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INVESTIGATION OF WESTERN BURROWING OWLS (*ATHENE CUNICULARIA HYPUGAEA*) AS HOSTS FOR FLEAS INFECTED WITH *YERSINIA PESTIS*

by

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Abstract

INVESTIGATION OF WESTERN BURROWING OWLS (ATHENE CUNICULARIA HYPUGAEA) AS HOSTS FOR FLEAS INFECTED WITH YERSINIA PESTIS

Thesis Abstract – Idaho State University (2015)

This thesis describes a portion of the work completed as part of a collaborative research project. We investigated the potential of Western Burrowing Owls (*Athene cunicularia hypugaea*) to serve as phoretic hosts for plague in the Pacific Northwest (ID, WA, OR, CO). We collected fleas from Western Burrowing Owls, taxonomically identified them, and tested them for *Yersinia pestis.* Blood samples from the Burrowing Owls were collected to test for exposure to *Y. pestis. Pulex irritans* represented 99.4% of the fleas collected. *Y. pestis* was not detected in the fleas and no evidence of plague infection was detected in the blood samples. We conclude that the potential for Western Burrowing Owls to serve as phoretic hosts to plague exists, but is not being realized at this time. We suggest that further studies of this potential involvement in areas experiencing plague epizootics are needed.

Chapter I: Introduction

Statement of Purpose

This thesis describes a portion of the work completed as part of a collaborative research project funded by the United States Fish and Wildlife Service (USFWS) Avian Health and Disease Monitoring Project.[1] In this project we investigated the potential of Western Burrowing Owls (*Athene cunicularia hypugaea*), to serve as phoretic hosts for plague in the Pacific Northwest (ID, WA, OR, CO). The project goals were to identify the species of fleas infesting Western Burrowing Owls, determine if the fleas harbored *Yersinia pestis*, and test whether the Burrowing Owls had current or past exposure to *Y. pestis.* This thesis describes the preliminary flea identification, *Y. pestis* culture results, and nucleic acid extraction procedures utilized to support the research questions in the study.

Hypothesis

Plague is a zoonotic bacterial disease caused by *Yersinia pestis*, and the source of three pandemics to date, with a death toll estimated as high as 200 million people.[2] *Y. pestis* is endemic in multiple areas of the world today, including the western United States.[3] Although human infections continue, plague is mainly a disease of wild rodents and their fleas.[4] The rodents involved in persistence of plague can be divided into two categories: susceptible (or epizootic) hosts, or non-susceptible (or enzootic) hosts.[5] In the United States, both types of hosts to plague are represented in numerous rodents and small mammals, including rats, prairie dogs, ground squirrels, mice and rabbits.[6] Enzootic hosts represent the primary explanation for plague's continued endemicity. Since they are not killed by the bacterium, these hosts provide for low-level sustained disease

transmission.[2] Plague may exist in this silent state, during which no detectable animal die-offs occur, for years at a time.[7] When plague is introduced to a susceptible host population through contact with an enzootic host or its infected fleas, epizootics (large scale animal die-offs) can occur.[7] These epizootic hosts provide amplification of the bacterium as they develop septicemia and succumb to the infection. Because of the high bacterial loads present in these hosts, fleas feeding on them are more likely to become infectious as a result, and to begin seeking other food sources when their natural host population has died. Rodent epizootics continue to be a public health concern today, due to the fact that the majority of human plague cases have been associated with them.[3,8,9] Therefore, continual surveillance of plague in endemic areas is emphasized by public health authorities in order to decrease the risk of human infection and the impact on wildlife.[3] Surveillance information is used, for example, to determine human activities that increase the risk of exposure, and the most appropriate control or prevention measures for a given focus, if they are to be used.[3] The most effective application of this information depends on the identification of the pertinent host and vector species in endemic areas.

The Western Burrowing Owl (hereafter, burrowing owl), is a small ground-dwelling owl found in desert or low vegetation habitats throughout western North America, including areas with endemic plague.[10] Burrowing owls forage for and prey on arthropods, small rodents, birds, amphibians and reptiles.[11] They prefer to nest in burrows dug by prairie dogs (*Cynomys* species), badgers (*Taxidea* species), or other fossorial mammals, rather than dig their own.[11] In the United States, prairie dog territory overlaps much of the burrowing owls' habitat in the Midwestern range, but does not extend into the western-

most states. This is notable because the burrowing owls residing in areas with smaller prairie dog populations, and therefore available burrows, such as in Idaho, Oregon and Washington, tend then to occupy more burrows fashioned by badgers, which are found throughout Western burrowing owl habitat.[1,11] Both prairie dogs and badgers have been associated with plague, and both carry fleas. Prairie dogs are highly susceptible to plague and suffer mortality rates of 85 to 100%.[12,13] Badgers, however, do seroconvert but are rarely known to die from the infection, which is thought to be acquired from consumption of infected prey or bites from their (infectious) fleas.[14–16] An earlier study of badgers in the Snake River Birds of Prey National Conservation Area (SRBPNCA) in southwestern Idaho reported finding 86% of those sampled as seropositive for plague.[15] This means that even though a burrowing owl may nest in a burrow produced by a badger instead of a prairie dog, it is still potentially at risk for exposure to plague-infected fleas.

