INVESTIGATIONS OF *LEPTOSPIRA* IN SMALL MAMMALIAN HOST SPECIES IN THE HAWAIIAN ISLANDS

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The mediocre teacher tells. The good teacher explains. The superior teacher demonstrates. The great teacher inspires. *--William Arthur Ward*

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Call it a clan, call it a network, call it a tribe, call it family. Whatever you call it, whoever you are, you need one. *-- Jane Howard*

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If you look deeply into the palm of your hand, you will see your parents and all generations of your ancestors. *--Thich Nhat Hanh*

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You don't know strength until strength is the only choice you have. *-- Cayla Mills*

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ABSTRACT

Considered the most widespread and prevalent of zoonoses, the emergent infectious leptospirosis disease is found throughout tropical regions in particular, with annual mean incidence rates in Hawaii consistently the highest in the United States. As a tropical archipelago with relatively low host and leptospiral diversity, Hawaii represents an exceptional opportunity for investigations in the ecology and evolution of this bacterial pathogen. In an effort to gain a better understanding of disease transmission dynamics and environmental drivers in Hawaii, the studies presented in this dissertation each take a distinct approach to examining the associations between three main components underlying the ecology of leptospirosis across the archipelago; namely, the *Leptospira* pathogen, animal hosts, and climate. First, I employed a longitudinal dataset of animal infection prevalence from a period of 14 consecutive years across five maintenance host species and three main islands to describe the epizootiological distribution of pathogenic leptospires in Hawaii. In a second study, I combined field biology and molecular lab techniques to characterize the 16S rRNA genetic diversity of *Leptospira* amongst a community of small mammals in a local rainforest. Finally, Hosmer-Lemeshow goodness-of-fit and Wald assessments of multivariate logistic regression models were used to investigate the association between rainfall and leptospiral animal infection prevalence at multiple spatio-temporal scales. The key findings in this dissertation address evolutionary patterns of host specificity, provide a preliminary examination of leptospiral genetic diversity in host vectors, and show that precipitation is an environmental driver of host infection

prevalence at specific spatial and temporal scales. These results shed light on leptospiral transmission dynamics in a tropical region enzootic for the bacterial pathogen, and lay the foundation for an integrated eco-evolutionary model of leptospirosis in Hawaii.

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INTRODUCTION

Zoonotic Emerging and Re-emerging Infectious Diseases (EIDs)

An emerging infectious disease is defined as a microbial threat that is novel (Morse and Schluederberg 1990), whose incidence is rapidly increasing (Morse and Schluederberg 1990), or has a greatly expanding geographic range (Morse 1995). The factors responsible for the emergence of infectious diseases affect the complex dynamics between pathogen and host in primarily four ways: genetic and biological; physical environmental; ecological; and social, political, and economic (Institute of Medicine 2003). Furthermore, the sources for emerging and reemerging pathogens can be either intra-species, environmental, or inter-species (Woolhouse 2002).

Emerging infectious diseases that originate from animal sources are known to represent a significant global human health burden. Originally restricted to pathogens that directly threaten human health, the definition of disease emergence was expanded a decade later in a follow-up to the first Institute of Medicine (IOM) (1992) report, *Emerging Infections: Microbial threats to health in the United States*, to acknowledge the impact of zoonoses in the second report, *Microbial threats to health: emergence, detection, and response*, (IOM 2003). The majority of emerging human pathogens are bacterial zoonoses from wildlife. Of the 1,415 microbial species tallied by Taylor et al (2001) to be known human pathogens, 61% are transmissible between humans and animals. Comparisons between emerging and non-emerging infectious diseases show that 12% are considered emergent diseases in the human population, with 75% of that

number a zoonosis. In a related approach, Jones et al's (2008) analysis of the total number of emerging infectious disease events occurring between 1940 and 2004 found 60.3% were due to zoonotic agents, 71.8% of which originated from wildlife. In addition, of the 335 total number of emerging infectious disease events from 1940 to 2004, 54% were found to be bacterial or rickettsial pathogens (Jones et al 2008).

Leptospirosis arguably is a good model for studying infectious disease emergence. It is considered an emerging infectious disease, is zoonotic, and its reservoirs include wildlife as well as peridomestic and domestic species. Moreoever, it is the most globally distributed of all zoonoses and the most prevalent (WHO 1999). It is also representative of most emerging zoonotic diseases because it shares many of the same emergence factors described previously. The etiologic agent is a bacterial pathogen that is carried by a diverse group of wild and peridomestic mammalian hosts and can be transmitted to humans either directly from reservoir animal species or indirectly from contaminated soil or water. The *Leptospira* disease ecosystem represents an opportunity for implementation of a comprehensive trans-disciplinary approach that can be used as a blueprint for other complex disease transmission research (Vinetz et al 2005).

The Evolutionary Ecology of EIDs

The study of emerging infectious diseases has benefited from a holistic perspective that views the association between etiologic agents and their hosts as an adaptive co-evolutionary relationship, and applies principles from a number of disciplines including ecology, evolutionary biology, medicine, molecular biology, and public health. This disease ecology approach uses as an organizing principle the theories of systems

biology (Holling 2001) and views disease as a complex dynamic system of relationships, mechanisms, and processes operating at multiple scales from the molecular to the ecosystem level (Horwitz and Wilcox 2005). The host-parasite system is considered malleable and subject to an intricate web of intrinsic biotic factors and extrinsic environmental effects (Horwitz and Wilcox 2005, Wilcox and Colwell 2005). Newly emerging and resurging infectious diseases are seen as the result of perturbations to the system that reverberate across temporal and spatial scales, and can originate from either direction of the hierarchy: at the highest organizing level (e.g., natural or anthropogenic environmental alterations) or at the lowest (e.g., single nucleotide polymorphisms) (Wilcox and Gubler 2005, Wilcox and Colwell 2005).

The application of an evolutionary ecology approach to studies of emerging infectious diseases has allowed for insights into the evolutionary and ecological processes underlying disease transmission. A review of emerging disease studies suggests that the primary factors responsible for the emergence of disease include evolutionary changes in pathogen virulence and host specificity, and ecological changes in the biotic and abiotic environments of both host and pathogen (Schrag and Wiener 1995).

Evolutionary Factors of Zoonotic EIDs

Pathogen Virulence

Virulence refers to the deleterious effect of a pathogen on infected hosts (i.e., disease severity) (Thomas and Elkinton 2004) and, from an evolutionary perspective, is an adaptive trait of parasite life history. The adaptive significance of virulence is in maximizing pathogen fitness through increased growth and reproduction. Anderson and

May's revolutionary theories on the evolution of virulence (1982) argued that, contrary to the prevailing views at the time, low or no virulence does not represent a pathogen's inevitable evolutionary endpoint. Rather virulence is viewed as an adaptive trade-off with transmission in order to optimize pathogen growth and reproduction rates. Because the evolution of pathogen virulence is concurrent with the evolution of strain resistance in the host, the degree of virulence exhibited by a parasite may differ amongst pathogen strains and between host species. Pathogen virulence may contribute to the severity or aseverity of a disease emergence event if an optimal level has not yet been reached because of novel host-pathogen association (Woolhouse et al 2001), such as spillovers (Daszak et al 2000) or other host shift events.

Host Specificity

Host specificity is another adaptive trait of pathogen life history that has direct relevance to disease emergence events. The evolution of generalism, in pathogens that have overcome the host species barrier and have developed the ability to infect multiple host types, leads to opportunities for disease outbreaks in the host species with which the parasite has the most recent evolutionary association (Woolhouse et al 2001). However, an understanding of host specificity patterns can be helpful for mitigating disease outbreaks. Because outbreaks of veterinary and public health concern usually involve host species with relatively recent evolutionary associations with an emerging pathogen and are therefore not usually capable of maintaining an infectious pathogen, even preliminary knowledge of reservoir host dynamics can be exploited for an infection

control strategy to 'ring-fence' or block all routes of disease transmission from pathogen sources (Haydon et al 2002).

Ecological Factors of Zoonotic EIDs

Host Biodiversity

Relative to evolutionary processes, ecological risk factors appear to be more relevant in the emergence and re-emergence of infectious diseases (Schrag and Wiener 1995, Woolhouse 2002). There is a large body of literature regarding the mechanisms by which host communities influence the transmission dynamics of a wide variety of diseases (Mills et al 2006, Carver et al 2009), and a number of studies regarding in particular the roles of host biodiversity on pathogen regulation in a disease ecosystem (Keesing et al 2010). The "Dilution Effect" refers to the protective effect of high host species diversity in disease systems where host reservoir competency is differential between species. The loss of host species that are incompetent disease reservoirs increases disease risk due to higher transmission rates of vector-borne (e.g., *Borrelia* etiologic agent of Lyme disease; LoGiudice et al 2003) and directly transmitted pathogens (e.g., rodent-borne hemorrhagic fevers; Mills 2006). A second mechanism by which host biodiversity can regulate pathogen prevalence is via classic ecological principles of interspecies competition due to limited food resources. As outlined by Mills (2006), an increasing number of animal species sharing the same limited food resources would lower the carrying capacity of the region for each species, leading to lower host population densities, and subsequently resulting in lower infection prevalence. Derne et

al's (2011) study of island ecosystems showed host biodiversity, particularly of land mammals, was inversely related to the annual incidence of human leptospirosis.

Environmental Drivers

Environmental drivers of leptospirosis emergence represent the abiotic extrinsic ecological factors that affect transmission dynamics and are a critical component of the leptospirosis disease ecology. In the Wilcox-Gubler-Colwell model (WGC) (Horwitz and Wilcox 2004; Wilcox and Colwell 2005; Wilcox and Gubler 2005), human societal factors are closely linked to, and have the same impact as, natural ecological systems. This framework for understanding the human-natural systems takes the distal view of disease emergence as ultimately caused by anthropogenic pressures such as human population and technology that are the initial impetus for regional landscape level change (Wilcox and Gubler 2005). The large-scale evolutionary ecological processes responsible for shaping host and pathogen inter-and intra-species dynamics are nested within a system that is at an even greater scale and includes human societal processes (Wilcox and Gubler 2005). A second main lesson to be learned from the WGC model is that the emergence of disease outbreaks is symptomatic of a destabilized adaptive host-parasite association that had co-evolved under a different series of ecological conditions (Horwitz and Wilcox 2004). The view of outbreak events as symptomatic of an unhealthy ecosystem that can no longer maintain human health is a fundamental concept underlying the disease ecology literature (Derne et al 2011; Millennium Ecosystem Assessment 2012; World Health Organization 2012).

History of Leptospirosis Research in Hawaii

Leptospirosis has long been known as a disease of humans and animals. The earliest references to symptoms consistent with leptospirosis have been found from ancient China and Japan (Faine 1994). These early observations described a febrile illness characterized by jaundice and fever, and recognized the occurrence of this illness coincident with wet seasons and specific environmental conditions such as exposure to rice paddies (Faine 1994). Although there had been descriptions of leptospirosis written in the early 1800s by other European researchers, Weil's published study in 1886 is the most widely known and was written during a time when recognition of the disease was more common in late 19th century Europe (Faine 1994). Isolation of the etiologic agent from ill human subjects was achieved in the early 1900s concurrently by independent researchers in Europe and Japan (Alston and Broom 1958, Faine 1994). It is during this time of discovery that rats, then dogs, and later other wild and peri-domestic species were recognized as animal carriers of the disease.

Hawaii likely has a long history of enzootic leptospirosis. The primary wild animal reservoirs of leptospirosis in Hawaii are rats, mice, and mongooses (Middleton et al 2001). The first rodent on the Hawaiian islands, the Polynesian rat (*Rattus exulans*), arrived with early Pacific Island explorers. Calibrated radiometric evidence suggests colonization of the Hawaiian archipelago to have occurred approximately 800 years prior to the present (Rieth et al 2011). Three other primary modern day host reservoirs of leptospirosis, the black rat (*R. rattus*), domestic mouse (*Mus musculus*), and the Norway rat (*R. norvegicus*), were likely introduced when tradeships from both U.S. and Europe began visiting the islands during the 1780s while traveling to and from China. The fourth

major carrier of leptospirosis, the Indian mongoose (*Herpestus auropunctatus*) was shipped in 1883 from an established population in Jamaica as a rodent control measure (Tomich 1986, Thulin et al 2006). Because a leptospirosis-like illness was not recorded to be amongst the early populations of island settlers, it is likely that the pathogen was introduced with European and North American rodent invaders if not already present on the islands via the Polynesian rats.

The earliest known history of leptospirosis in residents of Hawaii begins not long after the 1840s boom in sugarcane plantations resulting in the consequent increase in numbers of sugarcane workers and rodent pests along with other factors affecting emergence such as irrigation water and injuries (e.g., cuts and abrasions) resulting from handling sugarcane (Vinetz et al 2005). The first reports of illness, known at the time as Weil's Disease, came from sugar cane workers on the island of Hawaii in 1907 (Anderson and Minette 2001). J.E. Alicata was the first to provide laboratory evidence of leptospirosis by isolating spirochetes from a human case in Hawaii in 1936 (Alicata and Breaks 1943). Alicata also conducted the first widespread survey of leptospiral infection in humans and animals in Hawaii (Alicata and Breaks 1943). Epidemiological changes have been observed during the course of leptospirosis research in Hawaii. Originally known as an occupational disease primarily seen in sugar cane and macadamia nut workers and taro, shrimp, or freshwater fish farmers, infection risks have become associated with fresh water leisure activities since the decline of agriculture and the rise of tourism as the major revenue sources for Hawaii (Katz et al 2002).

