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Authors: Palmisano, Jenna N., Bockoven, Carson, McPherson, Samantha M., Ossiboff, Robert J., Walden, Heather D. S., et al.

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## Infection Experiments Indicate That Common Florida Anurans and Lizards May Serve as Intermediate Hosts for the Invasive Pentastome Parasite, *Raillietiella orientalis*

JENNA N. PALMISANO,<sup>1,3</sup> CARSON BOCKOVEN,<sup>1</sup> SAMANTHA M. MCPHERSON,<sup>1</sup> ROBERT J. OSSIBOFF,<sup>2</sup> HEATHER D. S. WALDEN,<sup>2</sup>  
AND TERENCE M. FARRELL<sup>1</sup>

<sup>1</sup>Department of Biology, Stetson University, DeLand, Florida, 32723, USA.

<sup>2</sup>Department of Comparative, Diagnostic and Population Medicine, College of Veterinary Medicine, University of Florida, Gainesville, Florida, 32608, USA

**ABSTRACT.**—*Raillietiella orientalis*, an invasive pentastome parasite, inhabits the lungs of snakes as adults and is rapidly spreading in Florida and Australia. The species that serve as intermediate hosts are currently unknown. We conducted laboratory infection experiments with insects (*Blaberus discoidalis*), lizards (*Anolis sagrei*), and anurans (*Anaxyrus terrestris*, *Lithobates sphenoccephalus*, *Osteopilus septentrionalis*) to develop an understanding of which species may serve as intermediate hosts and to determine the fitness consequences of infection by *R. orientalis*. Lizards and insects, but not anurans, were readily infected by consuming food that was contaminated with pentastome eggs. *Anolis sagrei* and *L. sphenoccephalus* were both infected after eating a single roach that was infected with *R. orientalis* larvae. Comparison with uninfected control animals revealed that pentastome infection did not significantly affect survival or growth in roaches, lizards, or anurans. The life cycle of *R. orientalis* in Florida is likely to involve a sequence of three hosts, with eggs hatching in coprophagous insects that infect lizards and anurans that infect the definitive host (snakes) after they are consumed. Our results indicate that the native species that serve as intermediate hosts are unlikely to experience major negative consequences from *R. orientalis*, unlike the native snake species that serve as the definitive hosts. The diversity of species that can serve as intermediate hosts and the potential for vehicular rafting by infected roaches and anoles indicate that the rapid geographic range expansion of *R. orientalis* will probably continue.

The introduction of species to ecosystems presents many potential threats to native populations. Introduced species can outcompete native species, decimate native prey populations, modify habitat, and cause the spread of parasites and other pests (Dorcas et al., 2012; Dunn et al., 2012). Nonindigenous species introduction is of particular concern in Florida, a major site of global pet imports (Krysko et al., 2011) and an area where geographic and climatic conditions create a high risk for invader establishment and spread (Schmitz, 2002). These attributes have allowed for establishment of more invasive reptile species in Florida than any other U.S. state (Krysko et al., 2016).

Miller et al. (2018) found *Raillietiella orientalis*, a pentastome parasite originating from Asia, in native snake species in Florida, probably indicating a parasite spillover from the invasive Burmese Python (*Python bivittatus*) that has become established since at least the 1990s in the southern portion of the state. The life cycle of pentastome parasites is heteroxic, typically involving both intermediate and definitive hosts (Chen et al., 2010). As adults, pentastomes are hematophagous and inhabit the respiratory systems of their definitive hosts, which are typically carnivorous reptiles. The definitive hosts acquire the parasite by consuming pentastome-infected intermediate or paratenic hosts. Ingested pentastomes leave the digestive tract of the definitive host, move to the lungs, and become sexually mature. Adult female pentastomes produce eggs that are released into the environment with the host's feces (Pare, 2008).

Miller et al. (2018) initially reported that the distribution of *R. orientalis* had not extended beyond the established range of *P. bivittatus* in Florida, but more recent studies (Farrell et al., 2019; Miller et al., 2020; Walden et al., 2020) have found that this pentastome has rapidly spread over 340 km north of the python's current range. In Florida, *R. orientalis* infections have been found in 16 snake species from many of the most

widespread North American snake genera including *Agkistrodon*, *Coluber*, *Crotalus*, *Lampropeltis*, *Nerodia*, and *Thamnophis* (Miller et al., 2018; Farrell et al., 2019; Metcalf et al. 2019). Furthermore, *R. orientalis* infections are of higher intensities in native snake species compared with the Burmese Pythons captured in Florida (Farrell et al. 2019; Miller et al., 2020). The parasite also achieves greater mean body mass in native snakes compared with *P. bivittatus* (Farrell et al. 2019, Miller et al., 2020). Recently, Farrell et al. (2019) documented several dead Pygmy Rattlesnakes (*Sistrurus miliarius*) that were infected by *R. orientalis* in central Florida. In Australia, Kelehear et al. (2014) found that *R. orientalis* was invasive, with very high prevalence and intensity of infections in several native snakes. Clearly, *R. orientalis* poses an important conservation concern for both Australian and North American snakes.