Belthoff and Smith observed that burrowing owls in the SRBPNCA predominantly carried a species of flea known to be capable of carrying plague: *Pulex irritans*.[17] Also called the "human flea," *P. irritans* can be found throughout most of the world, and besides humans, has a wide mammalian host range.[12] These fleas have also been collected, for example, from prairie dogs in Montana and from various carnivores.[12,18,19] *P. irritans* has also recently been studied as a potential human plague vector. The Lushuto District in Tanzania is a long-standing plague focus where *P. irritans* represented 72.4% of fleas collected from human dwellings during a study in 2005 and 2006.[20] Laudisoit et al reported that statistical analysis of the data from this study found the density of *P. irritans* to be strongly positively correlated with plague frequency ($F= 14.08$, $p=0.003$).[20] Following a small suspected bubonic plague outbreak in Madagascar

during January 2013, *P. irritans* was the predominant flea collected (73%) from that focus, and the only species of the five recovered that was found positive for *Y. pestis* DNA by polymerase chain reaction (PCR).[21] In fact, due to its association with humans and ability to thrive in cooler climates, *P. irritans* is now suspected as being the principal flea vector during the pandemics in northern Europe.[12]

Because of their habit of borrowing burrows from plague-associated mammals, and the populations of fleas they have been found to harbor, we hypothesized that burrowing owls could function as phoretic hosts to plague.[22] Birds are not widely known to be susceptible to plague, but the possibility of burrowing owls carrying plague has not been investigated previously.[23] Studies suggest that burrowing owls and other mammalian predators may temporarily acquire and transport plague-infected fleas, but the actual participation of burrowing owls, specifically in areas with low prairie dog populations, has not been determined. [1,24,25]

Description of Project

Researchers captured and released burrowing owls residing in natural and artificial burrows in five western states (Idaho, Oregon, Washington, Colorado and South Dakota) over the summers of 2012 and 2013. Fleas from burrowing owls in Idaho, Oregon, Washington and Colorado were collected in order to identify their species and to determine whether or not those fleas were harboring *Y. pestis*. (No fleas were found on burrowing owls captured in South Dakota.) We enumerated, identified and tested the fleas for presence of *Y. pestis* by culture and PCR. Researchers also collected owl blood samples in order to investigate plague infection in the owls. Serologic testing for antibodies against *Y. pestis* was conducted to determine if the owls had been exposed to plague. Additionally, in order to determine whether the burrowing owls had current

plague infections, the blood samples were tested for the presence of *Y. pestis* DNA by PCR.

Significance

This study was significant for several reasons. First, because burrowing owls are listed by the USFWS as a National Bird of Conservation Concern, it is important to determine if plague may be a contributor to their decline.[1,11] Second, persistence in enzootic animal reservoirs and their fleas is thought to be the main way that *Y. pestis* manages its "dormant" status in between epizootics, but the dynamics of these relationships are not well known. Over 200 hundred species of rodents and small mammals worldwide have been identified as plague reservoirs.[12] Therefore, evidence of an avian species serving in that capacity would impact current surveillance strategies. The species of fleas involved in plague transmission are important to identify not only because of their efficiency in transmission, but also because the period of time that a particular flea species is capable of transmitting plague has been found to vary.[26,27] If they are found to be involved in plague ecology, we suggest that fleas collected from burrowing owls could be useful to include in flea surveillance activities, which are typically limited to rodent fleas.[1] Additionally, if burrowing owls were found to be phoretically hosting plague, the suspicions of their involvement in the appearance of plague in new areas, or its re-emergence in old areas, could be validated.

The third area lies in the history of, and future potential for, plague's impact on humans. In the four states from which fleas were collected during this study, plague is endemic and continues to be a concern for both public and wildlife health.[28] This study may have implications to USFWS personnel who handle burrowing owls in field work, since they would then need to incorporate additional use of personal protective equipment (PPE) to

guard against infection, in the case that plague was found. Burrowing owls also often choose burrow sites on the edges of agricultural fields (as Figure 1, below, shows), dairies, or other areas that are kept mostly free of vegetation. If burrowing owls were found to be harboring plague, humans working in those areas could be at increased risk for exposure.

Figure 1. Burrowing owl family clustered near nest site located on edge of an agricultural field in Mountain Home, Idaho. Photo courtesy of Terry Ketterling. Used with permission.

The Centers for Disease Control and Prevention (CDC) reports that there are between 1000 and 2000 human plague cases reported worldwide each year, meaning that plague remains an international public health concern.[28] During the last several decades, the US has reported an average of seven human plague cases per year.[28] Therefore, plague also impacts those in the health care professions, including physicians, nurses, and medical laboratory scientists, who perform initial patient testing. Gage asserts the importance of

continual consideration of plague by health care professionals in their application of the differential diagnosis.[3] This is particularly important in areas where plague is endemic, but no less so in other areas, because imported human cases have occurred.[29] In the United States, the region including New Mexico, northeastern Arizona, southern Utah and southern Colorado is the most prominent plague focus today. A second smaller focus exists around the area encompassing southern Oregon, northern California and northwestern Nevada.[28]

Chapter II: Background

History of *Y. pestis*

The first plague pandemic, often referred to as the Justinian pandemic, originated in Ethiopia (Africa) and arrived in Pelusium, Egypt in 540 A.D.[2,30] It continued to spread: to Constantinople in 541 A.D., and on to the Middle East, Italy, southern France, Denmark, Ireland and the Mediterranean.[30] This pandemic is considered to have ended in 700 A.D., after plague swept through Europe, Asia and Arabia in ten successive epidemics.[2] Although it has been suggested that this pandemic caused more than one million deaths, Perry and Featherston note that while there was a population loss of 50 to 60% during that time, it "was not solely due to plague, since other epidemics such as smallpox probably occurred during this period."[2,6,30]

The second plague pandemic took the lives of an estimated 50 million Europeans, or a quarter of Europe's population, during the fourteenth and fifteenth centuries. The first epidemic in this period, now known as the "Black Death", is thought responsible for the deaths of 17 to 28 million Europeans alone.[2] The Black Death epidemic began in Sicily in 1347, after the arrival of plague from the Black Sea area, and continued until 1351.[2,30] Smaller-scale plague epidemics continued to add to the death toll afterward, in two- to five- year cycles from 1361 to 1480, affecting all of Europe and reaching to the edges of Great Britain, Scandinavia and Western Russia.[6]

