

PERSISTENCE OF PLAGUE: DETERMINING WHICH
ENVIRONMENTAL FACTORS CONTRIBUTE TO MAINTENANCE
OF *YERSINIA PESTIS* WITHIN SYLVATIC CYCLES.

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Doctor of Philosophy

by
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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

PERSISTENCE OF PLAGUE: DETERMINING WHICH ENVIRONMENTAL
FACTORS CONTRIBUTE TO MAINTENANCE OF *YERSINIA PESTIS* WITHIN
SYLVATIC CYCLES.

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ABBREVIATIONS

Democratic Republic of Congo (DRC)
early-phase transmission (EPT)
Yersinia murine toxin (Ymt)
post-infection esophageal reflux (PIER)
lipooligosaccharide (LOS)
lipopolysaccharide (LPS)
black-tailed prairie dog (BTPD)
Gunnison's prairie dog (GPD)
Kiowa and Rita-Blanca Grassland (KRB)
United States Department of Agriculture (USDA)
Natural Resources Conservation Service (NRCS)
Soil Climate Analysis Network (SCAN)
National Oceanic and Atmospheric Administration (NOAA)
United States Climate Reference Network (USCRN)
Parameter-elevation Regressions on Independent Slopes Model (PRISM)
calcium carbonates (CaCO_3)
electrical conductivity (EC)
sodium absorption rate (SAR)
available water supply (AWS)
organic matter (OM)
variance inflation factor (VIF)
relative humidity (RH)
post-infection (p.i.)
reactive oxygen species (ROS)
prairie dog (pdog)
colony forming units (CFU)
phosphate buffered saline (PBS)

Yersinia selective agar (YSA)
red, green, blue (RGB)
hue, saturation, brightness (HSB)
integrated density (ID)
relative fluorescent units (RFUs)
degrees of freedom (DF)
heart infusion broth (HIB)
heart infusion agar (HIA)
calcium chloride (CaCl_2)
magnesium chloride (MgCl_2)
region of interest (ROI)
generalized linear model (GLM)
generalized linear mixed model (GLMM)
polymerase chain reaction (PCR)
multiple sequence alignment program (MAFFT)
Burrow-Wheeler aligner (BWA)
BLAST ring image generator (BRIG)
basic local alignment search tool (BLAST)
single nucleotide polymorphisms (SNPs)

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ABSTRACT

Plague is a prioritized zoonotic disease caused by *Yersinia pestis*, a gram-negative bacterium. Sylvatic cycles of rodent host and flea vectors have established endemic foci responsible for the long-term maintenance of *Y. pestis*. Within the geographic range of prairie dogs, it is not understood why regions have recurrent epizootics while others remain plague-free. Abiotic factors have been correlated to epizootics and field surveys have led to hypotheses on the role other rodent species and flea or host population fluctuations may have in initiating these occurrences. Field and laboratory studies analyzing how these variables may impact vector competency have led to contradictory results and disparity in experimental design. Vector competency is often studied in the context of early-phase transmission or biofilm-mediated transmission applying temporal or genetic restrictions to the design and subsequent analysis. Corresponding analyses with these transmission models results in the neglect of unique geographic variables and flea:host interactions associated with the natural system.

The purpose of this work was to investigate edaphic factors associated with epizootic occurrences throughout the geographic range of prairie dogs and determine if these factors influence vector competency of the flea. Soil moisture, temperature, and

characteristics influencing water infiltration and storage had positive associations to plague occurrences. The depth at which these factors were associated, and the soil taxonomy varied based on geographic location, leading to site specific soil sensor installation for long-term evaluation. Investigating the infection in the flea under different abiotic and biotic components indicated host bloodmeal, temperature, and humidity influenced proventricular colonization and transmission efficiency. Variation in these components can alter vector competency, suggesting ecological factors drive disease spread or long-term maintenance. Another mechanism for long-term maintenance was discovered and strong evidence that *Y. pestis* leaves the alimentary canal and is transovarially transmitted is provided. The presence of bacteria throughout development from egg to F₁ progeny illustrates processes independent of defined transmission models contribute to disease spread and maintenance.

1 INTRODUCTION

1.1 Global Persistence of Plague and Human Cases

The causative agent for plague is the gram-negative bacteria, *Yersinia pestis*. Its divergence from *Yersinia pseudotuberculosis* within the last 1,500-20,000 years led to the acquisition of 3 plasmids critical for its unique arthropod borne transmission, pCD1, pFra/pMT, and pPla/pPCP [1]. This is a reemerging prioritized zoonotic disease that persists within the environment due to natural foci maintained by sylvatic cycles of various rodent hosts and their associated flea vectors. The pFra/pMT plasmid facilitates survival and colonization in the flea. The infective dose is as few as 10 bacteria from a flea bite which accounts for most human cases [2, 3]. Bubonic plague is the resulting form from a flea bite which has a 50% mortality rate if left untreated. The transition from the 25°C flea to the 37°C mammal leads to the upregulation of *pla*, needed to evade the mammalian host response and encoded on pPla/pPCP and the type III secretion system encoded on pCD1. Septicemic forms of plague can occur from the progression of the bubonic form if the bacteria enter the bloodstream or through interactions with animals. Human to human transmission occurs with the pneumonic form in which transmission occurs through droplets or aerosols and has a 90% mortality rate with death occurring in a matter of days [2]. Humans are an incidental host but due to proximity to rodent hosts and the persistence of plague in the environment, it is a cosmopolitan disease resulting in annual human mortality.

More than 90% of human plague cases occur in Africa, primarily Madagascar and the Democratic Republic of Congo [4]. In Madagascar, 13,234 suspected cases were reported from 1998-2016 and 4,630 cases were recorded in the DRC from 2004-2014 [4, 5]. In the United States, the first human plague case was documented in 1900 in a port city in

California [6, 7]. The first case outside of California occurred in 1934 and was linked to rodents [6]. It began to spread toward the East, integrating into native rodent populations and occurs throughout the Western and Midwestern regions today, primarily in California, New Mexico, Arizona, and Colorado [8]. There have been 482 human cases that occurred between 1970 and 2017 [9].

Long-term persistence of the bacterium in sylvatic cycles at low levels constitute enzootic periods. These periods are the source of continual global maintenance of plague foci [5]. There are sporadic occurrences when the number of bacteria transmitted from flea vectors to the rodent hosts increases to a level that causes high host mortality rates within a few days. These epizootic periods increase the potential for zoonotic transmission. In the United States, the annual occurrence of epizootics in rodent populations in conjunction with documented human cases, has resulted in plague being identified as the fourth prioritized zoonotic disease [10]. This led to increased interest in understanding factors associated with human cases, epizootic events, and vector competency of fleas. Correlations between human case frequency, epizootics, and climatic variables such as temperature and precipitation were reported [11]. The risk of plague in humans was shown to increase 2.8-fold in dry seasons which also exhibited increases in flea populations [12]. Fleas are ectotherms directly impacted by abiotic factors; therefore, it is necessary to determine how these correlated factors to human plague cases influence flea vectors.

1.2 Flea Ecology and Populations

As an ectotherm, a fleas' temperature is regulated by external environmental factors which directly alter physiological and metabolic processes. The habitat of a flea is crucial

for its survival and consists of the host in which it parasitizes, the hosts' habitat and the associated abiotic factors. For fleas that parasitize burrowing rodents, the habitat includes the microclimate of the burrow [13]. The burrow conditions provide a stable environment with minimal temperature and relative humidity fluctuations, supporting increased flea survival, particularly for the larval and pupae developing life stages [14-18]. Humidity levels of > 65% are important for maturation and survival in adults as well but very high levels can also shorten the life span [18, 19]. The burrow conditions differ from those outside but can still be impacted by seasons to some extent [20-22]. The soil structure, habitation by mammals, and presence of nest materials are likely of higher importance in defining the microclimate of the burrow than external climatic conditions [21, 22].

There is an underlying assumption that flea populations will be at higher levels during an epizootic event to facilitate the rapid spread of disease. Epidemiological modeling indicates high flea burdens are necessary due to the short period of infectivity of the mammalian host [3, 23]. Additional studies have shown that to sustain an epizootic event and dependent on mammalian susceptibility, 4-11 fleas may be needed for host-specific parasitism and 8-20 may be needed if fleas fed on non-host specific mammals [24]. In contrast, 5 or fewer are needed for enzootic periods [24]. Climatic variables have been positively associated with epizootic events as well as increased resources for mammalian hosts. This increase in resources leads to population increases subsequently followed by increased flea populations. Even if host population density is high, a high flea burden per host is needed for a plague epizootic to occur [3]. Surveying the number of fleas on hosts and swabbing burrows provides indices for measuring flea populations in rodent populations and there is a positive correlation between fleas on host and in burrows [25-

27]. However, there are aspects that need to be considered when using these measurements as indices. Burrows have a carrying capacity and even if host populations increase, there are still limitations for how many hosts can occupy the burrow system. This could have a positive influence on flea populations due to increased food sources, but it will also increase organic matter and alter the temperature and humidity of the burrow. Flea populations will fluctuate based on reproduction and absolute numbers on the body of a host will not always be an accurate determinant of how many are in the burrow. Lastly, with hosts that are parasitized by more than one flea species, the species composition, and therefore, the number of fleas, will fluctuate throughout seasons [28].

Burrowing rodents are associated with higher flea populations making it difficult to assess actual increases in flea populations [29]. Estimates of flea abundance in single species parasitizing the same mammalian host are also repeatable across geographic ranges [29]. There have been differences in flea populations in relation to epizootic events and indications that rodent and flea abundance were not factors in the occurrence of an epizootic [30-32]. Mathematical modeling also indicated that number of fleas is not as important as blockage of fleas to drive an epizootic event [24]. Notably, an increase in fleas also does not equate to introduction of bacteria into the mammalian population. Though flea population numbers will likely impact disease spread, additional mechanisms must be driving the initiation of an epizootic event. Overall, transmission is dependent on the colonization and survival of the pathogen within the flea. Factors that lead to increased flea populations are likely those that favor survival of *Y. pestis*. Determining how those factors, particularly those associated with the microclimate of the burrow, directly influence the flea bacteria interface is necessary.

1.3 Vector Competency and Transmission of *Yersinia pestis* by the Flea

Multiple flea species have been identified as capable of transmitting *Y. pestis*, but transmission efficiency varies [3, 33]. The oriental rat flea, *Xenopsylla cheopis*, was identified as the most efficient vector [3, 34]. In the United States, *Oropsylla montana* is the primary vector for humans and is found on rock squirrels and ground squirrels [35]. In order to be a competent vector, the pathogen must be acquired from an infected host, maintained until the next bloodmeal, and subsequently transmitted into the host during that bloodmeal [33]. To acquire the pathogen, a flea must feed from a bacteremic mammal and take in $\sim 10^6$ - 10^7 CFU/ml [3, 36]. To date, there are two transmission models that have been described, early-phase transmission, EPT, and biofilm-dependent regurgitative transmission [37]. In EPT, fleas are infectious for the first 4 days post-infection with day 3 identified as the most efficient for transmission. The ability to transmit during EPT is variable between flea species but both *X. cheopis* and *O. montana* are capable of EPT [36-38]. Transmission is thought to occur through regurgitative mechanisms dislodging bacteria colonized on the proventriculus. Regurgitation from the midgut is unlikely due to the proventriculus blocking backwards flow. The mechanisms behind this early colonization of the proventriculus are still unclear, especially considering it occurs in a biofilm independent manner and to date, no adhesins or other factors associated with bacterial autoaggregation have been identified in *Y. pestis* [35, 37, 39, 40]. Recent studies have indicated that a soft cast forms in the proventriculus facilitating early colonization but subsequent bloodmeals can lead to a dislodged cast [41]. Over time, a cast can solidify, leading to partial or full blockage of the proventriculus. For biofilm-dependent regurgitative transmission, *Y. pestis* proliferates in

the midgut forming biofilm 7-21 days post-infection, eventually migrating to the proventriculus causing partial or full blockage. Ingestion of a bloodmeal mixes with the bacterial laden biofilm on the proventriculus and is regurgitated back into the mammalian host [19, 37, 42-44]. As these transmission models were being described, two important transmission factors were identified in *Y. pestis*, the *hms* operon and *Yersinia* murine toxin, Ymt. Midgut survival is reliant on Ymt, a phospholipase D enzyme, and synthesis of an extracellular polysaccharide matrix needed for biofilm production is reliant on the *hmsHFRS* operon [37, 45, 40]. The pigmentation locus of *Y. pestis* stores exogenous hemin and houses the *hmsHFRS* operon which synthesizes poly β -1,6-N-acetyl-d-glucosamine (PNAG) exopolysaccharides.

Currently, neither of these transmission models can be conclusively linked as the driving force for either epizootic or enzootic occurrences in sylvatic cycles. However, research is beginning to indicate potential overlap of these models and associated mechanisms. Certain parameters have been implicated as necessary factors for epizootic events including susceptible hosts, bacteremic hosts, and high loads of infected fleas per mammal [23]. Due to the transmission capabilities of fleas shortly after intake of bloodmeal and flea survival during the transmission window, EPT was suggested as the transmission model supporting epizootic events [36]. In addition, mathematical modeling indicates that biofilm-mediated transmission was not sufficient due to the long incubation time necessary for blockage to form and reduced flea survival [36]. However, the efficiency of EPT for driving epizootics has been called into question, indicating the need for an increased number of fleas feeding on naïve susceptible hosts to successfully transmit unlikely [40]. It was suggested that EPT may support enzootic phases by

establishing immunological resistance in hosts through low level repeated exposure [24]. However, further evaluation with mathematical modeling indicated EPT was insufficient for enzootic cycles unless fleas were blocked [24].

Transmission efficiency is increased in blocked fleas but variation in blockage rates occurs between flea species [34]. Since transmission is regurgitative, colonization of the proventriculus is necessary [40, 46]. Host bloodmeal can play a significant role in early colonization of the proventriculus and subsequent blockage. Fleas fed on rat blood developed post-infection esophageal reflux, PIER, where colonization of the esophagus and proventriculus was observed as early as 1-day post-feeding [47]. Increased transmission efficiency on day 3 compared to fleas infected with *hms* mutants indicates the possible overlap of biofilm mediated proventricular colonization and EPT [47]. Temperature can also have a significant impact on blockage. In temperatures exceeding 23°C, there is a decline in blockage and full inhibition occurs at 30°C [48, 49]. Although partial and full blockage likely play an important role, not all blocked fleas will transmit. Approximately 36-47% of flea bites from a blocked flea will result in transmission [3, 23]. Unfortunately, minimal investigations into the natural sylvatic cycles during both enzootic and epizootic events in conjunction with the disparity in design of flea experiments between labs, has made it extremely difficult to make an inference on mechanisms behind enzootic and epizootic cycles.

1.4 *Yersinia pestis* and Environmental Cues

The transition from mammal to flea results in a drop in temperature from 37°C to 25°C and entrance into the proventriculus and midgut during intake of a bloodmeal. In order to survive and colonize the midgut, *Y. pestis* responds to the flea environment in a

multitude of ways. An oxidative stress regulator, *oxyR*, is resistant to reactive oxygen species and is important for initial colonization of the flea [50, 51]. PhoPQ and OmpR-EnvZ are two component systems that respond to environmental stresses by modifying the outer surface of the bacteria. The pH in the flea midgut can fluctuate, particularly after a bloodmeal, and PhoPQ senses a decrease in pH within the flea gut [40, 52, 53]. This results in lipid A modifications to prevent disruption of the LOS, providing protection against pH and AMP production. Acidic environments and nutrient depletion are sensed by the OmpR-EnvZ system resulting in biofilm formation within the midgut [54]. Biofilm formation within the flea midgut not only provides protection but also facilitates colonization within the proventriculus and the midgut.

Biofilm production occurs in response to environmental stressors and increased c-di-GMP concentrations. Intracellular levels of c-di-GMP are regulated by diguanylate cyclase (DGC) enzymes HmsT and HmsD which synthesize it and the phosphodiesterase (PDE) enzyme HmsP which degrades it. A periplasmic protein, HmsC senses environmental signals and interacts with HmsD leading to synthesis of c-di-GMP [55]. The regulation of *hms* genes is temperature dependent. A temperature dependent small regulatory RNA, HmsB, positively regulates the expression of *hmsCDE*, *hmsT*, and *hmsHFRS* [55]. The *hmsHFRS* operon synthesizes exopolysaccharides necessary for the formation of biofilm. Additional molecules are important to respond to environmental signals within the flea and involve the regulation of *hms* genes for biofilm production. A transcriptional regulator, RovM, senses nutrient limitations, and upregulates *hmsHFRS* genes to increase biofilm production for increased colonization.

Additional genes that respond to environmental stimuli can impact biofilm production by influencing the synthesis of c-di-GMP or impacting the structure of the LPS [56, 57]. Modification of LPS provides protection and *gmhA* promotes biosynthesis of LPS by catalyzing the first step in the heptose biosynthesis pathway. This is necessary for the attachment of sugars to the LOS inner core [58]. Additional modification of LPS is facilitated by *YrbH* and *WaaA*, both of which are involved in the synthesis of the core sugar Kdo. Activation of *gmhA* and *WaaA* can occur through CRP, a cAMP regulator that utilizes an alternative carbon source since glucose levels are low in the flea midgut, which in turn promotes biofilm production [57, 59].

1.5 Knowledge Gaps and Environmental Persistence of *Yersinia pestis*

Vector competence relies on the ability of *Y. pestis* to persist and colonize the flea. The auto aggregation event supporting the initial bacterial colonization of the proventriculus, or midgut is unknown. To date, there are no identified adhesins, outer membrane proteins, or bacterial fimbriae that have been identified as crucial for flea colonization [40]. The outer surface protein Ail and six of the nine known gene clusters encoding fimbriae have been targeted for deletions in mutant strains, none of which showed deficiencies in the flea [40]. The Hms polysaccharide which can facilitate cohesion and aggregation in *Y. pestis*, does not play a role in the initial colonization of the flea [40]. The lysis of cells from the bloodmeal upon intake could be a potential source for adhesion upon entry but that is unknown.