In the over half a century since Alicata's initial survey, a number of investigations of leptospirosis in wild and domestic animals across the islands of Oahu (Alicata and

Breaks 1943, Higa and Fujinaka 1976, Anderson et al 1982), Hawaii (Minette 1964, Shimizu 1984, Tomich 1980), and Maui (Zahn 1968, Anderson et al 1982) have been conducted. These studies offer valuable information regarding leptospiral transmission factors in Hawaii such as host density, host community composition, pathogen diversity, and disease prevalence. However with the exception of Shimizu's (1984) and Higa and Fujinaka's (1976) island-wide studies on the island of Hawaii and Oahu, respectively, trapping efforts by the majority of studies typically were confined to one site or at most two districts. Although these study limitations are certainly understandable given limited resources and manpower, it is difficult to make inter- or intra-island comparisons without concurrent investigations at multiple spatial scales.

A molecular basis for understanding ecological patterns of host specificity allows for elucidation of the broader question of zoonotic transmission. Past endeavors to survey pathogenic leptospires in animal and environmental sources of Hawaii have been limited to primarily darkfield examination of cultures and the microscopic agglutination test (Alicata and Breaks 1943, Minette 1964, Higa and Fujinaka 1976, Tomich 1979, Anderson et al 1982, Shimizu 1984, Bahunga 1992), although southern blot analysis of the 5s rRNA gene and whole genome restriction endonuclease analysis have been also evaluated (Bahunga 1992). Up to this point, there has not been an attempt at a genetic characterization of leptospiral diversity in Hawaii that is phylogenetically informative.

Global leptospirosis has long been associated with a number of environmental factors such as latitude, substrate, and rainfall. Greater leptospiral diversity has been attributed to the high species richness found in rural tropics as compared to temperate or urban environments with fewer potential reservoir hosts (Bharti et al 2003, Derne et al

2011). Pathogenic *Leptospira* have been isolated from contaminated soil (Slack et al. 2009) and surface water (Ganoza et al 2006), and are thought to grow and multiply at approximately neutral pH in clay-like soils that retain moisture in dry periods (Faine 1994). Water and mud are known transmission sources in high-risk occupations such as taro farming (Katz et al 2002), freshwater recreational events (Katz et al 2002), and poor living conditions (Ganoza et al 2006). Retrospective longitudinal studies have directly linked flooding and increased rainfall with human and veterinary leptospirosis in a number of regions including Brazil (Kupek et al 2000), French West Indies (Herrmann-Storck et al 2005), India (Pappachan et al 2004), United States and Canada (Ward 2002). However few studies have explored the association between rainfall and *Leptospira* in maintenance host populations, and none has extensively tested regional patterns of infection prevalence and climate until now.

Dissertation Aims and Research Significance

This dissertation aims to contribute to a better understanding of the ecology and transmission dynamics of leptospirosis in Hawaii as a model for emerging infectious disease in a tropical region enzootic for this bacterial pathogen. The three studies presented in this dissertation each take a distinct approach to examining the associations between three main components underlying the ecology of leptospirosis across the Hawaiian archipelago; namely, the *Leptospira* pathogen, small mammal maintenance host species, and climate.

Chapter Two is an epizootiological description of leptospiral distribution across small mammal species, islands, and years. This study employs a large-scale Hawaii State

Department of Health infection prevalence dataset comprising 15,171 animals collected over a period of 14 consecutive years, with 8 years of concurrent trapping across three major islands. Evidence of regional host specificity and biogeographic patterns at the serovar level is presented.

The second study combines field biology and molecular lab techniques to investigate the genetic diversity of *Leptospira* amongst a community of small mammals in a local rainforest. Chapter Three reports on the molecular-phylogenetic characterization of leptospiral isolates found in the kidneys of two rat species.

Chapter Four takes an environmental approach to understanding pathogenic *Leptospira* in Hawaii. The third study investigates whether and how a climatic factor such as precipitation affects leptospiral infection prevalence amongst wild animal maintenance hosts. Multivariate logistic regression models of multiple temporal and spatial scales were constructed and evaluated. Specific spatio-temporal scales relevant to the association between total monthly rainfall and infection prevalence were identified for each host genus.

In Chapter Five, I summarize the merits of this dissertation and incorporate the broader implications of my research with a discussion of recommendations for future leptospiral research in Hawaii.

Dissertation Organization

Each of the three main study chapters is written in the format of a specific scientific journal, as they are either published (Chapter Two), or soon to be submitted (Chapters Three and Four).

Chapter Two was published in an August 2012 edition of the American Journal of Tropical Medicine and Hygiene 87(2):337-341. M Wong conceived and designed the study. A.R. Katz and D. Li conducted the data analyses. M. Wong, A.R. Katz, and D. Li made substantive contributions to the analysis and interpretation of the data. M. Wong was responsible for drafting of the manuscript. A.R. Katz, D. Li, and B.A. Wilcox made substantive contributions to revise the manuscript critically for important intellectual content.

Chapter Three is formatted for submission to the journal Tropical Medicine and International Health. M. Wong conceived and designed the study. M. Wong and S.N. Bennett conducted the data analysis. M. Wong, A.R. Katz and S.N. Bennett made substantive contributions to the analysis and interpretation of the data. M. Wong was responsible for drafting of the manuscript. A.R. Katz, S.N. Bennett, and B.A. Wilcox made substantive contributions to revise the manuscript critically for important intellectual content.

Chapter Four is formatted for submission to the online journal PLoS Neglected Tropical Diseases. M. Wong conceived and designed the study. K.R. Kodama contributed meteorological data and GIS spatial data maps. D. Li was primarily responsible for the data analysis. M. Wong, A.R. Katz, K.R. Kodama, and B.A. Wilcox made substantive contributions to the analysis and interpretation of the data. M. Wong was responsible for drafting of the manuscript. A.R. Katz, D. Li, K.R. Kodama, and B.A. Wilcox made substantive contributions to revise the manuscript critically for important intellectual content.

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CHAPTER II

LEPTOSPIRA INFECTION PREVALENCE IN SMALL MAMMAL HOST POPULATIONS ON THREE HAWAIIAN ISLANDS

Leptospira Infection Prevalence

In Small Mammal Host Populations on Three Hawaiian Islands

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Abstract. We describe the geographic distribution and variation in host-pathogen specificity for *Leptospira* infected small mammals collected concurrently from three Hawaiian islands over a period of 14 years: 1990--2003. Four serogroups (Icterohaemorrhagiae, Ballum, Sejroe, and Australis) were identified from the 15,171 animals tested. Serogroup prevalence differed across host species and islands ($p < 0.0001$ for each), but not across years. The host associations and biogeographic patterns of *Leptospira* in Hawaii indicate a pathogen community shaped by ecological factors.

INTRODUCTION

Leptospira are bacteria of the order *Spirochaetales* and the etiologic agent of leptospirosis, a zoonotic disease transmitted to humans usually via exposure to soil or water contaminated with urine from infected mammalian host animals. Leptospirosis is considered the most widespread of all zoonoses[1] and an emerging infectious disease by the World Health Organization.[2] Pathogenic *Leptospira* form a large globally distributed complex of antigenic variants known as serovars with broad geographic and host species affinities.

The genus has a broad mammalian host range, yet host-serovar specificity often exhibits relatively high fidelity, especially among commonly studied peridomestic genera (e.g., *Rattus* spp, *Mus* spp). Recent research showing high *Leptospira* species diversity in correspondingly diverse natural mammalian communities in the humid tropics[3,4] suggests an ancient co-evolutionary host-parasite history, with humans (and later domestic animals) as accidental hosts.

The Hawaiian islands provide a rare *Leptospira* study opportunity. The archipelago has a limited number of *Leptospira* host reservoir species, nearly all of which are human commensals brought by early Polynesian voyagers in 1219--1266 A.D.[5] or introduced more recently by European traders in the late 18th century.[6] The archipelago has only one native land mammal, the Hoary Bat (*Lasiurus cinereus semotus*), whose endangered status makes it an unlikely reservoir, at least of public health significance. The remainder of the introduced land mammalian fauna includes six species common to nearly all main Hawaiian Islands: mongoose (*Herpestes auropunctatus* [HA]), mouse

(*Mus musculus* [MM]), brown rat (*Rattus norvegicus* [RN]), roof rat (*Rattus rattus* [RR]), Polynesian rat (*Rattus exulans* [RE]), and feral pig (*Sus scrofa*).

Of the over 200 *Leptospira* serovars currently known, a total of 11 have been recorded in Hawaii to date.[7,8,9] Human leptospirosis cases have been documented on each of the seven main inhabited Hawaiian islands: Oahu, Hawaii, Maui, Kauai, Molokai, Lanai, and Niihau, with the highest incidence on Hawaii, Kauai, and Oahu.[7,8] Pathogenic leptospires have been found in both non-domestic and domestic animals in previous surveys on Oahu in 1936—1942,[10] 1970—1973;[11] Hawaii in 1959--1961,[12] 1969—1973,[13] 1969—1974;[14] and Maui in 1968 (Zahn A, unpublished data). However with the exception of Shimizu's[13] and Higa and Fujinaka's[11] island-wide studies on the islands of Hawaii and Oahu, respectively, trapping efforts typically were confined to one site or at most two districts. Difficulty in making inter-island comparisons of leptospiral infections in non-domestic host populations is further compounded because data were not collected concurrently on multiple islands.

Because maintenance of this pathogen is reliant on nonhuman hosts, public health prevention efforts have typically focused on animal control measures in conjunction with public education to increase awareness of common exposure risks. Understanding specific patterns of host-serovar associations assists in informing public health efforts by providing insight into which animal carriers are associated with the *Leptospira* variant of interest.

Using a large-scale dataset comprised of 15,171 animals collected over a period of 14 consecutive years, with 8 years of concurrent trapping across Oahu, Kauai, and Hawaii islands, this retrospective summary represents the largest and longest study of

leptospirosis amongst non-domestic animal populations in Hawaii and (i) provides an update on leptospirosis in animals on Oahu and Hawaii since Higa and Fujinaka's[11] and Shimizu's[13] studies respectively, (ii) is the first description of animal leptospirosis on Kauai, and (iii) offers host specificity information to assist in public health management of this important communicable disease.

MATERIALS AND METHODS

Animal Sampling. As part of a statewide initiative for leptospirosis monitoring and surveillance by the Hawaii State Department of Health (HDOH) Vector Control Branch, five primary animal reservoirs from Oahu, Kauai, and Hawaii islands were trapped and tested for evidence of leptospiral infection: mongoose (HA), mouse (MM), brown rat (RN), roof rat (RR), and the Polynesian rat (RE). Animal trapping on Oahu was conducted from 1990 to 2003, while trapping on the islands of Hawaii and Kauai was conducted from 1991 through 1998. Trapping was opportunistic and was conducted at residential or business sites in response to rodent pest complaints or at field sites (e.g., waterfalls, streams, or taro plots) temporally associated with a confirmed human case. Live captures were brought to a HDOH Vector Control facility and killed via carbon monoxide gas then immediately weighed, sexed, and dissected. Harvested homogenized kidneys were used to inoculate Ellinghausen-McCullough-Johnson-Harris culture media followed by incubation at room temperature in the absence of ambient light. Cultures were inspected weekly for 6 weeks via dark-field microscopy (HDOH unpublished data).

Serogroup identification. Serogroup identification of isolates was performed at the HDOH Vector Control Branch (Halawa Valley, Oahu) via the microscopic agglutination test (MAT)[15] which screens the live unknown cultured isolate against a panel of rabbit antisera selected for the Pacific region and obtained from the U.S. Center for Disease Control and Prevention (CDC), Atlanta, GA (Table 1). In the case of cross-reactions of an unknown isolate to multiple antisera, identification was determined according to the antisera with the highest titer (i.e., greatest dilution) reaction. When a particular serovar became temporarily unavailable, a different serovar of the same serogroup was used in its stead. Therefore identification is accurate only to the serogroup level.

Isolates were considered of undetermined serogroup if the kidney culture contained leptospires as well as other bacteria ('mixed') despite the presence of fluorouracil in the growth media, or were of insufficient quantity for use in the MAT ('insufficient growth'), or did not match any of the known antisera in the panel ('unable to type').

Statistical analyses. Leptospiral serogroup distributions were measured within and across host species, and within and across all three islands. Calculations of summary prevalence proportions used counts of all leptospiral culture positive animals, including those of undetermined serogroup, in the numerator, and counts of all trapped animals (both infection negative and positive animals) in the denominator. The chi-square test was used to test the frequency distributions in host and island contingency tables.

A generalized estimating equations (GEE) model[16] with the identity link function was performed using the PROC GENMOD procedure in SAS version 9.2 (SAS institute,

Cary, NC) to examine the differences in leptospiral serogroup prevalence between serogroups, host species, or islands. For these analyses, the prevalence proportions for each serogroup were calculated relative to the frequencies of the other serogroups found in each host species or island. Counts of only animals infected with an identified serogroup were considered. Host species data were partitioned into five levels: mongoose (HA), mouse (MM), and the three rat species (RN, RR, RE). Location data was grouped by island: Kauai, Oahu, Hawaii. Pairwise comparisons and linear contrasts were used to compare the differences of leptospiral serogroup prevalence proportions among hosts and islands. The Bonferroni procedure was used to adjust the p-values to control the family wise error rate (FWER) at 5%. All comparisons with adjusted p-values of ≤ 0.05 were considered significant.

The GEE model with the identity link function was also employed to examine the temporal changes in leptospirosis infection prevalence across years adjusted for serogroup, host species, and island. To enable comparisons across the explanatory variables (i.e., serogroup, island, host species, trap year), prevalence proportions were calculated using the total count of animals trapped as the denominator.