The third pandemic, although declining, is considered as still ongoing.[2] It originated in China around 1855, probably in Yunnan, a southern province of China.[2,6] In 1898, it reached Bombay, and by 1918, more than an estimated 12 million people in India had died.[2] By 1900, plague had spread to Egypt, Portugal, Japan, Paraguay, Eastern Africa, the Philippines, Scotland, and Australia.[6]

In June of 1894, during the Hong Kong epidemic, *Y. pestis* was first "discovered" as the etiological agent of plague. Two independent researchers, Alexander Yersin and Shibasaburo Kitasato, are both credited for the discovery since they published their success in isolating *Y. pestis* only three days apart.[30] Yersin developed an antiserum that cured several plague patients in 1896, and later identified the relationship between plague and rats.[31]

Y. pestis entered the United States when a ship from China that was carrying flea-infested rats docked at San Francisco in 1900.[6] From 1924 to 1925, Los Angeles experienced an urban plague epidemic due to human contact with infected rats and their fleas, but that has been the last such epidemic the US has had to date.[32]

Plague Ecology & Transmission

At least 125 different species of fleas worldwide have been found to be capable of being plague vectors.[12] In North America, twenty-eight species of fleas have successfully transmitted plague, at least in a research setting, so far.[33] The precise mechanisms by which fleas transmit *Y. pestis* to their hosts have not been fully elucidated yet. However, researchers have determined that transmission by both biological means and mechanical means occurs.

Both transmission models begin as a flea feeds off a septicemic rodent, ingesting the plague bacteria along with the blood meal. Bacot and Martin described the biological means of plague transmission, in which a blockage results from multiplication of the bacteria inside the flea's gut.[34] Specifically, the blockage (also described as a biofilm) is formed on the surface of the valve that connects the esophagus and midgut, called the proventriculus.[34] Fleas in which this blockage develops are referred to as "blocked fleas." In subsequent attempts to feed, the bacterial blockage prevents the new blood

meal from reaching the flea's stomach, and the hungry flea exhibits frantic feeding behaviors, which have been observed in the laboratory to include abnormally long periods of sucking and frequent adjustments of location.[35] When the minor location changes fail to yield a meal for the flea, or the host dies from plague, it abandons the host in search of a "better" food source. Several species of fleas have been found to readily seek and bite unnatural hosts after starving for about three days.[36] Once the blocked flea has found a new host, which may not be a natural host, it attempts to feed again, but the bacterial mass continues to block the entrance to the flea's midgut.[12] The new blood meal backs up in the flea's throat, distending the esophagus and washing some loosely-attached plague bacteria off of the mass as it tries to swallow, and upon ceasing its attempt, the flea essentially regurgitates the plague-contaminated blood back into the bite wound.[7] Thus, this model relies on the blockage for infectivity, and explains the biological means by which the new host may become infected.

Until recently, the blockage model dominated literature and was widely accepted, which left alternative transmission routes relatively ignored, despite evidence for their existence. Eskey and Haas found that formation of a blockage after an infectious meal can take from five days up to 130 days, or may not actually occur at all, varying by and depending on flea species.[35] The oriental rat flea, *Xenopsylla cheopsis*, is noted as an incredibly efficient vector of plague compared to other fleas, and is implicated in transmission in many foci worldwide.[7,37] *X. cheopsis* also typically blocks.[27,34,35] *P. irritans*, however, rarely blocks, but is still able to transmit plague.[27,35] Also, Burroughs observed that blocked fleas are able to clear the mass in some instances, and may, after a variable feeding period, block again.[27] Fleas who are unable to clear their blockages do not

usually survive beyond five days, which equates to a very short period of infectivity as explained by this model. Unblocked fleas, however, can live and remain infectious for much longer periods.

Recent research suggests that fleas are capable of infecting a new host nearly immediately after ingesting their first contaminated blood meal, independent of blockage. This model of immediate infectivity is called early-phase transmission (EPT). EPT does not exclude the blockage model of transmission, but offers an explanation for a more rapid rate of disease spread, as was observed during previous pandemics.[37,38]

Because previous studies with an assortment of flea species have noted that blockages are rarely formed earlier than five days after fleas consume infected blood meals, the time frame designated as "early-phase" is typically the four day window immediately following a flea's ingestion of a contaminated meal. [27,35,38] Eisen et al demonstrated that *Oropsylla montana*, a common ground squirrel flea that is rarely blocked, is capable of efficiently infecting naïve mice within 24 hrs or less after an infectious blood meal, and may remain infectious for several weeks.[26,38] The precise mechanism of this mechanical transmission model is still not known, but transfer of *Y. pestis* has been suggested to occur by contaminated flea mouthparts (i.e. the proboscis).

Additional reservoirs

Computer models constructed by Webb et al to study the spread of plague through prairie dog colonies led them to conclude that transmission by vector alone cannot adequately sustain an epizootic, and that "a short-term reservoir is required for epizootic maintenance."[39] Research has established that potential short-term reservoirs present during epizootics include the carcasses of hosts that died from plague, contaminated soil, and other non-susceptible animal species that are nearby.[40,41]

Etiology of Plague

Bacteriological characteristics of *Y. pestis*

Y. pestis is a member of the *Enterobacteriaceae* family. It is gram-negative, non-motile, and does not form spores.[2] On a gram stain or Wright-Giemsa stain, the plague bacteria are small, plump coccobacilli and may display bipolar staining.[6,42] *Y. pestis* is facultative and nonfastidious. It will grow on sheep blood agar (SBA), chocolate agar, and MacConkey agar. *Y. pestis* prefers a lower incubation temperature than most other bacteria, optimally 25- 32°C, which is a difference that can be exploited when trying to isolate it from other species.[6] On SBA, colonies will be pinpoint at 24 hours, but grow to gray-white convex colonies that measure 1-2 mm in diameter upon 48 hours of incubation at room temperature.[6] Older *Y. pestis* colonies have been described as having a "beaten copper" finish, and may resemble "fried eggs."[6,42] On MacConkey agar, *Y. pestis* will grow as small non-lactose-fermenting colonies.[42] Biochemically, *Y. pestis* is oxidase negative, indole negative, urease negative, and catalase positive. It is also OPNG positive and produces an acid slant with little or no change in the butt of TSI agar.[2,6]

Virulence Determinants.

