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Occurrence of Rat Lungworm (*Angiostrongylus cantonensis*) in Invasive Coqui Frogs (*Eleutherodactylus coqui*) and Other Hosts in Hawaii, USA

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ABSTRACT: The rat lungworm (*Angiostrongylus cantonensis*) has emerged as an important human and animal health concern in Hawaii, US. Although the life cycle of the parasite requires both rat and gastropod hosts, other animals acting as paratenic hosts, such as frogs and centipedes, have been identified as sources of infection. We investigated the occurrence of rat lungworm infections in potential paratenic hosts in Hawaii to provide information on how they might be involved in transmission of angiostrongyliasis. We confirmed the presence of rat lungworm in 87% (21/24) of introduced Puerto Rican coqui frogs (*Eleutherodactylus coqui*) in Hilo, Hawaii, by real-time PCR. Additionally, four Cuban greenhouse frogs (*Eleutherodactylus planirostris*), two cane toads (*Rhinella marina*), and three centipedes (*Scolopendra subspinipes*) were found to be infected. In the frogs and toads, multiple tissue types were positive, including stomach and intestine, muscle, liver, heart, and brain, indicating larval migration. We identified rat lungworm infections in frogs, toads, and centipedes in Hawaii and highlighted the lack of knowledge of the role paratenic hosts may be playing in the transmission and life cycle maintenance of rat lungworm in Hawaii.

Key words: Cane toad, centipede, frog, Hawai'i, nematode, parasite, paratenic.

The rat lungworm (*Angiostrongylus cantonensis*) is a tropical and subtropical parasitic nematode that causes angiostrongyliasis (rat lungworm disease) in humans and other animals. Infections typically occur as a result of intentional or unintentional ingestion of animals or animal parts that contain infective third-stage parasite larvae. The life cycle of the rat lungworm requires both rat definitive hosts and gastropod intermediate hosts. Additionally, paratenic (transport) hosts can play an important role in transmission, acting as disease reservoirs, but in which no development of the parasite occurs. For example, frogs and centipedes have been identified as a source of human infection by rat lungworm (Cuneo et al. 2006; Wang et al. 2018). One study of an introduced frog species in New Caledonia reported 53% (23/43) of frogs sampled from market gardens to be infected with rat lungworm (Ash 1968). Natural infections of rat lungworm larvae have been found in multiple tissue types of frogs, including muscle, liver, digestive tract, and the heart-lung complex (Ash 1968; Asato et al. 1978). Other paratenic hosts of rat lungworm have also been identified, including some crustaceans, flatworms (planarians), and lizards (Wang et al. 2008).

In Hawaii, a high level of human cases of angiostrongyliasis have recently been reported, as well as high infection levels in both rat and gastropod hosts (Kim et al. 2014; Jarvi et al. 2017, 2018); however, little research has been conducted on paratenic hosts in Hawaii, except a brief mention of flatworms by Qvarnstrom et al. (2013). To our knowledge, there have been no studies of rat lungworm in amphibians in Hawaii. Although Hawaii has no native amphibians, multiple species have been introduced, including the invasive Puerto Rican coqui frog (*Eleutherodactylus coqui*), Cuban greenhouse frog (*Eleutherodactylus planirostris*), and cane toad (*Rhinella marina*). In particular, coqui frogs have one of the highest densities of terrestrial amphibians worldwide, with densities in Hawaii reaching 91,000 frogs/ha (Beard et al. 2009). Deter-

mining the role that paratenic hosts may be playing in the transmission and life cycle maintenance of rat lungworm is an important component in the overall understanding of the epidemiology of rat lungworm disease. Here, we report observations of rat lungworm infections in coqui frogs in Hawaii, as well as preliminary findings in greenhouse frogs, cane toads, and the Chinese red-headed centipede (*Scolopendra subspinipes*).

