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ELUCIDATING THE LIFE HISTORY AND ECOLOGICAL ASPECTS OF *ALLODERO HYLAE* (ANNELIDA: CLITELLATA: NAIDIDAE), A PARASITIC OLIGOCHAETE OF INVASIVE CUBAN TREE FROGS IN FLORIDA

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ABSTRACT: Given their ubiquitous nature, it is surprising that more oligochaete annelid worms (Annelida: Clitellata) have not adopted an endoparasitic lifestyle. Exceptions, however, are the understudied members of the genus *Dero* (*Allodero*) that parasitize the ureters of tree frogs and toads. This study experimentally explores the life cycle and host specificity of *Allodero hylae*, the worm's use of chemical cues in host searching, and its seasonal prevalence and abundance over a year-long collection period on the Florida Southern College campus. A total of 2,005 *A. hylae* was collected from the ureter, urinary bladder, or expressed urine of wild *Osteopilus septentrionalis*; a significant positive correlation was found between host snout–vent length and parasite intensity for female but not male hosts. Monthly prevalence of *A. hylae* reached a peak of 58% in April, but never dropped below 20% in any month; mean abundance peaked March–May, whereas few worms were recovered in December and January. Confirming a parasitic lifestyle, wild-collected hosts with intense infections, typically >40 worms, showed obvious dilatation of the ureter wall, and some young-of-the-year *O. septentrionalis* exposed to *A. hylae* in the laboratory were killed by the apparent rupture of the host's ureter. The worm has a direct life cycle: worms expelled in the host's urine are capable of locating and re-infecting other hosts within aquatic microhabitats such as bromeliad tanks, and worms can survive for weeks in a free-living environment, even undergoing a morphological change. Further, chemotaxis assays found a positive response to a tree frog attractant for worms recently removed from hosts. Overall, this study provides the first multifaceted investigation on the life history and ecology of any *Allodero* spp., which offers new insights into an understudied endoparasitic oligochaete.

Given the ubiquitous nature of oligochaetes and their obvious physical and physiological attributes that are reminiscent of many helminths (Stunkard, 1937, 1940; Goodchild, 1951), it is surprising that more oligochaete worms (Annelida: Clitellata) have not adopted an endoparasitic lifestyle. Indeed, endosymbiotic oligochaetes are rare, being restricted to 2 genera in the Naididae (Gelder, 1980). In the Naididae, *Chaetogaster limnaei*, a parasite of freshwater snails and mussels (Vaghin, 1946; Gruffydd, 1965; Buse, 1971; Conn et al., 1996), has received relatively generous study when compared with members of the genus *Dero* that parasitize tree frogs and toads. Although frogs and toads have a long history as study organisms in parasitology, with a recent resurgence in life-history and ecology studies (Brooks et al., 2006; Bolek and Janovy, 2007; Johnson et al., 2008; Langford and Janovy, 2009, 2013; Pizzatto and Shine, 2011; Rhoden and Bolek, 2011; Langford et al., 2013; Vhora and Bolek, 2013), few experimental studies have been conducted on the unique *Dero* spp. that parasitize anurans (Lutz, 1927; Harman and Lawler, 1975). Indeed, it is unfortunate that so little is known about these worms (Harman and Lawler, 1975), as life-history and ecological data would provide unique insights into the evolution of endoparasitism in annelids (Gelder, 1980), and add to the growing knowledge of life-cycle strategies used by parasites of amphibian hosts (see Bolek et al., 2009; Langford and Janovy, 2009).

Of the numerous genera in the Naididae, only members of the genus *Dero* use vertebrates (frogs) as phoretic agents (subgenera *Dero* and *Aulophorus*) or as endoparasitic or endocommensal hosts (subgenus *Allodero*) (Lopez et al., 1999). *Allodero* contains 6 species that infect the ureters (Wolffian ducts) or eyes of anurans (Gelder, 1980; Pinder et al., 1998); however, the exact nature of these symbiotic relationships is debatable. Michaelson (1926a, 1926b) and Lutz (1927) considered *Allodero lutzi* to have a parasitic relationship with its tree frog host because it fed upon host cells, whereas other authors argued for a commensal relationship because *Allodero* spp. are not known to cause visible inflammation or damage to the host's ureters (Harman, 1971; Pinder et al., 1998).