Y. pestis is believed to have originated from the much less-pathogenic *Yersinia pseudotuberculosis*, in Africa between 2000 and 20,000 years ago.[43] *Y. pseudotuberculosis* is also enzootic in many animal species, but human infections are few and usually associated with patients who have underlying conditions.[6] Despite being highly similar to *Y. pseudotuberculosis* genomically, all known biotypes of *Y. pestis* possess three plasmids that encode virulence factors.[2,44]

The plasmid pMT1, also known as pFra, is named for a murine toxin it encodes and measures 100 to 110 kb in size.[2] The murine toxin is thought to be necessary for *Y. pestis'* ability to colonize and survive in fleas.[31,44] pMT1 also contains the gene encoding the fraction 1 (F1) protein capsule or envelope. At 37°C, *Y. pestis* induces production of the F1 protein, which forms a "large gel-like capsule or envelope."[2] Expression of the F1 protein continues during growth restriction phases, but ceases at 22°C, which means it is not produced while *Y. pestis* is flea-bound.[2]

The second plasmid, pPCP1, is 9.5 kb and named for genes encoding pesticin, coagulase, and plasminogen activator.[2] The *pla* (plasminogen activator), *pst* (pesticin), and *pim* (pesticin immunity protein) genes on this plasmid are upregulated at 37°C, the mammalian host body temperature.[44] Pesticin is a bacteriocin that degrades the murein found in the cell walls of many bacterial species, and is suggested to enable *Y. pestis* to eliminate competition by other bacteria for resources in a host post-mortem.[45] Beesley et al demonstrated that no loss of virulence was observed in mutants lacking pesticin, aside from those factors specifically produced by *pla* that were removed also.[46] As indicated in the name, the *pim* gene produces the pesticin immunity protein that protects *Y. pestis* from being destroyed by its own bacteriocin.[44] Plasminogen activator (*pla*) is an outer membrane protease that triggers plasminogen activation in a mammalian host, thereby inducing fibrin degradation. Plasminogen activator also cleaves C3, a component of the host complement system, and degrades several *Yersinia* outer proteins (Yops).[2] This is believed to prevent the host's immune system from sequestering the infection. This plasmid is often referred to as pPst in other strains.[2]

The third plasmid, pCD1, is a 70- to 75-kb virulence plasmid named for genes concerning calcium dependence.[2] This plasmid is also found in *Y. pseudotuberculosis* and *Y. entercolitica*, where it is referred to as pCad or pYV.[2] pCD1 contains the low-calcium response stimulon (LCRS), which includes genes that encode V antigen (*LcrV*), Yops, and elements that regulate their expression.[2] Expression of the LCRS operon is stimulated by low calcium levels, and is also significantly up-regulated in response to a 37°C temperature.[2] *LcrV* provides negative feedback regulation to the LCRS operon, which inhibits transcription. *LcrV* also induces Interleukin-10 secretion in the host, which inhibits cytokine production by macrophages, dampening the host's immune response to the invading bacteria.[2,47]

Clinical disease presentation

Three main clinical presentations of plague, which are based on the route of exposure, are recognized.[48] Bubonic plague, named for the characteristic development of buboes (infected lymph nodes) in victims, represents 80 to 95% of worldwide cases today.[28] Septicemic and pneumonic plague are the second and third most common presentations. Far less commonly, plague may manifest in a meningeal (usually in children), pharyngeal (through ingestion; appears like tonsillitis), or in an abortive or asymptomatic form (as result of existing antibiotic regimens or high antibody titers).

Bubonic plague

Bubonic plague develops from the bite of an infected flea and causes a small ulcer or pustule at the site, which depending on its size, may not be noticed by the patient. The sudden onset of initial symptoms occurs two to six days after the flea bite, and is characterized by fever, malaise, shaking chills, headache, weakness, and development of buboes (painful swollen lymph nodes).[48,49]

The buboes develop as *Y. pestis* cells migrate from the bite site to the closest cervical, axillary or inguinal lymph node, where they proliferate rapidly to cause inflammation and necrosis. The buboes eventually blacken (which helped earn plague the "Black Death" nickname) due to hemorrhaging. Without effective antibiotic treatment, the bacteria can escape from the lymph nodes into the bloodstream, producing rapidly fatal septicemia in 50-75% of patients, or pneumonic plague when it reaches the lungs, in about 5% of cases.[50]

Pneumonic plague

The primary route by which pneumonic plague, the most severe clinical form, is acquired is through inhalation of aerosolized infectious droplets.[48] Sources of contaminated respiratory droplets include humans and household pets who have pneumonic plague, aerosol-producing laboratory procedures, or purposeful release as in a bioterrorist attack.[32]

The incubation period for primary pneumonic plague ranges from one to three days.[48] Initial symptoms include fever, malaise and a feeling of tightness in the chest.[50] Without immediate treatment, the disease progresses quickly, indicated by onset of dypsnea, cough, hemoptysis, chest pain, and terminal cyanosis.[48,50] Untreated pneumonic plague is fatal, often within two to three days after development of initial symptoms.[50]

Septicemic plague

Septicemic plague can occur from direct seeding of the blood through a flea bite or by direct contact with broken skin, or secondary to bubonic or pneumonic plague as bacteria are released into the bloodstream from the lymph nodes or lungs.[6] Septicemic plague manifests with fever, chills, and extreme weakness, with the addition of tachycardia and

hypotension (in septic shock).[48] Abdominal pain is also most commonly reported with this presentation.[48] Extensive hemorrhaging can cause skin and affected tissues to turn black and die. Development of overwhelming sepsis leads to disseminated intravascular coagulation (DIC), multiple organ failure and adult respiratory distress syndrome.