Contrasting findings on determining which transmission model supports enzootic or epizootic cycles are common throughout literature. Epizootic events have historically caused high mortality and extensive population losses in a matter of days. Susceptibility

to plague in some of these rodent host populations is changing based on if the geographic region supports endemic plague throughout or in geographically sporadic areas [60]. Nonetheless, epizootics still occur within a relatively short time frame. These characteristics have led to the implication that EPT occurs during epizootics. Modelling using ideal conditions defined as high density of host and fleas, every blocked flea finds a new host if the previous one dies, and each flea feeds at least twice on new hosts, indicated that minimum estimates of flea density needed per host was 9.4 for an epizootic [3]. This makes EPT unlikely to support epizootics but on the other hand, the rapid spread of disease and low survival of blocked fleas in the biofilm-mediated transmission model make it incapable of supporting an epizootic as well. Recent hypotheses indicate that EPT facilitates immune resistance in rodent populations by exposing mammals to low bacterial loads due to poor transmission efficiency. Biofilm-mediated transmission has higher efficiency due to the partial or full blockage of the proventriculus, making it more likely to support disease spread.

Regardless of transmission model, the flea can only become infected if it takes bloodmeal from a bacteremic host and the source of that host within these populations is unclear. The hypothesis on secondary host species, such as mice, in close proximity to these populations was formed, providing a potential explanation for the introduction of an infected flea or bacteremic mammal [61]. The host-specificity of fleas and ability to access fleas in the burrows then come into question. There is conflicting research on the presence of *Y. pestis* in enzootic and epizootic cycles [6, 34, 62, 63]. Other potential sources include *Y. pestis*' ability to survive in soil for extensive periods of time and remain viable [64]. Long-term colonization in fleas has been documented [34, 65].

Previous research in our lab showed bacteria within a flea 5.5-month post-infection. It may be possible that low levels of bacteria are maintained in fleas until certain environmental conditions occur leading to proliferation and increased bacterial loads.

Although there is current interest in understanding the role each transmission model has on either enzootic or epizootic cycles, to date, neither model has been adequately shown to facilitate the transition into an epizootic event. Early phase transmission is variable, considered to be rather inefficient, and potentially requires high numbers of fleas or an extent of blockage to occur for increased efficiency. Biofilm-mediated transmission is considered to be more efficient due to the increased bacterial loads associated with proventricular blockage. However, based on temporal requirements alone, this model seems implausible for an adequate number of fleas to be concurrently blocked and simultaneously dispersed throughout the expansive burrow system to cause large mortality events within days. The replication of the flea:host interactions seen in the natural system do not always occur within laboratory studies. Nonetheless, strong evidence supports abiotic and biotic factors on vector competency, and it is established that *Y. pestis* responds to environmental conditions and stimuli.

Epidemiology is impacted by ecological factors with disease occurrences typically associated with specific climates, ecological interactions, geographic regions and the necessary biotic interactions for survival [65]. Ecological factors directly impact vector competency; however, these factors are unknown for most sylvatic cycles due to heterogeneous ecosystems across geographic regions. In particular, the microclimate which has the greatest impact on fleas parasitizing burrowing rodents is largely disregarded. A large majority of the focus for epizootic and enzootic cycles has been on

transmission models, flea populations, and temperature influences on transmission efficiency. Studies including these factors in addition to other important ecological parameters are needed to begin to fill in these knowledge gaps. In this research, we attempt to address multifactorial influences on vector competency and investigate possible mechanisms for long-term maintenance of *Y. pestis* in flea populations.

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2 ASSOCIATIONS OF SOIL PARAMETERS AND PLAGUE

EPIZOOTICS IN DOCUMENTED PRAIRIE DOG COLONIES

*A large portion of this work has been published: Pauling, C.D., D.L. Finke, and D.M. Anderson. 2021. Interrelationship of soil moisture and temperature to sylvatic plague cycle among prairie dogs in the Western United States. Integrative Zoology: doi:10.1111/1749-4877.12567

Background: *Yersinia pestis* is the causative agent of plague, a notorious disease that has caused 3 pandemics and widespread human mortality [1]. Plague is a zoonotic, flea-borne disease that can also be spread between individuals through inhaled respiratory droplets. The pandemic potential of modern plague has been greatly reduced due to effective antibiotics that reduce the mortality rate as well as improved diagnostics and public health practices that have largely eliminated human-to-human transmission. Nevertheless, plague exposure and human mortality occur annually in endemic areas, which include the Western United States [2]. For these reasons, the US Centers for Disease Control and Prevention classify plague as a significant human and animal health concern. Rodents are highly sensitive to *Y. pestis* infection and are thought to be important to enzootic maintenance of plague throughout the world [3]. Wildlife, particularly omnivorous species, as well as humans and domestic animals are incidental hosts whose risk of exposure increases during times of high sylvatic plague activity. Rats are important hosts, known to develop disease, but are also found to be seropositive indicating non-lethal infection is likely common in nature [4]. Seroconversion rates of rats do not appear to correlate with human risk of plague. Fossorial rodents, namely prairie dogs, ground squirrels, gerbils, and marmots, are highly sensitive to plague and are fundamental to plague sylvatic cycles throughout the world [5-9]. Plague epizootics in US prairie dogs, for example, can involve >90% mortality to the colony, and these

can be so widespread that there is an impact on the ecosystem, particularly noticeable in the endangered black footed ferret, a predator of prairie dogs. Flea and host density are positive drivers of epizootics, which occur annually in some areas. In contrast, considerably longer periods of no observable sylvatic plague activity, up to 80 years, have occurred [10]. These observations indicate additional ecological conditions are necessary for epizootic outbreaks of disease.

In the United States, plague is reported every year in human and/or animal populations. Cases are typically seasonal and more widespread in the summer and early fall when the density of fleas on rodents is high [11, 12]. Studies on *Oropsylla hirsuta* (Baker, 1895) and the black-tailed prairie dog (BTPD), *Cynomys ludovicianus* (Ord, 1815), suggest that *Y. pestis* spread during an outbreak, as measured by its prevalence in *O. hirsuta* and gene flow calculations, is influenced by fleas migrating on prairie dogs as well as other rodent species [13]. Rodent behavior and abundance are influenced by climate, with conditions that favor rich vegetation typically correlating with increased rodent populations and incidence of plague [14, 15]. In addition, as ectotherms, flea populations are directly influenced by environmental conditions. For example, temperature can impact bloodmeal digestion rates as well as flea development thereby influencing overall fecundity [16]. Humidity can also affect survival of adult fleas [17]. Temperature, pH, and nutrient availability influence gene expression in *Y. pestis* to facilitate its transmission [18]. Rainy seasons generally precede years of high plague activity, likely because of vegetation and other food sources that increase the abundance of susceptible rodent hosts and their fleas [19]. Elevation and proximity to water are also factors that optimally support the sylvatic plague cycle [20, 21].

Decline of prairie dog colonies occurs when they become infected with *Y. pestis*, with sharp colony die-offs attributed almost exclusively to plague [9]. In the United States, black-tailed (*C. ludovicianus*), Gunnison's [*Cynomys gunnisoni* (Baird, 1855)], white-tailed (*Cynomys leucurus* Merriam, 1890), and Utah (*Cynomys parvidens* J. A. Allen, 1905) prairie dog colonies have been decimated by outbreaks of plague, as they are not only susceptible to flea transmission of plague, but may also transmit directly between individuals cohabitating the burrow of an infected animal [22]. Frequent low levels of plague incidence among prairie dogs indicates optimal conditions for dispersion through the colony and into neighboring colonies need to be present to trigger an epizootic event [9]. Eradication of flea infestation using insecticides was appreciated more than 50 years ago as a successful way to reduce the incidence of plague and is still in use today [23, 24]. However, plague epizootics have been recorded in areas with insecticide usage suggesting rapid resistance develops and/or that parameters other than total number of fleas are important in determining risk of outbreak [25]. Furthermore, the most notably impacted rodent associated with sylvatic plague cycles is the prairie dog, illustrated through yearly documented plague occurrences and colony extirpations, indicating something important involving prairie dogs and infected fleas drives the level of plague activity [26]. Environmental temperature and precipitation are known to correlate with human plague cases in the United States as well as to epizootics in prairie dogs [9, 15, 19, 27-29]. However, these are not predictive of epizootics in prairie dogs. Within prairie dog burrows where fleas develop, soil temperature and moisture are subject to less fluctuation than surface soils associated with ambient temperature and humidity.

Hypothesis: Environmental factors of the prairie dog habitat, including surface and

burrow parameters, are associated with the occurrence of epizootic outbreaks of plague in prairie dog colonies.

2.1 Soil Parameters from Prairie Dog Colonies Across Their Geographic Range

To begin to understand whether these parameters may be predictive of plague epizootics, in this work, we collected data from state and federal agencies and published data on plague cases in black-tailed prairie dogs (BTPD) or Gunnison's prairie dogs (GPD) from a 20-year period. We then used these data to query the relevance of abiotic soil conditions to plague epizootics in prairie dogs in the Western United States, on a local and regional level. Due to intermittent surveillance of prairie dog colonies throughout their geographic range, we focused on areas with historic multi-annual surveillance events and included only the years in which these occurred.

Database search and retrieval of historical plague data in prairie dogs. We collected information on climate as well as studies documenting locations of prairie dog colonies and the incidence of plague from 1999–2020 from numerous sources, which are detailed in Table S1, Supporting Information. Information on the location of colonies was collected at the county level for multiple states (AZ, CO, KS, NE, NM, ND, SD, TX, and WY) and at the colony level for the Kiowa and Rita Blanca grasslands (KRB), which span NM, OK, and TX (Figs 1 and 2). We assigned plague occurrences if information provided indicated direct detection of *Y. pestis* in prairie dogs or prairie dog fleas and/ or documented substantial declines in colony size including extirpations. The occurrence of plague within an area was assigned on a monthly basis. If a plague occurrence was detected within a specific area at the beginning of the surveillance period and at the end, all months within that period were assumed to be plague positive (Table S2, Supporting

Information). Additional information on seropositivity in wildlife other than prairie dogs was also obtained at the county level.

Monthly values of abiotic soil conditions were obtained from the US Department of Agriculture (USDA) Natural Resource Conservation Services (NCRS) Soil Climate Analysis Network (SCAN) stations and the National Oceanic and Atmospheric Administration (NOAA) US Climate Reference Network (USCRN) stations. In the case of the KRB, information was collected from OK Mesonet stations. Soil data collected in the KRB included percent volumetric soil moisture and soil temperature at depths of 2 through 24 in. We identified soil variables related to chemical composition including the percent carbonates by weight in fraction of soil less than 2 mm in size (CaCO_3), concentration of water-soluble salts (EC), amount of sodium relative to calcium and magnesium (SAR), and pH, as well as a few variables related to moisture and transmission of water including the volume of water in the soil between field capacity (amount of moisture being held in the soil after drainage of excess water) and the wilting point for plants (AWS), transmission of water in soils (K_{sat}), the change in soil volume from moist to dry, the shrink-swell potential (linear extensibility percent), and organic matter (OM).

In the multi-state analysis, there was some variation in the availability and depth of measurement for the soil data. SCAN stations also provided data on relative humidity. For extrapolation of SCAN/USCRN data to nearby prairie dog colonies within the same county, we first evaluated soil taxonomy defined by physical and chemical properties, including characteristics such as parent material, texture, structure, and the soil's moisture and temperature regime [30, 31], before extrapolating the data. Databases, including

gSSURGO [32] and the USDA NRCS Websoil survey [33], were used to determine soil characteristics, soil map units, and the soil taxonomy associated with each soil station and prairie dog colony. In a few cases, colony soil conditions were extrapolated from stations located in an adjacent county, but only when soil taxonomy was the same and the elevation and climate were similar. If there was no adjacent SCAN/USCRN station with the same taxonomy, the prairie dog data were not included in the analyses. In total, we collected information from 18 prairie dog locations within a 16-mile radius of a single USCRN station in Arizona, 56 locations within a 55-mile radius of a SCAN station in Colorado, 146 locations within a 40-mile radius of an OK Mesonet on the KRB, 27 locations within a 70- mile radius of a USCRN station plus 16 locations within a 32-mile radius of a SCAN station in Kansas, 5 locations within a 23-mile radius of a SCAN station in Nebraska, 32 additional locations within a 100-mile radius of a SCAN station in Nebraska, 13 locations within a 7-mile radius of a USCRN in New Mexico, 17 locations within a 14- mile radius of a USCRN station in South Dakota, 127 locations within a 40-mile radius to one of three SCAN stations in Texas, and 10 locations within a 25-mile radius of a SCAN station in Wyoming. Our strict criteria for extrapolation of colony soil characteristics resulted in the removal of much of the New Mexico region (analyzed only 13 out of 365 documented prairie dog locations) as well as South Dakota (analyzed only 17 out of 433 locations). Climate data, including precipitation and temperature, were obtained from PRISM [34].

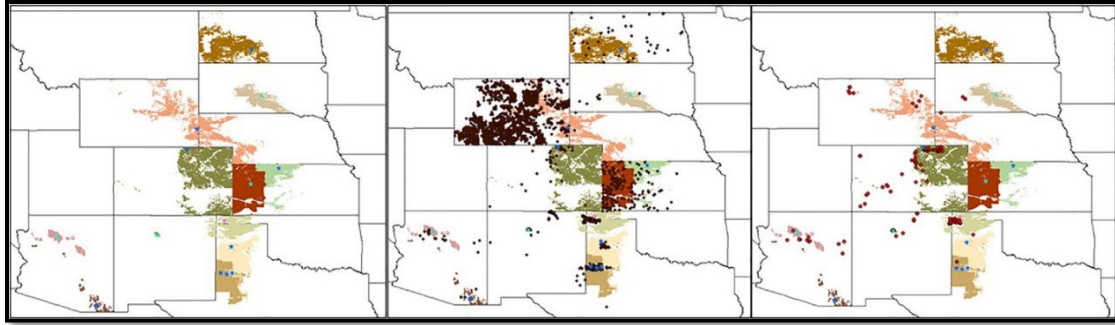


Figure 1 Documented locations of prairie dog colonies and nearby soil stations in plague endemic areas in the western United States. Left: The map represents USDA SCAN (blue stars) or NOAA USCRN (aqua stars) overlaid onto soil taxonomies in the area to show the region considered for each soil station. The Oklahoma mesonet is represented with a pink star. Center: Map shows all documented prairie dog colonies (brown circles) overlaid onto the soil taxonomy (colored regions) and soil station map. Right: Map represents only the prairie dog colonies with documented plague occurrences (red circles) over 20 years, overlaid onto the soil station/soil taxonomy map.

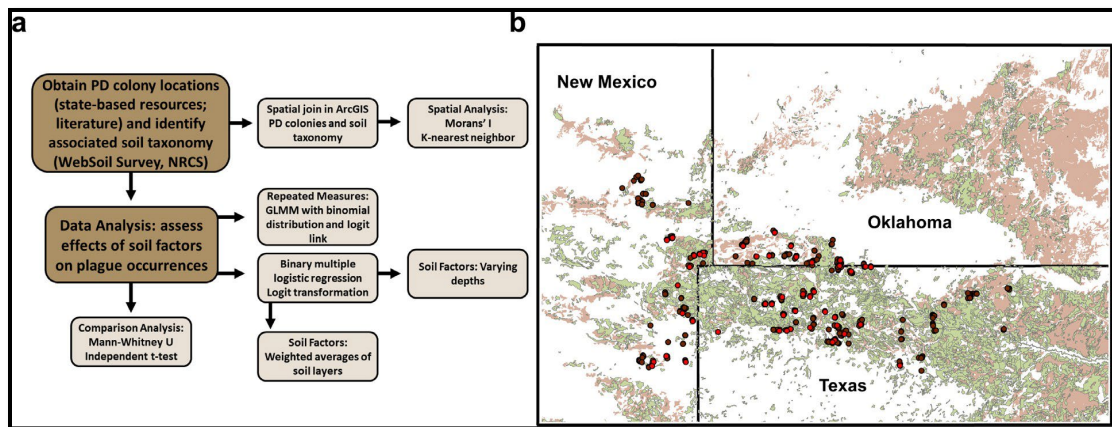


Figure 2 Assessment of soil parameters and their association with plague occurrences in prairie dogs in the Kiowa and Rita Blanca Grasslands. (a) Flow chart of overall analysis of plague associations to soil abiotic, chemical, and physical properties. (b) Map shows all documented prairie dog colonies (brown diamonds) and all documented plague occurrences in colonies (red diamonds) from a discontinuous population study spanning 1999–2016. Tan soil represents the soil taxonomy associated with the OK mesonet used to obtain soil moisture and temperature data. Green soil represents the 11 mapunits highly associated with plague.

Soil taxonomy classification. Prairie dogs primarily inhabit grasslands. Soil taxonomy classification followed the guidelines laid out in Soil Taxonomy, US Department of Agriculture ([31], Table 1). Soils in all study areas had an ustic moisture class, indicating that moisture is limited overall but present in the system during times of plant growth. The soil moisture subclass was determined by comparing mean annual summer and winter soil temperatures at a depth of 20 inch. Higher temperatures with minimal differential between summer and winter were classified as dry, while lower temperatures with greater differences were considered moist. The aridic (torric) subclass included soil

that is typically found in arid climates with little to no leaching. Soil taxonomy was further subdivided by temperature ranges: frigid was associated with mean annual soil temperatures between 32°F and 46.4°F, with 42.8°F or more difference in summer and winter soil temperatures; mesic was associated with soil temperatures at 46.4–59°F with a difference of 42.8°F or more between summer and winter. For particle size, loamy-skeletal particles indicate that weight by volume is 35% or more of rock fragments and less than 35% of clay. Fine-loamy is 18–35% clay with 15% or more of particles with diameters of 0.1–75 mm and fine soils have <60% of clay.

Analyses: Spatial autocorrelation in the occurrence of plague epizootics was measured using Moran's I in ArcGIS, which indicated clustering (z-score = 10.458, P-value = 0.000). Therefore, spatial weights were calculated using the binary K-nearest neighbor strategy and added to the regression model as a spatially explicit variable. The Box–Tidwell (1962) procedure was utilized to verify the linearity of the continuous independent variables with the logit of epizootic occurrence prior to analysis. Variance inflation factors (VIF) were used to detect the presence of multicollinearity among independent variables. Those variables with high VIF values were removed one at a time until VIF values were <7 with a tolerance value >0.10 and the model was a good fit based on the Hosmer–Lemeshow goodness-of-fit test, the –2 Log Likelihood, and R² values provided in SPSS 26.

