should not be surprising that the free-living generation of *Rhabdias* thrive in and are adapted to moist soil conditions.

Previous studies on entomopathogenic nematodes show that soil type can drastically alter survivorship (Georgis & Gaugler 1991). Finer textured soils, i.e., clay or silt, have decreased pore space between soil particles, which may constrict nematode movement (Gray & Lissmann 1964), create anoxic conditions, and retain soil moisture (Kung et al. 1990). Alternatively, larger particle soils, i.e., sand, provide sufficient pore size to facilitate movement, provide aerobic conditions, but dry quickly (Koppenhofer & 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. While a few juveniles developed in clay and sand treatments, the numbers were significantly lower than those produced in other soil treatments. In clay treatments, 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 & Janovy 2009), but the offspring normally survive the mother's death and emerge from her cuticle as infective juveniles. It is unclear why the offspring failed to rupture their mother's cuticle upon her death; however, we suspect that the offspring's death may be attributed to a lack of oxygen in the clay soils and thus in utero.

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) is a limiting resource in sandy soils from western Nebraska, thus restricting lungworm growth and development. Our assertion is supported by Chu (1936), who found that free-living juvenile lungworm (from snakes) growth and development is limited in the laboratory by bacterial diversity and abundance; indeed, several species of bacteria failed to support lungworm growth and such cultures did not produce infective juvenile worms. In combination, our studies suggest that soil composition and associated bacterial communities can limit the distribution of *R. joaquinensis* in nature when compared to its host's range. Alternatively, or in combination, survival of free-living worms could be limited by reduced locomotor capabilities (Hunt et al. 2001) or greater predation pressure (Karagoz et al. 2007) on sandy soils when compared to loam soils.

An example of predation is the disturbance of fecal pellets, which was common in sandy soils along the Platte River. We attempted to repeat our mesocosm study and determine anuran defecation patterns along the Platte River in western Nebraska, but removal of feces was rapid when compared to our studies at Lake Pawnee in eastern Nebraska. Feces along the Platte River in western Nebraska were often disturbed within 12 h (overnight) by beetles and possibly other invertebrates, whereas feces at Lake Pawnee in eastern Nebraska remained relatively undisturbed, sometimes for several days. The consumption or removal of feces by invertebrates may kill free-living worms or eggs in the fecal pellet and remove a source of bacterial food for worms; thus, forcing surviving worms to subsist in poor environmental microhabitats. Previous studies have found that beetles function as biological controls on nematode eggs, in which beetles macerated trichostrongylid eggs while feeding on cattle and sheep feces (Bergstrom et al. 1976).

Many soil-inhabiting nematodes are transferred to hosts via ascending clumps of vegetation (Anderson 2000). This behavior appears to increase transmission opportunities (Holasova et al. 1989; Saunders et al. 2001; Smith 1990) and offers favorable microhabitats for nematodes (Boff et al. 2002). In this study, the presence of vegetation nearly doubled the mean abundance of lungworms in toads when compared to bare soil, which provides the first evidence of vegetation facilitating *Rhabdias* transmission. Our laboratory experiments did not

suggest this behavior, likely because infective juveniles were only provided a simple container of water with glass surfaces that prevented this behavior. 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. Overall, it appears low-lying vegetation within the zone of infection should increase the probability of transmission to anuran hosts, but future studies are needed to establish the importance of this behavior in other lungworm species and in areas outside the zone of infection.

Previous authors have established a precedent for using seasonality data to establish transmission patterns in lungworms (Baker 1979; Barton 1998; Bolek & Coggins 2000). In this study, the constant presence of worms in both the lungs and body cavity suggests consistent transmission during the cricket frog's active season from March to October, which is not surprising given that the frogs primarily reside in the parasite's zone of infection. Apparently, exposure to skin-penetrating nematodes is a trade-off for maintaining a territory in shoreline habitats for cricket frogs, which provides the frogs' access to water, food, cover, and mates (Ferguson et al. 1967). Interestingly, YOY toads at our site inhabit this shoreline habitat for a few weeks after metamorphosis, in which time they acquire relatively intense lungworm infections, whereas adult toads that pass through our sites rarely enter the zone of infection and thus are rarely infected with lungworms (GJL unpub. data). Apparently, toads in Nebraska acquire initial lungworm infections as YOY while seeking cover and moisture in shoreline environments and rarely reacquire infections as they adopt a terrestrial lifestyle. Further exposure to *R. joaquinensis* in adult toads likely only occurs during their short, explosive breeding season during the spring. Similar observations have been made for species of toads infected with lungworms from Europe (Goater & Ward 1992), Australia (Barton 1998), and South America (Kloss 1974); thus, it appears that anurans adopting a permanent or near-permanent terrestrial lifestyle should reduce their exposure to infective lungworm parasites. However, collections of Eastern American toads (*Anaxyrus americanus*) in Wisconsin by Bolek & Coggins (2003) found mostly adults infected, which suggests that other species of lungworms, i.e., *Rhabdias americanus*, may be adapted to infecting hosts in more terrestrial habitats, which was previously suggested by Langford & Janovy (2013).

Our study emphasizes the importance of conducting ecological studies on the free-living stages of parasitic nematodes to understand their distribution and transmission. Such studies are needed to not only elucidate the parasite's ecology and life history but also understand how these worms impact host populations. For example, lungworms introduced to Australia with their invasive host (*R. marina*) have received substantial attention as potential biocontrol agents (Saunders et al. 2010), yet there is concern these exotic lungworms may adapt to infect native frogs (Pizzatto & Shine 2011). If the free-living ecology of the introduced lungworm (*R. pseudosphaerocephala*), which is native to the Americas, is similar to that of *R. joaquinensis*, then the ecological requirements of free-living stages may constrain, at least in part, their geographical distribution in Australia. If confirmed, this insight would provide researchers with additional information to establish where these introduced lungworms are likely to occur and if native frogs encounter infective juvenile worms in a zone of infection. Thus, we encourage other researchers to conduct ecological studies on the free-living stages of parasitic nematodes that infect wildlife, especially worms that spend a substantial portion of their life cycle in the soil.

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