RESULTS

Overall prevalence. Table 2 provides a summary of leptospiral prevalence by serogroup, host species, and island. A total of 15,171 animals were tested, with 2,766 animals found to harbor culture positive *Leptospira* in their kidneys (18.2%). Host-specific prevalence ranged from 11.4% for RE to 26.7% for RN. Leptospiral infection prevalence varied significantly among the five host species ($\chi^2 = 250.2$, df = 4, p <

0.0001). Overall prevalence also differed significantly across the main Hawaiian islands with the highest prevalence on Hawaii (25.1%), followed by Oahu (10.9%), and Kauai (10.3%) ($\chi^2 = 523.2$, df = 2, $p < 0.0001$). Of the 2,766 culture positive animals, four leptospiral serogroups were identified. Icterohaemorrhagiae was the most common (37.7%), followed by Ballum (28.4%), Sejroe (20.1%) and Australis (0.4%). Undetermined serogroups comprised 13.4% (371 animals) of the *Leptospira* culture positives. Undetermined samples were not included in further counts and statistical analyses. Therefore 2,395 out of a total of 2,766 culture positive results (86.6%) were considered in additional tests of host and island distributions (Table 2).

Serogroup prevalence by host species. Four serogroups were identified in the carrier species studied: Sejroe, Ballum, Icterohaemorrhagiae, and Australis (Figure 1). All four serogroups were observed in mongooses (HA), brown (RN) and roof rats (RR). All serogroups except Australis were identified in mice (MM) and Polynesian rats (RE). Icterohaemorrhagiae, Sejroe, and Ballum were found in all host species tested.

Serogroup dominance by host species. Although the host range of the four leptospiral types was broadly inclusive of all animal carrier species tested in this study, each species appeared to be strongly associated with one predominant serogroup and to a lesser degree with multiple secondary serogroups. The most common serogroup was found to differ by host species, with Sejroe the primary serogroup in mongoose (73.7%), Ballum in mice (82.2%), and Icterohaemorrhagiae in rats (87.4% RN, 69.8% RR, 55.2% RE) (Figure 1).

Statistical tests of relative serogroup prevalence showed evidence of host specificity. Within each species, linear contrasts between the numerically dominant

serogroup and each of the other serogroups observed in that species were highly significant ($p < 0.0001$ for all comparisons).

Serogroup distribution by island. Serogroups differed in composition and relative prevalence across islands. All four serogroups were documented on Oahu and Hawaii while leptospiral diversity was narrower on Kauai due to the absence of Sejroe (Figure 1).

Serogroup dominance by island. The dominant serogroup also differed by island. The most common serogroup on the island of Kauai was Ballum (57.7%), Sejroe (44.9%) on Oahu, and Icterohaemorrhagiae (49.0%) on Hawaii (Figure 1).

Tests of serogroup prevalence by islands showed a significant association between island affiliation and serogroup prevalence. For each island, pairwise comparisons among serogroups showed highly significant differences in prevalence proportions ($p < 0.0001$).

Geographic distribution of leptospiral host associations. Host specific associations apparent in the pooled all-islands dataset remained primarily consistent when examined at the island level (Figure 2). Sejroe is the dominant serogroup in mongooses (HA) on the islands in which mongooses are established (i.e., Oahu and Hawaii). Ballum is the dominant serogroup in mice irrespective of which island was examined. Icterohaemorrhagiae is the primary serogroup for all rats (RN, RR, RE) in the pooled data, as well as for the majority of rats on each island.

Statistical tests under the GEE model of host specificity patterns for each island were, in general, consistent with the pooled dataset. For each island, there is a significant difference in the distribution of relative prevalence across hosts overall (Kauai: $\chi^2 =$

3566.8, df = 9, $p < 0.0001$; Oahu: $\chi^2 = 18417.1$, df = 12, $p < 0.0001$; Hawaii: $\chi^2 = 11080.4$, df = 12, $p < 0.0001$). Detailed scrutiny of each host pairwise comparison for Ballum and Icterohaemorrhagiae are consistent for the pooled dataset and for each island's dataset. The only exceptions to host specificity trends in the pooled dataset are for Australis and Sejroe host comparisons on Kauai. The only animals on Kauai found to harbor Australis were brown rats (RN, 2.8% prevalence) hence comparisons of Australis prevalence between all other host species on Kauai were not significant. Similarly, Sejroe was absent from all animals tested on Kauai hence comparisons of prevalence for this serogroup were not significant.

Temporal analyses. After adjusting for serogroup, host species, and island, changes in prevalence across years were found to be not significant ($\chi^2 = 13.2$, df = 13, $p = 0.43$).

DISCUSSION

The application of an epidemiological approach (i.e., epizootiology) that includes ecological and evolutionary considerations can help provide insights into disease factors that may influence the dynamics of zoonotic diseases such as leptospirosis. Host associations and biogeography are two important factors that can have direct effects on the patterns of infectious zoonotic disease.

Host effects such as the preferential association of a serogroup with a particular animal species (i.e., host specificity) were found to be significant in this study. Each serogroup was associated with one primary host species and multiple ancillary hosts. The patterns of host specificity in the Hawaiian Islands are consistent with leptospiral

serogroup-host associations that have been generally observed worldwide, for example *Rattus* spp. are known carriers of Icterohaemorrhagiae and mice of Ballum.[17,18,19] Sejroe in Hawaii is common only in mongooses, which indicates this animal is the likely sole primary maintenance host for Sejroe although other animal species are susceptible. Ballum was observed to be relatively common in all four rodent species but not mongooses. As the mongooses are phylogenetically quite distant from rodents, and *Rattus* and *Mus* are considered closely related genera, Ballum may be less well adapted to mongooses as a host. Australis was not observed in mice despite a large sample size of tested animals (n = 3,171) therefore indicating that mice may not be susceptible to Australis. Icterohaemorrhagiae appears to be the least host specific since it was amply represented in all animals. Host-specificity has been attributed to host related biological compatibility factors, genetic factors mediating resistance, and age-related immunological factors.[20]

Host availability was a second mechanism by which hosts were found to contribute to the pattern of serogroup prevalence in Hawaii. The availability of appropriate maintenance hosts appears to determine which leptospiral serogroups were present. A serogroup is not likely to be found on an island if its primary maintenance host is absent. For example in this study no evidence of Sejroe was found on Kauai, one of three islands in Hawaii (along with Lanai and Niihau) where mongooses have not been established. Furthermore, the apparent absence of Sejroe on the island of Kauai indicates that the other four major animal carriers, while susceptible to Sejroe, are either not compatible reservoir hosts capable of maintaining this serogroup in the long term or that Sejroe has not yet been introduced to this island.

In regards to biogeography, there was a significant effect of insularity on leptospiral community richness, serogroup abundances, and prevalence. Community richness as indicated by serogroup diversity was found to be lower on Kauai than on Hawaii and Oahu, with one less serogroup on Kauai as compared to the other islands. The dominant serogroup also differed across islands. Analyses of prevalence within and across islands highlight the uniqueness of each island's leptospiral profile.

Our data shows that leptospiral infection prevalence proportions in this study varied significantly across serogroups, host species, and islands, but not across years. Of note, a significant temporal trend in infecting serogroups has been identified in humans in Hawaii with a decrease in cases related to infections with Icterohaemorrhagiae and an increase in infections related to Australis from 1974--2008 ($p < 0.0001$ for each).[9] The increasing impact of Australis may be related to the increasing population of feral swine and their impingement into urban population centers. Studies are planned to investigate the contribution of feral swine on human leptospirosis in Hawaii.

Because the prevalence data in this study are based on opportunistic trapping as opposed to a systematic survey, extrapolations and generalizations from the results should be made judiciously. Trap efforts varied widely with uneven sample sizes between islands and animal species and greater sampling efforts in urban centers than rural sites. Another caution is that infection prevalence in animals may not necessarily translate directly into risk predictors for humans since contact rate, persistence of the bacteria in the environment, and other factors determine the probability of pathogen transmission.[20]

Leptospiral diversity and prevalence can be affected by a number of environmental influences.[21] This study illustrates how two ecological factors, host-

pathogen interactions and geography, can shape the community ecology of a pathogen. An understanding of the mammalian reservoirs of leptospirosis can provide a basis for the management of disease risks by targeting potential transmission sources and pathways.[19]

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TABLE 1. Panel of 10 reference strains* of *Leptospira* used by the Hawaii State Department of Health Vector Control Branch in the microscopic agglutination test (MAT).

	Serogroup	Serovar	Strain
1	Canicola	canicola	Hond Utrecht IV
2	Australis	australis	Ballico
3	Autumnalis	autumnalis	Akiyama A
4	Ballum	ballum	Mus 127
5	Bataviae	bataviae	Van Tienen
6	Sejroe	hardjo	Wolffi 3705
7	Mini	georgia	LT 117
8	Icterohaemorrhagiae	copenhageni	M20
9	Pomona	pomona	Pomona
10	Pyrogenes	pyrogenes	Salinem

*The following strains were discontinued from the panel when either the antisera was depleted and not replaced, or the type culture used for quality control was lost: Bataviae bataviae Van Tienen (1990s), Mini georgia LT 117 (1990s), Autumnalis autumnalis Akiyama A (2003).

TABLE 2. Leptospiral prevalence by serogroup, host species, and island, state of Hawaii, 1990--2003.

		Total Sampled (n = 15,171)	Culture Positives (n = 2,766)	Serogrouped *Leptospira** (n = 2,395)
		n	n (%)	n (%)
Serogroup				
	Sejroe		555 (20.1)	555 (23.2)
	Ballum		786 (28.4)	786 (32.8)
	Icterohaemorrhagiae		1,043 (37.7)	1,043 (43.5)
	Australis		11 (0.4)	11 (0.5)
	Undetermined		371 (13.4)	
Host Species				
	Mongoose (HA)	4,405	811 (18.4)	744 (31.1)
	Mouse (MM)	3,171	740 (23.3)	656 (27.4)
	Brown Rat (RN)	1,686	451 (26.7)	382 (15.9)
	Black Rat (RR)	5,200	683 (13.1)	546 (22.8)
	Polynesian Rat (RE)	709	81 (11.4)	67 (2.8)
Island				
	Kauai	2,313	238 (10.3)	137 (5.7)
	Oahu	4,922	538 (10.9)	461 (19.2)
	Hawaii	7,936	1990 (25.1)	1797 (75.0)

*Culture positive samples with undetermined serogroup are not included in this set of counts