Single state/area model. For data collected from the KRB, a generalized linear mixed model using a binomial distribution with a logit link was used to analyze associations between plague occurrence, climatic variables, and abiotic soil conditions. Climatic and soil variables were fixed effects and colony identification was a random effect to account

for repeated measures. Additional analyses for the KRB included chemical and physical soil attributes of map units (N = 49) spanning the regions that were associated with individual prairie dog colonies and plague occurrences. Soil map units, which define areas of similar soil properties, were assigned as positive for plague if there was a record of colony decline during that month in that location. Comparisons of soil attributes at defined soil depths and as weighted averages (0–80 in) between plague and no plague map units were conducted using either Mann–Whitney U or Independent t-test depending on normality of the data. For locations other than KRB, the effects of measured soil parameters on the occurrence of plague epizootics in prairie dog colonies were analyzed using a binary multiple logistic regression with a logit transformation in SPSS 26 (IBM, New York, USA).

Table 1 Soil taxonomy and associated soil characteristics for the KRB

Soil taxonomy	Soil characteristics		
	Moisture subclass	Temperature regime	Particle size
Fine, mixed, superactive, mesic Torrtic Paleustolls†	Aridic (torric)	Mesic	Fine
Fine-loamy, mixed superactive mesic Aridic Paleustolls	Aridic (torric)	Mesic	Fine-loamy
Coarse-loamy, carbonatic, mesic Aridic Calcistolls	Aridic (torric)	Mesic	Coarse-loamy

†Associated with OK Mesonet.

Multi-state analysis. Relationships between soil parameters and plague epizootics were investigated for each state individually and in an overall multi-state analysis. Some colonies were not included in the multi-state analysis due to inconsistent availability of data (e.g. Coconino, AZ only had soil measurements at 2 and 4 in.). In addition, South Dakota did not have relative humidity measurements. Therefore, in order to include SD in the multi-state model, vapor pressure deficit minimum (the difference between moisture in the air and the moisture holding capacity of the air) and precipitation measurements

were substituted for relative humidity data. Sampling intensity also varied across some locations (e.g. extensive prairie dog surveillance has been conducted in Weld County, CO). To account for sampling variation, separate regression models were constructed for AZ, CO, NM, and SD. Relative humidity was used in the individual state analyses for AZ, CO, and NM and vapor pressure deficit minimum was used in state analysis for SD.

KRB model suggest soil moisture and temperature at different depths are associated with plague occurrence. The KRB spans the southern OK panhandle, TX and NM, where BTPDs dominate the landscape (Fig. 2). The area has experienced several plague epizootics over the past 20 years, occurring from 2002–2004, 2006–2009, and 2014–2016 [35]. In addition, in the summer of 2011, 10 BTPD colonies (about 13% of the total) in the KRB were dusted for fleas, allowing an assessment of the potential impact of insecticides on plague occurrence. During this period, one large BTPD colony appeared to experience a collapse in number suggesting that insecticide usage did not prevent the occurrence of plague, but there was otherwise no reported incidence of disease within these colonies.

The available soil information in the KRB included daily soil measurements of moisture and temperature taken at depths from 2–24 in. To assess the potential role of soil moisture and temperature in the epizootic spread of plague, we averaged monthly soil moisture and temperature at varying depths and asked if there was association with plague occurrence in the BTPDs in the KRB. These data showed that RH, soil moisture at 2, 10, and 24 in., and soil temperature at 2 in. had significant associations with plague occurrences (Table 2). An increase in temperature at 2 inches increased the likelihood of plague by 12%. A one-unit increase in RH and soil moisture at 2 and 24 in. were

associated with increased likelihood of plague (4630% and 239% respectively); however, with soil moisture at 10 in., the effect was opposite, with a decrease in the likelihood of plague occurrence associated with a one-unit increase in soil moisture. This surprising result led us to further investigate soil characteristics associated with the different depths.

Table 2 General linearized mixed model for the KRB analysis for the relationship between abiotic variables, soil moisture, soil temperature, and plague occurrences

Variable	Exponentiated coefficient	SE	Significance	95% CI [†]	
				Lower	Upper
Intercept	2.802×10^{-12}	4.6124	0.000	3.286×10^{-16}	2.389×10^{-8}
Relative Humidity	1.130	0.417	0.003	1.041	1.227
Precipitation	0.937	0.1888	0.732	0.647	1.358
Moist 2 in.	47.292	1.0195	0.000	6.396	349.664
Moist 10 in.	0.056	0.8341	0.001	0.011	0.288
Moist 24 in.	3.396	0.4455	0.006	1.417	8.140
Temp 2 in.	1.120	0.0239	0.000	1.069	1.174
Spatial	1.816	0.2772	0.032	1.054	3.129

[†]Sample size = 1025; AIC = 9065.952.

Overall model suggests soil moisture and soil temperature are associated with plague

occurrence in the Western United States. Given the associations between weighted

averages of soil moisture and soil temperature in the KRB, we asked if this was true throughout the prairie dog landscape. For this assessment, we were somewhat limited by the coverage of the soil and weather stations. For example, in New Mexico, there was only a limited area with soil taxonomy data, and therefore prairie dog colony data from outside the area were not included in the analysis (see Fig. 1 for map of PD colonies and soil stations included in the multi-state analysis). For CO, KRB, and TX, however, soil taxonomies covered large areas, allowing the inclusion of more prairie dog surveillance data. We generated an overall Western US model to evaluate soil moisture and temperature at different depths in a multi-state analysis. Within this broad region, there

are large climate and elevation differences, variable ecology, and variable soil taxonomy. Nevertheless, the model detected significant associations between soil moisture and the likelihood of plague, albeit not at all depths (Fig. 4). Further, according to the multi-state model, a one unit increase in soil moisture at 8 in. depth was associated with an increase in the likelihood of plague occurrence in a prairie dog colony by ~9% (Table 3). Similarly, temperature at 4 in. showed significant association with plague in the overall model. A one degree (F) increase in soil temperature was associated with an increase in the likelihood of plague occurrence in prairie dogs of ~9%. Since these differences are much less pronounced than they were in the KRB and we lost associations in the upper and lower soil depths, the multi-state model suggests critical ecological differences within the region such as other rodent hosts or edaphic variations.

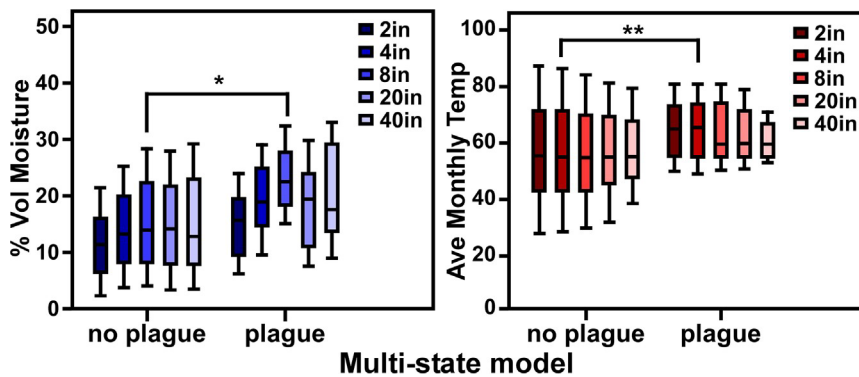


Figure 3 Overall multi-state model shows increased soil moisture and soil temperature are associated with increased plague occurrence in prairie dogs in the western United States. Boxplots showing the range of measurements (75th percentile in boxes, whiskers show 90th percentile) for depths of the percent volumetric moisture in the soil (left panel, blue) and soil temperature measurements (right panel, red) in the overall model that includes data from all plague endemic states. Bars indicate median; * $p < 0.05$, ** $p < 0.01$ for that variable between areas with plague and those without plague.

Table 3 Model for the multi-state analysis of the relationship between soil parameters and plague occurrences in prairie dog colonies

Model Summary [†]			Goodness of Fit [‡]		
Sample Size	-2 Log Likelihood	R ²	Chi Square	df	Sig
1126	312.265	0.362	6.829	8	0.555
Variable	Exp (β)	P-value			
pH	1.181	0.644			
PPT	0.815	0.113			
VPD _{min}	0.747	0.055			
Moist 2 in.	0.983	0.572			
Moist 8 in.	1.087	0.015			
Moist 20 in.	1.040	0.109			
Moist 40 in.	0.984	0.374			
Temp 4 in.	1.092	0.005			
Temp 40 in.	0.986	0.715			
Spatial[§]	4.951	0.000			

Bold type indicates significant association detected. [†]Nagekerke R². [‡]Hosmer and Lemeshow Goodness of Fit. [§]Spatial lag variable calculated with k-nearest neighbor binary weights.

Local areas had varying relationships between plague occurrence and soil parameters of

temperature and moisture and different depths. To reduce the impact of widely variant

soil taxonomies and available data on the analysis of soil temperature and moisture, we

developed models for individual states (Table S3, Supporting Information). On the basis of

the same soil taxonomy, not more than 50 miles from a soil station, soil data were

extrapolated to nearby prairie dog colonies to increase the power of the data that went into

the model. According to the New Mexico model, a one-unit increase in relative humidity

(RH) increased the odds of plague occurrence by 45.3%, but in contrast, there was no

significant association between RH and plague occurrence in AZ, CO, or SD (Table 4).

In the AZ model, higher soil moisture content at 4 inches depth was associated with a

65.9% increase in plague (Fig. 5a; Table 4). Deeper measurements in AZ were not

available, even though BTPDs are found there. For CO, a one-unit increase in moisture

at a depth of 20 in. was associated with a 71.8% increase in likelihood of plague (Fig. 5b).

There were no associations between soil moisture and plague occurrences in NM (Fig. 5c). In SD, moisture at 20 in. was also found to significantly associate with a 64.9% increase in likelihood of plague (Fig. 5d). Thus, these individual state models are consistent with a major role for the environment in the prairie dog burrow as a driver of plague outbreaks. Soil temperature in the shallow depths was often a significant predictor of plague occurrence. For example, soil temperature at a depth of 2 in. was a significant predictor of plague in AZ and NM, but its impact was varied. An increase in soil temperature of one degree (F) increased the odds of a plague occurrence by 7.2% in AZ and 40.6% in NM, with limited data available at lower depths. Therefore, observation at the state level varied from the overall model, and from the KRB, and likely reflects unique aspects of soil taxonomy and/or plague ecology found in individual geographic areas.

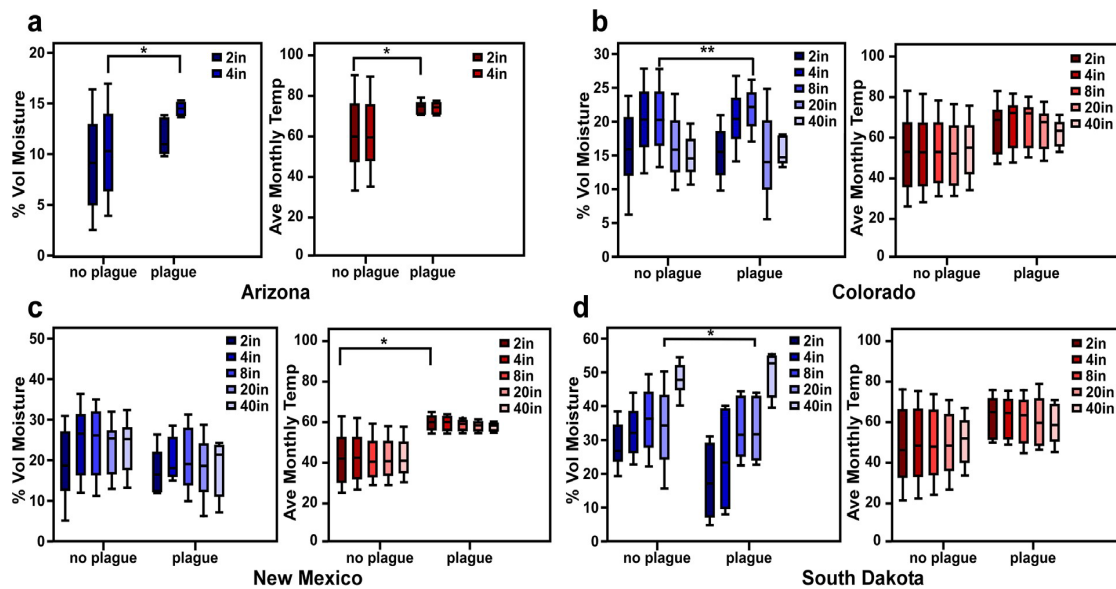


Figure 4 Individual states varied in their associations between soil parameters at multiple depths and plague occurrence. Boxplots showing the range of measurements (75th percentile boxes with 90th percentile whiskers) for depths of soil moisture (blue, left panel) and soil temperature measurements (red, right panel) associated with plague occurrences in individual states: (a) AZ; (b) CO; (c) KRB; (d) NM; (e) SD. Bars indicate medians; data from individual states were compared between prairie dog colonies where plague has been documented (plague) and those where it has not been documented (no plague); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 4 Model evaluating the relationship between soil parameters and plague occurrences in prairie dog colonies for individual states

Location	Sample Size	Variables			Model Summary [†]		Goodness of Fit [‡]		
		Variable	Exp (β)	P-value	-2 Log likelihood	R ²	Chi square	Df	Sig
Arizona	223	RH [§]	1.016	0.688	79.5044	0.506	4.246	8	0.834
		PPT	1.412	0.455					
		Moist 2 in.	0.650	0.065					
		Moist 4 in.	1.659	0.017					
		Temp 2 in.	1.072	0.028					
		Spatial[¶]	1.683	0.000					
Colorado	120	RH	0.966	0.637	59.968	0.566	3.160	8	0.924
		PPT	1.788	0.171					
		Moist 2 in.	0.817	0.229					
		Moist 8 in.	0.766	0.150					
		Moist 20 in.	1.718	0.002					
		Moist 40 in.	0.888	0.152					
		Temp 4 in.	1.130	0.115					
		Temp 40 in.	0.975	0.792					
		Spatial	1.119	0.824					
New Mexico	108	RH	1.453	0.024	21.808	0.582	1.291	8	0.996
		PPT	0.032	0.090					
		Moist 2 in.	0.621	0.066					
		Moist 8 in.	1.330	0.523					
		Moist 40 in.	1.107	0.764					
		Temp 2 in.	1.406	0.029					
South Dakota	100	Spatial	14.753	0.020	29.054	0.832	2.605	8	0.957
		PPT	0.786	0.616					
		VPD _{min}	1.322	0.826					
		Moist 2 in.	0.869	0.418					
		Moist 4 in.	0.835	0.347					
		Moist 20 in.	1.649	0.035					
		Moist 40 in.	0.928	0.765					
		Temp 4 in.	1.001	0.995					
		Temp 40 in.	1.040	0.789					
		Spatial	35.839	0.001					

Bold type indicates significant variables for the model. [†]Nagekerke R². [‡]Hosmer and Lemeshow Goodness of Fit. [§]RH, relative humidity. [¶]Spatial lag variable calculated with k-nearest neighbor binary weights.

2.2 Associations Between Chemical and Physical Composition of Soil and Plague

Occurrences in Prairie Dog Colonies in a Specific Geographic Range, the KRB.

The Kiowa and Rita Blanca (KRB), which is heavily populated by BTPDs with long-term historical surveillance and multiple documented epizootics, was examined in greater detail to layer the potential correlation between physical and chemical soil characteristics and plague occurrences. Soil characteristics were obtained from WebSoil Survey which included chemical and physical characteristics. The analysis was done on the weighted average of the characteristics across all soil layers but also for 6 defined soil

layers at different depths. In addition, we compared the KRB to Lubbock with KRB representing a plague associated area and Lubbock representing a non-plague associated area. Location maps were generated using ArcMap (Esri, California, USA).

Analysis of soil characteristics indicates significance of calcium carbonates on plague.

Soil characteristics were classified as weighted averages across layers at three depths, 0–4 in., 8–12 in., and 21–25 in. Chemical soil characteristics, including pH, were then added to the model and tested for multi-collinearity between variables before calculating potential associations with plague occurrence. A one-unit increase in pH at the lowest depth of 21–25 in. was strongly associated with an increase in plague occurrence ($P = 0.001$). Overall, there was a significant difference in pH between map units associated with plague and those that were not ($P = 0.000$). We removed soil moisture and temperature from the model, and soil characteristics for all map units associated with documented prairie dog colonies were analyzed. This allowed us to add soil depths that increased in approximately 20 in. increments and went to the depth of BTPD nests. Soil characteristics related to chemical composition (CaCO_3 , EC, pH, SAR), moisture (AWS, OM), and transmission of water (linear extensibility percent, percent clay, percent silt, K_{sat}) were analyzed for overall weighted average from 0–80 in. and for the weighted averages of defined soil layers. Throughout the entire time period covered, there were 62 documented plague occurrences within colonies. Eleven of the 49 map units were associated with more than half of the plague occurrences ($n = 39$) (Fig. 1b). Comparing these 11 map units to the remaining indicated that the weighted average (0–80 in.) of K_{sat} ($P = 0.028$) and percent clay ($P = 0.048$) were significantly different, with higher K_{sat} and lower percent clay associated with the 11 map units where plague was most common.

Comparing layers indicated K_{sat} 75–80 in. ($P = 0.031$) and OM 35–40 in. ($P = 0.028$) were significantly different. Importantly, when we compared map units without any plague occurrences ($n = 21$) to map units with plague occurrences ($n = 28$), there was a significant difference in the weighted average of CaCO_3 ($P = 0.05$), and more specifically at 55–59 in. ($P = 0.006$) and at 75–80 in. ($P = 0.010$). Modeling the weighted average of soil characteristics, we found that a one-unit increase in CaCO_3 and AWS increased the odds of plague occurrence by 29.8% and 47.8%, respectively (Table 5). When we modeled each soil layer independently, additional soil characteristics showed significant associations with plague (Table 6), including CaCO_3 and pH (Fig. 6d). This analysis supports a strong association between soil characteristics in the burrow and the occurrence of plague in BTPDs of the KRB.

