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Survey of Ranavirus and *Batrachochytrium dendrobatidis* in Introduced Frogs in Hawaii, USA

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ABSTRACT: Ranaviruses and the fungus *Batrachochytrium dendrobatidis* are globally important agents of emerging infectious amphibian diseases. Amphibians on Oahu, the Hawaiian Island with the greatest potential for disease introduction through the movement of goods and people, have never been surveyed for ranaviruses or *B. dendrobatidis*. We surveyed all five species of frogs on Oahu, Hawaii, US for these pathogens. Of 325 individuals sampled from six sites, none were positive for ranavirus. However, we found *B. dendrobatidis* in a total of four individuals of three species, the cane toad (*Bufo marinus*), the American bullfrog (*Rana catesbeiana*), and the greenhouse frog (*Eleutherodactylus planirostris*), but not in the green and black poison dart frog (*Dendrobates auratus*) or the Japanese wrinkled frog (*Rana rugosa*). The apparent lack of ranavirus and low prevalence of *B. dendrobatidis* are noteworthy given how widespread these pathogens are in terms of both global distribution and host range. Surveillance should continue to document any changes in *B. dendrobatidis* prevalence or the arrival of ranaviruses in Hawaii.

Key words: Amphibian disease, *Bufo marinus*, *Dendrobates auratus*, *Eleutherodactylus planirostris*, *Rana catesbeiana*, *Rana rugosa*.

The fungus *Batrachochytrium dendrobatidis* and ranaviruses are emerging infectious diseases causing population declines and die-offs of ectothermic vertebrates globally (Daszak et al. 2003; Fisher et al. 2009; Gray et al. 2009). Both pathogens are found on all continents except Antarctica and infect hundreds of species (Olson et al. 2013; Duffus et al. 2015). The increasing incidence of die-offs and extirpations attributed to *B. dendrobatidis* and ranaviruses in recent decades could be due to factors such as introduction of novel strains into the existing pathogen ranges, climate change, or additive and synergistic effects of multiple stressors on hosts (Berger et al. 2005; Lesbarrères et al. 2012; Schloegel et al. 2012; Price et al. 2017). Although the distribution and impact of *B. dendrobatidis* has been well documented, the study of ranaviruses has been less extensive (Fisher et al. 2009; Olson et al. 2013; Gray and Chinchar 2015). Finding either *B. dendrobatidis* or ranavirus in the early stages of colonizing an island, as might be suggested by occurrence in only a single location or species, could allow study of establishment patterns and factors affecting the success and rate of spread in a new system. Also, the smaller land mass of an island could allow for exhaustive sampling or manipulation of host and pathogen populations that is not feasible in larger areas (Bosch et al. 2015).

To provide a baseline of pathogen occurrence for future comparison, we surveyed frogs on Oahu (Hawaii, US) for presence of *B. dendrobatidis* and ranavirus. Although no amphibians are native to Hawaii, five introduced amphibian species are well established on Oahu: Cane toad (*Bufo marinus*), American bullfrog (*Rana catesbeiana*), Japanese wrinkled frog (*Rana rugosa*), greenhouse frog (*Eleutherodactylus planirostris*), and green and black poison dart frog (*Dendrobates auratus*). Whereas *B. dendrobatidis* has been documented in the coqui frog (*Eleutherodactylus coqui*) on the islands of Maui and Hawaii (prevalences of 1.1% and 3.3%, respectively; Beard and O'Neill 2005), it has not been surveyed elsewhere in the Hawaiian Islands, and ranavirus has not yet been investigated in Hawaii. Although coqui frogs are not established on Oahu, we sampled the greenhouse frog, a congener that is widespread on Oahu.