We collected 24 coqui frogs on 21 June 2018 from a site in Hilo, Hawaii. The site is at an elevation of approximately 25 m and is in an area known to have high levels of rat lungworm infections in wild rats (Jarvi et al. 2017). Additionally, four greenhouse frogs, two cane toads, and three centipedes were collected opportunistically between February and June 2018 within 20 km of the coqui frog collection site. Tissue samples taken from frogs and toads comprised approximately 25–100 mg of each of five distinct regions: digestive tract, muscle, liver, heart, and brain. Typically, the brains and hearts were collected in their entirety, whereas digestive tract samples consisted of tissue from both stomach and lower intestine. Liver samples comprised tissue from each of the three lobes, and muscle samples included tissue from both thigh muscles. The centipede samples consisted of scrapings of internal organs from within the exoskeleton. Snout-vent length, or the length from tip of the snout to the posterior end of the backbone, was taken for each coqui frog to the nearest millimeter. For each individual sampled, separate instruments were used for the dissection of each organ after soaking in 10% bleach (Clorox 8.3% v/v sodium hypochlorite diluted 10:1) for a minimum of 10 min to remove any potential DNA contamination (Prince and Andrus 1992). Tissue samples were stored in 500 µL of DNA lysis buffer (0.1 M Tris HCl, 0.1 M ethylenediaminetetraacetic acid, 2% sodium dodecyl sulfate) with 0.2 g of 0.5-mm zirconia-silica beads (BioSpec Products, Bartlesville, Oklahoma, USA) and six 3.0-mm zirconia beads (OPS Diagnostics, Bridgewater, New Jersey, USA) at –80 C. Samples were homogenized (one to four cycles, 8 m/s, 40 s) in a FastPrep-24 5G bead-beater (MP Biomedicals, Santa Ana, California, USA), cooled on ice for 5 min, and centrifuged at 6,200 × G for 3 min. DNA was extracted from 50 µL of tissue homogenate using the Qiagen DNeasy Blood and Tissue Kit (Valencia, California, USA) according to the manufacturer's animal tissue protocol. Tissue samples smaller than 25 mg were digested directly in 180 µL of DNA lysis buffer without homogenization. Real-time PCR followed Jarvi et al. (2012), with thermal cycling conditions of one cycle of 50 C for 2 min, 95 C for 10 min, followed by 40 cycles of 95 C for 15 s, 60 C for 1 min. Initial data showed amplification of some samples beginning after 35 cycles, so the number of cycles was increased to 50 cycles to allow for these samples to reach the plateau phase of amplification. Samples were considered positive for rat lungworm if two or three replicates within a run showed exponential amplification in both the ΔRn vs. cycle and Rn vs. cycle plot types that crossed a set threshold of 0.25 fluorescence units. One small brain sample was evaluated at a 0.015 threshold because of a lack of sample available to rerun at 50 cycles. Most replicates of a sample had a cycle threshold (C_T) SD<0.5 within one run; however, because consistent C_T replication is challenging with very low target DNA concentrations, a C_T SD of 0.5–1.0 within one run was accepted for sample replicates with cycle thresholds >35. Despite repeated attempts, replicates of two brain samples were unable to produce a C_T SD<1.0 within one run; however, multiple replicates were consistently positive in repeated runs (Table 1). Samples with low reproducibility were also deemed positive if one replicate per run showed amplification and was reproduced in multiple runs (Table 1). Samples with exponential amplification in only one replicate that was not reproduced in multiple runs were categorized as undetermined. Negative samples were determined by lack of exponential amplification in all replicates. All animal procedures followed the approved Institutional Animal Care and Use Committee protocol (QA-2835, US Department of Agriculture, National Wildlife Research Center).

TABLE 1. Presence (+) or absence (–) of rat lungworm (*Angiostrongylus cantonensis*) in different tissue types from invasive coqui frogs (*Eleutherodactylus coqui*) in Hilo, Hawaii, USA, by real-time PCR.