The spread of *R. orientalis* indicates that suitable intermediate hosts occur in both Florida and Australia, but almost nothing is known about which species are competent intermediate or paratenic hosts in these areas or in this parasite's native range. The only known case of intermediate host infection was a single larva taken from an African shrew (Miller et al., 2018). Kelehear et al. (2014) and Miller et al. (2020) suggested that terrestrial frogs are a likely intermediate host of *R. orientalis* in Australia and Florida as snake species that consume anurans are most frequently infected, but the intermediate hosts of this pentastome remain unknown on both continents. It seems likely that coprophagous insects, including roaches (Blattodea), consume eggs in snake feces and then serve to infect entomophagous species. The life cycle of several congeners of *R. orientalis* that infect lizards and an amphibisbaenian includes roaches or other insects as intermediate hosts (Lavoipierre and Rajamanickam, 1973; Ali and Riley, 1983; Winch and Riley, 1985; Bosch, 1986).

Determining species that can serve as intermediate hosts of *R. orientalis* would indicate the fauna that might be harmed by infective larvae. For reptile and amphibian species there are no studies on the health consequences of serving as an intermediate

<sup>3</sup>Corresponding author. E-mail: jpalmisano@stetson.edu  
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host for pentastomes. Fish (*Gambusia affinis*), however, exhibited few health effects even with heavy infections by nymphs of the pentastome *Levinsunguis subaequalis* (Woodyard et al., 2019). Controlled infection studies are needed to determine if pentastomes, including *R. orientalis*, have major impacts on both definitive and intermediate host fitness or if the hosts tolerate infection with few health consequences as in some other parasite infections of reptiles (Mayer et al., 2015; Paterson and Blouin-Demers, 2020). Determining the intermediate hosts for *R. orientalis* will also allow diet-based predictions of the snake species likely to be adversely affected by the reproductive parasites, as species that frequently consume the intermediate host should be subject to more frequent and intense pentastome infections. An understanding of intermediate hosts could facilitate predicting the speed and ultimate geographic range of the parasites as *R. orientalis* can only maintain populations where a suitable intermediate host is abundant. The most problematic intermediate host species would be those easily transported and abundant because they would enable the rapid expansion of this pentastome's geographic range.

We conducted controlled infection studies with lizards and anurans to determine if these species are viable hosts for *R. orientalis* larvae. We hypothesized that common lizards (*Anolis sagrei*), anurans (*Anaxyrus terrestris* and *Osteopilus septentrionalis*), and roaches (*Blaberus discoidalis*) could all be infected with *R. orientalis* if exposed to viable eggs and that they would not exhibit adverse health effects (reduced survival and growth rate). We also fed *Anolis sagrei* and *Lithobates sphenoccephalus* roaches that had consumed *R. orientalis* eggs to determine if coprophagous insects could serve as competent intermediate hosts for pentastome infection of lizards and anurans. We expected few health consequences in the intermediate hosts because pentastome larvae are often quite small and therefore unlikely to cause major tissue damage or to exploit host resources (Pare, 2008; Woodyard et al., 2019).

#### MATERIALS AND METHODS

*Anolis sagrei* Exposure to *R. orientalis* Eggs.—We collected 20 Brown Anoles (*A. sagrei*) from DeLand, Florida in May 2019. We measured the snout–vent length (SVL) and mass of the lizards at the start of the experiment. We placed the lizards into individual plastic 6.6-L containers and provisioned each container with a climbing branch, a few layers of paper towels as substrate and shelter spaces, and a shallow water bowl. We positioned one end of the enclosures on top of heat strips. We allowed the animals to acclimate to the lab for 5–7 days preceding the treatments. The lab room air temperature was approximately 21–24°C and lab lighting was approximately a 12 : 12; L : D photoperiod. After acclimation, we randomly assigned the anoles to one of two treatments (pentastome egg exposed or control). We used an unexposed control group to determine if animals in the experiment were previously infected or unintentionally infected through lab contamination or diet. Unfortunately, we could not ensure that animals at the start of the experiment were not infected by larval pentastomes as larvae can only be detected through dissection.