Laboratory Diagnosis

Samples from plague patients typically submitted to clinical laboratories may include peripheral blood, sputum, blood cultures, and bubo aspirates. Peripheral blood samples from plague patients typically display white blood cell counts of 10,000-25,000 cells/mm^3, with notable neutrophilia marked by immature cells. Leukemoid reactions ($\geq$50,000 cells/mm^3) occasionally occur. Platelet counts may be normal to low in early stages, but drop to thrombocytopenic levels in septic patients.[51] *Y. pestis* can often be seen in peripheral smears made from septic patients' blood, due to such intense bacteremia, and is likely to be recovered from blood cultures also. In advanced stages of plague, laboratorians will observe abnormal results correlating with impaired liver and renal function and development of DIC.[50]

Isolation of *Y. pestis* through the culturing of patient samples is necessary for confirmatory testing, as well as antimicrobial resistance testing. Because of its status as a potential agent of bioterrorism, and the dangers of handling *Y. pestis*, the American Society of Microbiology (ASM) and the Centers for Disease Control & Prevention (CDC) urge clinical laboratories to proceed with work on any isolate they suspect is *Y. pestis* no further than the point at which they cannot rule it out.[42,52] The CDC has established a national program, the Laboratory Response Network (LRN), that designates clinical laboratories as "sentinel laboratories," which represent sites at which patients

would first present.[53] These sentinel laboratories use standard laboratory tests (gram stain, colony morphology, catalase, urease, oxidase, etc) in an attempt to rule *Y. pestis* out as the probable identification of an isolate.[42] Upon being unable to rule-out an isolate, the sentinel laboratory is instructed to contact their assigned referral (or LRN) laboratory, which is often the public health laboratory. The LRN labs are capable of rapid, standardized, and more advanced testing, and also attempt to rule out the suspicious isolates. In the event that a presumptive identification of *Y. pestis* is made, the isolate will be forwarded to the CDC for final confirmation, and an epidemiological (and/or criminal) investigation of the case is launched.

Use of an automated identification system in cases where *Y. pestis* has not been ruled out is discouraged for several reasons. First, the danger of aerosol production during processing by an automated system creates a risk for laboratory personnel, and second, because *Y. pestis* is known for being falsely reported by these systems as *Shigella*, *Y. pseudotuberculosis*, H_2S-negative *Salmonella* or *Acinetobacter*.[42] False identification of *Y. pestis* creates opportunity for an incorrect or delayed patient diagnosis, which is one of the top causes for death due to plague in the US. For these reasons, clinical microbiologists must remain alert and careful to consider their automated identification results in light of the isolate's colony morphology, gram stain results, and the patient's clinical condition and case history. In Idaho, training on the identification and proper handling of suspected *Y. pestis* isolates is provided to clinical laboratorians by the Idaho Bureau of Laboratories, in conjunction with the CDC.

Treatment

Y. pestis is susceptible to modern antibiotics, but success of treatment depends on prompt diagnosis and administration. If treated with antibiotics early, most bubonic plague

victims recover. The mortality rate with treatment is 4-15%, but increases to 30-75% without treatment. Research suggests that for septicemic and pneumonic plague cases, antibiotic treatment must begin no later than 12 hours after fever onset to be successful. Thus, rapid and accurate diagnosis of plague infection, particularly in pneumonic and septicemic cases, is crucial to patient survival.

Streptomycin and gentamicin are the primary drugs of choice, but tetracycline (not recommended for children), doxycycline, chloramphenicol, and levofloxacin have also shown efficacy.[51] Doxycycline and levofloxacin are recommended for prophylactic use, and chloramphenicol is specifically recommended for treatment of meningitis.[2,51] Confirmatory lab specimens, blood cultures and lymph node specimens should be collected, if possible, before beginning treatment.

Chapter III: Materials and Methods

Flea collection

Researchers collected fleas from individual nests of burrowing owls (method not described here) in Idaho, Oregon, Washington, and Colorado, and placed them in Ziploc bags or scintillation vials labeled by nest site.[22] The fleas were kept on ice during transport to the laboratory, where they were stored at -20°C until they could be examined and processed.

Identification

Under a dissecting microscope, we enumerated, sexed, and taxonomically identified the fleas, using a key (order *Siphonaptera*).[54] Nearly all of them keyed as *Pulex* species (see results). The morphological features that distinguish a male *Pulex irritans* from a male *Pulex simulans* could not be visualized under these conditions, and no distinguishing features are known to exist between the two species in females, so we designated a small number of fleas from each nest site to undergo clearing and mounting (for males) and molecular analysis (for females) in order to complete the identification.[22,54]

For sites in which 50 or less fleas were recovered, we reserved ten male *Pulex* fleas, photographed and cut them in half, approximately at the top of the abdomen. We placed the front half of the flea individually in a labeled 1.5 mL screw-cap vial to be triturated and cultured, and preserved the rear half of the flea individually in 70% ethanol for subsequent clearing and final identification.[22] Ten representative female *Pulex* fleas were also randomly selected for final identification and placed into individual labeled vials for trituration and subsequent nucleic acid extraction. We pooled the remaining fleas by species in 1.5 mL screw-cap vials.

For nest sites from which more than 50 fleas were recovered, we preserved ten male and ten female *Pulex* fleas in 70% ethanol for final identification. The remaining fleas were pooled by species in a 1.5 mL screw-cap vial for trituration.

Trituration

To promote trituration of the flea tissue, we added two sterile glass beads to each vial of fleas. We adapted a trituration method described by Gabitzsch et al, and added sterile horse infusion broth (HIB) with 20% glycerol: 100 µL to vials containing less than 10 fleas, or 10 µL per flea, up to 500 µL, to vials containing 10 - 200 fleas.[55] Pools containing more than 200 fleas were split into two smaller aliquots and processed accordingly. We triturated the pooled fleas using a mini bead-beater: 30 -45 seconds on medium-high speed for vials containing less than 10 fleas, or 1-3 min on the highest speed setting for vials containing more than 10 fleas.