Currently, the life cycle of *Allodero* spp. is conjectural, on the basis of limited experimental observations (Lutz, 1927) and field collections (Goodchild, 1951; Harman and Lawler, 1975; Pinder et al., 1998). To our knowledge, Lutz's (1927) experiments are the only attempt to culture a species of *Allodero* in a free-living environment, and he suggested that these worms may require a free-living stage in their life cycle. However, Lutz (1927) was unable to determine the mechanism for transition from parasitism to free living, as he did not witness worms naturally leaving the host, nor did he find sexually reproducing or egg-laying worms. Further, it is unknown whether worms infect tadpoles or adult frogs (or both) or in what environment transmission occurs. Goodchild (1951) suggested that *Allodero hylae* relied upon the host's breeding season to prompt sexual reproduction and produce an infective generation that could infect tadpoles. Harman and Lawler (1975) conducted field studies to test Goodchild's hypothesis and found no infected tadpoles or young-of-the-year frogs from a small breeding pond with infected adult tree frogs, thus concluding that *A. hylae* is not transmitted in the breeding pond and may have developed a terrestrial life cycle.

Allodero in the Americas is restricted to tree frogs (Hylidae) and toads (Bufonidae) as hosts. *Allodero hylae* is only known to infect *Hyla cineria*, *Hyla squirella*, and *Hyla versicolor*, but only in subtropical regions of the Southeastern Coastal Plain (Harman and Lawler, 1975). More important, host specificity of *A. hylae* appears to vary geographically, with worms infecting *H. cineria*, but not sympatric *H. squirella* and *H. versicolor* in Mississippi (Harman and Lawler, 1975), whereas worms infected *H. squirella* and *H. versicolor* in Louisiana, but not sympatric *H. cineria*

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(Harman, 1973). Further, Goodchild (1951) found *A. hylae* infecting *H. squirella* but not sympatric *H. cineria*. Such local host specificity has not been explained in *A. hylae*, but previous studies (e.g., Bolek and Janovy, 2007; Langford and Janovy, 2013) have found that parasites of amphibians may exploit different subsets of hosts across their geographic ranges.

Seasonal prevalence and mean abundance data are lacking for most species of *Allodero*. Although a partial exception can be made for the collections of Harman and Lawler (1975), who found *A. hylae* only from March to July in Mississippi, despite collection attempts from July to November. Further, Harman (1973) only collected *A. hylae* in June and July from Louisiana, although it is unclear if he sampled during other months. Given the limited number and duration of previous field collections, a year-long collection of anurans is needed to establish the seasonal infection parameters and field host specificity for *A. hylae*.

The present study provides the first experimental evidence of the life cycle and host specificity of an endoparasitic *Allodero* sp., including evidence that worms can harm anuran hosts. In addition, we provide novel data on the use of chemical cues in host searching, and the first year-long data set of infection parameters in wild tree frogs. Our goals were to (1) establish the seasonal prevalence, abundance, and field host specificity of *A. hylae* in anurans from peninsular Florida, (2) use experimental and natural infections to elucidate the life cycle and potential physiological and ecological limitations on host specificity, (3) maintain free-living worms in the laboratory, and (4) run chemotaxis assays to determine whether worms readily recognize and locate hosts.

MATERIALS AND METHODS

Field collections and observations

To establish seasonal infection parameters of *A. hylae* the tree frog *Osteopilus septentrionalis* was collected from September 2012 to August 2013, whereas *H. cineria*, *H. versicolor*, *Rana* (*Lithobates*) *sphenocephala*, *Rana* (*Lithobates*) *catesbeiana*, *Bufo* (*Rhinella*) *marinus*, and *Bufo* (*Anaxyrus*) *terrestris* were captured from March to August 2013 to determine field host specificity of *A. hylae*. All anurans were captured opportunistically by hand in the vicinity of the Florida Southern College (FSC) campus (28.03°N, 81.94°E) from small ponds, vegetation, and a neglected swimming pool. The campus and surrounding neighborhood have seasonal and permanent water bodies that support aquatic larval amphibians and have a diversity of native, e.g., *Quercus* spp., and introduced tropical plants, e.g., bromeliads, that retain small pools (microhabitats) of water.