We euthanized crickets (*Acheta domesticus*) and removed fat globules from their abdomens. We fed control lizards unaltered fat bodies and egg-exposed lizards fat bodies dusted with powdered snake feces containing *R. orientalis* eggs. The eggs were collected from Pygmy Rattlesnake fecal samples from adult snakes collected at Lake Woodruff National Wildlife

Refuge. The fecal samples were examined microscopically to ensure the presence of pentastome eggs. To increase the sample size of exposed lizards, we later exposed an additional 11 *Anolis sagrei* to *R. orientalis* eggs from June to September of 2019.

Over the experimental period, we misted the anoles and changed their water daily, and cleaned their enclosures and fed them crickets (*Acheta domesticus*) weekly. Both egg-exposed and control lizards were euthanized at several intervals ranging from 5 to 168 days after treatment to allow us to determine when pentastome larvae became detectable. On the day of euthanasia, we recorded SVL and body mass and dissected the anoles under a stereomicroscope ( $\times 8$  to  $\times 32$ ). The body cavity was opened from the pelvis to the throat and examined for pentastome larvae in and adjacent to the body cavity. The location of the pentastomes was recorded.

*Osteopilus septentrionalis* Exposure to *R. orientalis* Eggs.—We collected 20 Cuban Treefrogs (*O. septentrionalis*) from western Volusia County, Florida in May and June of 2019. We measured the snout–ischium length (SIL) and mass of frogs before experimentation. We transferred the frogs to individual ventilated plastic containers provisioned with paper towels for substrate and a water bowl. We gave the frogs a 7-day acclimation period and then randomly assigned them to a treatment group (10 control frogs and 10 egg-exposed frogs). The lab conditions were the same as in the *Anolis sagrei* egg-exposure experiment.

We force-fed the egg-exposed group cricket fat globules dusted with egg-contaminated Pygmy Rattlesnake feces and the control group received unaltered fat globules. Over the experimental period, the husbandry techniques were similar to the *A. sagrei* exposure experiment. We euthanized frogs from 46 to 95 days after treatment and recorded body measurements preceding dissection. We used the same dissection techniques as in the *A. sagrei* exposure experiment to find pentastome larvae. To increase our sample size, we exposed eight additional *O. septentrionalis* to *R. orientalis* eggs in November 2019 and we euthanized these frogs 60 to 77 days after treatment.

*Anaxyrus terrestris* Exposure to *R. orientalis* Eggs.—We collected 24 Southern Toads (*Anaxyrus terrestris*) from two neighborhoods north of DeLand, Florida between late August and early September 2019. One site, Glenwood Reserve (GR), was a low-density residential neighborhood where we collected 3 male and 10 female toads. The other site, Brandywine (BW), was a higher-density residential neighborhood where we collected 11 toads (10 males, 1 female) from the edge of an ephemeral breeding pond. BW was 2,400 m from GR. After capture, we measured toad SIL and mass. We housed the toads in individual ventilated plastic containers. Each container had coconut husk substrate to allow the toads to burrow and a water bowl. We gave the toads a 7-day acclimation period and then randomly assigned them to treatment groups (8 control toads and 16 egg-exposed toads). The lab conditions, egg exposure techniques, and husbandry were as described for the *O. septentrionalis* exposure experiment. We euthanized toads 32–145 days after treatment. We recorded body measurements after euthanasia and utilized the same dissection techniques as in the first experiments.

*Blaberus discoidalis* Exposure to *R. orientalis* Eggs.—We commercially ordered juvenile discoid cockroaches (*B. discoidalis*) that were 20–30 mm long. The cockroaches were housed in groups of 10 in five plastic containers (38.1 length  $\times$  15.2 width  $\times$  17.8 height cm). Each container had two paper egg cartons, a damp paper towel for substrate, and a petri dish with water. The roaches were fed diced food that consisted of a variety of produce (lettuce, carrots, radishes) and fruit (apples and strawberries). We