For nest sites containing less than 50 fleas, we removed 50 µL of triturate from each individually processed flea and combined it with the pooled triturate for that nest site prior to culturing. The remaining volumes of the individual triturates were frozen at -20°C.

Culture

A 20 µL aliquot from each freshly-triturated pool was inoculated onto sheep blood agar and the plates were incubated at 28°C for 48-72 hr. We then visually examined them for colonies displaying morphological features suggestive of *Y. pestis.* We re-plated triturates from which significant, obscuring background growth was recovered using a 1:1000 dilution. We also plated *Y. pestis* (strain A1122) on SBA and incubated it with

the flea triturate cultures, in order to confirm that *Y. pestis* could grow under these conditions.

Nucleic Acid Extraction

Nucleic acids were extracted from an aliquot of each of the pooled flea triturates using the MagNA Pure Compact Instrument (Roche). We used the MagNA Pure Compact Nucleic Acid Isolation Kit I with the manufacturer-supplied DNA Blood External Lysis protocol. Sample volumes of less than 200 µL were adjusted to that volume with phosphate buffered solution (PBS) prior to extraction. DNA was eluted in volumes of 100 or 200 µL.

In order to validate our extraction method, we prepared several spiked aliquots of flea triturate for nucleic acid extraction and real-time PCR analysis. Two aliquots of 40 µL were taken from a flea pool with a large volume of triturate. The aliquots were spiked with 10 µL or 20 µL of genomic DNA extracted from *Y. pestis* (strain A1122). The spiked samples were then included in a typical batch of flea triturates prepared for nucleic acid extraction, and processed according to the same DNA extraction protocol. The two spiked samples were tested by real-time PCR for a single target known to be present in all *Y. pestis* strains. We added genomic DNA from *Y. pestis* (strain A1122) to the positive control reaction tube; sterile water was added to the negative control reaction tube. At conclusion of the PCR assay, the negative control was negative. All other samples produced appropriate signatures for the target we tested for. These results indicate that the PCR assay was not contaminated during setup, and that we were indeed able to recover *Y. pestis* DNA from flea triturates using our extraction method. These results

also suggest that our ability to detect this target by PCR was not hindered by inhibitors potentially present in the flea triturates.

The processing of all fleas post-identification was completed in a BSL-3 laboratory, and all relevant personnel protection and infection control procedures were observed.

Chapter IV: Results

We collected fleas from 86 burrowing owl nests in 2012 and 2013. The number of fleas collected and examined totaled 4821, and we identified 99.4% of them as *Pulex irritans*. We determined that the remaining 0.6% of fleas belonged to *Meringis* sp., *Dactylopsylla* sp., and possibly *Oropsylla thrassis.* (See Figure 2.)

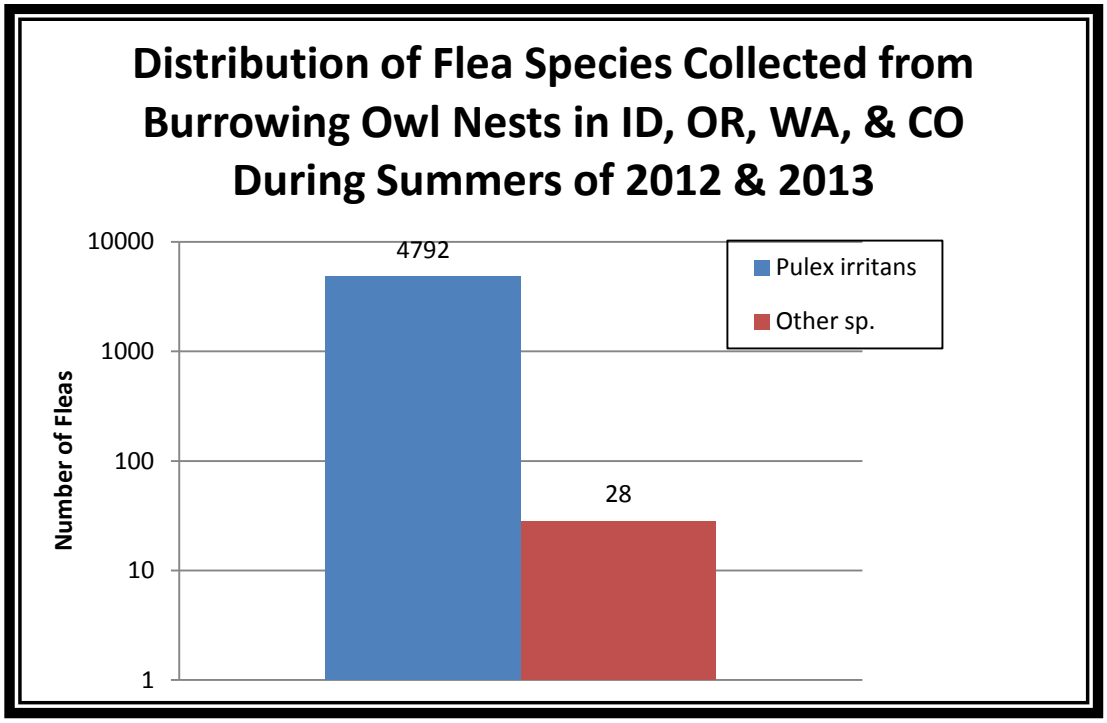

Figure 2. Distribution of flea species collected from burrowing owl nests in ID, OR, WA, and CO. *Pulex irritans* represents 99.4% of the population. "Other" species include *Meringis* sp., *Dactylopsylla* sp., and possibly *Oropsylla thrassis*.