Frogs were sampled from six sites on the island of Oahu: James Campbell National Wildlife Refuge (21°41′36″N, 157°57′13″W), Kawainui Marsh (21°23′29″N, 157°45′31″W), Lyon Arboretum (21°19′57″N, 157°48′7″W),

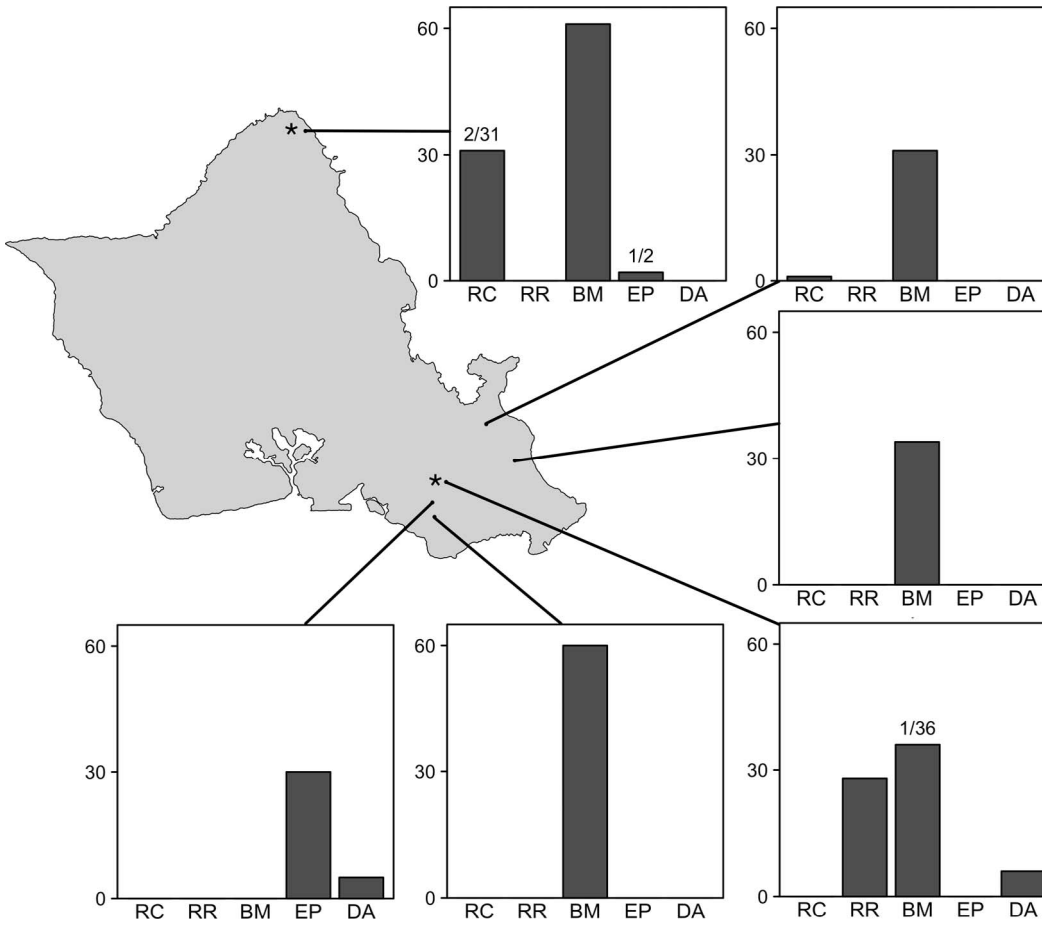

FIGURE 1. Map of localities wherein five frog species were sampled for ranavirus and *Batrachochytrium dendrobatidis* on Oahu, Hawaii, USA during 9 July 2015 to 27 July 2015 and 30 March 2016 to 14 April 2016. Insets show number of individuals sampled per species per site. RC=*Rana catesbeiana* (American bullfrog); RR=*Rana rugosa* (Japanese wrinkled frog); BM=*Bufo marinus* (cane toad); EP=*Eleutherodactylus planirostris* (greenhouse frog); DA=*Dendrobates auratus* (black poison dart frog). Asterisks mark the two sites where *B. dendrobatidis* was found, and numbers above bars indicate the number of frogs infected with *B. dendrobatidis* out of the total frogs sampled. Bars without numbers indicate 0 infected individuals. Ranavirus was not detected at any site in any species. Sites from north to south are James Campbell National Wildlife Refuge, Kawainui Marsh, Olomana Golf Course, Lyon Arboretum, Residential Manoa, and the University of Hawaii at Manoa campus.