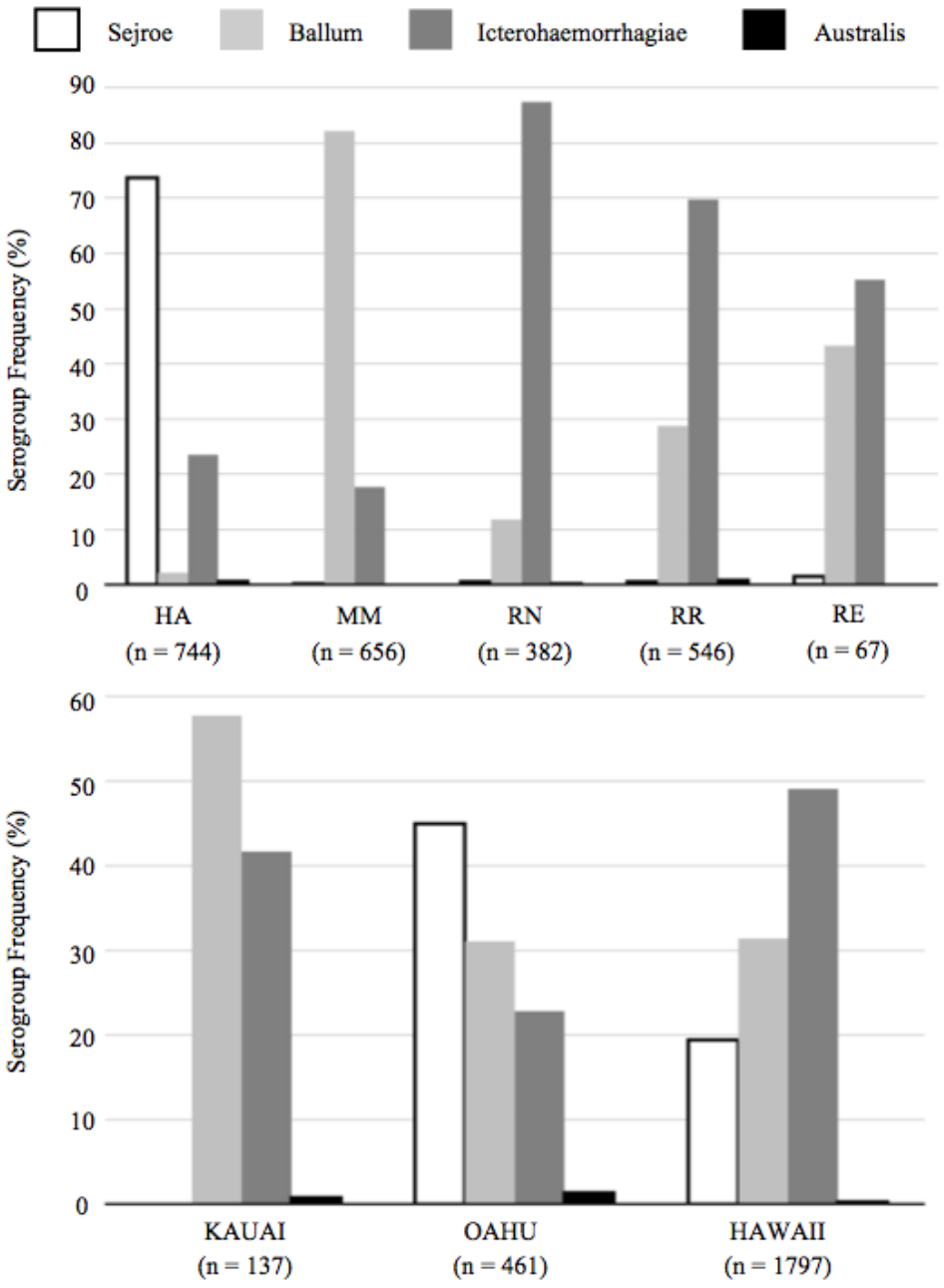
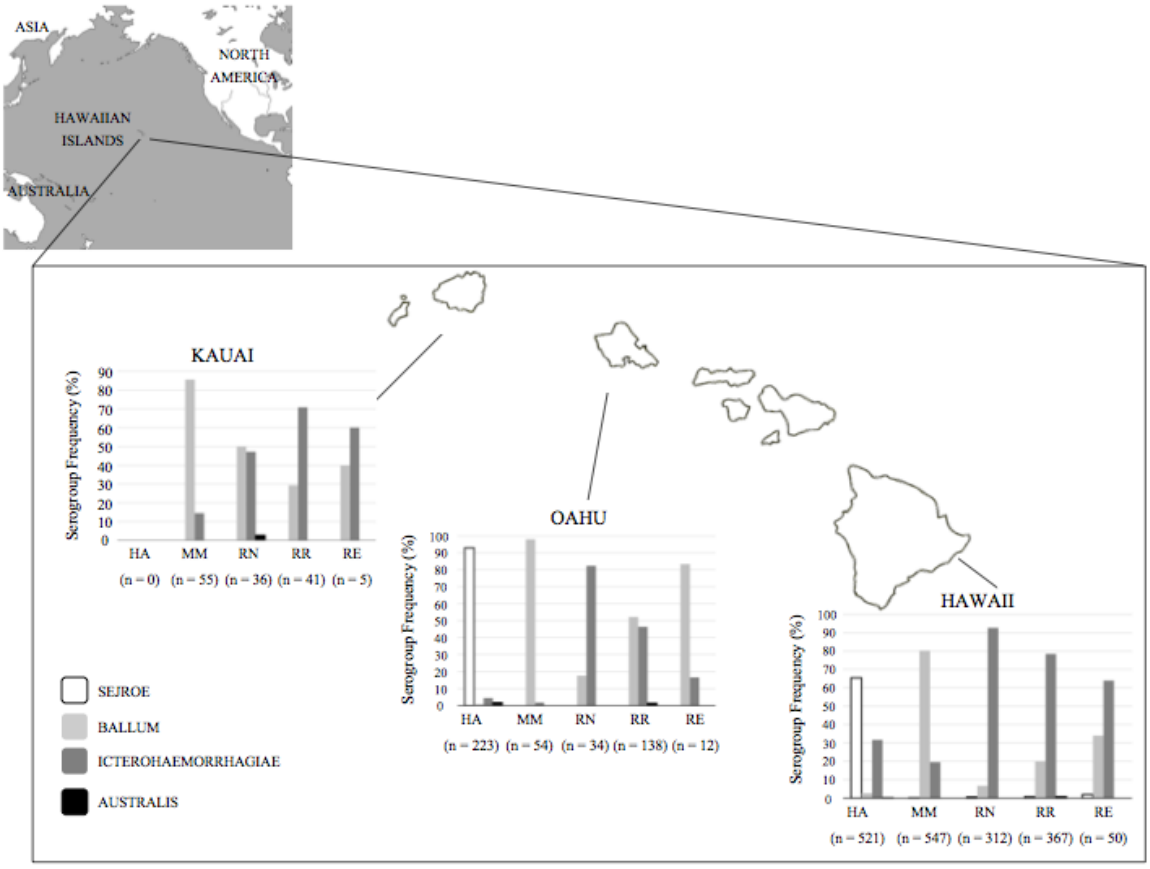


FIGURE 1. Distribution of leptospiral serogroups in five animal species (mongoose, *Herpestes auropunctatus* [HA]; mouse, *Mus musculus* [MM]; brown rat, *Rattus norvegicus* [RN]; roof rat, *Rattus rattus* [RR]; Polynesian rat, *Rattus exulans* [RE]) and three Hawaiian islands, 1990--2003.

FIGURE 2. Distribution of leptospiral serogroups in five animal species (mongoose, *Herpestes auropunctatus* [HA]; mouse, *Mus musculus* [MM]; brown rat, *Rattus norvegicus* [RN]; roof rat, *Rattus rattus* [RR]; Polynesian rat, *Rattus exulans* [RE]) on the islands of Kauai (1991--1998), Oahu (1990--2003), and Hawaii (1991--1998).



CHAPTER III

PATHOGENIC *LEPTOSPIRA* IN SERA AND KIDNEY OF NATURALLY INFECTED MAINTENANCE ANIMAL HOSTS IN HAWAII

Pathogenic *Leptospira* In Sera and Kidney of Naturally Infected Maintenance Animal Hosts in Hawaii

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Abstract

OBJECTIVE

To describe the molecular phylogenetics of pathogenic leptospires in maintenance hosts of the Manoa watershed, Honolulu, Hawaii.

METHODS AND RESULTS

Paired kidney and sera samples from 43 naturally infected non-domestic individuals of three animal species (mongoose, *Herpestes auropunctatus*; roof rat, *Rattus rattus*; and Polynesian rat, *Rattus exulans*) were tested using genus-specific G1/G2 PCR and 16S rRNA real-time PCR assays. We detected leptospiral DNA from the kidneys of four *Rattus spp.* individuals, of which two were verified by direct sequencing of PCR amplicons to be pathogenic *Leptospira*. Subsequent phylogenetic analyses identified the *R. rattus* and *R. exulans* leptospiral isolates as of the *Leptospira interrogans* lineage.

CONCLUSION

This study is the first to report genetic sequences of Hawaii leptospiral isolates. The finding of *Leptospira interrogans* from animal samples is consistent with the observations of previous recent human and animal leptospiral investigations in Hawaii.

keywords real-time PCR, *Leptospira*, 16S rRNA DNA sequences, maintenance hosts, Hawaii,

Introduction

Because mammals serve as reservoir hosts for *Leptospira* bacteria that are pathogenic to humans, animal surveillance is an important tool for understanding transmission of this global zoonosis. Maintenance hosts are asymptomatic so a definitive diagnosis of leptospiral infection often requires direct testing of tissue samples. Since reservoir hosts harbor lifelong *Leptospira* infections in their kidneys, detection of an infection in animals has typically involved harvesting kidneys.

Sacrificing animals in order to harvest kidney tissue for diagnostic testing may not be a feasible option. Alternative no-kill detection methods such as the culturing of *Leptospira* from urine are available (Faine 1993) but oftentimes the sterile collection of urine is not practical, particularly for large-scale wild animal investigations. A minimally invasive method of testing for *Leptospira* infections would aid in animal studies in addition to benefiting human diagnosis. Testing of animals who have been naturally infected is important because the bacteria burden in natural infections is likely to be lower than in laboratory subjects who generally require high dosage intraperitoneal innoculation (Haake 2006; Ko *et al.* 2009; Athanazio *et al.* 2008) in order to induce infection.

The aim of this study is to attempt to isolate and identify pathogenic *Leptospira* in sera and kidney tissues of naturally infected wild animal carriers in an urban watershed associated with recent human leptospirosis cases (Gaynor *et al.* 2007) and contaminated surface stream water (Walker *et al.* 2008). We also seek to characterize the genetic

diversity and molecular phylogenetics of leptospires infected small mammalian host populations.

Material and methods

Animal samples

Live trapping of the five major carrier species in Hawaii (i.e., the mongoose, *Herpestus auropunctatus*; roof rat, *Rattus rattus*; Norway rat, *R. norvegicus*; Polynesian rat, *R. exulans*; and mouse, *Mus musculus*) was conducted from December 16, 2007 to December 21, 2007 at Manoa Falls, an easily accessible tropical rainforest locale popular with recreational hikers on the island of Oahu, Hawaii (Figure 1). Individuals were brought to a biosafety level 2 facility for processing on the same day of capture, and anaesthetized via isoflurane. Animal trapping and sample collection activities were conducted under University of Hawaii Institutional Care and Use Committee approval of 2006 protocol 05-020-2.

DNA extraction

Immediately upon sacrifice, whole blood was collected via cardiac puncture using a sterile glass pipet and centrifuged in 2.0mL tubes for 5-10 minutes at 2100 x g to collect the sera supernatant fraction. Sterile scissors were used to excise and mince half of a kidney from each individual. Sera and kidney samples were kept frozen at -17C until DNA extraction. Total genomic DNA was prepared from scissored kidney tissue and

100uL starting volumes of sera using standard protocols for the DNeasy blood and tissue extraction kit (Qiagen, USA).

Traditional PCR analysis

Amplification of leptospiral DNA from 2uL of blood and kidney extractions was performed using previously described primers G1 and G2 and PCR conditions (Gravekamp 1993). PCR reagents used were from the AmpliTaq Gold® DNA Polymerase PCR system for amplification of bacterial templates and very low target sequences (Applied Biosystems). The following cycling profile was carried out on a Mastercycler gradient thermacycler (Eppendorf): 94°C for 5 minutes, followed by 46 cycles of 95°C for 15 seconds, 61°C for 1 minute, and 72°C for 2 minutes, with a final extension of 72°C for 7 minutes, and then held at 4°C. The entire 25uL amplification volume for each reaction was electrophoresed on an ethidium-bromide stained 2% agarose gel to check for the presence of amplification products.

In addition, DNA extracts were amplified with mitochondrial d-loop primers RJ3R and EGL4 (Robins *et al.* 2007) to verify host species as well as to confirm template quality for PCR. To speciate rodent hosts, a restriction fragment length protocol developed by Matisoo-Smith and Allen (2001) was followed in which enzymes HaeIII and DdeI were used to digest RJ3R/EGL4 amplification products.

Real-Time PCR analysis

A TaqMan assay for the leptospiral 16s ribosomal gene was performed as previously described (Ganoza *et al.* 2006) using *L. interrogans* primers developed by Smythe *et al.* (2002). Quantification was accomplished on an Opticon2 Real Time PCR Machine (MJ Research, USA) with standards of known concentrations of *L. interrogans* serovar Icterohemorrhagiae strain M20 (i.e., 10^2 , 10^3 , 10^4, and 10^5 leptospires/mL) prepared by direct cell counts via a counting chamber as previously described by Ganoza et al. (2006). Standards were first run in duplicates under the same conditions as replicate unknown samples. Positive samples were run a second time in triplicates with triplicate standards to quantify the amount of leptospiral DNA present. All runs included two negative controls with sterile water as template.

Amplification runs were analyzed using the software provided with the Opticon2 detection system. The minimum detectable amplification product was produced by the 10^2 standard and was nearly indistinguishable from the 10^3 amplification product. Since both of the last reliable standards produced an amplification signal by PCR cycle 40, unknown samples amplifying after 40 cycles were considered negative for pathogenic *Leptospira*.

16s ribosomal RNA gene PCR and sequencing

To obtain 16S gene sequences, leptospiral DNA qPCR amplification products were used as template. The nested-PCR lepto16S11f and lepto16S1338r primers previously described by Ganoza *et al.* (2006) were used as delineated in conjunction with the HotStarTaq Master Mix kit (Qiagen). The published amplification conditions were

performed on a PCT-200 Peltier thermalcycler (MJ Research, USA) and modified by using a 64°C annealing temperature. The amplification product band from each electrophoresed nested 16S reaction was extracted from 1% agarose using the MinElute PCR purification kit (Qiagen, USA). 5uL of the extracted PCR product was cycle sequenced with the same lepto16S11f and lepto16S1338r primers used for amplification, at a 2.5uM concentration.

Resultant sequences were edited and assembled using Sequencher 4.7 (Gene Codes Corp, Ann Arbor, MI). Pairwise sequence identity information was obtained using NCBI Blastn 2.2.27 (Zhang *et al.* 2000). The 16S rRNA gene sequences of 42 type strains for pathogenic, intermediate, and saprophyte species of *Leptospira* were obtained from Genbank for use as phylogenetic references (Table 1). Multiple sequence alignments were performed using Clustal Omega with default parameters (Sievers *et al.* 2011). Modeltest (Posada 2008; Guindon *et al.* 2003) was run on the subsequent alignment to verify that the GTR model of nucleotide substitution is appropriate. A phylogenetic tree was constructed on the RAxML BlackBox (Stamatakis *et al.* 2008) server using gamma distributed rate heterogeneity with no estimate of invariant sites. The resultant tree was visualized in FigTree v1.3.1 (Rambaut 2009).

Results

A total of 43 individuals were sampled; 1 mongoose (*Herpestes auropunctatus)*, 38 roof rats (*Rattus rattus*), and 4 Polynesian rats (*Rattus exulans*). Neither Norway rats (*Rattus norvegicus*) nor mice (*Mus musculus*) were captured during the trapping period.

PCR analyses

All 43 blood samples were negative for 16s rRNA leptospiral DNA by qPCR. The one putative G1/G2 PCR positive from a *R. rattus*, was not confirmed by 16S qPCR.

Of the 43 kidney samples, four individuals were found to be 16S qPCR positive for the presence of leptospiral bacteria. qPCR confirmed a previous G1/G2 PCR positive *R. rattus* and amplified three other samples that were G1/G2 PCR negative: two *R. exulans*, and a *R. rattus*. Bacterial counts for the four positive animals ranged from 1,469 to 18,770 leptospires/mL (mean = 8,615 leptospires/mL; 95% CI = 2,852 to 14,378 leptospires/mL).