Although we were able to obtain growth of *Y. pestis* when it was cultured alongside the flea triturate plates, none of the cultures from flea triturates displayed growth suggestive of *Y. pestis*. Figure 3 (below) shows colony morphology typical of *Y. pestis* compared to that of a typical flea triturate culture plate. On three occasions, we picked a single colony

(each from a separate nest) that appeared suspicious for *Y. pestis* and streaked for isolation onto fresh SBA. After incubation, we examined them, finding that all were pure cultures, but none produced morphology characteristic of *Y. pestis*. The species represented in the growth recovered from the flea triturates tentatively included, on the basis of colony morphology alone, numerous Gram negatives (large mucoid colonies), *Staphylococcus* species, *Bacillus* species and several fungi.

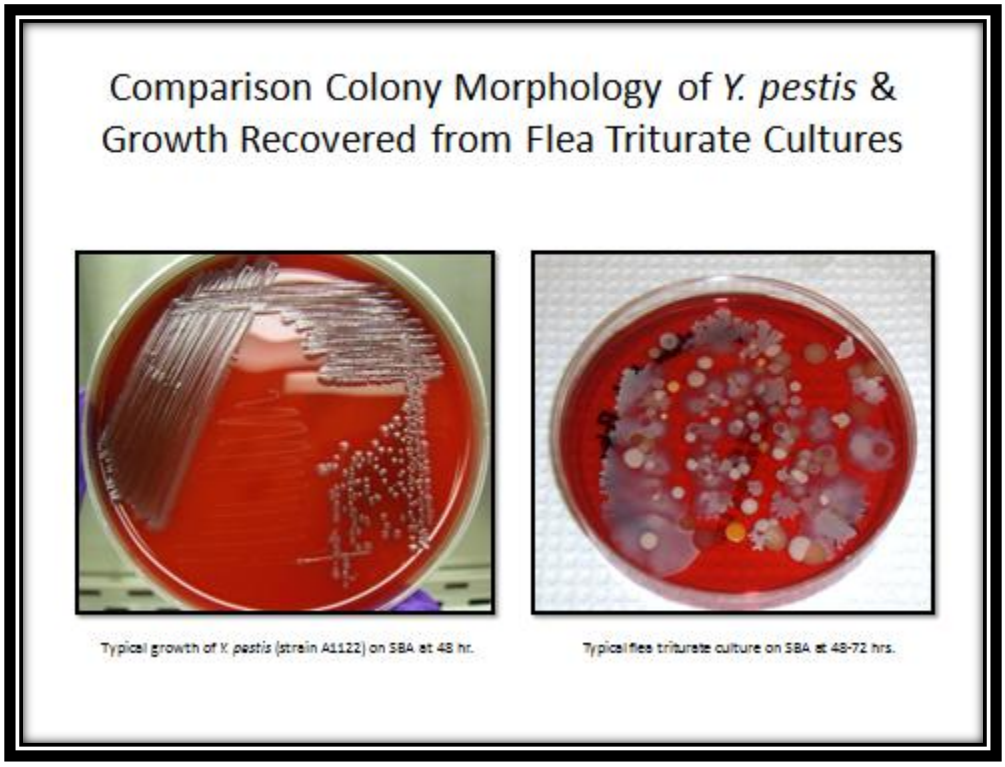

Figure 3. Comparison of colony morphology of *Y. pestis* (strain A1122) and typical flea triturate growth. Both cultures shown on SBA after 48-72 hr incubation at 28°C.

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Chapter V: Discussion

We found that burrowing owls in this study predominantly (99.4%) carried a single species of flea: *P. irritans.* We also observed that the fleas were abundant on the owls in most areas. These results support the previous findings by Smith and Belthoff of the human flea on burrowing owls in Idaho, and extend knowledge of the geographic range in which *P. irritans* appears to be associated with burrowing owls.[17] The remaining 0.6% of fleas that we collected represented three other species that are typically associated with rodents, which indicates that the burrowing owls do host other flea species, but infrequently.

These findings also support our hypothesis that burrowing owls could be serving as phoretic hosts to plague. The two populations of fleas, if separated as *P. irritans* and "other," harbored by the owls each represent potential for the burrowing owls to be exposed to plague, or to transport infectious fleas. We will first explore why the presence of *P. irritans* is particularly notable.

First, and perhaps most importantly, *P. irritans* has been shown to be capable of transmitting plague, especially en masse.[27] Because *P. irritans* tended to be plentiful on the burrowing owls sampled during this study, we conclude that these capable fleas could transmit plague to the burrowing owls if they were infectious. Also, because the fleas were retrieved from the burrowing owls' plumage, we conclude that it is indeed possible for them to be transported to new locations by the owls during migratory or hunting movements.

Second, *P. irritans* is said to be a "nest flea," meaning that it spends time in its host's nest or bedding, as opposed to "fur fleas" who reside mainly on-host.[36] And third, *P,*

irritans infests a wide range of hosts, which includes plague-associated fossorial mammals. Considering these points, we suggest that burrowing owls could encounter infectious *P. irritans* that were residing in the burrows they chose. Alternately, the fleas harbored by the burrowing owls could disengage once the owls settled in a burrow, in favor of the nest environment, and potentially encounter a new host, if the owls inhabited the burrow concurrently with another species. If the new host was infected with plague, there exists an opportunity for the owl's flea(s) to acquire and transmit it back to the owl. In the case of prairie dog burrows, burrowing owls actually prefer to occupy active burrows and will eventually stop returning to abandoned ones, which may put them at close enough proximity for the potential of host-sharing by fleas to exist.[56,57] Although we focused on burrowing owls who nested in burrows produced by mammals other than prairie dogs, we suggest that our hypothesis can be applied to areas where prairie dogs are prevalent, in which there may be increased potential for involvement of burrowing owls in plague ecology when they are in close proximity to this epizootic species. *P. irritans* has been detected in prairie dog colonies in Montana, so future studies would be useful in determining whether or not burrowing owls are involved.[19]

The other species of fleas recovered from the burrowing owls also signify potential for burrowing owls to phoretically host plague. Given that they typically reside on rodents, many of which are enzootic hosts of plague, these fleas could infect the burrowing owls if they fed on them. However, Benedictow states that fleas in general are reluctant to feed on hosts outside their natural range, so it is possible that these fleas might not feed on the owls, but only occupy them temporarily.[36] In either case, these foreign fleas have the opportunity to be transported outside their normal range, where they could spread plague

to new areas if they were infectious. Therefore, we conclude that the potential for burrowing owls to phoretically host plague or plague-carrying fleas does exist.