Animals were transported to the FSC Biology Department, euthanized by double pithing, measured for snout–vent length (SVL) and total length. All organs and body cavities were examined for parasites within 48 hr of collection. Specifically, frogs were opened along their length to avoid nicking the urinary bladder, then the gastrointestinal tract was removed to expose the ureters, and finally the ureter and urinary bladder were opened to remove worms. Specimens of *A. hylae* were removed and relaxed in 0.1% tricaine methane-sulfonate (MS222), then fixed in 70% ethanol; other worms were placed in aged tap water before being used in experimental infections or to culture free-living *A. hylae* (see below). Representative specimens were dehydrated, cleared in clove oil, mounted in damar balsam, and examined with phase-contrast microscopy. *Allodero hylae* was the only species of *Allodero* collected from frogs during this study, which were identified by comparisons with paratypes (from the U.S. National Parasitology Collection and Smithsonian Institution) and drawings from Goodchild's (1951) original description. Specifically the worms were separated from other *Allodero* spp. by reviewing hair and needle setae (bundles and sizes), including the absence of pronounced intermediate teeth, which all closely aligned with Goodchild's description. Voucher specimens of *A. hylae* from *O. septentrionalis* have been deposited in the H.W. Manter Parasitology Collection, University of Nebraska, Lincoln, Nebraska (HWML 64746).

To assess the ability of *A. hylae* to undergo a morphological change in a free-living environment, worms collected from the ureters of *O. septentrionalis* were placed in groups of 10–30 worms into 5 cm petri dishes with aged tap water. Tree frog skin and feces, or filtered pond water (Dietz and Alvaredo, 1970), was added to the dish to provide a food source. To document the morphological changes in the worms, 4–8 worms were collected at weekly intervals for the first 2 mo in culture. Special attention was given to documenting development of dorsal and ventral setae, fossa and gills, zones of fission, gut development, and sexual organs.

It was observed that tree frogs with intense infections voided worms in their urine, especially when attempting to escape a perceived predator, i.e., a human hand. This was first discovered when G.J.L. hand-captured a female *O. septentrionalis* that expelled at least 12 worms onto his hand as it tried to escape; the frog was quickly returned to the lab where dissection revealed an additional 87 and 7 worms in the ureters and urinary bladders, respectively. To determine whether *A. hylae* might reside in a ground-level *O. septentrionalis* breeding pond, a 500-L plastic-lined pond with a sand and soil bottom, aquatic vegetation, numerous invertebrates, *O. septentrionalis* tadpoles, and *Gambusia* (fish) was thoroughly searched by sifting through the substrate and vegetation with a fine screen and white entomological pans for all aquatic oligochaetes. In a similar effort, the cups of tank bromeliads (*Guzmania lingula*) that grew adjacent to and above the ground-level pond were thoroughly washed (after removal from the substrate) with tap water from a squirt bottle. The water was collected in a bucket and strained through a fine screen to expose any naidid worms. In both environmental sampling efforts, naidid worms were preserved (see above) and identified according to standard methods.

Experimental infections

To test the potential route(s) of transmission and host specificity, 40 (10-day-old) lab-reared *O. septentrionalis* (Hylidae: n = 40) and wild-collected (from the FSC campus) *B. terrestris* (Bufonidae: n =21), *H. cineria* (Hylidae: n = 24), *R. catesbiana* (Ranidae: n = 15), *Rana utricularia* (Ranidae: n = 15), and the exotic *Eleutherodactylus planirostris* (Eleutherodactylidae: n = 24) were exposed to *A. hylae*. Each species was divided into 3 equal groups and assigned to time-0 controls, experimental, or time-*T* controls. All time-0 control tree frogs were euthanized and examined for the presence of *Allodero* spp. before the start of the experimental infections. Each anuran was placed in a 5-cm petri dish with a thin layer of aged tap water; in the experimental group, 4 *A. hylae* were added to each petri dish. The worms were obtained directly from the ureters of wild *O. septentrionalis* and maintained in aged tap water for 24 hr before use in experimental infections. During *O. septentrionalis* infections, tree frogs were intermittently monitored under a dissection microscope for the first 12 hr to note worm behavior; all other species were monitored for less than 6 hr. After 72 hr the experimental and time-*T* control for all groups were dissected and the number and location (including the petri dish) of *A. hylae* were recorded.

To determine if *A. hylae* voided during host urination can subsequently infect a cohoused uninfected tree frog, 25 (30-day-old) lab-reared *O. septentrionalis* were divided into 3 groups and assigned to time-0 controls (n = 5), experimental (n = 15), or time-*T* controls (n = 5). All time-0 control tree frogs were euthanized and examined for the presence of *Allodero* spp. before the start of the experimental infections. Experimental animals were placed individually into 1-L plastic containers with a donor tree frog (10 donors total; 5 frogs were used twice) known to void *A. hylae* in its urine, whereas time-*T* controls were housed individually with an uninfected lab-reared adult tree frog. Donor frogs were captured daily within their enclosure to simulate a predation attempt and ensure that adults forcefully voided their urinary bladder at least once during the experiment. The experiment was conducted in the dark to discourage cannibalism upon the smaller tree frogs. After 72 hr the experimental and time-*T* control groups were dissected and the number and location of *A. hylae* were recorded.