The minimum template concentration for successful amplification of leptospiral DNA using the primers of this study was 10^2 leptospires/mL with 16S rRNA qPCR primers (Ganoza *et al.* 2005) and 10^5 leptospires/mL with G1/G2 conventional PCR primers.

Molecular identification

16S leptospiral gene sequences were obtained from leptospires in the kidneys of two *R. rattus* individuals and an *R. exulans*. Attempts to sequence 16S amplicons from the fourth positive individual, a *R. exulans*, were not successful despite pooling of weak amplification products from the same template. Consensus sequences ranged in length from 1211 to 1276bp, with overlaps of between 414-477bp.

Phylogenetic analysis of 16S leptospiral type strain sequences (Table 1) resulted in three monophyletic groups consistent with prior published phylogenies (Matthias *et al.* 2005; Ganoza *et al.* 2006; Morey *et al.* 2006) of pathogenic, intermediate, and saprophytic leptospiral strains. Unknown leptospiral sequences from a *R. rattus* male (i.e., K8) and a *R. exulans* female (i.e., K35), clustered with the known pathogenic *L. interrogans* species (51%, Figure 2). Total sequence length of the two isolates was 1203 base pairs, with 99% sequence similarity. The third sample, from a *R. exulans* female, appears to be a closely related contaminant from the genus *Treponema* (data not shown).

Discussion

qPCR detection of pathogenic leptospires in host tissue was found to be more sensitive than traditional PCR amplification in this study. Although PCR may theoretically amplify using just one target template DNA molecule, the actual sensitivity threshold is much higher and appears to be dependent on the target gene. Additionally we found that *Leptospira* infections were more likely to be detected via kidney tissue than blood of naturally infected maintenance hosts.

Molecular detection of leptospiral DNA in blood is likely more challenging than direct amplification from host kidneys because a high concentration of *Leptospira* in peripheral blood is only present for a relatively short period of time compared to the lifespan of a host. A previous study of experimental infections in *R. norvegicus* laboratory rats showed that *Leptospira* were detectable in organ tissues beginning on day 3 post-inoculum and that peak renal colonization was established between days 7 and 9 in

maintenance hosts (Athanazio *et al.* 2008). Our study indicates that any residual spirochetes remaining in the bloodstream beyond this very brief acute bacteremic period are likely below the detection level for current molecular amplification techniques.

The results of this study are consistent with the findings of one other previous study that compared leptospiral detection methods across various tissue types of wild caught animals. Tulsiani *et al.* (2011) showed that of 213 wild-caught rats from Queensland, Australia, 57 individuals had sera that were real-time PCR negative for 16S leptospiral DNA and had concurrently collected kidney samples that were real-time PCR positive by the same assay. Of the 7 matching kidney and sera samples from fruit bats in their study, one individual was PCR positive for both kidney and sera, while the rest had PCR positive kidneys with PCR negative sera. These results underscore the difficulty of detecting infected individuals by sera alone due to the ephemeral nature of leptospiremia in reservoir hosts.

The use of only sera for molecular detection of leptospiral infection is further hampered by the small sample volumes inherent with no-kill sampling of small mammals such as rats and mice. IACUC standards recommend blood volumes of no more than 1% of body weight in any 3 week period (University of Pennsylvania Office of Regulatory Affairs 2008) for humane sampling from live animals. In this study, the 1% rule represents 0.2 – 1.7 mL of total blood volume allowable for *R. rattus* and 0.2 – 1.2 mL for *R. exulans* (data not shown). For the two individuals with *Leptospira* sequence confirmed infected kidneys in our study, cardiac puncture post-euthanasia produced 0.9

and 0.5 ml of sera (i.e., the yield from 1.5 and 2.5 ml of total blood, respectively), which was subsequently found to be insufficient for successful detection of leptospiral DNA.

This study is the first to report genetic sequences of Hawaii leptospiral isolates. We used sequences from the phylogenetically informative 16S rRNA leptospiral gene to identify *Leptospira* directly amplified from the kidneys of non-domestic animals collected on Oahu. Both isolates in our study were found to belong to the *L. interrogans* lineage. *L. interrogans* serogroup Icterohaemorrhagiae is known to cause human disease in Hawaii (Katz *et al.* 2002; Katz *et al.* 2011) and is enzootic in all five of the major small mammal maintenance hosts in Hawaii (Wong *et al.* 2012). On Oahu, the island where this present study was located, the prevalence of Icterohaemorrhagiae in *R. rattus* and *R. exulans* across 1990-2003 was 46.4% and 16.7%, respectively, with Icterohaemorrhagiae the second most dominant serogroup in both rat species after Ballum (Wong *et al.* 2012). Additional animal surveys are needed to describe the genetic diversity of pathogenic *Leptospira* in maintenance hosts of Hawaii, and thereby provide a basis for comparisons with human leptospires.

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Table 1. List of 16S rRNA *Leptospira* reference gene sequences used in this study.

Accession Number	Species	Serovar	Strain	Class
AY631885	*L. fainei*	Hurstbridge	BUT 6^{T}	Intermediate
AY631896	*L. inadai*	Lyme	10^{T} ATCC 43289^{T}	Intermediate
AY796065	*L. broomii*	(not described)	5399^{T}	Intermediate
EF025496	*L. wolffii*	Khorat	Khorat-H2^{T}	Intermediate
EF612284	*L. licerasiae*	Varillal	VAR010^{T}	Intermediate
AB279549	*L. kmetyi*	Malaysia	Bejo-Iso9^{T}	Pathogenic
AY631877	*L. weilii*	Celledoni	Celledoni^{T} ATCC 43285^{T}	Pathogenic
AY631880	*L. alexanderi*	Manhao 3	L 60^{T} ATCC 700520^{T}	Pathogenic
AY631881	*L. alstoni*	Sichuan	79601^{T} ATCC 700521^{T}	Pathogenic
AY631883	*L. santarosai*	Shermani	LT 821^{T} ATCC 43286^{T}	Pathogenic
AY631886	*L. noguchii*	Panama	CZ 214^{T} ATCC 43288^{T}	Pathogenic
AY631894	*L. interrogans*	Icterohaemorrhagiae	RGA^{T} ATCC 43642^{T}	Pathogenic
AY631895	*L. kirschneri*	Cynopteri	3522 C^{T} ATCC 49945^{T}	Pathogenic
AY887899	*L. borgpetersenii*	Javanica	Veldrat Batavia 46^{T} ATCC 46292^{T}	Pathogenic
AY631876	*L. biflexa*	Patoc	Patoc I^{T} ATCC 23582^{T}	Saprophyte
AY631897	*L. vanthielii*	Holland	WaZ Holland^{T} ATCC 700522^{T}	Saprophyte
AY631879	*L. wolbachii*	Codice	CDC^{T} ATCC 43284^{T}	Saprophyte
AY631888	*L. tepstrae*	Hualin	LT 11-33^{T} ATCC 700639^{T}	Saprophyte
AY631878	*L. meyeri*	Ranarum	Iowa City Frog^{T} ATCC 43287^{T}	Saprophyte
AY631882	*L. yanagawae*	Saopaulo	Sao Paulo^{T} ATCC 700523^{T}	Saprophyte
*AY714984	*Leptonema illini*	Illini	3055^{T} NCTC 11301^{T}	

^{T}type strain
*Outgroup

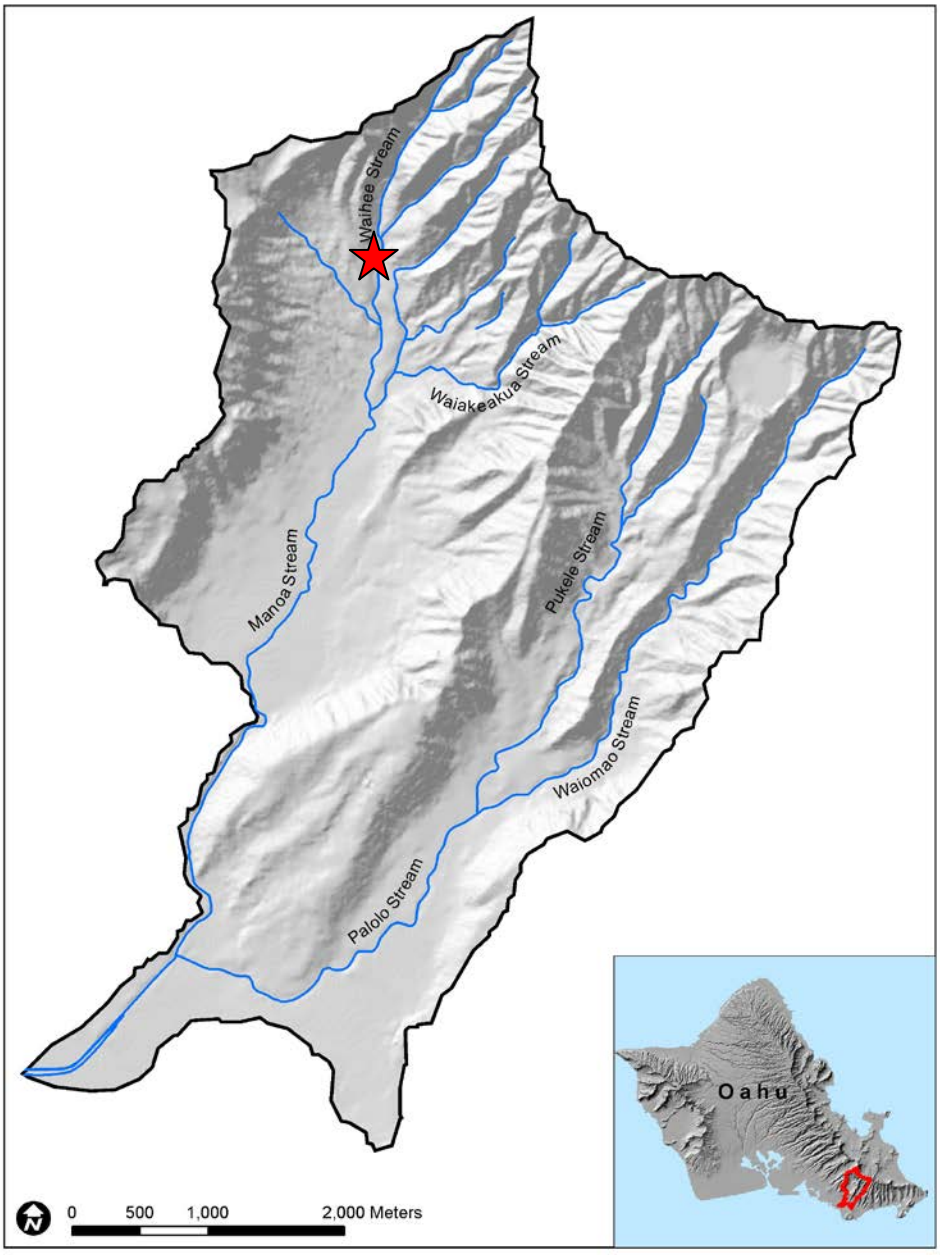

Figure 1. Manoa Falls study site in the Manoa watershed Honolulu, Hawaii.

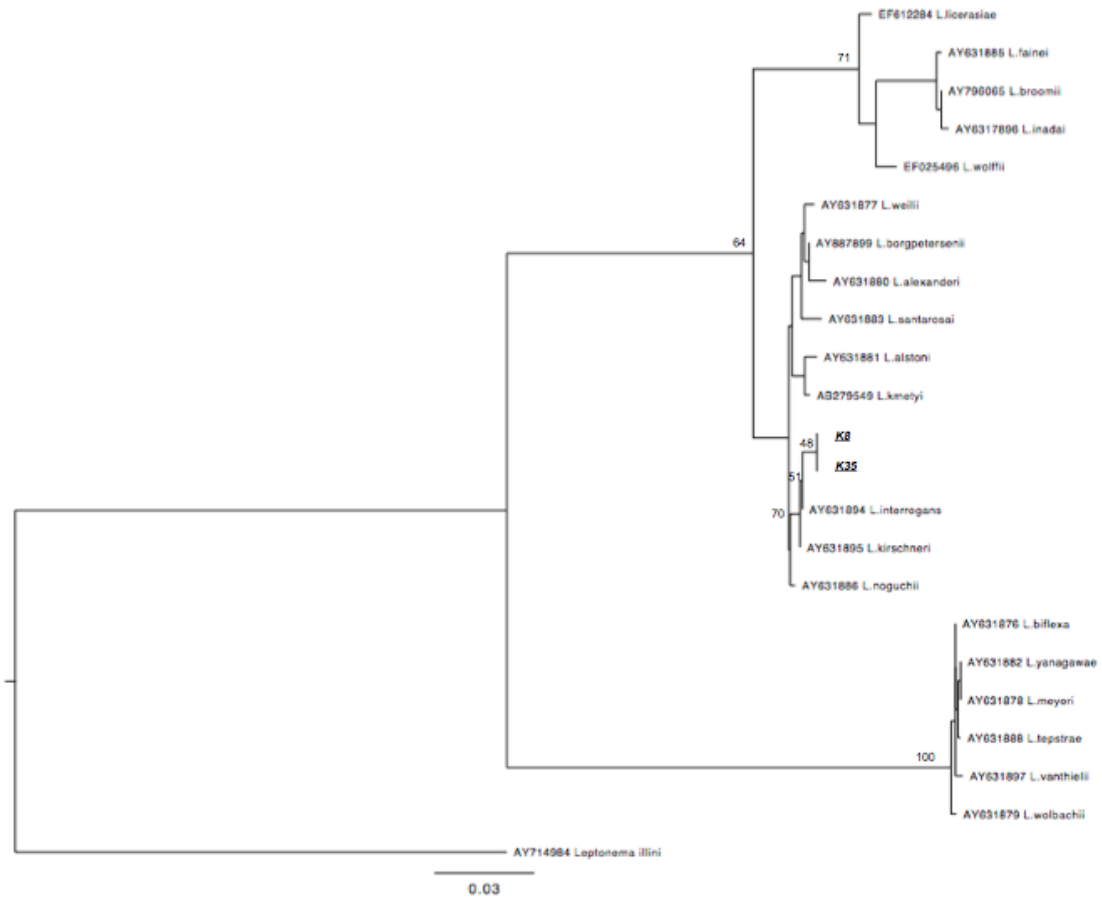

Figure 2. Maximum Likelihood (ML) phylogeny of *Leptospira* species based on 16S rRNA gene sequences. Hawaii rat leptospiral isolates are highlighted (bold underlined italics). Support from 1000 ML bootstrap replicates are indicated as percentage values at selected nodes. The fraction of nucleotide substitutions per site is shown.