Olomana Golf Course (21°21′17″N, 157°43′43″W), University of Hawaii, Manoa (21°17′57″N, 157°48′49″W), and residential Manoa (21°18′39″N, 157°49′5″W). Field sites included a range of habitat types from urban or suburban to those that were more natural and which were chosen based upon accessibility and known presence of the host species. We collected up to 30 individuals per life stage from one or more populations of the five species on Oahu during 9 July 2015 to 27 July 2015 and 30 March 2016 to 14 April 2016 (six sites total; Fig. 1). Nitrile gloves were worn and changed between individuals, which were collected and housed in individual bags or containers. All equipment was scrubbed and disinfected between field sites, and instruments were scrubbed and sterilized between individuals using a 1% chlorhexidine diacetate (Nolvasan, Zoetis Inc., Kalamazoo, Michigan, USA).

All sample collection occurred within 24 h of capture. We sexed individuals, measured

body size with calipers (snout-urostyle length), and measured mass with an electronic balance (to 0.1 g for adults and 0.01 g for tadpoles and metamorphs). To determine *B. dendrobatidis* infection status, we collected skin swabs from adult frogs from all species except for greenhouse frogs due to their small size. We swabbed the ventral surface, arms, legs and digits of each adult frog with a sterile cotton swab according to the standard for *B. dendrobatidis* sampling (Boyle et al. 2004). We preserved the whole bodies of smaller specimens (adult greenhouse frogs, all tadpoles, and recent metamorphs of all species) in 95% ethanol. All frogs were humanely euthanized by submersion in 1% solution of buffered tricaine methanesulfonate (Tricaine-S, Western Chemical Inc., Ferndale, Washington, USA), followed by pithing. We collected samples of liver, intestine, and kidney for ranavirus testing. All tissue and swab samples were stored at –80 C.

We extracted DNA from swabs using PrepMan Ultra (Applied Biosystems, Foster City, California, USA) following established protocols (Retallick et al. 2006). For tissue samples, DNA was extracted using Qiagen (Valencia, California, USA) DNeasy Blood and Tissue Kits. We used quantitative PCR to test for the presence of *B. dendrobatidis* in DNA extracted from swab samples or from whole body tissues following Hardy et al. (2015). For ranavirus testing, we used 50 ng genomic DNA per individual as determined using an Epoch spectrophotometer (Biotek, Winooski, Vermont, USA), and used quantitative PCR targeting a 70 base pair region of the *MCP* gene following Gray et al. (2012). For both *B. dendrobatidis* and ranavirus tests, all samples were run twice on a StepOne Real-Time PCR machine (Applied Biosystems) using water as a negative control. For *B. dendrobatidis*, we used DNA extracted from cultured fungal zoospores as a positive control. The amount of *B. dendrobatidis* on swabs was expressed as zoospore equivalents (ZE), based on standards generated from each plate using dilutions of the control corresponding to 1,000, 100, 10, and 1 zoospores. For ranavirus, we used two positive controls: gBlocks® gene fragments (Integrated DNA Technologies, Inc., Coralville, Iowa, USA) and DNA extracted from a cultured FV3 ranavirus strain originally isolated from a captive amphibian.