A final experimental exposure was conducted to simulate transmission of *A. hylae* in a natural habitat, e.g., tank bromeliads. Ten tank bromeliads were acquired from FSC's Horticulture Greenhouse, washed and inspected thoroughly for naidid worms (although some worms may have remained, it is doubtful), potted in commercial potting soil, and

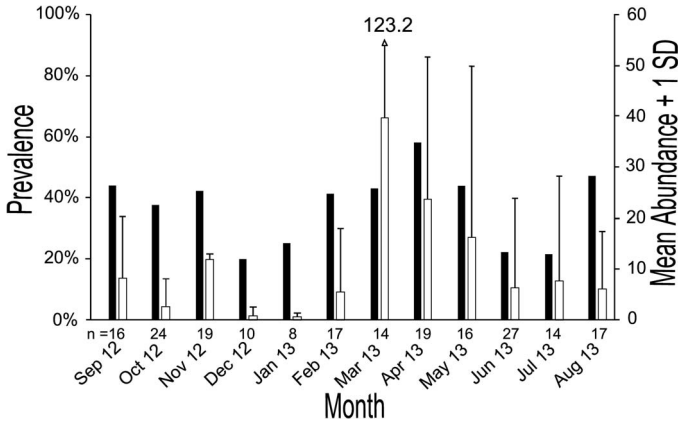

FIGURE 1. Seasonal prevalence and mean abundance of *Allodero hylae* collected from the Cuban tree frog (*Osteopilus septentrionalis*) in the vicinity of the Florida Southern College campus in Lakeland, Florida.

placed individually inside 30- × 24- × 24-inch bug dorms (BioQiup®) within FSC's Frank Lloyd Wright Greenhouse. A donor *O. septentrionalis* (reused from the experiment above) was released into each bug dorm for 1 wk (each was stimulated to urinate on multiple occasions) and replaced with 3 (40-day-old) lab-reared, uninfected Cuban tree frogs. A control (n = 15 frogs in 5 cages) was established as described above, but without the addition of a donor frog. After 1 wk the experimental and control tree frogs were dissected and the number and location of *A. hylae* were recorded. During this experiment, tree frogs were fed 3–4 appropriately sized commercial crickets (Ghann's Cricket Farm, Augusta, Georgia) or flightless fruit flies (Carolina Biological Supply, Burlington, North Carolina) daily and the 'tanks' of the bromeliads were maintained at half capacity with aged tap water. Of note, donor and uninfected tree frogs were not cohoused because preliminary attempts found a high occurrence of cannibalism on the juvenile frogs.

Chemotaxis assays

To test for any attraction of *A. hylae* to frogs, we modified a chemotaxis assay developed for *Caenorhabditis elegans* by Ward (1973). In brief, 8-cm petri dishes were spread with 10 ml of melted 1.5% agarose in tap water; once agarose gel was cooled, 3 ml of tap water were placed on top (Ward [1973] did not add water), which resulted in thick slurry that permitted worm movement without sacrificing gradient formation by the attractant. The placement of attractants and controls followed Bargmann et al. (1993), who placed the attractant and control on opposite ends of the petri dish on the surface of the agar substrate; worms were then placed in the center of the dish and thus provided a choice between the control and attractant. Worms in the vicinity of the control and attractant were counted at 15 and 30 min. The attractant used in this study was acquired by placing a host animal in tap water in a small container for 24 hr. The resulting anuran-scented water was used as the attractant, and aged tap water was used as a control. Chemotaxis assays were assessed using the chemotaxis index (from +1.0 to –1.0) developed by Bargmann et al. (1993): Chemotaxis index = (# moved to attractant − # moved to control)/#total. Two ages of worms were tested in these assays: <7 days and >14 days since they were removed from a host.

Statistical analyses

Ecological measures of infections are reported in accordance with Bush et al. (1997), with means reported ± 1 SD. A *z*-test for equality of proportions was used to compare the prevalence (P) of infection between male and female Cuban tree frogs, and a *t*-test or Mann–Whitney test was used to measure differences in mean abundance (MA) and mean intensity (MI) between host sexes; a *t*-test with a Grubbs' test for outliers was used for differences in mean chemotaxis index (MCI) (Sokal and Rohlf, 1981). Spearman's rank correlation was used to calculate possible relationships between host SVL (normalized with the natural log) and MI; male and female tree frogs were analyzed separately.