Table 5 Model for the KRB analysis of the relationship between map unit specific soil characteristics (weighted averages all layers) and plague occurrences in prairie dog colonies

Model Summary [†]			Goodness of Fit [‡]		
Sample Size	-2 Log Likelihood	R ²	Chi Square	Df	Sig
44	38.406	0.530	6.149	8	0.631
Variable	Exp (β)	P-value			
Ksat	0.950	0.448			
CaCO_3[§]	1.298	0.013			
EC [¶]	58.084	0.062			
AWS	1.478	0.021			
OM	5.614	0.519			
pH	0.230	0.130			
SAR	0.767	0.385			
% Clay	1.043	0.745			
% Silt	1.014	0.836			
Kw	0.00001	0.304			
Spatial ^{**}	1.605	0.458			

Bold type indicates significant association detected. [†]Nagekerke R^2 . [‡]Hosmer and Lemeshow Goodness of Fit. [§]Percentage of carbonates by weight in fraction of soil less than 2 mm in size. [¶]Concentration of water-soluble salts in soils measured in decisiemens per meter at 25°C. ^{**}Spatial lag variable calculated with k-nearest neighbor binary weights.

Table 6 Significant results from analysis of soil attributes at varying depths with plague occurrences in prairie dogs in the KRB

Layer	Variable	Exp (β)	p-value
0-4 in [‡]	CaCO3[‡]	1.354	0.049
	pH	0.017	0.024
	SAR	0.001	0.046
	OM	19.189	0.039
	Ksat	0.924	0.046
	% Silt	0.810	0.034
8-12 in [§]	% Silt	0.866	0.040
22-26 in [¶]	Ksat	0.763	0.063
	% Clay	0.816	0.062
35-40 in ^{††}	OM	0.0001	0.030
	Linear Ext	7.926	0.045
	% Clay	0.777	0.061
55-59 in ^{‡‡}	CaCO3^a	1.227	0.043
	pH	783.784	0.056
	SAR	0.190	0.049
	Linear Ext	17.085	0.034
	% Silt	0.902	0.073

Bold type indicates significant associations detected. [‡]Percent- age of carbonates by weight in fraction of soil less than 2 mm in size. [‡]N = 48, -2 Log Likelihood = 43.203, R² = 0.503, Chi-square = 7.392, df = 7, Sig = 0.389. [§]N = 46, -2 Log Likelihood = 50.627, R² = 0.36, Chi-square = 12.357, df = 8, Sig = 0.136. [¶]N = 45, -2 Log Likelihood = 54.112, R² = 0.211, Chi-square = 7.715, df = 8, Sig = 0.462. ^{††}N = 46, -2 Log Likelihood = 39.860, R² = 0.530, Chi-square = 9.158, df = 7, Sig = 0.242. ^{‡‡}N = 44, -2 Log Likelihood = 35.491, R² = 0.576, Chi-square = 10.427, df = 8, Sig = 0.236.

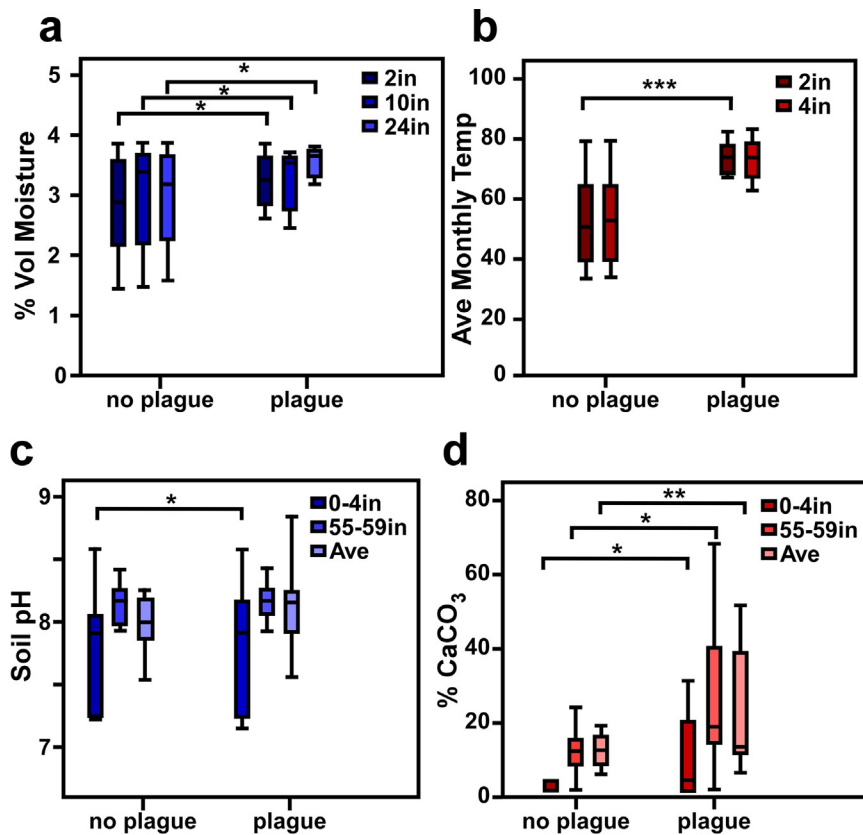


Figure 5 Soil moisture, temperature, pH, and carbonates associated with reported outbreaks of plague in black-tailed prairie dogs in the KRB. (a–d) Available data on soil moisture (a), soil temperature (b), pH (c), and carbonates (d) at different soil depths or the weighted average of the layers (ave) was compared between areas with documented plague during 1999–2016 and areas where no plague epizootics in prairie dogs were documented. Boxplots show the range of measurements (75th percentile in boxes, whiskers show 90th percentile) for the indicated depths; bars indicate medians; $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

2.3 Analysis of Soil Parameters and the Occurrence of Plague in Other Wildlife

Finally, due to documented occurrences of prairie dog-specific flea species on other mammalian hosts pre- and post-epizootic, indicating the potential for other animals to mediate flea dispersion, we assessed the relationship of these parameters to the potential spillover exposure of plague to other wildlife [36, 37]. We analyzed available data on seroconversion or plague incidence from the KRB region (where prairie dog plague is reported annually) and 200 miles away in Lubbock, TX (where there are prairie dogs but no documented recent plague outbreaks), which has similar climate, and an established SCAN station. Binary multiple logistic regression was used to assess the effects of soil abiotic conditions and relative humidity on the presence of plague cases in other wildlife.

Other wildlife included non-rodent hosts (e.g. mesocarnivores) and other rodents (e.g. rats or ground squirrels). County level data were provided in most instances rather than exact coordinates and/or description of the wildlife capture. Wildlife within the same county as the prairie dog colonies or in adjacent but climatically similar counties were used in the analysis. In this model, we found a likely correlation between prairie dog epizootic outbreaks of plague in the KRB and spillover of plague into other wildlife in the KRB (Fig. 7, Table 7). However, with no documented plague epizootics in prairie dogs located in Lubbock, yet reported seroconversion in other wildlife, there may not be strict association between plague exposure/seroconversion of wildlife and nearby prairie dog epizootic disease. Comparison analysis of soil attributes between the KRB and Lubbock indicated significant differences in composition, organic matter, CaCO_3 and pH (Tables 8, S4 and Fig. S1, Supporting Information). This suggests overall differences may be present in soil moisture retention and the microclimate of the burrow that may be important contributors to plague outbreaks in this area.

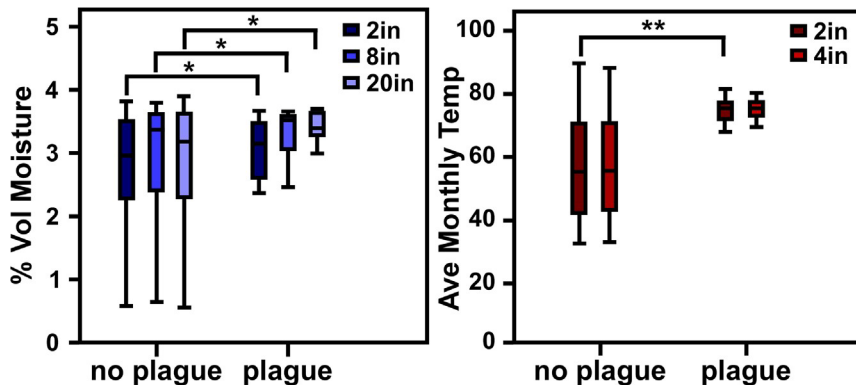


Figure 6 Spillover of plague into other wildlife during epizootic episodes in the KRB. Boxplots showing the range of measurements (75th percentile in boxes, whiskers show 90th percentile) for depths of soil moisture (left panel, blue) and soil temperature measurements (right panel, red) associated with plague occurrences in wildlife other than prairie dogs at the KRB. Data were compared between areas without plague in the prairie dog colonies and those with plague; lines indicate medians; * $p < 0.05$, ** $p < 0.01$ for the indicated parameters at the indicated soil depths.

Table 7 Model for KRB analysis of the relationship between soil parameters and plague occurrences in wildlife in close proximity to documented prairie dog colonies

Model Summary ^a			Goodness of Fit ^b		
Sample Size	-2 Log Likelihood	R ²	Chi Square	df	Sig
1025	193.915	0.331	3.084	8	0.929
Variable	Exp (β)	P-value			
RH	1.060	0.103			
PPT	1.628	0.000			
Moist 2 in	0.208	0.023			
Moist 10 in	3.578	0.020			
Moist 24 in	3.007	0.016			
Temp 2 in	1.096	0.005			

Bold type indicates significant variables for the model. ^aNagekerke R². ^bHosmer and Lemeshow Goodness of Fit.

2.4 Soil Taxonomy May Influence the Sylvatic Cycle of Plague.

We made qualitative comparisons between soil taxonomy in regions with prairie dog colonies without recent documented plague, which was found in parts of Kansas and North Dakota. As in the KRB, particle size and texture of the soils tended to show trends, such that fine-silty soil (e.g. with <15% of particles with diameters of 0.1– 75 mm and <18% of clay by weight) appeared to predominate. We therefore generated a model to compare soil moisture and soil temperature data in these regions where plague is only rarely documented to all those where plague is common in prairie dogs. This model showed an overall higher percent volumetric moisture at all depths in areas without endemic plague compared to those with plague (Fig.8). Furthermore, these plague-free areas had higher relative humidity and mean precipitation over the time period that was assessed. There were no detectable differences in soil temperature trends in this analysis. Overall, the analysis is consistent with a requirement for an optimal burrow environment to support rapid spread of plague in prairie dogs.

Table 8 Soil taxonomy and associated soil characteristics for areas without reported plague in prairie dogs in the Western United States during 1999–2020

Location	Soil taxonomy	Soil characteristics		
		Moisture subclass	Temperature regime	Particle size
Phillips, Kansas	Fine-silty, mixed, superactive, mesic Typic Argiustolls	Typic	Mesic	Fine-silty
Logan, Kansas	Fine-silty, mixed, superactive, mesic Aridic Haplustolls	Aridic (torric)	Mesic	Fine-silty
North Dakota	Fine-silty, mixed, superactive, frigid Typic Haplustolls	Typic	Frigid	Fine-silty

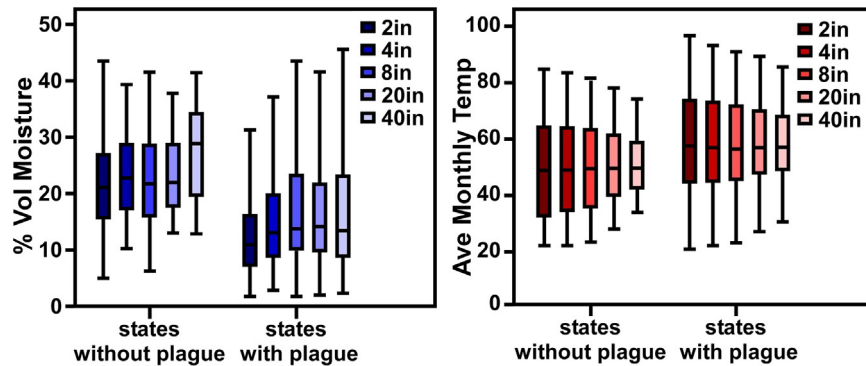


Figure 7 Overall decreased soil moisture occurs in areas with endemic plague in the prairie dog population. Boxplots showing the range (boxes indicate 75 percentile, whiskers show 90th percentile) of measurements for depths of soil moisture (left panel, blue) and soil temperature measurements (right panel, red) associated with states with documented plague occurrences within our analysis (AZ, CO, NE, NM, SD, TX, WY) versus states without documented plague occurrences in prairie dogs (KS and ND). Data were compared for all documented prairie dog towns used in our analysis; lines indicate medians; no significant interactions were detected.

Soil taxonomy is important to understanding the overall soil temperature and moisture profile within geographic areas that support prairie dog colonies. Using soil taxonomies overlayed with prairie dog colonies, we were able to extrapolate soil data from sensors established in adjacent counties. Data analysis indicated positive associations between soil moisture and temperature with the likelihood of plague occurrences. An increase in these parameters would directly influence the relative humidity of the burrow microclimate. Although macroclimate associated variables may influence the microclimate, it is primarily established based on edaphic factors and host mammalian population use of the burrow system. Prairie dogs expand across a large geographic range throughout midwestern portions of the United States. Local climatic

variables, organisms associated with each ecological system and overall population sizes and established metapopulations will create distinct differences in microclimates with the potential to influence plague occurrences in each localized region across this range. To examine these potential differences as well as investigate large-scale similarities, we installed soil sensors in an ungrazed unit on the KRB located in Clayton, New Mexico.

Due to the estimated depth of prairie dog burrows of 80 inches deep we installed two soil sensors 3 inches apart. The sensors are Gropoint® soil moisture and temperature profile sensors that take measurements over the entire length of the probe at 10cm increments. Measurements are logged through a SDI-12 interface to a data logger. Installation was done with a pilot rod attached to a slide hammer that was fabricated to the exact shape of the soil sensors to avoid any excessive disturbance or alteration to the soil layers. The sensors are 90cm and 120cm in length, with one installed with the top at 90cm below ground to reach a depth of 210cm. The shorter sensor was installed with the top at the soil surface and the lowest point reaching 90cm below ground.

Using data collected from the last day of July through the end of October, we compared the soil temperature and moisture between the installed soil sensor at the KRB and a Mesonet sensor located approximately 117 miles away. There were no statistically significant differences in soil temperature measured at three different depths (Fig.9). There were statistically significant differences in soil moisture between the locations, which could be attributed to outliers within the data from the KRB (Fig.10,11). Clayton, New Mexico experienced abnormal events of increased precipitation, in addition to a snowfall event in early November. Monthly precipitation in July was 4.26 inches compared to 0.5 inches at the Mesonet followed by another month of significant

difference in August with 1.69 inches of rainfall at the KRB versus 0.57 inches at the Mesonet. In September, this dropped to 0.08 inches at the KRB which was lower than the 0.17 inches at the Mesonet. We saw high peaks in soil moisture throughout the month of August, peaks in September and two large peaks in October (Fig.10). These peaks likely correlate to days with 0.01 inches or more (9 in August, 3 in September, 5 in October) or days with 0.1 inches or more (3 in August, 2 in October) of precipitation. The volumetric water content remains increased for a period of time after these peaks (Fig.11). The amount of precipitation can help explain immediate differences in soil moisture, however; it was not retained (Fig.10). The volumetric water content at the KRB fell below that of the Mesonet during the month of October, despite monthly precipitation of 0.52 inches versus 0.26 inches at the Mesonet. The KRB experienced moderate to high amounts of precipitation during the months of July and August but was followed by months with low to normal amounts. Only those latter months were comparable to the amount of precipitation occurring at the Mesonet station. Investigating site specific edaphic factors, we noted there are differences which may influence the soils' ability to retain or move water. The two sites have clay loam classified as well drained but there is a difference of 3% in both the sand and silt components with higher silt at the KRB. In addition, the hydrologic soil group differs with KRB classified as C with a slow infiltration rate when thoroughly wet and a slow rate of water transmission in comparison to a classification group of B with moderate infiltration and rate of water transmission. The slower infiltration of water into the soil at the KRB could explain the temporally lagged occurrence of peaks seen in overall soil moisture post precipitation events. The soil attributes could also help explain why soil moisture fell below that of the

Mesonet during October as minimal precipitation occurred prior, allowing the water to infiltrate the soil without any subsequent exposure to high precipitation.