CHAPTER IV

THE RELATIONSHIP BETWEEN *LEPTOSPIRA* INFECTION AND RAINFALL IN SMALL ANIMAL HOSTS IN HAWAII: THE ROLE OF SPATIAL AND TEMPORAL SCALE

The Relationship between *Leptospira* Infection and Rainfall in Small Animal Hosts in Hawaii: The Role of Spatial and Temporal Scale

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Abstract

Background: The association between leptospirosis and a number of abiotic factors, in particular rainfall, has been well documented for humans and domestic animals. However, only a limited number of studies have examined rainfall data as a predictor of leptospiral infections in non-domestic host populations critical to the maintenance of the pathogen in the environment.

Methodology/Principal Findings: Using a large-scale dataset composed of 15,171 rats, mice, and mongooses collected over a period of 14 consecutive years, with 8 years of concurrent trapping across three Hawaiian islands, we utilized logistic regression model analyses to assess the association between *Leptospira* infection and total monthly rainfall at three spatial (rainfall gauge station, forecast area, island) and three temporal (trap month, season, year) levels.

For all genera, there was a good fit with observed data at one spatiotemporal levels: rainfall gauge station-season. The station-month and forecast area-trap month predictive models were found to be relevant for mongooses and rats but not mice, while the station-year model, reflecting the coarsest temporal scale, was a good fit only for mongooses.).

Conclusions/Significance: Finding significant associations between an infectious disease and rainfall at markedly fine temporal and spatial scales is uncommon. We discuss

potential intrinsic and extrinsic factors that may be responsible for the spatiotemporal scales relevant for each maintenance host group.

Introduction

Leptospirosis is an infectious disease caused by *Spirochaete* bacteria (Family *Leptospiraceae*, Order *Spirochaetales*) of the pathogenic *Leptospira* spp. Considered the most widespread zoonosis in the world [1], leptospirosis is prevalent in tropical regions [2], including Hawaii [3,4]. Hawaii's high incidence of leptospirosis is likely related to environmental factors, including its tropical climate and high rainfall. The disease is transmitted via water contaminated with urine as well as through direct contact with the urine of mammalian reservoir host populations shedding leptospires [5].

The association between leptospirosis and abiotic environmental factors, in particular climate and rainfall, has been well documented. In Hawaii, the focus of this study, incidence of human leptospirosis has been shown to follow a seasonal pattern with peak incidence during the wetter winter months [6]. In other parts of the world, episodic heavy rainfall and seasonal flooding has been linked to an increased risk of leptospirosis outbreaks in humans [7, 8] as well as in domestic animals [9,10]. It follows that rainfall sufficient to produce standing water or surface flows can promote intra-maintenance host leptospire transmission, contributing to increased host infection prevalence, and thus transmission amplification [11]. This effect should subsequently attenuate during lower rainfall periods.

A limited number of studies have examined the association between rainfall and leptospiral infections in non-domestic animal host populations critical to the maintenance of the pathogen in the environment. Rodent host population abundance and leptospiral carriage rates were found to be highest in regions of high annual rainfall on the island of

Hawaii [12], and in a wet hot season as compared to the subsequent dry hot season in New Caledonia [13]. A study based on a parameterized demographic model using life history attributes of a African rodent showed that transmission rates for both maintenance and dead-end hosts when two-season (wet and dry) cycles are encompassed are defined by episodic peaks for both pathogen and host populations. Transmission was found to exceed the constant values seen under conditions of no seasonal variation, particularly in regions where rainfall is periodic [14].

To test whether maintenance host infection varies with rainfall, we examined the infection status of three animal genera encompassing the maintenance hosts thought mainly responsible for human cases. Our study covers three of the four main islands of the Hawaiian archipelago known to be enzootic for leptospiral infection for which host prevalence data as well as meterological data are available over a span of 14 years.

Methods

Animal data

Leptospira prevalence data obtained from the Hawaii Department of Health Vector Control Branch was employed for this study. Five primary animal reservoir hosts were opportunistically trapped on the islands of Oahu (1990-2003), Kauai and Hawaii (1991-1998) and tested for evidence of leptospiral infection: mouse (*Mus musculus*), small Indian mongoose (*Herpestus auropunctatus*), roof rat (*Rattus rattus*), brown rat (*Rattus norvegicus*), and Polynesian rat (*Rattus exulans*). Harvested kidney homogenates

were used to inoculate Ellinghausen-McCullough-Johnson-Harris culture media. Details of the animal sampling and laboratory procedures have been published previously [15].

To establish a uniform geographic scale of reference, all trap locations were first grouped according to the nearest rain gauge station. At the next level, animal trap locations were grouped into Forecast Areas (Table 1). A forecast area is a geographic zone delineated by the National Oceanic and Atmospheric Administration (NOAA) to mitigate wide ranges that would result from the extreme spatial variability in Hawaii's weather conditions [16]. Finally for each trap location, the island of origin represented the broadest geographical scale used. In this study, three main Hawaiian islands are represented: Hawaii, Kauai, and Oahu.

Rainfall data

Rainfall data was obtained from the NOAA National Weather Service Honolulu Forecast Office. Real time precipitation data are collected via Hawai'i Hydronet, a network system of 70 tipping bucket rain gauges and data loggers located throughout the Hawaiian Islands that provides rainfall data in 15-minute increments [17]. Total monthly rainfall data starting from January 1991, the earliest archived records available, were appended to each trap record according to the nearest rain gauge station and trap date.

We examined three spatial (i.e., rain gauge stations, forecast areas, and islands) and three temporal levels (i.e., month, season, and year) of total monthly rainfall. The two seasons recognized by climatologists in Hawaii are a 5-month summer (May-September) and a 7-month winter (October-April) [18,19].

Statistical analyses

Multivariate logistic regression models were employed to estimate the associations between *Leptospira* prevalence and three factors: total monthly rainfall, a temporal variable and a spatial variable. Analyses were conducted separately for mice, mongooses, and rats in order to detect any spatial or temporal differences in the relationship between rainfall and infection that may be due to biological differences between the three genera. To determine the scales relevant to rainfall and infection patterns, three temporal levels (i.e., the month, season, and year in which the animal was trapped) and three spatial levels (i.e., rain gauge station, forecast area, and island) were evaluated. The 9 possible temporal and spatial combinations were assessed with Hosmer-Lemeshow goodness-of-fit tests [20]. A model with good fit has no significant difference between the observed and expected prevalence of leptospiral infection in animals, and is therefore indicated by a small χ^2 value and a correspondingly large p-value ($p > 0.05$). Wald chi-squared tests were further used to examine the association between total monthly rainfall and *Leptospira* prevalence after adjusting the effect of the temporal and spatial variable within each selected model with good fit. Model construction and testing were performed using SAS v9.2 (SAS Institute, Cary, NC). All significance levels were set at 5%.

RESULTS

Leptospiral infection status data were available for 15,171 animals, trapped across three islands (Hawaii, Kauai, Oahu) from 1990 to 2003. The largest proportion of animals trapped and tested for leptospiral infections were rats (50.1%), followed by mongooses (29.0%), and lastly mice (20.9%) (Table 1).

Of the total number of animals sampled, the majority was collected from Hawaii island (52.3%), followed by Oahu (32.4%), and Kauai (15.2%). The spatial extent of sampling efforts on Hawaii island covered three out of the six forecast areas on the island and was in the vicinity of 14 out of 18 Hydronet rain gauge stations on that island (Figure 1). Animals on Kauai were sampled in two of the three forecast areas covering 11 stations (Figure 2); seven of which are represented in this study. On Oahu, animals were trapped near 26 of the 31 stations located across six of the seven forecast areas (Figure 3). The highest prevalence of infected animals regardless of species was found in the North and East forecast area of Hawaii island (Table 1), historically the wettest of the forecast areas sampled in this study [18].

Table 1 shows the sample sizes of animals collected in each month and season. The month with the highest prevalence of *Leptospira* infected mice (25.8%) was May, while April was the highest prevalence month for mongooses (32.4%), and October for rats (19.1%). For all animals, the highest *Leptospira* prevalence was found among animals trapped in the winter season compared with the summer season.

Model fit

Table 2 shows the spatial and temporal variable pairs that resulted in the best-fitted logistic regression models as determined by Hosmer-Lemeshow tests. For mongooses, there was a good fit for four of the nine spatio-temporal variable pairs and observed leptospiral prevalence proportions. Three of the nine rat logistic regression models, and only one of the nine mice logistic regression models demonstrated a good fit. Models incorporating all other variable pairs (i.e., forecast area-season and –year, and all island combinations) did not fit the observed prevalence data. The station-season model was the sole good fit for all three genera. The station-month and forecast area-month models demonstrated a good fit for mongooses and rats but not mice, while the station-year model was a good fit only for mongooses.

The Wald chi-squared tests revealed highly significant associations between total monthly rainfall and infection prevalence in each of the three animal groups ($P < 0.0001$, All). The significant effect of monthly rainfall differed by trap month.

Discussion

For all groups, a good fit was observed between total monthly rainfall and *Leptospira* prevalence at the level of stations and seasons. For mongooses and rats, a good fit was also detected at the station and month levels, the finest spatiotemporal scale examined in this study. The Wald tests indicate that for all animals, leptospiral infection prevalence is significantly associated with total monthly rainfall.

We utilized a ‘bottom-up’ approach [21] to describe the spatiotemporal relationship between climate and leptospiral infections amongst host animals in Hawaii.

Identification of a relevant scale is critical to investigations of linkages between environment and disease patterns [21-23]. In some systems, the relationship between climate and disease is discernable only at the largest resolution on either the temporal (e.g., cholera [24]) or spatial (e.g., meningococcal meningitis [25]) scale. One reason for this difficulty is because the spatiotemporal scale of climate patterns frequently does not match those observed in disease dynamics [22]. A second reason is because variability at the finest scales may obscure emergent patterns that are readily evident only at a macro scale [23].

There are at least two explanations for our findings that finer rather than coarser spatiotemporal scales demonstrate the best fit in our models. The orographic nature of Hawaii's tropical rainfall results in very wide spatial variability within each island, with some of the world's steepest gradients in average precipitation [18]. Intrinsic host factors are a second mechanism contributing to the localized spatial scale of the leptospiral disease dynamics seen in Hawaii. The maintenance hosts in this study are known to exhibit high site fidelity and have small home ranges, particularly for urban populations with access to stable year-round food resources (R Sugihara, unpublished data). High rainfall variability across small geographic within-island regions and sedentary hosts with minimal territory sizes appear to result in the highly localized effects of climate on leptospiral infection pattern observed in this study.

Host factors may be responsible for the temporal scales relevant to this study. Rainfall effects on infection prevalence of rats were seen at the monthly level and, in mice, at the seasonal level but not at the annual level for either genera possibly because

the average lifespan of the wild rodent species in this study is generally one year: roof rats, 11 months [26]; brown rats, 1 year [27]; Polynesian rats, 12 months [28,29]; mouse, 12-18 months [30,31]. For mongooses, rainfall was a significant predictor of *Leptospira* infections at the yearly level in addition to the finer temporal scales, likely because the average lifespan of this species is on the order of years in the wild (i.e., 4 years [32]). Of note, the predictive logistic regression model for mongooses improves with decreasing scales. The seasonal level was the only temporal scale found to be a good fit in all animals. The timing of births and population density fluctuations in roof rats [33-35], Polynesian rats [33,36-38], brown rats [39], and mongooses [40-44] are known to occur in seasonal cycles of various extents in Hawaii. Seasonality in host population dynamics can drive recurrent changes in pathogen populations and can thereby lead to the spread and persistence of infectious diseases [45,46].

Although predictive models were found to be generally a better fit with observed infection prevalences at the finer end of the spatiotemporal gradient tested in this study, it is not clear why some models were not found to be more strongly supported. While multiple scales were found to be relevant for the association between rainfall and prevalence in mongooses and rats, rainfall was found to be predictive of leptospiral infections in mice at only the station-season level. The logistic regression models for mongooses and rats were not well supported at the forecast area-season level even though seasonality was shown to be an important temporal scale at the station level for both groups. Rat infection prevalences appear to be associated with total monthly rainfall more similarly to mongooses than to mice although mongooses are the least biologically

similar in comparison to the other four mammals in this study. Future studies should investigate the mechanisms by which rainfall differentially influences the infection prevalences of various host genera.