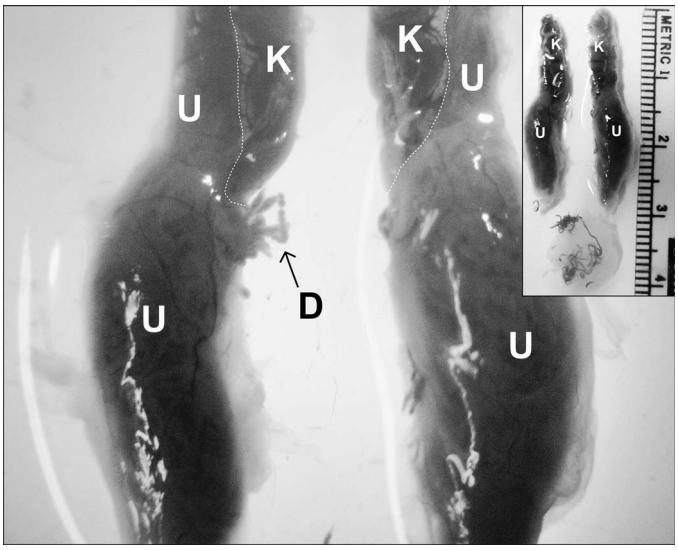

FIGURE 2. Expanded ureters from an adult female Cuban tree frog (*Osteopilus septentrionalis*) infected with 256 *Allodero hylae* that anteriorly displaced the frog's kidneys ~13 mm. Kidney (K), ureter filled with *A. hylae* (U), *A. hylae* emerging from rupture in ureter (D). The white dotted lines delimit the juncture of the kidney and ureter. Insert: Full view of the same urinary tract, including the urinary bladder (below); the ureters are expanded to a width of 7 mm, whereas uninfected ureters have a width less than 1 mm in similarly sized tree frogs.

RESULTS

Field collections and observations

A total of 2,005 *A. hylae* was collected from the ureter, urinary bladder, or expressed urine of wild *O. septentrionalis* (88 males, 109 females) on or near the FSC campus (P = 37.8%, MA = 10.3 ± 26.5, MI = 27.3 ± 35.2 [range = 1–256]). P (female = 40.4%, male = 34.1%; $z = 0.94$, $P = 0.36$), MI (female = 27.1 ± 41.9, male = 27.8 ± 34.4; $U = 631.5$, $P = 0.75$), and MA (female = 10.8 ± 29.7, male = 9.3 ± 23.8; $t = 0.4$, $P = 0.35$) were not significantly different between host sexes. A significant positive correlation was found between host SVL and parasite intensity for females ($r_s = 0.26$, df = 42, $P = 0.04$), but not males ($r_s = 0.24$, df = 28, $P = 0.09$). In addition, 2 worms were collected in July from a single male *H. cineria* (n = 15), whereas *H. versicolor* (n = 10), *R. sphenocephala* (n = 23), *R. catesbeiana* (n = 18), *B. marinus* (n = 9), and *B. terrestris* (n = 24) were not infected with *A. hylae*. In *O. septentrionalis*, the monthly P of *A. hylae* reached a peak of 58% in April, but never dropped below 20% in any month; MA peaked March–May, and few worms were recovered in December and January (Fig. 1). More important, some wild-collected hosts with intense infections, typically >40 worms, showed obvious dilation of the ureter (Fig. 2).

Free-living experiments were successful in maintaining worms removed directly from the ureters in aged tap or filtered pond water for up to 5 mo (worms died from apparent senescence starting at 4 mo). Worms collected at 1 wk had started to develop hair setae and dorsal setae on some, but not all, segments, but the fossa was unaltered, whereas worms collected at 2 wk and beyond were distinctly different. Specifically, worms developed hair setae (generally 1 per bundle, but sometimes 2), dorsal setae on all segments from IV, and a fossa with well-developed gills; these 3 characters are absent (or nearly so) in parasitic individuals. Zones

of fission in parasitic worms were most commonly noted in the spring and summer months, with 23% of worms collected in April displaying fission; fission was not noted December–February. Worms transferred to a free-living environment completed ongoing fission, which was typically complete by the end of the first week, but did not initiate further division outside of a host. Free-living worms formed a well-developed gut no later than the second week that contained free-living food organisms, e.g., bacteria and diatoms, which was distinctly different from the underdeveloped gut of parasitic individuals that contained mucus and host cell fragments. No sexual development was noted. Worms survived longer when host feces were used to inoculate aged tap water with bacteria and other free-living food sources.