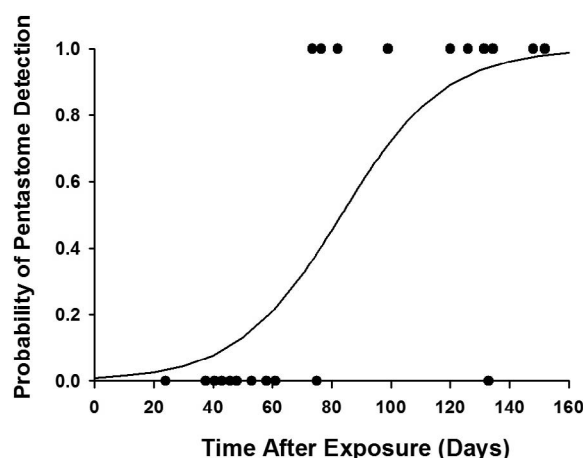


FIG. 1. Results of logistic regression to determine the probability of pentastome larvae detection in *Anolis sagrei* (yes = 1, no = 0) fed pentastome eggs vs. the number of days after exposure when the lizard was examined.

replaced the food and water two to three times a week and misted the containers to maintain a moist environment. We placed the containers in an environmental chamber set at 26°C and gave the roaches a 2-wk acclimation period before implementing treatments.

We made a mix of egg-laden *S. miliaris* feces and diced carrot, pear, potato, and corn. We repeated this same process a week later with the other half of the infected feces and similar food. After being exposed for the second time, we left the containers in the controlled environment and fed the roaches uncontaminated food to allow the pentastome parasites time to develop in the roaches. Two months after infection, we dissected 10 roaches and examined them with a light microscope ( $\times 100$ ).

*Anolis sagrei* Exposure to Roaches Infected with *R. orientalis*.—In March 2020 we captured 24 *A. sagrei* from Deland, Florida. We duplicated the husbandry practices used in the *A. sagrei* egg-exposure experiment. We fed 16 of the anoles fat bodies taken from pentastome-infected roaches from the *B. discoidalis* exposure study. We euthanized the lizards 87–127 days after treatment, measured their mass and SVL, dissected the lizards, and counted and recorded the location of pentastome larvae.

*Lithobates sphenoccephalus* Exposure to Roaches Infected with *R. orientalis*.—In May 2020, we captured 16 Southern Leopard Frogs (*L. sphenoccephalus*) in Volusia County, Florida. Frog husbandry was identical to the *O. septentrionalis* exposure study. We exposed 10 of the leopard frogs to *R. orientalis* larvae through the consumption of infected roaches from the *B. discoidalis* exposure study. Four frogs died 57 to 104 days after treatment and those remaining were euthanized on day 117. We took body measurements and dissected frogs on the day of their death. As in the previous studies, we counted the number of pentastome larvae and recorded their locations.

*Deoxyribonucleic Acid (DNA) Analysis*.—During dissections, we collected one isolated pentastome larva from both a *L. sphenoccephalus* and an *A. sagrei* that had consumed infected roaches. For these larvae, we used polymerase chain reaction and Sanger DNA sequencing of the 18S ribosomal ribonucleic acid gene, as in Farrell et al. (2019), to determine if the larvae were *R. orientalis* or another pentastome species.

*Statistical Analysis*.—For each experiment, we used Fisher's exact test to determine if there was a significant difference between the frequency of infected anoles in the pentastome-

exposed and control groups. For the *A. sagrei* in the egg-exposure experiment we determined if there was a relationship between the number of days after egg exposure and the probability that a lizard had detectable pentastome infection among all experimentally egg-exposed lizards using a logistic regression model in JMP version 10, SAS Institute Inc.

To calculate growth of *A. sagrei* in the experiment involving exposure to larvae from roaches, we calculated both  $\Delta$ SVL (final SVL minus initial SVL divided by number of days) and  $\Delta$ mass (final mass minus initial mass divided by number of days). We used similar methods to calculate the growth rate in terms of mass for *L. sphenoccephalus*. To determine if pentastome infection affected growth rate we used *t*-tests comparing mean growth rate ( $\Delta$ SVL or  $\Delta$ mass) in the control and exposed treatment groups. To determine if parasite intensity (the number of pentastome larvae found in a frog or lizard) affected growth rate, we used linear regression models with growth rate ( $\Delta$ SVL or  $\Delta$ mass) as the dependent variable and pentastome larvae number as the independent variable.

## RESULTS

*Anolis sagrei* Exposure to *R. orientalis* Eggs.—Ten of the 21 *A. sagrei* that were exposed to eggs had detectable pentastome infections. None of the unexposed anoles had detectable pentastomes. Exposure to pentastome eggs significantly increased the likelihood of the lizards being infected (Fisher's exact test,  $P = 0.012$ ). The earliest larvae were seen in euthanized lizards 75 days after treatment (Fig. 1). The nine lizards that were exposed to pentastome eggs and euthanized between days 39 and 61 were not detectably infected; we suspect they had larvae that were not visible. Only one egg-exposed *A. sagrei* that was dissected after day 79 lacked detectable pentastome larvae. Logistic regression indicated that the probability of pentastome infection significantly increased over time in the experimental period ( $\chi^2 = 6.25$ ,  $P = 0.0124$ ). The logistic regression equation indicated that the 50% probability of detectable infection occurred 83.3 days after egg exposure.