This study provides evidence of an additional mechanism by which rainfall can influence the ecology of a zoonotic disease heretofore not suggested. Extreme weather events have been recognized as a potential driving factor that can lead to an increase in the contact rate between human susceptibles and animal sources of zoonotic pathogens by altering reservoir host population densities and behavior [10]. The results of our study are consistent with the hypothesis that rainfall influences infection prevalence in maintenance host reservoir populations, thereby increasing the number of infectives even in the absence of flooding or large-scale natural disasters. Larger numbers of infected hosts will increase the concentration of environmental leptospiral pathogens. This, in turn, may counteract pathogen dilution also associated with increased rainfall and prolong the period of environmental risk to humans. These findings provide a clearer understanding of the potential interactions between animal sources of pathogens and their environment [47], and highlight the role of rainfall as a modulator of leptospiral infection prevalence in host reservoir populations and thus potentially leptopirosis incidence in humans.

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Figure Legends

Figure 1. Trap locations on Hawaii island (1991-1998).

Figure 2. Trap locations on Kauai island (1991-1998).

Figure 3. Trap locations on Oahu island (1990-2003).

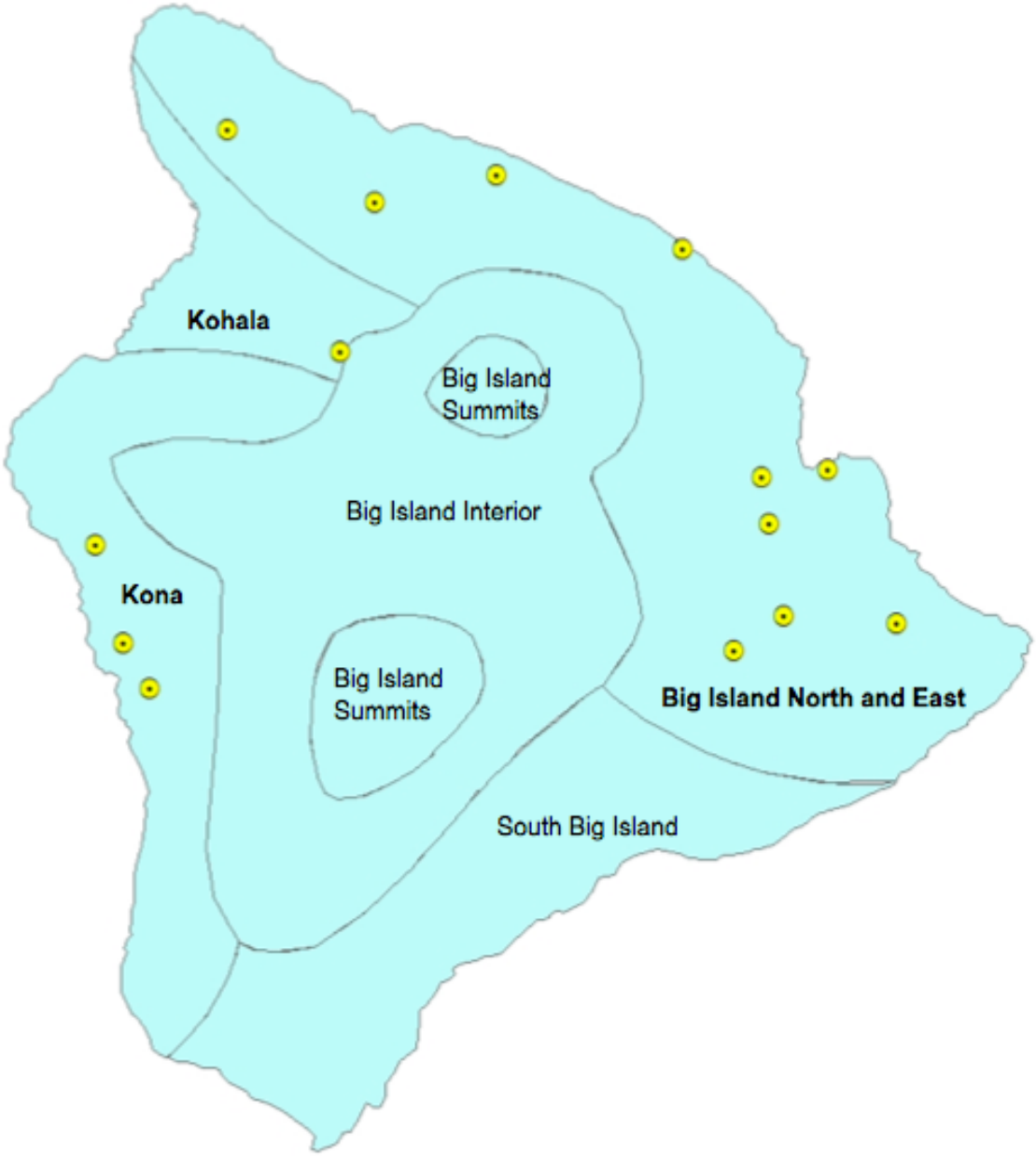

Figure 1. Trap locations on Hawaii island (1991-1998). Trap sites were in the vicinity of 14 Hydronet rainfall gauge stations across three forecast areas (bold).

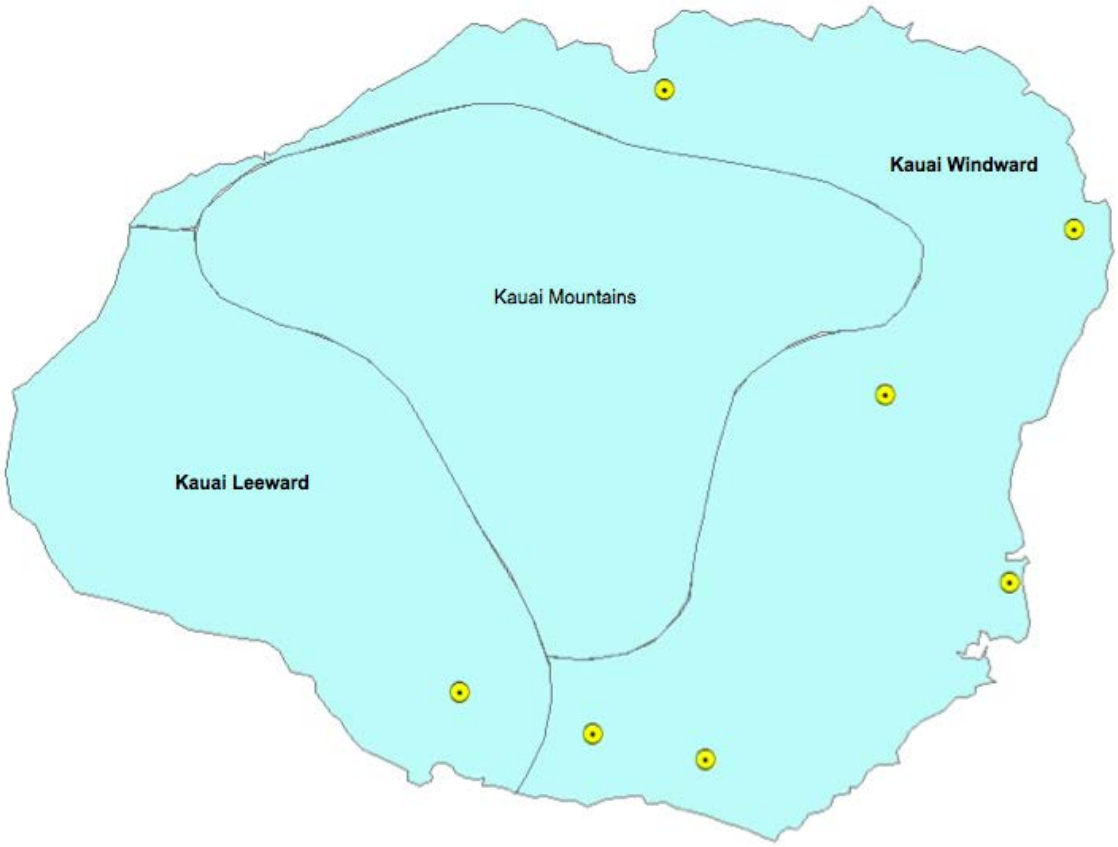

Figure 2. Trap locations on Kauai island (1991-1998). Trap sites were in the vicinity of seven Hydronet rainfall gauge stations across two forecast areas (bold).

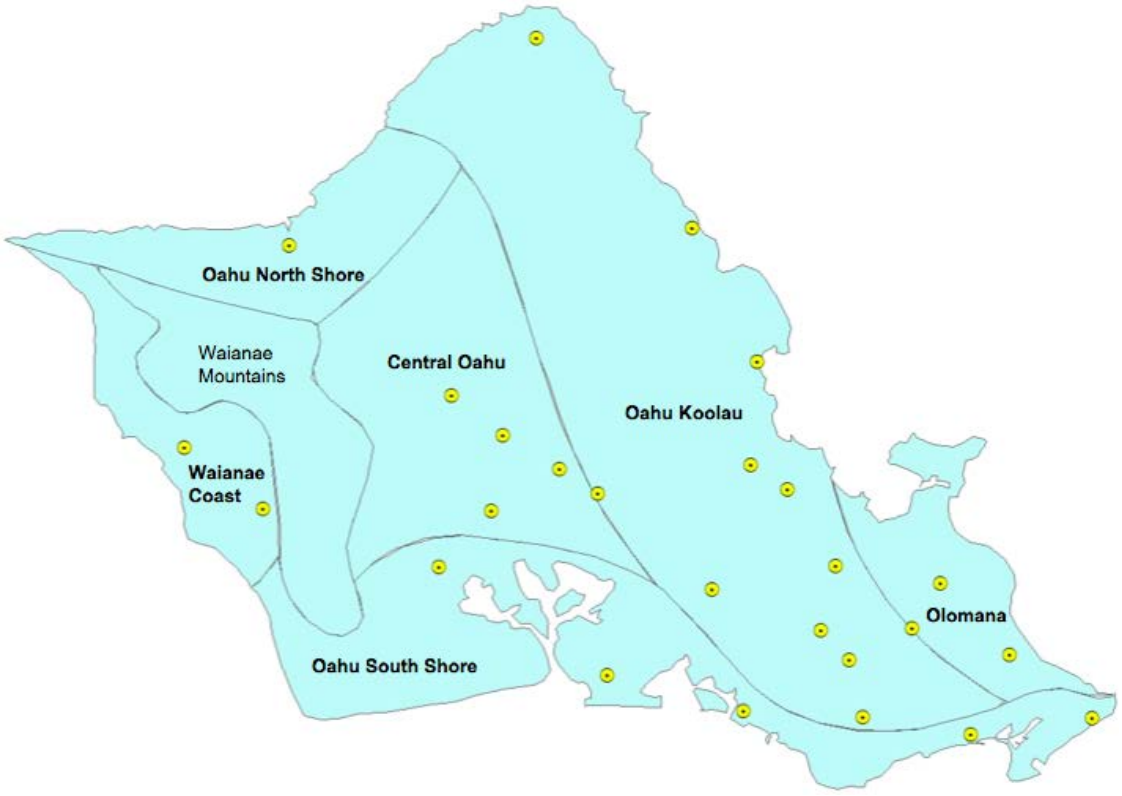

Figure 3. Trap locations on Oahu island (1990-2003). Trap sites were in the vicinity of 26 Hydronet rainfall gauge stations across six forecast areas (bold).

Table 1. Spatial and temporal distribution of animals tested for *Leptospira* infection on three Hawaiian islands.

	Mouse (*Mus musculus*)		Mongoose (*Herpestus auropunctatus*)		Rat (*Rattus exulans, rattus, norvegicus*)	
	N	n (%)	N	n (%)	N	n (%)
Leptospira infected	3171	740 (23.3)	4405	811 (18.4)	7595	1215 (16.0)
Trap Month						
Jan	126	24 (19.0)	143	28 (19.6)	433	63 (14.5)
Feb	243	93 (38.3)	344	75 (21.8)	840	154 (18.3)
Mar	263	96 (36.5)	379	116 (30.6)	853	141 (16.5)
April	324	69 (21.3)	445	144 (32.4)	868	164 (18.9)
May	380	98 (25.8)	367	62 (16.9)	807	110 (13.6)
Jun	169	36 (21.3)	303	56 (18.5)	455	70 (15.4)
Jul	284	69 (24.3)	384	65 (16.9)	667	110 (16.5)
Aug	352	57 (16.2)	370	41 (11.1)	623	73 (11.7)
Sept	349	79 (22.6)	515	79 (15.3)	546	78 (14.3)
Oct	359	63 (17.5)	573	82 (14.3)	633	121 (19.1)
Nov	158	25 (15.8)	282	36 (12.8)	424	58 (13.7)
Dec	164	31 (18.9)	300	27 (9.0)	446	73 (16.4)
Season						
Summer	1534	339 (22.1)	1939	303 (15.6)	3098	441 (14.2)
Winter	1637	401 (24.5)	2466	508 (20.6)	4497	774 (17.2)
Forecast Area						
Hawaii						
Kohala	1166	56 (4.8)	2	0 (0)	21	0 (0)
Kona	19	4 (21.1)	1146	38 (3.3)	575	60 (10.4)
North&East	964	536 (55.6)	1638	535 (32.7)	2405	761 (31.6)
Kauai						
Leeward	124	4 (3.2)	0	0 (0)	172	3 (1.7)
Windward	611	78 (12.8)	0	0 (0)	1406	153 (10.9)
Oahu						

Central	56	15 (26.8)	319	55 (17.2)	439	24 (5.5)
Koolau	98	25 (25.5)	670	127 (19.0)	1598	158 (9.9)
North Shore	6	2 (33.3)	56	9 (16.1)	120	13 (10.8)
Olomana	46	12 (26.1)	134	16 (11.9)	375	27 (7.2)
South Shore	46	5 (10.9)	369	29 (7.9)	418	13 (3.1)
Waianae Coast	35	3 (8.6)	71	2 (2.8)	66	3 (4.5)

Number of sampled animals (N), number of *Leptospira* positive individuals (n) & prevalence (%) are indicated

Table 2. Evaluation of candidate logistic regression models via the Hosmer-Lemeshow goodness-of-fit test.