Efforts to find free-living *Allodero* spp. in an *O. septentrionalis* breeding pond were not successful, whereas 11 worms that conformed to free-living *A. hylae* reared in the laboratory were collected while washing the bromeliads, along with a variety of other naidid worms, including *Dero digitata*.

Experimental infections

Experimental infections were unable to establish infections in *B. terrestris*, *R. catesbeiana*, and *E. planirostris*; however, *H. cineria* (P = 37%, MI = 1.6 ± 0.6 [1–2]) and *O. septentrionalis* were infected (P = 93%, MI = 2.4 ± 1.1 [0–4]) with *A. hylae*. Control anurans were uninfected. Five of the *O. septentrionalis* died during the experimental infection, whereas no controls died during the experiment. Necropsy revealed that 1 or both ureters had been ruptured in the dead tree frogs, which appeared to be the cause of host death. In all 5 dead tree frogs (MI = 3.2 ± 0.8 [1–4]), at least 1 ($\bar{x}$ = 1.4 ± 0.5 [1–2]) *A. hylae* was discovered wandering through the frog's body cavity. During careful observations at the beginning of the infections, it was noted that worms quickly located hosts (within minutes); subsequently worms moved onto the frog's rear legs and torso before entering the host through the cloaca. Given that the ventral area of these juvenile tree frogs is transparent (to some extent), we were able to note the quick (often within 30 min after cloaca penetration) movement of these worms out of the urinary bladder and into the ureter. No worms were seen entering the mouth. During exposures, it was noted that *B. terrestris* recognized the worms as a food source and promptly consumed all worms in their dish. Apparently the soft-bodied worms were digested, as worms did not infect toads and setae were found in feces during dissections. No other anurans were seen consuming the worms, and all worms were recovered in the petri dishes of *R. catesbieana* and *E. planirostris* at the experiment's conclusion. Indeed, worms displayed little interest, i.e., random movement was apparent, when exposed to all nonhylid hosts.

Experimental exposures that exposed uninfected *O. septentrionalis* to cohoused donor tree frogs in small containers were successful (P = 73%, MI = 1.6 ± 0.5 [1–4]), but controls were uninfected. During the final experimental exposure, which occurred in an enclosure with a tank bromeliad, *O. septentrionalis* acquired infections (n = 30, P = 60%, MI = 4 ± 2.3 [1–7]) from previously housed donor hosts. No control frogs were infected. None of the juvenile tree frogs in these final two exposures died during exposure to *A. hylae*, which probably reflects their greater size, i.e., 30+ days old, when compared with the 10-day-old tree frogs used in the initial exposures (see above).

Chemotaxis assays

In this study, chemotaxis assays found a positive response to the tree frog attractant for worms <7 days removed from a host at 15 and 30 min (n = 20; MCI15 = 0.24 ± 0.3 [–0.33–1], MCI30 = 0.30 ± 0.3 [–0.33–1]), but not for worms >14 days (n = 20; MCI15 = –0.02 ± 0.1 [–0.3–0.2], MCI30 = 0.00 ± 0.1 [–0.2–0.2]). Within assays, MCI on the basis of time (15 vs. 30 min) was not significantly different (<7 days: $t = -0.51$, $P = 0.30$; >14 days: $t = -0.43$, $P = 0.33$), whereas a significant difference was found on the basis of days free living at 15 (MCI: $t = 3.29$, $P = 0.001$) and 30 min (MCI: $t = 3.35$, $P = 0.001$); which supported observations that chemotactic response is reduced in long-term free-living worms that undergo conspicuous morphological changes (see above). Although worms kept long term displayed a neutral chemotaxis score, it was noted that ca. 20% of worms showed a positive response to the attractant (ceasing movement at the attractant), whereas the remaining worms appeared to wander aimlessly throughout the assay.