Our survey suggested that Oahu is currently free of ranavirus, despite the ubiquity of ranaviruses globally and across amphibian taxa. None of the 325 tissue samples tested for ranavirus DNA yielded cycle threshold values below 30, whereas both types of positive controls consistently yielded cycle threshold values below 25. Therefore, we were confident that no ranaviral DNA was present in our samples. Prevalence of ranavirus in all sites was 0%; however, 95% confidence intervals indicate the possibility of ranavirus occurrence in low levels that might have been undetected by our sampling (Table 1). Ranaviruses have been documented in three of our study species elsewhere: American bullfrog (Galli et al. 2006; Une et al. 2009; Schloegel et al. 2009), cane toad (Zupanovic et al. 1998), and green and black poison dart frog (Miller et al. 2008; Kik et al. 2012). Lack of ranavirus in our study could reflect its absence in Oahu, its extremely low prevalence, or its occurrence only in unsampled sites.

We detected *B. dendrobatidis* DNA at low prevalence in three out of five species at two out of six sites: one adult greenhouse frog (19 ZE) and two adult American bullfrogs (5 and 6 ZE) at James Campbell National Wildlife Refuge, and one cane toad metamorph at Lyon Arboretum (3 ZE; Table 1 and Fig. 1). Estimated prevalence of *B. dendrobatidis* in these two sites across all species was 2.1% and 1.4%, respectively, and 0% in the other sites (Table 1). Reported prevalence of *B. dendrobatidis* was similarly low in coqui frogs on Hawaii Island and Maui (Beard and O'Neill 2005). Because there are no previous surveys of *B. dendrobatidis* on Oahu, we are unable to hypothesize the history of residence or whether it was introduced to the island via host or free-living zoospores. All five of our study species are known to be infected by *B. dendrobatidis* elsewhere (Berger et al. 1999; Miller et al. 2008; Rizkalla 2010; Fong et al.

TABLE 1. Ranavirus and *Batrachochytrium dendrobatidis* fungus prevalences in five frog species (*Bufo marinus*, cane toad; *Eleutherodactylus planirostris*, greenhouse frog; *Dendrobates auratus*, black poison dart frog; *Rana catesbeiana*, American bullfrog; and *Rana rugosa*, Japanese wrinkled frog) sampled on six study sites on Oahu, Hawaii, USA during 9 July to 27 July 2015 and 30 March to 14 April 2016. Total is the number of individuals of all species sampled from each site.[a]

Study site	Total	Ranavirus DNA		Fungal DNA	
		Positives	Prevalence (95% CI)	Positives	Prevalence (95% CI)
James Campbell NWR	94	0	0.0 (0.0–3.9)	3	2.1 (0.4–8.2)
Kawainui Marsh	32	0	0.0 (0.0–10.7)	0	0.0 (0.0–10.7)
Lyon Arboretum	70	0	0.0 (0.0–5.2)	1	1.4 (0.3–7.7)
Olomana Golf Course	34	0	0.0 (0.0–10.2)	0	0.0 (0.0–10.2)
UH Manoa campus	60	0	0.0 (0.0–6.0	0	0.0 (0.0–6.0)
Residential Manoa	35	0	0.0 (0.0–9.9)	0	0.0 (0.0–9.9)

[a] NWR = National Wildlife Refuge; UH = University of Hawaii; CI = confidence interval.

2015). The rarity of *B. dendrobatidis* in our sites could have been due to the recency of amphibian colonization, species-specific variation in infection rates, or influence of lowland climate and habitat. Our results differed from those of Kolbe et al. (2015), who detected ranaviruses, but not *B. dendrobatidis*, in amphibians on the island of Madagascar.

Hawaiian amphibians could experience population spread or growth due to limited impacts of *B. dendrobatidis* and ranaviruses, and these nonnative species could prey on or compete with native species (Kraus 2009). Additionally, ranaviruses have low host specificity and could impact native fishes (Gray and Chinchar 2015). In an increasingly globalized world, preemptive disease surveillance is important for early detection of pathogens, both for rapid response to outbreaks and to identify opportunities to study incipient emerging infectious diseases. Despite lacking native amphibians, Hawaii could play an important role in the global spread of disease because it is one of the most invaded states in the US and is a hub for the movement of goods and people between Asia, the Pacific, and North America (Kraus 2003, 2009).

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