Pentastome larvae were found throughout the body cavities of infected anoles, associated with the dorsal and ventral body wall, liver, and heart (Fig. 2A). Larvae were approximately 0.6–0.7 mm in length and had the four short, hooked appendages characteristic of pentastomes (Fig. 2C). The number of larvae in the egg-exposed anoles was variable, ranging from 1 to over 40 larvae per lizard.

*Osteopilus septentrionalis* Exposure to *R. orientalis* Eggs.—One of 18 egg-exposed *O. septentrionalis* was pentastome infected and we found only one larva in it. None of the 10 control *O. septentrionalis* had detectable pentastomes. There was no significant effect of egg exposure on *R. orientalis* infection rate (Fisher's exact test,  $P = 1.0$ ).

*Anaxyrus terrestris* Exposure to *R. orientalis* Eggs.—None of the 24 Southern Toads died before they were euthanized, and 45.8% were infected with pentastomes. We found pentastome larvae associated with the viscera (i.e., the heart, lungs, stomach, and liver), mesenteries, and floating free in the abdominal cavity (Fig. 2B). There was not, however, a significant relationship between treatment (egg exposure or control) and pentastome infection status (Fisher's exact test,  $P = 0.68$ ). There was a significant difference in the prevalence of pentastome infections at the two sites as 15.4% (2 of 13) of toads collected at the GR site and 81.8% (9 of 11) of toads collected at the BW site were pentastome infected (Fisher's exact test,  $P = 0.003$ ).