DISCUSSION

Our study is the first multifaceted investigation on the life history and ecology of any *Allodero* spp. that provides new insights into an understudied endoparasitic oligochaete. Early experiments by Lutz (1927) failed to elucidate the life cycle of *A. lutzi*, although he discovered that the life cycle likely contains a free-living stage. Subsequent studies supported a free-living stage and suggested that the worms may only reproduce asexually (Gelder, 1980), thus requiring worms to leave one host to infect another, likely in the host's breeding pond (Goodchild, 1951). Our experimental infections in both a laboratory and a natural setting found that *A. hylae* entered tree frogs directly through the cloaca and traveled to the ureter. Further, during our observations of wild and laboratory animals, we found that intensely infected tree frogs expelled *A. hylae* in their urine; worms that spill over into the urinary bladder (apparently crowded out of the ureters) from the ureter are susceptible to loss during urination. Thus, this spillover effect provides a natural explanation for worms leaving a host.

In addition, our findings support the suggestion of Harman and Lawler (1975) that transmission does not occur in the host's breeding pond, but in a terrestrial environment. We failed to discover *A. hylae* in an extensive search of a common *O. septentrionalis* breeding pond, whereas a search in nearby arboreal bromeliad tanks (an aquatic microhabitat) found several free-living worms that appeared to be similar to *A. hylae* that we previously reared in a free-living environment in the laboratory. In support of transmission occurring in aquatic microhabitats, our experimental infections designed to replicate a natural environment found that uninfected tree frogs can acquire infections from a previously housed donor host in a bromeliad environment. Whereas this site of transmission may seem surprising, examples exist of nonparasitic *Dero* spp. living their entire lives in bromeliad tanks and using tree frogs as phoretic hosts to transfer between tanks (Lopez et al., 1999; Sabagh and Rocha, 2014). This observation supports the assertion that phoretic relationships may provide a preadaptation to an endoparasitic lifestyle (Anderson, 2000; Roberts and Janovy, 2008), which suggests that these worms would be a useful group

to study the transition from a free-living lifestyle to an endoparasitic one.

Before our study, the type of symbiotic relationship seen in *Allodero* species was debatable, with some authors suggesting a parasitic relationship (Michaelson, 1926a; Lutz, 1927; Gelder, 1980), whereas others consider the relationship to be commensal (Harman, 1971; Pinder et al., 1998). Our results clearly define the *A. hylae*–tree frog relationship as parasitic (defined by Roberts and Janovy, 2008); we found that worms can cause physical changes to the host's ureter in intense infections, e.g., ureter dilatation. Although it is unclear to what extent this physical response alters the function of the frog's urogenital system, as these frogs seemed healthy and reproductively active, e.g., females held developing eggs and large fat bodies. Clearly, further studies are warranted on the immune response that *A. hylae* elicits on adult tree frogs. Further, in our experimental infections, we found that worms can kill young *O. septentrionalis* by rupturing the ureter and wandering through the host's body cavity. Our results provide an obvious departure from the relatively benign relationship found by previous studies (see above), yet our study is the first to conduct experimental infections that permit such observations. Interestingly, all studies to date (ours included) have collected few young, wild hosts that are infected with *A. hylae*. Given that young tree frogs share habitats with adults (Donnelly and Guyer, 1994), it seems reasonable to expect young tree frogs to encounter and become infected with *A. hylae*. Thus, we propose that young tree frogs are readily infected in nature, as seen in our experimental infections, but that a higher number of young hosts may die from a ruptured ureter when compared with older frogs that possess larger, thicker ureters. It is important to point out that our study only found harm in the exotic and invasive Cuban tree frog (*O. septentrionalis*); thus, we have no evidence to suggest that *A. hylae* harms native anurans, but we did not test young individuals of native species.

Our novel experimental infections to test host specificity showed that *A. hylae* displays strict host specificity within the tree frogs (Hylidae); the worms infected both species of hylids, *O. septentrionalis* and *H. cineria*, but were unable to infect members of the Ranidae, Bufonidae, or Eleutherodactylidae. The results of our host specificity experiments are supported by our field collections, which found infections primarily in *O. septentrionalis*, with a single infection occurring in *H. cineria*, whereas *H. versicolor* and all nonhylids were uninfected. Thus, it appears that worms on the FSC campus rely on *O. septentrionalis* to play host, but occasionally infect *H. cineria*. It is unclear whether the difference in infection parameters between *O. septentrionalis* and *H. cineria* has a physiological or ecological basis. The relatively low P and MI of worms found in *H. cineria* during our experimental infections, when most ecological determinants of infection are eliminated (see Langford and Janovy, 2013), suggest a physiological explanation for the worm's preference of *O. septentrionalis*.