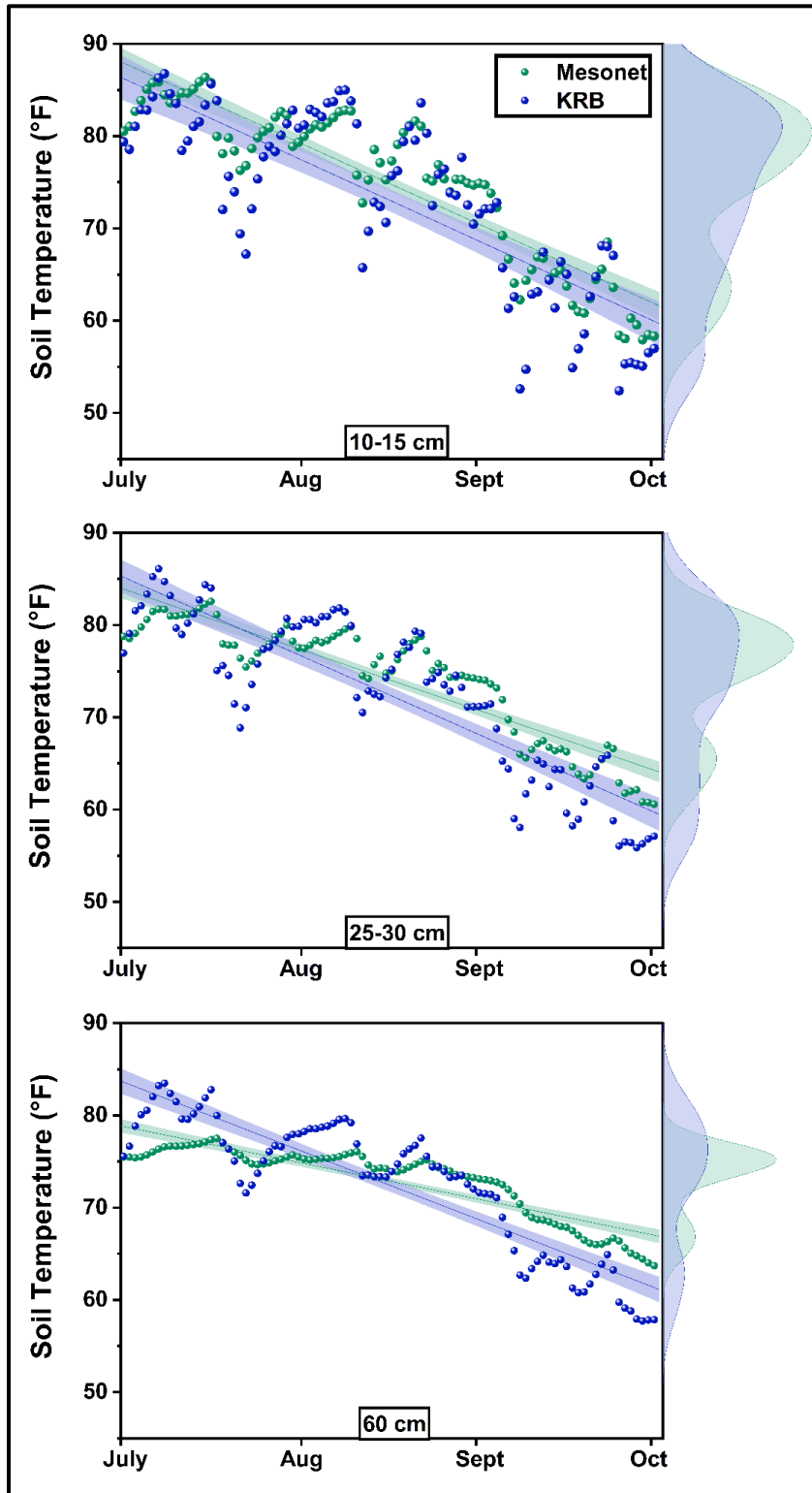


Figure 8 Installed soil sensors on the KRB are not significantly different in soil temperature readings than sensors installed on the same soil taxonomy in adjacent counties. Soil temperature measurement from soil sensors installed on the KRB (blue dot) and from a Mesonet sensor established in an adjacent county (green dots) showing data points along a linear gradient. Curves are plotted on the secondary Y-axis to show distribution of the soil temperatures for each sensor, with the green curve showing temperature distribution at the Mesonet and the blue curve show temperature distributions from the KRB sensor. Soil temperature measurements from July 31st to October 31st for different depths of 10-15cm (Top), 25-30 cm (Middle) and 60 cm (Bottom) were taken every hour and the dots represent daily averages.

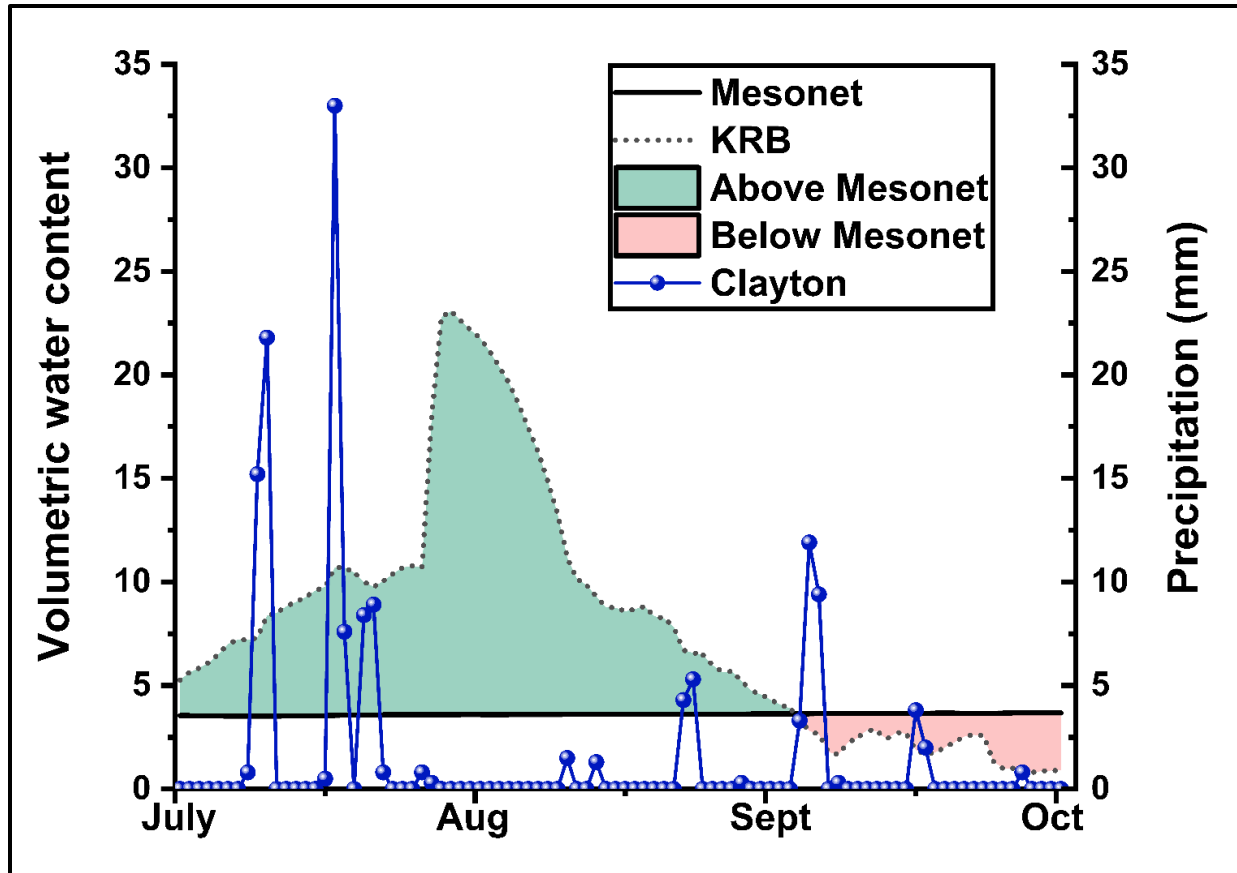


Figure 9 Significant differences in soil moisture measurements at 25-30 cm between Mesonet and installed sensors on the KRB. Volumetric water content measured at 25-30 cm at the KRB (dotted line) and from the Mesonet (solid line) from July 31st to October 31st, represented by the left y-axis. The green filled in area shows measurements from the sensor at the KRB above those from the Mesonet and the pink area indicates measurements that fall below those from the Mesonet. The blue line connects measurements of precipitation in mm (blue dots) obtained from the NOAA Global Historical Climatology Network (GHCN[39]) for Clayton, NM, represented by the right Y-axis.

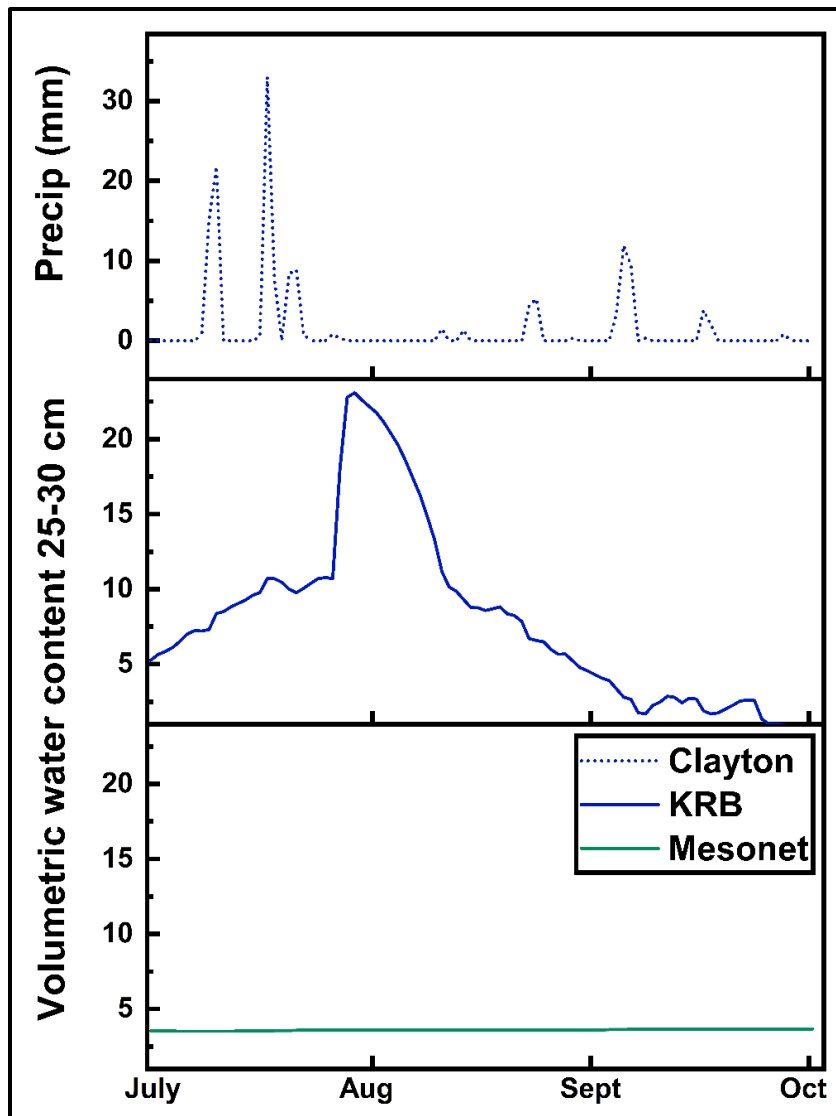


Figure 10 Differences in soil moisture measurements at 25-30 cm from the KRB follow local abnormal precipitation events. Precipitation (mm) from Clayton, NM (Top), volumetric water content of soil for the KRB obtained from installed soil sensors (Middle) and volumetric water content of soil measured from a Mesonet in an adjacent county (Bottom) from July 31st to October 31st. The significant differences measured in the KRB follow the pattern of precipitation events occurring in Clayton, NM.

2.5 Discussion

Plague ecology has been studied in some detail, and the stable sylvatic cycles that occur throughout the world have commonalities that allow predictions to be made on possible risks of exposure. With more than 100 years of data in the endemic plague areas of 4 continents, it has been well established that multiple rodent hosts and multiple flea vectors are competent for enzootic maintenance of plague. Yet the ability to accurately predict the risk of exposure or the risk of an epizootic outbreak is lacking. The KRB model highlighted several soil variables that could have a potential role in plague

persistence and outbreaks within the area. Logistic analysis indicated a significant role for CaCO_3 and AWS overall; however, only CaCO_3 was significant at specific soil layers relevant to the burrow. AWS had to be excluded from some of the layer models due to collinearity analysis. For example, AWS is influenced by soil characteristics, including porosity, which is dependent on particle size, organic matter, and carbonates within the soil. A decrease in organic matter and carbonates reduce the capacity of soils to store moisture. Therefore, although AWS was not included or significant in the individual soil layer analyses, multiple soil variables that influence AWS were.

Silt retains moisture longer than sand but does not store water to the same extent as clay, which typically has smaller pores. SAR influences the dispersion or aggregation of clay particles thus influencing overall soil structure and permeability. An increase in SAR decreases the permeability and is thus interrelated to K_{sat} , which is dependent on porosity. In the KRB model, a one-unit increase in both SAR and K_{sat} decreased the likelihood of plague occurrences. Taken together, a decrease in permeability of the soil decreases the likelihood of plague occurrences whereas an increase in factors that have a positive effect on moisture storage and water infiltration, (OM and CaCO_3), increase the likelihood of plague occurrences. Therefore, as moisture storage at 2 in. likely impacts vegetation abundance, it is likely that soil characteristics near the surface directly influence host species density and abundance, and as a consequence, the probability of an outbreak. There was a negative association between moisture and plague occurrences at 10 in. depth, with the only significant variable percent silt. The overall soil composition changed in the transition from 0–4 in. to 8–12 in., with decreased silt and sand and increased clay. It is conceivable that the increase in clay with silt staying overall

the same could lead to increased moisture retention, which may have a negative impact on important chemical components of the soil or overall water infiltration throughout the entirety of soil horizons. Going deeper into the soil, where prairie dog nests are, an increase in OM decreased the likelihood of plague, but an increase in linear extensibility, which is influenced by the percent clay, predominated by increasing the probability of plague by 692.6%. This is counter to surface level observations and seems to suggest that lower moisture conditions in the burrow favor plague outbreaks. However, linear extensibility and OM have an interrelationship with the overall moisture retention, water infiltration, and nutrient availability, therefore, making it difficult to assess the influence of singular variables on this dynamic system. Further complications may be introduced by organic matter which would be predicted to facilitate the retention of moisture within specific areas of the burrows, leading to sodden soil and an environment where survival of nesting fleas, eggs, and larva are decreased due to predation or disease.

The multi-state model showed overall that soil moisture at 8 in. depth and soil temperature at 4 in. depth were associated with plague outbreaks in the prairie dog populations, though these associations were not strong. This could reflect an impact on vegetation or other nutrient availability for the prairie dogs if moisture does not move to deeper soil layers due to the soil chemical composition and interrelated water storage and infiltration capabilities. In the individual state models, South Dakota and Colorado (as well as the KRB), we found that increased soil moisture at the lower depths (20 or 40 in.), but not 4–8 in., significantly impacted plague occurrence. Soil moisture increases, especially when combined with increased temperatures, could cause increased humidity, with downstream impacts on flea development and survival that may increase overall flea

abundance [39, 40]. Other significant impacts of humidity in the burrow are likely, many of which could influence transmission, host susceptibility of spread of disease, further complicating the relationships.

Soil data availability decreased the power of the analysis models, and likely caused variable associations in the different depths of soil due to the need to extrapolate data across the landscape. In addition, prairie dog colony and wildlife data were reported at the county level. Further limitations in the dataset occurred for colonies that exist on private lands, where prairie dog surveillance does not get reported. In Texas, for example, numerous plague seropositive cases have been documented in other wildlife and prairie dogs are prevalent throughout the region, but there is limited information on colony populations over time. Voles and other rodents who live in above-ground nests or in shallow burrows also contract plague and may contribute to the occurrence of outbreaks, particularly in regions where prairie dogs are less commonly found. For these animals, it is known that the abundance of fleas on and off host is influenced by soil moisture content, with very low and very high moisture levels representing unfavorable conditions [41, 42]. An increase in moisture would likely result in increased humidity within the burrows of these fossorial hosts of plague, especially when soil temperature increased in conjunction with soil moisture, though this relationship was not investigated here. If humidity is increased within the burrow, there could be a significant impact on flea reproduction, development, and feeding behavior, and could even directly influence the probability of transmission of *Y. pestis* at the molecular level [17, 43].

Due to the significance that the microclimate environment can have on fleas within this system, it is important to investigate localized variables such as precipitation,

temperature, and edaphic factors as they can lead to site specific characteristics of prairie dog colonies. With the installation of soil sensors on the KRB, we were able to capture local abnormal precipitation events that had month long impacts on soil moisture and identified differences in soil characteristics that were not investigated in the large-scale analysis. This highlights the importance of investigating a subset of colonies on a small scale to capture these locally influenced characteristics and abnormal weather events that can have impacts on the vector and thus transmission cycle of sylvatic plague. At the same time, similarities in temperature within soil taxonomies spanning a large geographic range were established, adding to the validity of the extrapolated data. Investigating the prairie dog sylvatic cycle is logistically challenging as each individual colony cannot be monitored continually. Confound this with the types of data needed to fully investigate the factors influencing epizootics such as long-term climatic data, ecological associations and edaphic factors and it is necessary to implement multiple research strategies.

Overall, the data indicates an interrelationship exists between soil moisture, soil temperature, soil chemical and physical characteristics, and the occurrence of plague in prairie dogs. These results justify further investigations into the interactions between soil parameters as significant predictors of plague epizootic outbreaks and the direct impact of abiotic conditions, on the flea:pathogen:mammal interface. These results, in addition to the continual accrual of new data on prairie dog locations and plague occurrences, will supplement ongoing investigations that may be useful in predicting risk of exposure and for strategies designed to reduce sylvatic plague transmission.