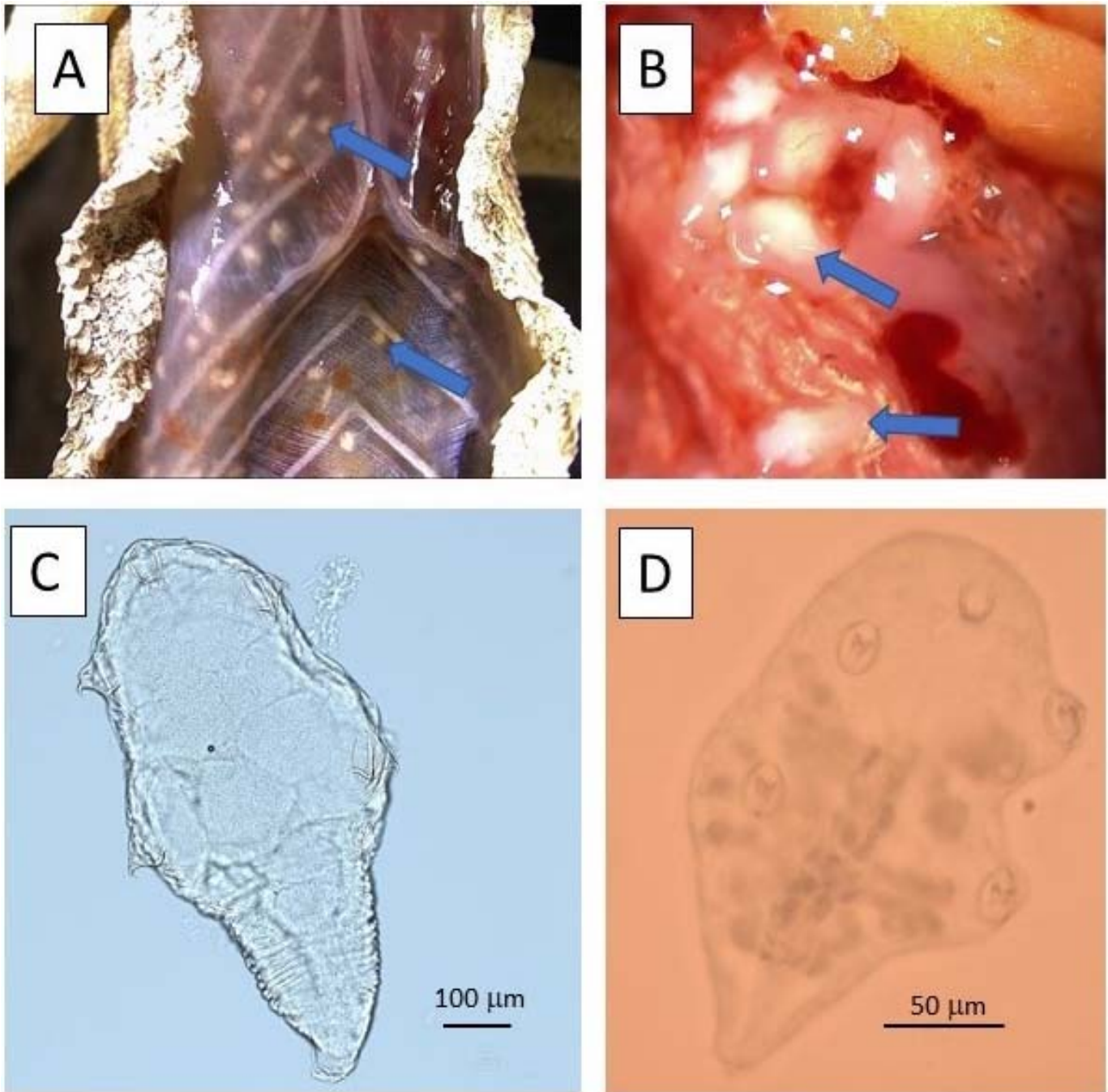


FIG. 2. (A) Ventral surface of an *Anolis sagrei* from the *Raillietiella orientalis* egg-exposure experiment with many pentastome larvae visible through the body wall ( $\times 10$ , blue arrows indicate two of the larvae). (B) A dissected *Anaxyrus terrestris* showing six pentastome larvae on the surface of the lung ( $\times 20$ , two larvae marked with blue arrows). (C) An isolated pentastome larva taken from *A. terrestris* ( $\times 400$ ). (D) An isolated *R. orientalis* larva collected from a roach fat body ( $\times 400$ ).

*Blaberus discoidalis* Exposure to *R. orientalis* Eggs.—We found living pentastome larvae in 9 of the 10 discoid cockroaches that we dissected. The larvae were always found in the fat bodies of the roaches and were 200–300 μm long (Fig. 2D).

*Anolis sagrei* Exposure to Roaches Infected with *R. orientalis*.—Survival of lizards in this study was high, with only 2 of 24 *A. sagrei* dying before their scheduled euthanasia date. A control lizard died 47 days after exposure and an exposed lizard died 95 days after exposure. There was no significant difference in the percentage of control lizards (87.5%) and exposed lizards (93.8%) that survived (Fisher's exact test,  $P = 1.0$ ). Our dissections

revealed that 93.5% (15 of 16) of *A. sagrei* that consumed infected roach tissue were infected by pentastome larvae. DNA sequencing of one of the larvae identified it as *R. orientalis*. None of the control lizards was pentastome infected, resulting in significantly higher *R. orientalis* prevalence in the exposed group (Fisher's exact test,  $P < 0.0001$ ). Infection intensity was high in the exposed lizards, with a mean of 9.86 pentastome larvae per infected lizard ( $n = 15$ ,  $SD = 8.75$  pentastomes), but variable, with four infected lizards having only a single pentastome larva and the three most heavily infected lizards having 18, 21, and 31 pentastome larvae.

TABLE 1. Mean growth rates for length and mass (SE in parentheses) for *Anolis sagrei* and *Lithobates sphenoccephalus* from the infected-roach consumption studies.

Variable	Control	Exposed	<i>t</i>	df	<i>P</i>
<i>A. sagrei</i> $\Delta$ mass (g/mo)	0.24 (0.08)	0.20 (0.04)	0.42	22	0.69
<i>A. sagrei</i> $\Delta$ snout-vent length (mm/mo)	1.40 (0.33)	0.91 (0.17)	1.23	21	0.38
<i>L. sphenoccephalus</i> $\Delta$ mass (g/mo)	2.21 (0.43)	2.98 (1.01)	-0.85	12	0.41

Infection by pentastomes had no discernable impact on *A. sagrei* growth. On average, both control and exposed lizards gained mass and increased in SVL during the experiment, and there were no significant differences between the two treatment groups (Table 1). Regression analysis indicated that pentastome intensity also had no significant effect on lizard  $\Delta$ mass ( $R^2 = 0.031$ ,  $F_{1,20} = 0.63$ ,  $P = 0.44$ ) or lizard  $\Delta$ SVL ( $R^2 = 0.042$ ,  $F_{1,21} = 0.92$ ,  $P = 0.34$ ).