			Tissue type analyzed[a]				
Frog no.	Snout-vent length (mm)	Status[b]	Brain	Heart	Liver	Muscle	Stomach-intestine
1	14	P	ND	–	+[c]	–	–
2	15	A	ND	ND	ND	UD	–
3	22	A	–	UD	–	–	–
4	26	P	ND	+	+	–	+
5	27	P	+	ND	+	+	+
6	28	P	–	–	–	–	+
7	28	P	–	+[c]	–	–	+
8	29	P	+	UD	–	–	+
9	30	P	–	+[c]	–	+	+
10	30	P	+	+	+[c]	–	+
11	31	A	–	–	–	–	–
12	31	P	+	+	+	+	+
13	32	P	+[c]	–	+	+	+
14	32	P	UD	–	+	+	–
15	33	P	+	+[c]	–	+	–
16	34	P	–	+	–	+	+
17	36	P	+	+	+	ND	+
18	37	P	ND	–	+	–	–
19	37	P	+	–	–	+	–
20	37	P	+[d]	+	+	+	+
21	38	P	+[d]	+	+	+	+
22	39	P	+	+	+	–	+
23[e]	39	P	–	+	+	+	+
24	42	P	–	–	+	+	+
% Positive[f]		87 (21/24)	58 (11/19)	60 (12/20)	61 (14/23)	55 (12/22)	67 (16/24)

[a] ND = no data; UD = undetermined (samples with exponential amplification in only one replicate that was not reproduced in multiple runs).

[b] Presence (P) and absence (A) of *A. cantonensis* from one or more tissue types.

[c] Samples with low reproducibility, having one replicate per run reproduced in multiple runs.

[d] Samples with consistently positive replicates in repeated runs but a cycle threshold SD>1.0 across replicates within one run.

[e] Frog with semislug (*Parmarion martensi*) found in stomach that also tested positive for *A. cantonensis*.

[f] Total number of samples positive/total number of samples analyzed in each category.

A total of 87% (21/24) of sampled coqui frogs were positive for rat lungworm in at least one tissue type per individual. Overall, parasite presence was detected in each of the five tissue types sampled (stomach-intestine, muscle, liver, heart, and brain), with 14 frogs positive for at least three tissue types (Table 1). Snout-vent lengths of coqui frogs ranged from 14 to 42 mm, with positive individuals found at both extremes. Of note, a whole semislug (*Parmarion martensi*) was found in the stomach of one of the frogs sampled, with both the frog and slug testing positive (Table 1). The invasive *P. martensi* can carry heavy parasite burdens and has been identified as a highly competent intermediate host of rat lungworm in Hawaii (Hollingsworth et al. 2007). Additionally, the four greenhouse frogs, two cane toads, and three centipedes were all positive for rat lungworm, with the latter previously identified as a paratenic host in China (Wang et al. 2018).

Although experimental infections may be necessary to confirm these species' role as

paratenic hosts definitively, our findings suggest that they have the potential to be players in rat lungworm epidemiology in Hawaii. Although molecular analysis only confirms the presence of the parasite DNA and not life cycle stage or viability, positive detections from muscle, liver, heart, and brain (as opposed to stomach-intestine) indicate larval migration within the host's body, with some of these tissue types previously identified as the source of human infection from other frog species (Cuneo et al. 2006). Although the species discussed here are not known to be intentionally consumed by humans in Hawaii, the ingestion of infected hosts could still pose a threat to other animals, because rat lungworm can infect both domestic and wild animals such as dogs (*Canis lupus familiaris*), horses (*Equus caballus*), and birds (Spratt 2015).

If these species are indeed capable of acting as reservoirs for infective larvae, then there may also be spillover risk to rat definitive hosts, ultimately aiding in the completion of the parasite life cycle in the wild. Rats have been documented scavenging coqui frog and cane toad carcasses on Hawaii Island (Abernethy et al. 2016), where rat species have high rat lungworm infection levels (Jarvi et al. 2017). These rat species are also known to consume centipedes, cane toads, and *Eleutherodactylus* spp. elsewhere (Marples 1955; Fitzgerald 1990; Stewart and Woolbright 1996). Concern also exists regarding the potential spread of infected paratenic hosts to other locations, especially in areas where competent definitive and intermediate hosts are already present. Multiple reports exist of frogs, including coqui frogs, being spread from Hawaii to other locations such as Guam and the continental US (Beard et al. 2009; Olson et al. 2012). Although our report of rat lungworm infections in frogs and centipedes implicates them as possible disease reservoirs, further investigations are warranted to better understand the role paratenic hosts may be playing in angiostrongyliasis transmission in Hawaii.

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