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2.7 Supporting Information:

Table S1 Sources for soil data, prairie dog locations, and plague occurrences used in this study

States	Weather Station	Prairie Dog Location	Plague occurrences	
			Prairie dogs	Wildlife/humans
Arizona	USCRN, SCAN	(AZGF 2020, Busch J <i>et al.</i> 2013, Girard J <i>et al.</i> 2004, Hale S <i>et al.</i> 2013, Roche T <i>et al.</i> 2017, Russell R <i>et al.</i> 2018, Sackett L 2018)	(Girard J <i>et al.</i> 2004, ProMED 2020)	(AZDH 2020, AZGF 2020, ProMED 2020)
Colorado	SCAN	(Collinge S <i>et al.</i> 2005, Cully JJ, Williams E 2001, Eads D, Biggins D 2017, Eads D, Hoogland J 2017, Pauli J <i>et al.</i> 2006a,b, Roche T <i>et al.</i> 2015, Roche T <i>et al.</i> 2017, Roche T <i>et al.</i> 2012, Sackett L 2018, Salkeld D <i>et al.</i> 2010, Savage L <i>et al.</i> 2011, Seglund A, Schnurr P 2010, Stapp P, Salkeld D 2009, Tripp D <i>et al.</i> 2009)	(Cully JJ, Williams E 2001, Eads D, Biggins D 2017, Pauli J <i>et al.</i> 2006a, Tripp D <i>et al.</i> 2009)	(CDPHE 2020, ProMED 2020, Wild M <i>et al.</i> 2006)
Kansas	USCRN, SCAN	(Cully JJ <i>et al.</i> 2000, Kansas Department of Wildlife PaT 2020, Vanderhoof J <i>et al.</i> 1994)	(Cully JJ <i>et al.</i> 2000, Cully JJ <i>et al.</i> 2010, Cully JJ, Williams E 2001)	(USFWS 2020)
KRB (TX/OK/NM)	OK Mesonet	(Cully JJ <i>et al.</i> 2010)	(Cully JJ <i>et al.</i> 2010)	(USFS 2015)
Nebraska	SCAN	(Morrison L, Peitz D 2011, Sidle J <i>et al.</i> 2001, USDA 2020)	(Virchow D 1992)	(ProMED 2020, Veenendaal T 2020)
New Mexico	USCRN, SCAN	(Chipault J <i>et al.</i> 2012, Cully JJ <i>et al.</i> 1997, Eads D 2017, Eads D <i>et al.</i> 2013, Eads D <i>et al.</i> 2015, Friggens MM, Beier P 2010, Hoogland J <i>et al.</i> 2018,	(Cully JJ <i>et al.</i> 1997, Cully JJ, Williams E 2001, Friggens M <i>et al.</i> 2010, Hoogland J <i>et al.</i> 2018, Melman S <i>et al.</i>	(NHNM 2006, NMDH 2020, ProMED 2020)

		Melman S <i>et al.</i> 2018, Sackett L 2018, Sidle J <i>et al.</i> 2001)	2018, NHNM 2006)
North Dakota	SCAN	(Shaughnessy MJ 2014, Sidle JG <i>et al.</i> 2001)	(Dyke S <i>et al.</i> 2015)
Oklahoma	OK Mesonet (Brock F <i>et al.</i> 1995, McPherson R <i>et al.</i> 2006)	(Cully JJ <i>et al.</i> 2010)	(Cully JJ <i>et al.</i> 2010) (OKDWC 2020, ProMED 2020)
South Dakota	USCRN	(Creekmore T <i>et al.</i> 2002, Eads D <i>et al.</i> 2015, Eads D <i>et al.</i> 2020, Mize E <i>et al.</i> 2017, Locke T <i>et al.</i> 2008, Locke T <i>et al.</i> 2017, Locke T <i>et al.</i> 2012, Russell R <i>et al.</i> 2018, Sidle J <i>et al.</i> 2001)	(Kempema S <i>et al.</i> 2015, Longmire C 2014, ProMED 2020, USFS 2015)
Texas	SCAN	(Nascarella M <i>et al.</i> 2005, Parks and Wildlife T 2020, Locke T <i>et al.</i> 2017, Locke T <i>et al.</i> 2012, Russell R <i>et al.</i> 2018, Singhurst J <i>et al.</i> 2010)	(Cully JJ <i>et al.</i> 2010) (ProMED 2020, TDSHS 2020, TPWD 2020)
Wyoming	SCAN	(Baroch J, Plume D 2004, Locke T <i>et al.</i> 2017, Russell R <i>et al.</i> 2018, Sidle J <i>et al.</i> 2001, Wyoming Uo 2020)	(Anderson S, Williams E 1997, Cully JJ <i>et al.</i> 2010, Cully JJ, Williams E 2001, Pauli J <i>et al.</i> 2006a, Thiagarajan B <i>et al.</i> 2008, Ubico SR <i>et al.</i> 1988) (ProMED 2020, USFS 2015, USFWS 2020, UW 2020, WDH 2020)

Table S2 Counties with documented plague occurrences in prairie dog fleas or prairie dogs, and the months and year associated with the plague occurrence and surveillance

State	County	Surveillance	Year(s)
Arizona	Coconino	April, October	2013–2015, 2017
Colorado	Weld	August, October	1994–1995, 1997, 1999–2000, 2003–2005
Nebraska	Scotts Bluff	October	2005
New Mexico	Sandoval	July, September	2016
South Dakota	Lyman	June, October	2012
New Mexico (KRB)	Union	June, September	2005, 2016
Oklahoma (KRB)	Cimarron	June, September	2004, 2005, 2016
Texas (KRB)	Dallam	June, September	2004, 2005, 2014–2016

Table S3 Soil taxonomy and associated soil characteristics for individual area analysis

Location	Soil Taxonomy	Soil Characteristics		
		Moisture Subclass	Temperature Regime	Particle Size
Arizona	Loamy-skeletal, carbonatic, mesic Lithic	N/A	Mesic	Loamy-skeletal
Colorado	Ustollic Calciorthids Fine-loamy, mixed, superactive mesic Aridic Argiustolls	Aridic (torric)	Mesic	Fine-loamy
New Mexico	Loamy-skeletal, mixed Typic Paleborolls	N/A	Frigid	Loamy-skeletal
South Dakota	Fine, smectitic, mesic Vertic Calciustepts	Typic	Mesic	Fine

N/A: not available.

Table S4 Significant results of comparison analysis of soil attributes at the KRB to soil attributes at Lubbock, TX as weighted averages for 0-80 in and at varying depths. Significant *P*-values (bold) and marginally non-significant variables are shown

Variable	Weighted Avg	0-4 in	8-12 in	22-26 in	35-40 in
% Clay	-----	-----	0.038	0.053	-----
% Sand	0.031	-----	0.038	-----	-----
% Silt	0.038	-----	-----	-----	-----
CaCO ₃ ^a	-----	0.028	0.006	0.010	-----
pH	-----	-----	0.031	-----	-----
OM	-----	0.056	0.016	0.016	0.006

a. % of carbonates by weight in fraction of soil less than 2mm in size

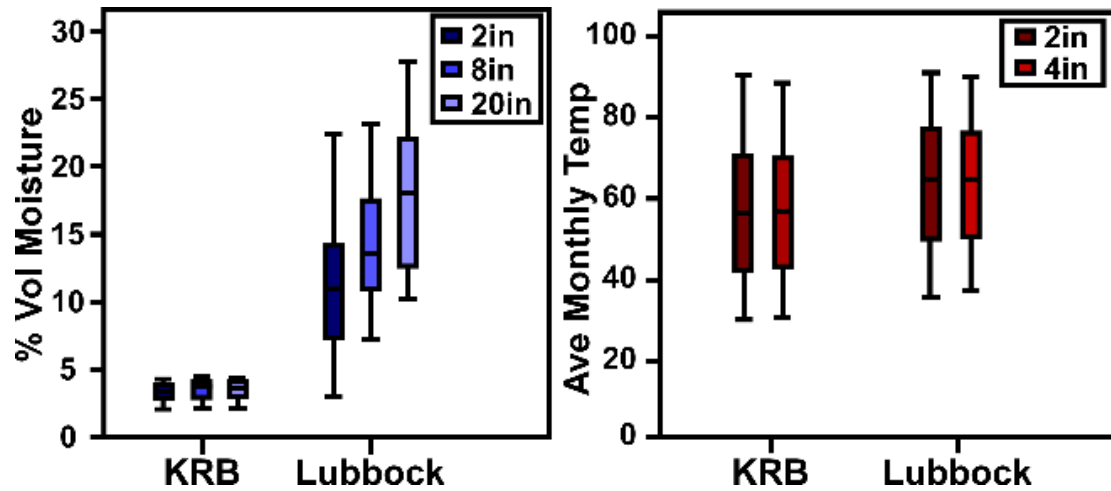


Figure S1 Interrelationships between soil parameters and prairie dog specific plague associated or non-associated areas. Boxplots showing the range of measurements (75th percentile in boxes, whiskers show 90th percentile) for depths of soil moisture (left panel, blue) and soil temperature measurements (right panel, red) associated with the KRB, a plague associated region and for Lubbock TX where plague has not been documented in prairie dogs but has comparable climate and geography to KRB. Data were compared between areas without plague in the PD colonies and those with plague; lines indicate medians; * $p < 0.05$, ** $p < 0.01$ for the indicated parameters at the indicated soil depths.

3 IMPACTS OF HOST BLOODMEAL, TEMPERATURE, AND HUMIDITY ON TRANSMISSION EFFICIENCY OF FLEAS AND BACTERIAL GROWTH IN INFECTED FLEAS.

Background: The persistence of plague within sylvatic cycles is facilitated by stable enzootic transmission cycles occurring between the flea vector and mammalian host [1, 2] [3-7]. However, there are epizootic occurrences with high mortality within these mammalian populations. In the United States, research has documented both enzootic and epizootic cycles within prairie dog colonies, a major sylvatic cycle for endemic foci of *Yersinia pestis*. The factors that cause an enzootic cycle to transition into an epizootic cycle are unknown, although studies have indicated correlations between epizootic events and biotic interactions as well as environmental conditions, specifically temperature and precipitation [8, 9]. The continual occurrence of enzootic cycles outside of epizootics indicate that optimal conditions need to be present to trigger such an event [3].

The flea vector is an imperative component for transmission and maintenance of *Y. pestis* within sylvatic cycles. Two transmission models have been described in fleas, early-phase transmission (EPT) and biofilm-dependent transmission [10-12]. Both of these models are regurgitative and reliant on bacterial colonization of the proventriculus. In EPT, fleas transmit the bacteria within the first 4 days post-infection (p.i.), and it has been shown to occur in all flea vectors including the laboratory flea, *Xenopsylla cheopis*, and those found on prairie dogs such as *Oropsylla hirsuta*, *Oropsylla montana*, *Oropsylla tuberculata cynomuris*, and *Pulex simulans* [13-15]. Due to the short time frame after infection, EPT has been implicated as the transmission model facilitating epizootic events [10]. EPT is independent of the mechanisms needed for biofilm formation and midgut

survival [16, 17]. For transmission to occur 7-21 days p.i., biofilm formation is needed for protection from midgut clearance, colonization, adherence, and eventual migration to the proventriculus resulting in occlusion of the proventricular valve. During subsequent feedings, intake of blood contacts the proventricular occlusion, dislodging bacteria and causing regurgitation and subsequent inoculation of the animal host. The time frame for this to occur, lends to the implication that biofilm-mediated transmission facilitates enzootic cycles [11,12]. However, recent studies have illustrated that this generalized temporal framework of transmission may not be adequate to implicate these models in either epizootic events or enzootic maintenance. This disparity is likely in part due to the amount of variability shown in fleas for blockage, transmission efficiency, number of fleas required for terminal disease or seroconversion, and the impact of bloodmeal on infection in each flea species. Transmission efficiency is variable between flea species but also between individual fleas of the same species [18-20].

Recent evidence indicates that in some species of fleas fed non-host bloodmeal, transmission efficiency is lower during EPT compared to blocked fleas which tend to have higher transmission efficiency with more incidence of terminal disease [20, 21]. This indicates an increased bacterial load within the flea and biofilm mediated proventricular colonization [20, 22]. These results have called into question the transmission mode associated with the dual phases of the sylvatic cycle. There is a possibility that an overlap occurs with biofilm-mediated and early-phase transmission [20, 24]. This suggests the possibility that identified mechanisms driving biofilm formation are occurring outside of the established timeline of 7-14 days p.i. of the flea. Ecological factors within the sylvatic cycle could be a source to facilitate or increase

biofilm production as seen in fleas fed with different bloodmeal sources. Host bloodmeal has been shown to introduce variation in proventricular colonization, blockage, and transmission. Digestion of the bloodmeal creates a harsh environment in the flea midgut for the bacteria to survive. There is an increase in oxidative stress leading to ROS production [22, 24, 25]. Digestion rates and the subsequent exposure of this harsh environment vary, for example > 20 hrs for rat blood versus 3-6 hrs for mouse blood [22, 28, 29].

The solubility of the hemoglobin associated with rat blood leads to PIER, post-infection esophageal reflux, which increases transmission during EPT due to partial or full blockage [20, 22, 26,27]. This appears to be counterintuitive considering the longer digestion times of rat blood and subsequent harsh environment for the bacteria to survive. On the other hand, it may be driving an increase in biofilm formation as a defense mechanism by the bacteria earlier in the infection. Fleas fed on various bloodmeals will have equitable transmission if they become blocked to some extent, however; bloodmeal may facilitate a higher percentage of fleas becoming blocked, particularly during EPT [20, 21, 24]. This is supported by investigations showing differences in overall clearance with up to 90% of fleas retaining infection when fed on rat blood versus those fed on mouse [21]. These implications are not applicable across all flea species and their associated sylvatic cycles. Studies have shown large differences in transmission efficiencies of fleas fed on rat blood, from zero to high transmission, particularly in *Oropsylla sp.* which are ground squirrel or prairie dog specific fleas [14, 15, 24].

It is established that *Y. pestis* must respond to the nutritional, environmental and stress associated factors within the flea environment. Gene regulation is influenced by

temperature and environmental signals, including an upregulation of *ymt* for protection within the flea midgut and *hms*HFRS operon for increased biofilm formation at 26°C [11, 22, 32]. As an ectotherm, the physiological processes within the flea, such as respiration, reproduction, and digestion, are influenced by abiotic factors [30-32]. Therefore, abiotic factors influence the midgut environment of the flea which is regulated by these processes. As such, the habitat of the flea, including the host, host burrow, and related environmental variables has the potential to influence the ability of *Y. pestis* to replicate and form an infectious biofilm in the flea, profoundly affecting transmission efficiency and spread of disease. Previously, we analyzed the associations between microclimate variables, particularly edaphic characteristics, and the occurrence of plague within sylvatic cycles. We found positive associations between soil moisture and temperature and plague occurrence. Variation in these two factors would subsequently impact relative humidity within the burrow system which has not been directly investigated on how it may influence the flea: pathogen interface. Therefore, we wanted to further investigate how both biotic and abiotic factors potentially introduce variation into the system and the subsequent impact on vector competency. We analyzed variations in relative humidity and temperature in concert with host specific and non-host specific bloodmeals.

Hypothesis: Increased temperatures reduce bacterial growth and biofilm formation in the flea reducing transmission efficiency whereas, increased humidity promotes bacterial growth and biofilm formation, increasing transmission efficiency.

3.1 Comparison of Bacterial Growth in Fleas Infected with Different Host Bloodmeals at Standard Maintenance Temperature and Relative Humidity

To investigate the influence of host bloodmeal source on overall acquisition and colonization in the flea, we established groups of rat fleas, *X. cheopis*, to be maintained at standard relative humidity and temperature levels of 70% RH and 25°C in the lab. Most fleas are specialized for a single host, however; there is a range on how specialized or generalized they are in relation to opportunistic feedings and host associations. Rat fleas are specialized for rat hosts but are opportunistic feeders, making them the most widely used laboratory species due to feasible maintenance and high survival rates. Therefore, the rat flea model gives us the opportunity to analyze host specific and non-host specific bloodmeals with equitable survival rates and without confounding factors. We assessed the influence of four host bloodmeals on *Y. pestis* survival in vivo and how each may be influenced by temperature and humidity. Two bloodmeals with relatively insoluble hemoglobin, rat and prairie dog, were used to represent host bloodmeal and to evaluate vector competency of fleas fed on prairie dog blood [23]. Prairie dog blood has not been used in recent lab investigations to analyze impacts of host bloodmeal on infection and transmission but has been hypothesized to have the same impact on infection as rat blood. Mouse bloodmeal was used to represent a secondary host based on an established hypothesis that an epizootic may be initiated due to introduction of fleas from highly bacteremic secondary hosts [33]. Finally, pig blood was used as a nutrient rich blood source due to the large increase in reproduction seen in cat fleas fed on porcine blood with the assumption that it would facilitate infection and flea survival [34]. For our investigations, we fed fleas once and did not provide any maintenance feedings throughout the infection. This provided us the opportunity to analyze the direct impacts of abiotic factors on a single infection event.

Methods: Rat and mouse host bloodmeal was purchased through BioChemed services and was either Sprague Dawley or Wistar laboratory rat strains or Swiss Webster, Balb/-C, or C57BL/6 laboratory mouse strains. Prairie dog (pdog) blood was collected from a euthanized male prairie dog trapped for population reduction purposes by wildlife management agencies in Texas. Pig blood was donated from Dr. Tharp's lab at the University of Missouri, Biomedical Sciences. All blood was collected in sodium heparin tubes, aliquoted, and stored at -80°C. Fleas were starved for 5-7 days and fed an inoculated bloodmeal, containing between 5×10^8 to 1×10^9 *Y. pestis*/ml, through an artificial feeding system. The KIM6+ (*pgm*+ *hms*+ *pla*+ pCD1-) attenuated strain carrying fluorescent plasmid tdTomato with an emission wavelength of 581nm was used. A mouse skin was mounted to the bottom of the glass feeder and the bloodmeal was introduced into the feeder contacting the skin. A container holding fleas was then placed on the bottom of the feeder, on the adjacent side of the skin and secured. The feeder was attached to a 37°C water bath to maintain bloodmeal temperature representative of the mammalian host. Fleas were permitted to feed for one hr, removed, and observed for presence of ingested bloodmeal. Only fleas that had ingested a bloodmeal were utilized in the analysis. Fleas were separated into 6 groups of 10 each based on different days p.i. including Days 1, 3, 4, 5, 7, and 14, to analyze timepoints associated with proventricular colonization and the transition to biofilm formation. Each group was housed in one 15ml conical tube containing sawdust until processing. The tubes were maintained upright at 70% RH, 25°C, covered with fine mesh which was then secured to allow for equitable conditions between the incubator and tube. Fleas were euthanized by placing the entire 15 ml conical tube into the freezer. Three fleas were used for CFU plating. Each flea was

rinsed 3 times in ddH₂O and the midgut was dissected out, resuspended in 10 µl sterile 1xPBS and homogenized by pipetting up and down. This resulted in a total of 30 µl containing 3 homogenized midguts that was serially diluted and plated on Yersinia selective agar (YSA). The remaining fleas were processed for confocal imaging. Each experiment was performed 3 times.

Confocal Microscopy: The remaining fleas from each group were processed for confocal imaging. Due to mortality of fleas or damage of midguts during processing, the number of midguts prepared and analyzed in each experiment varied. Overall, we attempted to obtain 10 representative images and subsequent analyses for each day p.i. for all bloodmeals. To process the midgut, fleas were removed from the sawdust and rinsed 3 times in ddH₂O and placed on sterile microscope slides. Midguts were dissected directly on the slides in sterile 1xPBS. When all fleas from that group were processed, midguts remained on the slide and were fixed with 4% paraformaldehyde for 30 minutes. They were then rinsed with PBS for a minimum of 30 minutes and this step was repeated 3 times. The placement of an image spacer and coverslip finalized the midgut preparation. Imaging was performed at the University of Missouri Advanced Light Microscopy Core on a Leica SP8 confocal microscope with image settings of 1024x1024 pixels, numerical aperture of 0.4, refractive index of 1, pinhole of 1 AU and emission of 580 nm for all images.