*Lithobates sphenoccephalus* Exposure to Roaches Infected with *R. orientalis*.—Four of the 16 *L. sphenoccephalus* died before their scheduled euthanasia. One pentastome-exposed frog died 70 days and another 86 days after treatment. One control frog died 57 days and another 104 days after treatment. Dissections revealed that 87.5% (7 of 8) of the *L. sphenoccephalus* that were fed infected roaches were pentastome infected, whereas none of the control frogs was infected, resulting in a significant effect of treatment on infection status (Fisher's exact test,  $P = 0.0014$ ). DNA sequencing of one of the larvae identified it as *R. orientalis*. In terms of body mass, the leopard frogs in both treatment groups grew slowly over the course of the experiment and there was no significant effect of pentastome infection treatment on growth rate (Table 1). There was also no significant effect of parasite intensity, measured as the number of larvae, on frog  $\Delta$ mass ( $R^2 = 0.083$ ,  $F_{2,11} = 0.49$ ,  $P = 0.62$ ).

#### DISCUSSION

The complete life cycle of *R. orientalis* is poorly documented and understood in both its native and introduced range. Although lists of the snake species that serve as definitive hosts are available for *R. orientalis* from native populations in Asia (Christoffersen and De Assis, 2013) and introduced populations in Australia (Kelehear et al., 2014) and Florida (Miller et al., 2020), the only well-documented case of infection of a paratenic or intermediate host involves a single larva found in a shrew collected in Kenya (Miller et al., 2018). Our results indicate that insects, lizards (*A. sagrei*), and two anurans (*Anaxyrus terrestris* and *L. sphenoccephalus*) can be infected by *R. orientalis* larvae. The infection of discoid roaches with *R. orientalis* by consuming pentastome egg-contaminated food is consistent with other raillietiellid pentastomes that also use roaches (Lavoipierre and Rajamanickam, 1973; Ali and Riley, 1983; Bosch, 1986) or other insects (Winch and Riley, 1985) as intermediate hosts. Given the diets of the Australian and Florida snake species with the highest prevalence of *R. orientalis*, Kelehear et al. (2014) and Miller et al. (2020) both suggested that terrestrial frogs were likely to be important intermediate hosts. That conclusion was supported by our results showing that both *L. sphenoccephalus* and *A. terrestris* can be infected with *R. orientalis* larvae. Brown Anoles also hosted *R. orientalis* larvae. It would be important to determine which other lizard species can be infected with *R. orientalis* given the great diversity of native and nonindigenous species that occur in Florida. We did not include mammals in our infection studies, but they are also likely to serve as intermediate hosts because snake species that typically consume

only endotherms, including *P. bivittatus* (Miller et al., 2020) and *Crotalus adamanteus* (Metcalf et al., 2019), have been found in Florida with *R. orientalis* infections.

Although roaches and *Anolis sagrei* were highly susceptible to infection from ingesting *R. orientalis* eggs, anurans seemed resistant to egg-initiated infections. In both *Anaxyrus terrestris* and *O. septentrionalis*, the experimental groups fed *R. orientalis* eggs did not have a significantly higher prevalence of *R. orientalis* than the control groups. We observed *R. orientalis* infect *A. terrestris* in approximately equal numbers in both our control and egg-exposed treatment groups. It appears that many of the *A. terrestris* were already infected by *R. orientalis* at the time of collection and that site BW had a much higher prevalence of *R. orientalis* than site GR. Further collection and immediate dissection of Southern Toads from site GR established that most were pentastome infected (T. Farrell, unpubl. data), indicating that the *R. orientalis* larvae in our control group were probably a result of field acquisition of parasites rather than inadvertent infections occurring in the laboratory.

Our experiments indicate that several species were often easily infected with *R. orientalis* and that few, if any, individuals had strong resistance to infection. The *Anolis sagrei* fed *R. orientalis* eggs did not have larvae we could detect using a dissecting microscope for several months after exposure, but 90.9% of the exposed lizards (10 of 11) that we examined 75 days after exposure were detectably infected by *R. orientalis*. This indicates that very few or none of the *A. sagrei* were completely resistant to *R. orientalis* infection, and furthermore, they maintained larval pentastomes for months, up to 152 days after consuming eggs. Similarly, 93.5% of the *A. sagrei* that were fed a single section of a roach fat body and 87.5% of all *L. sphenoccephalus* that ate one *R. orientalis*-exposed roach were infected by the parasite. These results indicate that the vast majority of exposed roaches were infected with competent *R. orientalis* larvae after consuming eggs and that eating either part of one infected roach (for *A. sagrei*) or a single roach (for *L. sphenoccephalus*) resulted in very high rates of infection.