We did not observe colonies consistent with *Y. pestis* morphology in any of the flea triturate cultures. Given that the majority of the fleas we examined are known to be capable of harboring plague, two possible circumstances could explain our "negative" culture results: (1) *Y. pestis* was not present, or (2) *Y. pestis* was present, but we could not detect it. We suggest that we did not recover *Y. pestis* in culture from the fleas during our study because it was not present.

Current accepted methods for testing fleas for *Y. pestis* include directly culturing flea triturates, or inoculating mice with flea material and isolating *Y. pestis* from the mouse tissue.[4] Recovery of an isolate is also necessary in investigations of human plague cases. Thus, culture is foundational in the confirmation of *Y. pestis* and is the "gold standard" in sensitivity, by which other detection methods are measured.[55,58,59]

Engelthaler and Gage demonstrated that fleas collected from rodent burrows in areas of endemic plague carried bacterial loads of 10^3 to $10^{6.9}$ *Y. pestis* per flea (mean= $10^{5.6}$).[60] If we applied these figures to our study, even at the lowest quantity of bacteria (10^3 *Y. pestis* per flea) reported, a single infected flea triturated in as much as 500 µL of HIB would provide enough bacteria (20 cfu/µL) to easily detect by culture, because we plated 20 µL of each flea triturate. Additional work completed as part of this project further supports our culture results.

All of the DNA samples extracted from the flea triturate pools tested negative for *Y. pestis* DNA by nested PCR.[22] These results support the absence of *Y. pestis* colonies we observed during culture testing. The negative PCR results are especially valuable in

demonstrating the absence of *Y. pestis* in the fleas, because although culture cannot detect non-viable bacteria, molecular methods like PCR can. Thus, the high sensitivity of culture to viable cells coupled with the capability of molecular analysis to detect DNA present in all bacterial cells, gives us confidence that we accurately detected the absence of *Y. pestis* in the fleas.

Results obtained from analysis of the burrowing owl blood samples collected during this study also support our conclusion that *Y. pestis* was not present. Serologic analysis of the burrowing owls' serum demonstrated that antibodies against *Y. pestis* were not detected. Belthoff et al reported that titers of $\geq$1:16 are considered positive for bloodstream infections, but titers of <1:32 were reported for all burrowing owl samples.[22] These results indicate that the burrowing owls had not been exposed to *Y. pestis* (through flea bites or otherwise) in the past, because they would have developed antibodies against it, even if they aren't susceptible. The burrowing owl blood samples were also tested by PCR for presence of *Y. pestis* DNA, and all samples were negative.[22] Negative serology results coupled with positive PCR results would have indicated current, first time *Y. pestis* infections in the burrowing owls. Thus, because both the serology and PCR results were negative, we conclude that these burrowing owls were not being exposed to *Y. pestis*, either currently or in the past, which correlates with our results that show that the proposed vector, the fleas, was not carrying *Y. pestis*. Taken together, the negative culture, PCR and serology results all support our explanation that we were not able to detect *Y. pestis* in this study because it was not present, in either the fleas or the burrowing owls.

There were, however, no known plague epizootics occurring during the period of time and in the areas in which this study was conducted. But because the potential for burrowing owls to phoretically host plague exists, there is also potential for impact on humans and wildlife. However, we have demonstrated that this potential, though it does exist, is not realized in these areas at this time. Thus, we suggest that people handling burrowing owls in the field, or working near them in agricultural settings, continue to be at minimal risk of exposure to plague from them. Future studies of fleas collected during a plague epizootic would be beneficial in determining whether the relative risk to humans increases during such events. We did not find evidence to suggest that plague contributes to burrowing owl mortality. We also recognize the need for future studies in epizootic settings in order to determine whether burrowing owls are involved in the maintenance or distribution of plague under those circumstances.

Suggestions for future research

Besides suggesting the need for further studies addressing the relationship between burrowing owls, fleas and plague, we can offer several additional recommendations for them. In future flea collections, we recommend placing collected fleas into microtubes, such as 1.5 mL Eppendorf tubes or similar, instead of Ziploc bags. The small size of the fleas, combined with static build-up on the plastic bags, often made it difficult and time-consuming to retrieve the fleas without any accidental loss. A possible alternative to this tedious step could involve the addition of sterile water or saline to a bag (or Eppendorf tube), suspending the fleas and preventing inadvertent loss due to static bounce, and providing a "wash" step in which some environmental bacteria and debris could be removed. No wash step was performed in this study, but was recommended by Engelthaler et al.[61]

Chapter VI: Conclusion

This project supports results from prior research that indicated that burrowing owls carry numerous fleas, and that *P. irritans* is the predominant species, and extends the geographic range of such knowledge to burrowing owls in Idaho, Oregon, Washington, and Colorado. Because they are infested by *P. irritans*, a capable plague vector, and nest in burrows that present the possibility for plague exposure, we conclude that the potential for burrowing owls to serve as phoretic hosts to plague exists. However, we did not find evidence to conclude that they are serving in this capacity, at least in the areas and time frame of our study, which was observed to be during an interepizootic period. We suggest that further studies are needed to determine whether or not this is still the case when the burrowing owls are nesting in areas experiencing epizootics.

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