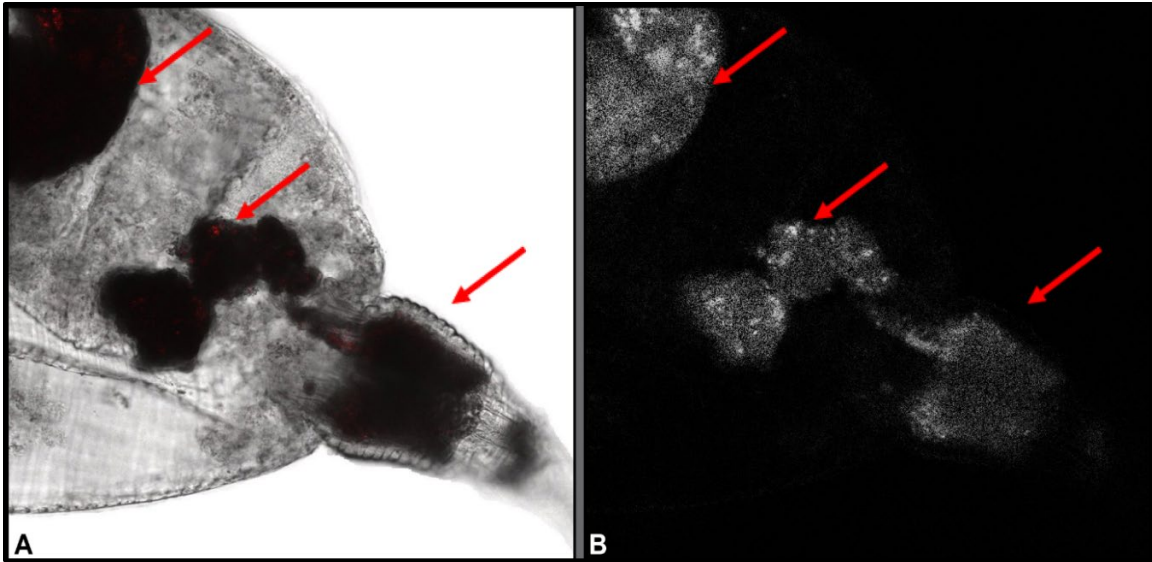


Figure 1 Illustration of the associated gray value with the fluorescent bacteria in the saturation channel in Image J showing proventricular colonization and biofilm formation within the midgut. Panel A shows an infected flea on Day 4 post-infection as a RGB image on the Leica SP8. The RGB image was converted to an HSB stack, and the saturation channel used to measure mean gray value and integrated density, shown in Panel B. Red arrows showing bacteria associated tissues and biofilm.

Image Analysis: Image J (Fiji) was used to analyze bacterial presence, localization, and overall quantity within the flea midgut. RGB images were uploaded into Image J and converted to HSB stacks. This converts the pixel intensity to gray values which can be used for analysis measuring a single fluorescent marker. The saturation channel was used to measure the mean gray value as it detects variations or shades of a color. This is important for our samples as the fluorescent signal is often quenched due to interaction within tissues, bloodmeal, and biofilm (Fig. 1). The integrated density (ID) measurement in Image J is the product of the area and the mean gray value measured in relative fluorescent units (RFUs). Individual fleas vary by size and accounting for the area of each flea midgut, we were able to compare all samples. Within the program, we manually outlined the region of interest and selected integrated density under the analysis menu. Each flea had two regions of interest, the entire tissue from esophagus to hindgut for an overall value, or the proventriculus only value. To calculate the ID, we first determined background measurements on midguts from uninfected fleas fed on rat blood and imaged

Day 1 post-feeding (N=10). The average of all negative samples was calculated for overall mean gray value (1.998) and proventricular mean gray value (2.84). The ID for either the proventriculus only or the overall tissue was calculated with the following formula: $ID = ID - (Area \times Background)$.

Statistical Analysis: For analysis, we investigated if there were differences in proventricular and overall RFUs between groups of fleas fed on different host bloodmeals during multiple days p.i.. Imaging results were grouped by bloodmeal for each day p.i. and tested for normality. The data were not normally distributed, and analysis was performed using the Kruskal-Wallis H followed by a Bonferroni correction for multiple comparisons using Dunn's (1964) procedure in SPSS 27.

Results: A total of 234 samples were analyzed for fleas maintained at 70% RH, 25°C. The proventricular and overall RFUs were statistically significantly different across bloodmeals on days 3, 4, 5, and 14 and pairwise comparisons showed significant differences between multiple bloodmeal groups (Table 1). In fleas fed on host-specific bloodmeals, the infection follows what has been established in literature with an increase on day 3, the most efficient day for EPT, followed by a decrease before the start of biofilm formation on day 7. Decreased proventricular colonization would be associated with decreased transmission which has been shown to occur with mouse blood. Rat bloodmeal has shown increased infection in both rat fleas and *Oropsylla* sp., indicating the hemoglobin characteristics of the blood facilitate this, however; pdog blood had a decreased infection in fleas, illustrating that host specificity may play an important role in the natural system.

Table 1 Proventricular and overall RFUs were statistically significantly different between groups of fleas fed on different bloodmeals on multiple days post-infection.

Proventricular RFUs					
Day p.i.	χ^2	p-value	Bloodmeals		p-value
Day 3	12.524	0.006	Pdog	Rat	0.003
Day 4	13.973	0.003	Pdog	Rat	0.001
Day 5	12.914	0.005	Mouse	Pig	0.003
Day 14	18.402	<0.001	Mouse	Pig	0.001
			Pdog	Pig	0.002
Overall RFUs					
Day 3	12.952	0.005	Pdog	Rat	0.028
			Pdog	Pig	0.015
Day 4	10.848	0.013	Pdog	Pig	0.008
Day 5	18.443	<0.001	Mouse	Pig	0.0003
			Rat	Pig	0.008
Day 14	17.518	<0.001	Pdog	Pig	0.001
			Mouse	Pig	0.006

All groups DF = 3, associated test statistic shown and 2-sided p-value.

For the duration of the infection, proventricular colonization remained higher in fleas fed with rat and pig blood. Fleas fed with rat blood follow a pattern that coincides with increased transmission on day 3 during EPT, and on day 7, biofilm-mediated transmission (Fig. 2). In fleas fed with pig blood, proventricular colonization increased through day 5 before it began to decrease around day 7 (Fig. 2). For the duration of the infection, both mouse and pdog had lower values, however; mouse followed the same pattern we see in rat. Proventricular RFUs were lower during EPT for fleas fed on pdog blood and increased later in the infection from days 5-7 (Fig. 2). The majority of fleas had proventricular colonization to some extent throughout the infection (94%) (Fig. 2). Bloodmeal appears to influence the progression of infection and biofilm formation needed for proventricular colonization and within the midgut, therefore; potentially influencing overall transmission efficiency.

In order to evaluate this in more detail, we assigned levels based on RFU value to determine the percentage of fleas with higher proventricular colonization in relation to day p.i. and bloodmeal (Figs. 3, 4). We determined these groups based on long-term

observations of over 1000 samples analyzed by our lab. There were 14 negative samples (6%) based on RFU values of less than 1 and 32 samples (13.7%) had minimal proventricular colonization with RFUs ranging from 1-10. The majority of fleas, n=144 (61.5%), were classified as having mild proventricular colonization with RFUs between 10.1–100, with 30.3% of these occurring during EPT. Moderate proventricular colonization with RFUs of 100.1-200 was seen in 31 fleas (13.2%) and only 13 (5.6%) had high colonization with an RFU value of over 200. For the moderate to high colonization, more than half of these were associated with EPT and were typically fleas fed on rat or pig blood. However, 8 were fleas infected with mouse blood and only 1 was fed on pdog blood. For host specific bloodmeal (rat), none of the fleas were negative and only 2 fell into the minimal category. Most had mild proventricular colonization (n=45) which was higher than any other bloodmeal at this level. In addition, these fleas made up 13 (30%) of the 44 samples that were at the moderate to high level (Figs. 3, 4). The same patterns exhibited in proventricular colonization are seen in overall RFUs, but the kinetics of infection are more pronounced as seen on day 3 in fleas fed on rat blood (Fig. 5). Pairwise comparisons showed multiple differences, but the majority were between pdog and pig or mouse and pig. Only day 3 had significant differences between bloodmeals associated with sylvatic plague cycles, rat and pdog (Table 1). CFUs showed similar patterns to colonization based on the median value, however; they did not have a precise correlated pattern (Fig. 5). The days that do not show CFU data points or less than 3 indicate there were no CFUs that grew. Some fell below the number of detection (30 CFUs), particularly with fleas fed on pdog blood. Bloodmeal appears to influence the kinetics of infection but introduces variation in proventricular colonization and biofilm

formation throughout the infection. There are fluctuations in the level of infection based on day p.i. with host-specific bloodmeal that correlate with the described transmission models. Additionally, it remains stable in proventricular colonization with all fleas having positive RFU values, mostly within the mild range of infection.

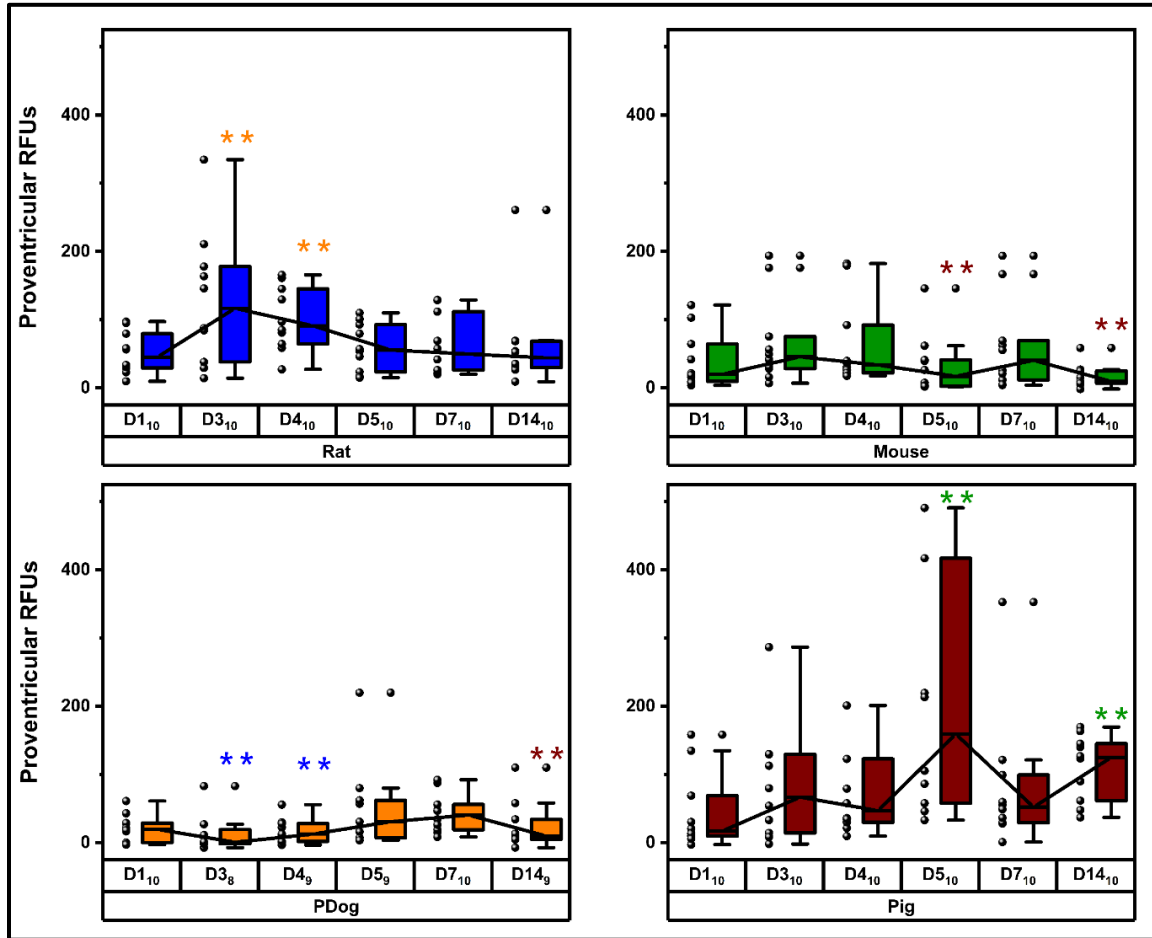


Figure 2 Bloodmeal source influences kinetics of proventricular colonization in *Xenopsylla cheopis* fleas. Significant differences in proventricular RFUs during EPT in fleas fed on different bloodmeals and maintained at standard relative humidity and temperature. Significant differences between groups of fleas fed on different bloodmeals for days post-infection are represented with asterisks colored to represent the bloodmeal with significant pairwise comparison (rat = blue, mouse = green, pdog = orange, pig = maroon), 2-sided $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Sample sizes for each group are subscripts on the day label along the X-axis. The Y-axis was adjusted for better visualization by removing one outlier for pig on D5 of 1657.143. Box plots illustrate the minimum and maximum values as whiskers and the box represents data that falls within 25-75% of all data with the median line. The line is connecting the median value for all sets of data. Data points that lie outside but are in line with the box and whiskers are outliers. Data points to the left represent each data point for the set.

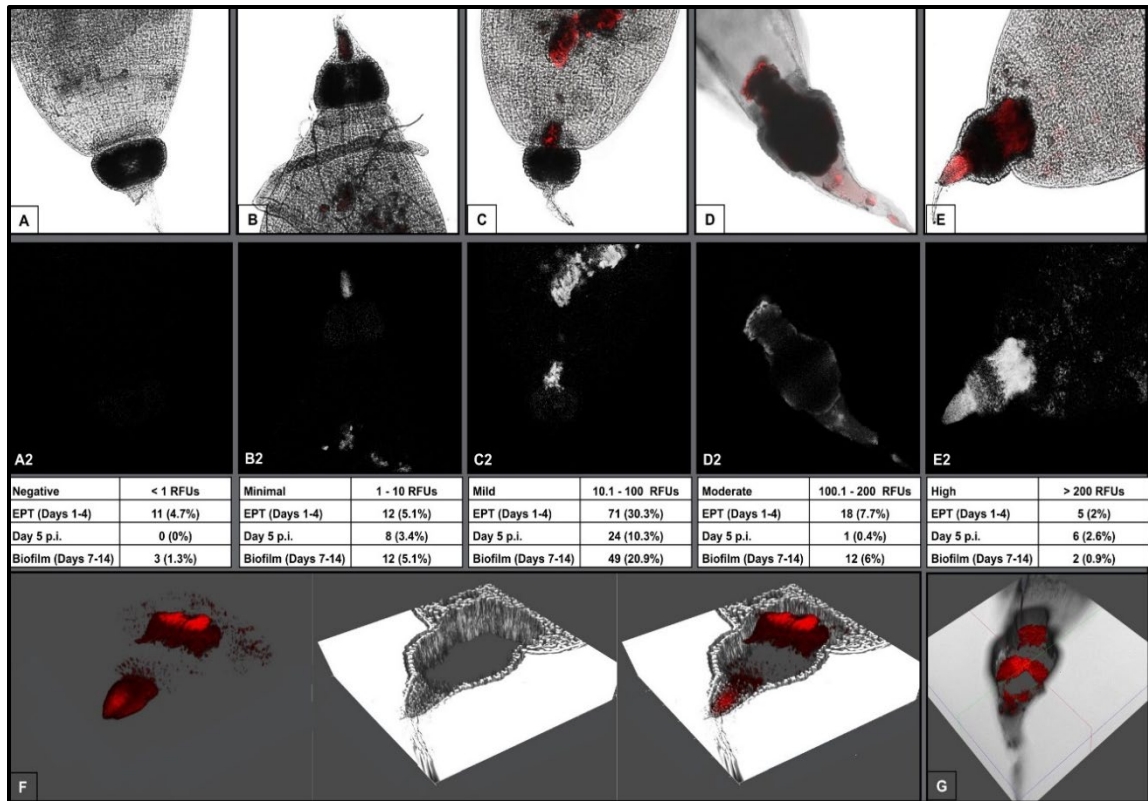


Figure 3 Variation in proventricular colonization is related to host bloodmeal, with stable colonization in host-specific bloodmeal correlating to established transmission models. Levels of proventricular colonization assigned to samples with representative images and associated RFU levels. The top row shows RGB images before being converted to HSB (A-E). The second row of images are the respective HSB saturation channels of the converted RGB images from the top row (A2-E2) showing the fluorescence as gray values. The table under each set are the respective classification level and ranges of RFUs assigned to that level (Negative: A-A2 RFU= - 3.4064, Minimal: B-B2 RFU= 3.22, Mild: C-C2 RFU= 15.15, Moderate: D-D2 RFU= 165.44, High: E-E2 RFU= 699). Each table also indicates how many samples classified under that level fell into the EPT, biofilm mediated transmission, or day 5 post-infection time frame, followed by that combination's percentage of the entire sample size (N=234). Panels F and G are z-stacks of the proventriculus provided to illustrate the efficacy of our method on using microscopy to quantify the proventricular colonization. The z-stacks create 3-D models of the tissue and highlights the association of fluorescence to bacteria only with no autofluorescence of the chitinous spines within this area.