Our results provide insight into the likely life cycle of *R. orientalis* in Florida. Whereas many species of snakes serve as definitive hosts for *R. orientalis* and release large numbers of embryonated eggs into the environment with their feces (T. Farrell, pers. obs.), how these eggs ultimately infect other snakes has been unknown. We suspect that egg-initiated *R. orientalis* infections in natural situations are rare in lizards and anurans since they are not coprophagous and are particularly likely to avoid snake feces. Roaches, however, are frequently coprophagous and are extremely abundant in almost all habitats in Florida. Insects are unlikely to directly infect snakes as none of the snake species with high adult *R. orientalis* prevalence (>40%) in South Florida, including *Agkistrodon piscivorous*, *Coluber constrictor*, *Nerodia clarkii*, *Nerodia fasciata*, *Pantherophis guttatus*, *S. miliarius*, and *Thamnophis sirtalis* (Miller et al. 2020), typically consume insects. Roaches, however, are common prey items for many species of lizards and anurans in Florida (e.g., Glorioso et

al., 2010) and could easily transmit pentastomes to hosts that are consumed by the snake species with high *R. orientalis* prevalence.

Laboratory infection studies provide evidence for which species can support parasitic infections, but they may not give a clear indication of actual parasite life cycles in more complex natural systems. Field collections of roaches, anurans, and lizards in Florida are needed to determine if the species we infected in the lab actually acquire *R. orientalis* infections in the field. Our study does not provide evidence of natural infections in *Anolis sagrei* and *L. sphenoccephalus* given the lack of infected individuals in our unexposed control treatment groups.

To determine if *A. sagrei*, *Anaxyrus terrestris*, and *L. sphenoccephalus* are competent intermediate hosts, rather than dead-end hosts, snake infection experiments need to be completed to determine if consumption of *R. orientalis*-infected lizards and anurans results in definitive host infection.

Many amphibian and reptile species host pentastomes, but the health consequences of these infections are largely unknown. Most of what we know involves case studies and anecdotal evidence (reviewed by Pare, 2008) of health impacts in definitive hosts. There has been no prior experimentation on pentastome infection that included uninfected control groups for either definitive or intermediate hosts. Our research indicates that infection by *R. orientalis* had no significant impact on survivorship or growth rate in *Anolis sagrei* or *L. sphenoccephalus*. Although some of these intermediate hosts had high parasite intensity, with maxima of 45 larvae in a single *A. sagrei* and 33 larvae in *L. sphenoccephalus*, there were still no observed health impacts. These larvae are approximately 1 mm in length and therefore much smaller than the adult female *R. orientalis* that average >50 mm long in native snakes in Florida (Farrell et al., 2019; Miller et al., 2020). Adult *R. orientalis* are also metabolically active, producing large quantities of eggs, and hematophagous, both of which might lead to rapid depletion of host energetic resources (Farrell et al., 2019).

Our results may help explain the extremely rapid expansion of the geographic range of *R. orientalis*, which has been recently found at several sites (Farrell et al., 2019; Miller et al., 2020; Walden et al., 2020) up to 340 km north of the areas where Miller et al. (2018) originally found the species. On the Florida Peninsula, roaches and Brown Anoles are found in close association with humans and are often inadvertently transported through human activity. *Anolis sagrei* used vehicular rafting and other human-facilitated methods of transport to greatly expand its range in the southeastern United States (Campbell, 1996, 2003). The ability of *R. orientalis* to use these synanthropic species of insects and lizards as intermediate hosts increases the likelihood of range expansion assisted by long-distance human-aided transport.

The spread of *R. orientalis* in North America is unlikely to be limited by the available hosts. The snake genera that are known to host *R. orientalis* in Florida include *Agkistrodon*, *Coluber*, *Crotalus*, *Lampropeltis*, *Nerodia*, and *Thamnophis* (Farrell et al., 2019; Metcalf et al. 2019; Miller et al., 2020), taxa that occur almost everywhere snakes are found in North America. Similarly, our finding that *Anaxyrus*, *Lithobates*, and *Anolis* can host *R. orientalis* larvae indicates that suitable intermediate hosts may occur throughout much of the continent. The potential for major rapid expansion of its geographic range and preliminary information on its deleterious health impacts on snakes (Farrell et al., 2019) indicate the importance of *R. orientalis* as a conservation issue. We need geographically widespread cen-

suses of both snakes and likely intermediate hosts to track both the spread and impact of this invasive parasite.

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