Our study corroborates the historical trend of variable local host specificity in field-collected tree frogs (see Goodchild, 1951; Harman, 1973; Harman and Lawler, 1975). Indeed, we found *A. hylae* to only infect a subset of the hosts it is known to infect throughout its range. It is unclear why *A. hylae* appears to prefer a different primary host species throughout its range, although we suggest that local adaptation on the most abundant host may provide an explanation. *Osteopilus septentrionalis* is indeed the most abundant tree frog on the FSC campus; unfortunately, no data are available on host relative abundance from previous studies. Admittedly, this explanation is problematic because sexual reproduction is required for local adaptation to occur (sensu Lively and Dybdahl, 2000), yet sexual reproduction is unknown in the subgenus *Allodero*. Alternatively, *A. hylae* may represent a species complex, which is not uncommon in oligochaetes (e.g., Pérez-Losada et al., 2009). To date no molecular studies have been conducted on the *Allodero*, yet such studies would be valuable in clarifying host specificity and species identity issues within these worms. Previous studies have found a combined molecular and experimental approach useful in elucidating issues of host specificity and untangling potential species complexes in parasites from amphibians (see Bolek et al., 2009; Pizzatto and Shine, 2011; Langford and Janovy, 2013).

During field collections we found that *A. hylae* infects *O. septentrionalis* in all months, always infecting at least 20% of collected hosts; however, we found no consistent seasonal peaks in P. Yet, we did find seasonal trends for the worm's MA, with a peak in spring (March–May) and a low in winter (December–January). Our study is the first to find *A. hylae* infecting tree frogs year-round. Harman (1973) and Harman and Lawler (1975) were able to collect *A. hylae* only in March–July from Louisiana and Mississippi, respectively, although it is unclear to what extent these authors attempted to collect worms throughout the year. We suggest that *A. hylae* overwinters in its tree frog host and limits asexual reproduction, which is supported by a lack of asexual reproduction in worms collected during the winter in our study. Such a reproductive strategy would explain the lower MA of worms collected in winter from our study; however, it is still unknown why previous studies have failed to collect *A. hylae* in fall and winter (August–February). Although it seems unlikely, worms from the temperate northern Gulf Coast may have adopted an alternative life-cycle strategy in which worms overwinter in a free-living environment and reinfect tree frogs before the host returns to its breeding pond.

Our study is the first to successfully maintain free-living *A. hylae* in the laboratory and document that the worms undergo a conspicuous morphological change, which includes the addition of hair setae, dorsal setae, and a fossa with gills. Lutz (1927) found remarkably similar morphological changes for free-living *A. lutzi* in Brazil. Apparently, these morphological changes provide the worms an advantage in a free-living environment, which is not necessary in the parasitic stage. Indeed, when free-living *A. hylae* infect a host, the worms lose their hair setae, most of their dorsal setae, and the gills in the fossa are minimized within 72 hr of entering a host (G. Langford, unpubl. data). In fact, the worms we removed at 72 hr conformed well to descriptions of a small subsection of parasitic *A. hylae* that have been described by previous studies on the *Allodero* (Goodchild, 1951; Harman, 1973; Harman and Lawler, 1975; Pinder et al., 1998), which suggests that the worms in these studies had recently infected their host. We also found that free-living *A. hylae* formed a well-developed gut and fed upon free-living food items, which provides additional support for a free-living stage being a natural component of the worm's life cycle. Finally, we noted that free-living (>2 wk) *A. hylae* are easily confused with nonparasitic species of *Dero*, which suggests that *Allodero* spp. may be mistakenly identified when collected outside of their anuran hosts.

In the wild, free-living worms reside in aquatic microhabitats, e.g., bromeliad tanks, where they encounter tree frog hosts (see above). Although host vibrations probably provide an initial cue that a large animal has entered the microhabitat, the worms must identify and locate a suitable host. Our chemotaxis results suggest that *A. hylae* are adept at identifying and locating tree frogs, but not other anurans. In other words, they actively seek to re-enter a hylid host, which confirms our expectation that, similar to most parasitic organisms, *A. hylae* possesses a strong host-seeking mechanism. Lopez et al. (1999) also noted a strong positive response by *Dero* sp. (subgenus not identified) when *Hyla truncata* was placed into an aquarium with the worms. Unexpectedly, our results also suggest that a majority of worms that morphologically acclimate to a free-living environment are unlikely to reinfect a host, at least within the time periods we tested. Given their clear acclimation to a free-living environment and reduced host-seeking behavior, we suggest that these acclimated worms may develop sexual organs under specific environmental conditions (see Parish, 1981); however, we have seen no evidence of sexual reproduction in these worms.

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