		Station		Forecast Area		Island	
		χ^2	P	χ^2	P	χ^2	P
Month	Mongoose (df = 8)	4.2058	0.8381*	9.3522	0.3135*	42.6257	<0.0001
	Mice (df = 8)	18.8311	0.0158	21.4366	0.0061	89.4451	<0.0001
	Rats (df = 8)	9.6569	0.2899*	9.5464	0.2983*	39.4897	<0.0001
Season	Mongoose (df = 8)	8.0962	0.4241*	18.783	0.0161	85.7145	<0.0001
	Mice (df = 8)	13.9315	0.0836*	27.5173	0.0006	31.4989	0.0001
	Rats (df = 8)	5.9213	0.6560*	27.5173	0.0006	37.4871	<0.0001
Year	Mongoose (df = 8)	11.9968	0.1513*	23.6978	0.0026	40.5129	<0.0001
	Mice (df = 8)	36.8699	<0.0001	1.9059	0.0051	43.8459	<0.0001
	Rats (df = 8)	23.1938	0.0031	29.4856	0.0003	15.8267	0.0449

*P-values > 0.05 indicate no significant difference between the observed and expected rates of leptospiral infection proportions in animals, and signifies high goodness-of-fit.

CHAPTER V

CONCLUSIONS

Dissertation Merits

The impetus for the research in this dissertation is based on previously developed working ecological models that use leptospirosis in Hawaii to illustrate transdisciplinary approaches to understanding the biocomplexity of emerging infectious disease systems (Kaneshiro et al 2005, Vinetz et al 2005, Wilcox and Colwell 2005). The fundamental achievement of the body of research in this dissertation has been to shed light on leptospiral pathogen transmission dynamics. The main findings follow up on some of the key recommendations previously suggested (Vinetz et al 2005) for leptospiral studies in Hawaii by: addressing ecological and evolutionary patterns of host specificity, providing a preliminary examination of leptospiral genetic diversity in host vectors, and showing that precipitation is an environmental driver of host infection prevalence at specific spatial and temporal scales. These results lay the foundation for a more integrated eco-evolutionary understanding of leptospirosis in Hawaii.

The Hawaii *Leptospira* Study System

Recent leptospirosis research has centered on the ecology of this disease in tropical regions where human incidence is highest (Levett 2001) with a number of empirical studies focused specifically on island ecosystems (Derne et al 2011, Desvars et al 2011a, Desvars et al 2011b, Desvars et al 2012, Foronda et al 2011, Lagadec et al

2012, Lau et al 2010a, Lau et al 2012a, Lau et al 2012b, Lau et al 2012c, Perez et al 2011, Wong et al 2012). As reviewed in Derne et al (2012), tropical islands are a productive setting for leptospiral ecology studies because: each island represents a discrete ecological unit, tropical latitudes have increased risk for leptospirosis as compared with temperate ones, and many islands are those of developing nations with more limited infrastructure in terms of public works and public health. This further contributes to increased risks of disease outbreaks.

The Hawaii Archipelago in particular has additional regional characteristics specifically favorable to eco-evolutionary investigations of pathogenic leptospires that have conservation and public health significance. Hawaii's island ecosystems are hierarchically organized based on naturally replicated units (e.g., islands, ahupua'a or watersheds, and watershed sub-basins) of biological and sociocultural significance (Kaneshiro et al 2005). Hawaii's rodent fauna comprises just four species (e.g., *R. norvegicus*, *R. rattus*, *R. exulans*, and *M. musculus*) that can occur sympatrically with other known *Leptospira* animal reservoirs under certain habitat conditions or in various mixed communities. Hawaii's few native mammalian species are endemic and furthermore, are known to be susceptible (e.g., Hawaiian Monk Seal; Aguirre et al 2007, Poet et al 1993) or are likely to be susceptible (e.g., Hawaiian Hoary Bat) to leptospiral infection. Lastly, Hawaii has consistently held the highest mean annual incidence rate for human leptospirosis in the U.S. (i.e., 1.63 per 100,000 population; Katz et al 2011), and remains a good comparative case study with other developed Asia Pacific regions since

rates in Hawaii are considered relatively moderate and large-scale outbreaks are rare despite sharing many of the same risk factors (Victoriano et al 2009).

Recommendations for future studies of *Leptospira* in Hawaii

The Hawaii study system represents an exceptional opportunity to investigate relevant hypotheses involving the ecology and evolution of *Leptospira* that have been previously suggested (Kaneshiro et al 2005, Vinetz et al 2005, Wilcox and Colwell 2005).

Host-Serogroup Dynamics

The groundwork laid by the epizootiological study in this dissertation (i.e., Chapter Two) should be continued with routine surveillance and extended to include more islands and other reservoir host species. Longitudinal epidemiological datasets such as the one used in this dissertation are valuable for tracking seroprevalence changes that are indicative of evolutionary changes in pathogen-host dynamics (Desvars et al 2011). The recent confirmation of mongoose on the island of Kauai (KHON2 2012) provides an opportunity to document the spread of pathogenic *Leptospira* in a novel host species and to study its impact on pathogen community dynamics in a replicate island system. The establishment of a stable mongoose population on the island of Kauai is particularly interesting in light of this dissertation's findings that the Sejroe serogroup is absent amongst the other main reservoir hosts of leptospires on the island, and would provide an extraordinary opportunity to test the hypothesis that mongooses are the sole

primary maintenance hosts for Sejroe in the state. Other than the three islands central to this dissertation (e.g., Hawai'i, Kaua'i, O'ahu) and the remote Northwestern Hawaiian Islands (Aguirre et al 2007, Poet et al 1993), very little is known about pathogenic leptospires on the remaining main islands (e.g., Maui, Lana'i, Moloka'i, Ni'ihau, Kaho'olawe) and minor islets of Hawaii. Nothing is known about leptospiral infections in any of the feral domesticates (e.g., pigs, goats, cattle) and wild mammalian species (e.g., axis and Columbian black-tailed deer, European and Armenian mouflon sheep, rock wallabies) that represent sizable reservoir populations whose range overlaps human settlement in both rural and urban areas of Hawaii.

The Hawaii *Leptospira* animal infection data set utilized in two of the three studies in this dissertation was collected with much painstaking labor by the State Department of Health Vector Control Branch over the course of more than 14 years and across three main islands. It is a feat that in light of current state finances and department interests is unlikely to be continued or replicated in the near future. This is unfortunate because a systematic statewide surveillance program specific for zoonoses is critical not only to monitor epizootiologic changes in animal host populations and infection prevalences but also for the timely detection of emerging infectious pathogens of public health and veterinary interest. A passive surveillance system that networks regional veterinary clinical data similar to the national Disease Watchdog program was recently implemented in Australia (Ward and Kelman 2011) with relevant national and state control programs. A similar program is much needed in Hawaii as a major U.S. portal of Asia Pacific commerce and tourism.

Molecular Characterization of Leptospira in Hawaii

A comprehensive multi-faceted research program that incorporates animal, human, and environmental components of the leptospirosis disease system has not been attempted in Hawaii. However, this approach has been successful in the Peruvian Amazon (Matthias et al 2005, Segura et al 2005, Ganoza et al 2006, Matthias et al 2008), American Samoa (Lau et al 2012a, 2012b, 2012c) and islands of the Indian Ocean (Desvars et al 2011a, 2012).

Chapter Three of this dissertation provides the beginning of a molecular characterization of leptospiral diversity in Hawaii that is phylogenetically useful. Phylogenetically informative molecular data such as 16S ribosomal RNA gene sequences not only provides the capability to discriminate amongst leptospiral types at high resolutions and facilitates rapid identification with ease relative to other *Leptospira* classification methods. It also allows for a uniform comparison of human, animal, and environmental isolates that is the basis for elucidation of leptospiral transmission dynamics (Vinetz et al 2005). Information regarding the molecular genetic relatedness of leptospiral types further enables testing of evolutionary hypotheses regarding Hawaii leptospires and the historical spread of this most widely distributed zoonosis.

Evolution of Optimal Virulence in Leptospira

Ebert and Bull (2008) describe the evolution of pathogen virulence as a progression that correlates with three phases of infectious disease emergence: 1.

Accidental infection in a new host; 2. Successful invasion; and 3. Establishment. While the virulence level of an emerging pathogen in phase 1 cannot be predicted, phase 2 virulence is theorized to change rapidly in either direction along the spectrum of avirulence or high virulence, while an established pathogen in phase 3 has achieved an equilibrium level of virulence that is optimized for maximal host transmission.

The large-scale longitudinal dataset employed to characterize host specificity patterns (i.e., Chapter Two) and elucidate the effects of rainfall on transmission dynamics (i.e., Chapter Four) can also be utilized to address questions of pathogen virulence. Specifically: Which stage of virulence evolution is *Leptospira* in Hawaii? Are all four serogroups at the same stage, or are different serogroups in different stages? Are the evolutionary patterns of serogroup virulence the same across the three main islands?

Chapter Two findings of host specificity patterns indicate that the leptospiral serogroups in Hawaii are likely to have progressed beyond phase 1. Ebert and Bull (2008) note that accidental infections are more likely when the reservoir host and the novel host are phylogenetically related. Based on serogroup distributions by host animal species in Hawaii, serogroup Ballum appears to have spilled over from its primary reservoir host, mice, to predominantly the three rat species, and secondarily to the more remotely related mongoose. Conversely, serogroup Sejroe appears to not have gone through much host switching and remains primarily associated with mongoose only.

Changes in the incidence of infection can signal the stage to which a parasite has progressed. According to the Ebert and Bull (2008) model, a hallmark of phase 2 is an epidemic phase of rapidly increasing incidence, followed by an endemic phase in which

the number of infected hosts reaches a plateau and the parasite is considered to have entered phase 3.

Chapter Two in this dissertation did not find significant changes in serogroup prevalence across years, but we did not test for differences in host associations across time. Temporal trends in serogroup prevalence by host species may give insight into the evolution of leptospiral virulence in Hawaii.

Transmission Dynamics

Despite the successful implementation of multiple integrated research programs in disparate locales, no study has yet explicitly addressed the relative roles of environmental versus direct animal transmission of the pathogen within maintenance and accidental host populations, which is central to an understanding of the problem of leptospirosis emergence globally. Taken together, Ganoza et al's (2006) and Matthias et al's (2005) studies of leptospires from humans, rodents, and environmental waters in the Peruvian Amazon indicate that, as would be expected, leptospiral diversity appears to be greater in the environment than within animals. However, Matthias et al (2005) found a novel clade of pathogenic bat leptospires not described in Ganoza et al's (2006) investigation of stream and standing water in the same city. If maintenance hosts not only multiply pathogenic leptospires but also act as filters of leptospiral diversity, it is likely that some host species are more efficient transmitters of leptospiral types that confer high virulence and some host species are better at transmitting leptospires of more intermediate virulence – in which case the latter of the two types of animal carriers might arguably be

considered more protective of public and veterinary health. Given the narrow range of potential maintenance host species in Hawaii relative to many other tropical regions enzootic for leptospires, Hawaii remains a particularly appropriate locale for clarifying the relative roles of the two routes of leptospiral pathogen transmission.

Anthropogenic Landscape Alterations

Kaneshiro et al (2005) describe an ecosystem resilience model in which leptospirosis is an example of how the health of the mountain-to-sea ecosystems intrinsic to Hawaii is linked with human health. The findings of Chapter Four support the model's underlying hypothesis that host infection prevalence is a function of environmental change such as rainfall variability. Future ecological studies of leptospirosis in Hawaii should investigate whether increased human disease risk is associated with urbanized development of natural drainage basins or other anthropogenic landscape-level factors. If so, these future findings would be in agreement with predictions of the ecosystem resilience model (Kaneshiro et al 2005).

Climate Change

Chapter Four also provides a historical backdrop against which future changes in precipitation regimes across the state might be readily recognized. As reviewed by Weltzin et al (2003), global warming is predicted to result in increased precipitation in the tropics over this century, with greater intensity and higher frequency of extreme weather events occurring in general around the world (Easterling et al 2000), and in

particular small islands (Mimura et al 2007). Small tropical islands are predicted by Lau et al (2010) to be the most vulnerable to leptospirosis outbreaks due to global warming because of a number of risk factors including small land mass, isolation, limited natural resources, fragile ecosystems, and high human population density. Changes in climatic and atmospheric trends and their impact on ecosystem processes via effects on species distribution and diversity, population structure, and community composition can only be detected with long-term monitoring (Weltzin et al 2003).

Besides long-term observations of community change, Weltzin et al (2003) recommend 'focused gradient studies' as an additional method of studying the role of stochastic precipitation patterns in shaping communities and ecosystems, and thereby predicting the effects of large-scale anthropogenic changes in abiotic environmental factors. As applied to disease systems, these cross-site comparisons along gradients of precipitation intensity and frequency allow for elucidation of the linkages between variability in precipitation. Chapter Four findings indicate that the spatio-temporal scales of relevance for the effects of rainfall patterns on leptospiral animal infection prevalence in Hawaii are primarily at the forecast area-month and station-season levels. Thus investigations across multiple stations or forecast areas of variable monthly and seasonal rainfall are anticipated to yield fruitful insights into the relative contributions of transmission rates, host population abundances, and host community compositions towards determining host animal leptospiral infection prevalence in Hawaii.

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