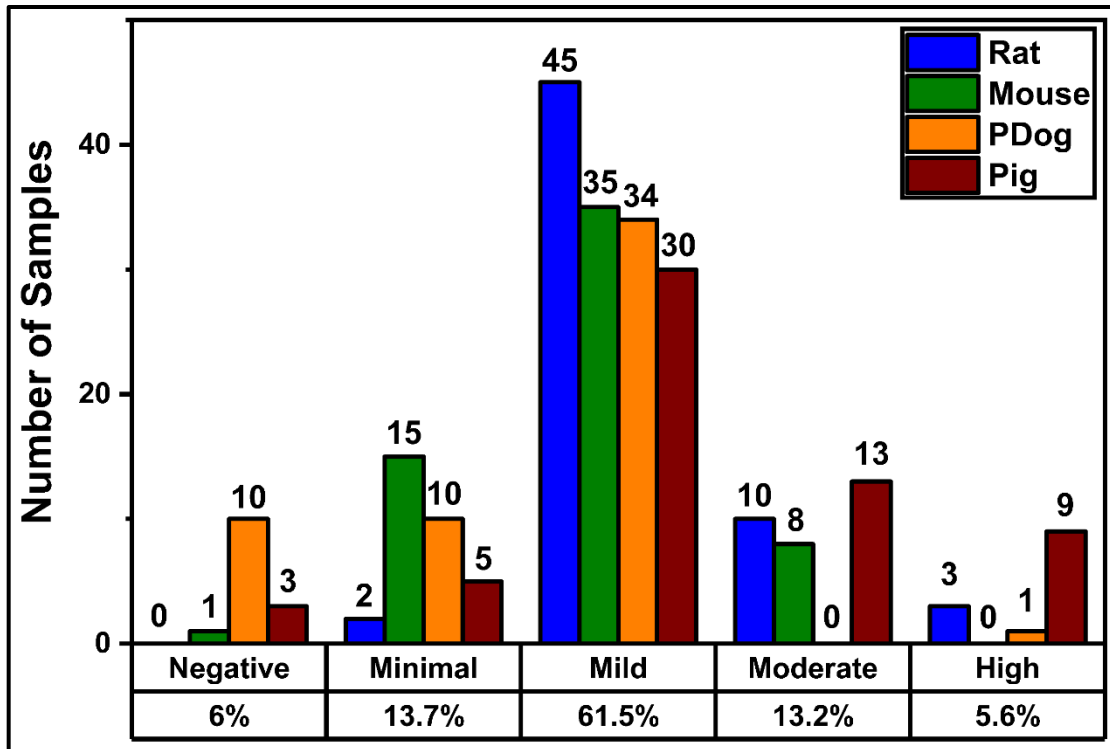


Figure 4 Host-specific bloodmeal facilitates stable proventricular colonization with minimal variation while non-host bloodmeal is associated with a greater distribution across all classifications throughout the infection and wider distribution of the extent of proventricular colonization. The chart is representative of all samples analyzed throughout the entire infection. Samples associated with each level were further categorized by bloodmeal with blue representing rat, green for mouse, orange for prairie dog and maroon for pig. The numbers above each bar indicate the sample size for that bloodmeal within that classification group. The percentages indicate the total number of fleas within that category out of all fleas analyzed (14 negative fleas out of 234 analyzed).

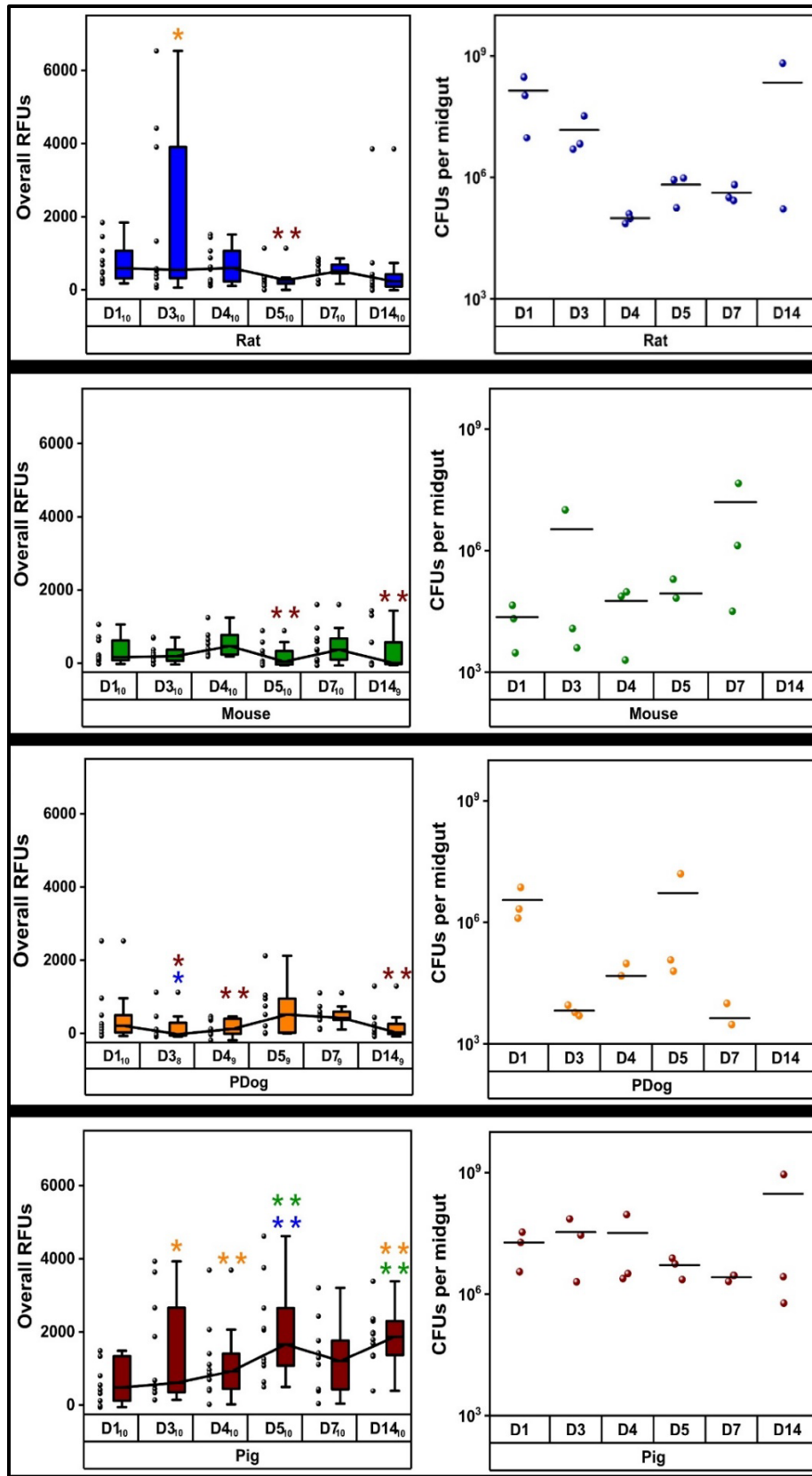


Figure 5 Bloodmeal influences overall infection of *Y. pestis* in *Xenopsylla cheopis* fleas. Significant differences in overall RFUs in fleas fed on different bloodmeals and maintained at standard relative humidity and temperature. Significant differences between groups of fleas fed on different bloodmeals within these days are represented with asterisks colored to represent the bloodmeal with significant pairwise comparison (rat = blue, mouse = green, pdog = orange, pig = maroon), 2-sided * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Sample sizes for each group are subscripts on the day label along the X-axis. Each dot on the CFU graphs represents a group of 3 midguts. X-axis was adjusted ..

3.2 Bacterial Temperature and Humidity Impact the Dynamics of *Y. pestis* Behavior in the Flea Midgut

In addition to host bloodmeal, habitat associated abiotic factors can greatly impact the vector. Therefore, we wanted to investigate how alterations in the environment may impact the kinetics of infection. In addition to analyzing this using a host specific bloodmeal, we continued to investigate the additional bloodmeals under altered conditions. Maintenance conditions for fleas in the laboratory are based on host geographic ranges and the flea life history traits. Continuing to consider the geographic ranges associated with sylvatic cycles known to have epizootics, there are higher temperatures in the SW region and increased precipitation to the N and NE regions. We introduced an increase in temperature to 30°C, an increase in RH to 80% and then a combination of both increased temperature and RH, 30°C, 80% RH. We hypothesize that increased temperatures will reduce bacterial colonization in both the proventriculus and midgut while increased humidity will facilitate it.

Methods: Based on the introduced parameters, we established groups of fleas to be maintained at increased temperature (30°C) and/or humidity levels (80%). We continued to analyze days 1, 3, 4, 5, 7, and 14 days p.i. The diagram below illustrates how the groups were constructed based on the different parameters (Fig. 6). The methodologies remained the same from what was utilized for fleas infected and maintained at standard temperature and humidities.

In vitro biofilm analysis: We analyzed biofilm formation of our KIM6+ strain carrying ptdTomato in vitro to validate biofilm formation and to analyze how it may be altered when grown under these different RH and temperature combinations. Overnights were

grown in heart infusion broth (HIB) with 4mM CaCl₂ and 4mM MgCl₂. Cultures were adjusted to 0.5 OD at 600 nm and 200 µl was placed into each well used in a 96-well plate with 100 µl of HIB supplemented with 4 mM CaCl₂ and 4 mM MgCl₂. The plates were then maintained at one of the four RH and temperature combinations for 24, 48, or 72 hrs on a rocking platform. Outside rows of the plate were left blank and one row of the plate was used as background containing media only within the wells. All assays were done in triplicate. Planktonic cells were removed, and wells were washed with 400 µl of ddH₂O twice, air dried and then stained with 0.02% crystal violet for 15 minutes. The stain was removed, and wells were washed with 400 µl ddH₂O four times and bound crystal violet was solubilized with a 20% acetone:80% ethanol solution and absorbance was measured at 570 nm.

Mass measurements in flea midguts: Using ImageJ, the region of interest (ROI) was outlined for the entire flea midgut to obtain the area. Any dark masses that contained bacteria were then selected as a region of interest and measured, and all measurements were combined for a total area of masses in the midgut (Fig. 7). The total area of masses was then calculated as a percentage of total midgut area. Only masses associated with bacteria were measured and these masses were treated as biofilm based on previous literature. This was then used to compare differences in mass formation between groups of fleas maintained at different conditions.

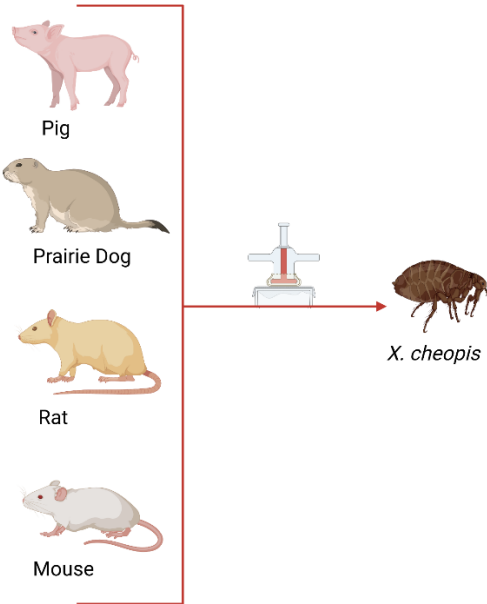
Days P.I.	Bloodmeals to use for each temperature and humidity combo	Different temperature and humidity combination for flea maintenance
EPT Day 1 Day 3 Day 4 Day 5		   
Biofilm Day 7 Day 14		

Figure 6 Experimental parameters for flea infections using four different bloodmeals and four environmental combinations. Each day p.i. will have four groups based on bloodmeal and within each bloodmeal group, there are four subgroups based on the abiotic factor combinations provided in the far-right column. Created with BioRender.com

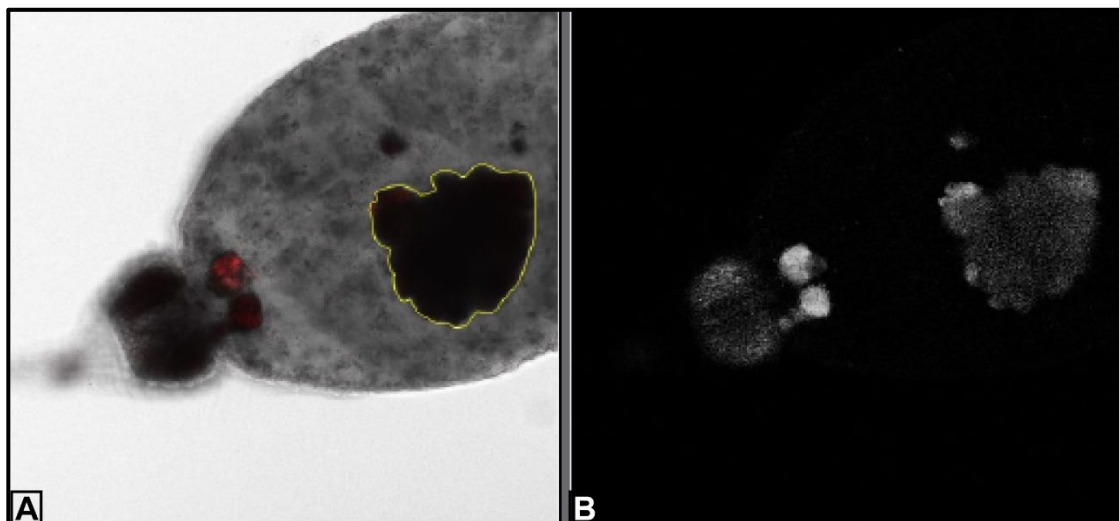


Figure 7 The area of masses within the tissues are outlined, creating ROIs that were measured in Image J. Panel A shows a RGB image with distinct masses. The ROI is outlined in yellow, and the area is measured and repeated for all masses present. Only those that are associated with bacteria based on the gray value seen in panel B were measured.

Statistical Analysis: We analyzed a total of 903 samples across 4 different bloodmeals, each with 4 different environmental combinations for 6 days p.i. for a total of 96 distinct groups. For each bloodmeal, we determined if there was a statistically significant three-way interaction between day p.i., relative humidity, and temperature, with a univariate

GLM. Due to the violation of homoscedasticity, we ran this analysis using parameter estimates for robust standard errors. A few distinct outliers skewed the distribution of some blood groups, particularly pig, however; we did not remove these from the analysis as individual variation in fleas is representative of the natural system. Due to this, we conducted a Kruskal-Wallis H to determine differences in proventricular RFUs or Overall RFUs between groups based on day p.i., relative humidity, and temperature. This was done with days 1-7 p.i. only as fleas maintained at 30°C and 70% humidity had high mortality rates at Day 14 causing low samples sizes for that group. Within each bloodmeal category, data was grouped according to the RH and temperature combination (e.g., 70_30) for each day p.i. within each bloodmeal category. Subsequently, for each day p.i. with significantly different groups, we conducted pairwise comparisons with a Bonferroni correction for multiple comparisons using Dunn's (1964) procedure to determine which groups were different for each day p.i. The same statistical analysis was done to compare the three time points for the in vitro biofilm assay. For total mass measurements within the flea midguts, we wanted to determine differences in groups based on RH and temperature within one bloodmeal for each day p.i. This allowed us to look at each day p.i. and determine differences in mass production between environmental conditions and correlate that to transmission model timelines for EPT and biofilm-mediated. Variability based on individual fleas was present, so we ran an independent samples median test in SPSS 27 to analyze if the percentage of values above and below the median were equal or significantly different.

Results Proventricular RFUs. *Host specific bloodmeal analysis (Rat):* In fleas fed with host specific bloodmeal there was a statistically significant three-way interaction between

day PI, RH, and temperature $F(5,205)=2.746$, $p=0.020$. Investigating each day p.i. indicated significant differences in multiple groups based on altered RH and temperature (Table 2, Fig. 8). Distinct differences are seen in proventricular colonization during EPT with increased RH, specifically day 3 where there is a drastic and continual reduction until day 7. The pattern during EPT is opposite from standard temperatures. Increased temperature had higher RFUs on day 1 but then had declines in infection starting day 3. With both increased RH at 80% and temperature at 30°C, a unique alternating pattern is seen. There was low proventricular colonization during EPT, but the pattern became cyclic after day 3 where there was an increase at day 4 followed by a decrease at day 5, increase at day 7 and another sharp decrease by day 14. This group was included in 7 of the 8 statistically significantly different groups. This indicates that abiotic factors impact proventricular colonization throughout infection in both an independent and joint manner.

Non-host specific bloodmeal: All bloodmeal groups were impacted by altered abiotic factors exhibiting patterns specific to the bloodmeal. For example, with rat we saw a general decrease throughout EPT with increased humidity, yet with mouse we saw a large increase on day 1 before a decrease, pdog was significantly impacted only during days associated with biofilm transmission, and there was a decrease throughout EPT except on day 3 with pig blood. For all bloodmeals we see differences in RFUs during days implicated in the transmission models (day 3, 7, 14). There are differences within groups that should not be ignored even though the median value is not significantly different because the variation in the data points within that group alters. It may alter towards more fleas having higher values of proventricular and overall RFUs or more on the lower end. It appears that clustering of the data points changes in some, either reducing or increasing

variation. For instance, with pdog blood, increased RH leads to higher RFUs on day 1 and then on day 7 and 14. If we use RFUs as a measurement for transmission potential, this indicates that at standard parameters these fleas would have low transmission potential throughout the infection. However, there is a drastic increase in potential for transmission later in the infection when maintained at higher RH. Though not significantly different from standard maintenance, there could be notable biological significance. Nonetheless, colonization is influenced by RH and temperature but to what extent and during what time in the infection is specific to bloodmeal.

Table 2 Proventricular RFUs were statistically significantly different between groups of fleas fed on different bloodmeals on multiple days post-infection.

Proventricular RFUs					
Rat Bloodmeal					
Day p.i.	χ^2	p-value	Groups		p-value
Day 1	24.345	<0.001	70 25	80 30	0.003
			80 25	80 30	0.007
			70 30	80 30	0.000015
Day 3	19.079	<0.001	70 25	80 30	0.000235
			70 30	80 30	0.010
Day 4	13.585	0.004	70 25	70 30	0.003
Day 5	19.640	<0.001	70 25	80 30	0.002
			70 30	80 30	0.001
Mouse Bloodmeal					
Day 1	13.544	0.004	70 30	80 30	0.002
Day 3	9.568	0.023	70 25	80 25	0.050
			70 25	70 30	0.040
Day 7	10.369	0.016	70 25	80 25	0.010
PDog Bloodmeal					
Day 1	12.083	0.007	70 25	70 30	0.018
			70 30	80 30	0.040
Day 4	9.280	0.026	70 30	80 30	0.025
Day 7	11.317	0.010	80 25	70 30	0.021
			80 25	80 30	0.031
Pig Bloodmeal					
Day 1	13.724	0.003	70 25	80 30	0.012
			80 25	80 30	0.008
Day 4	9.259	0.026	80 25	80 30	0.021
Day 5	15.315	0.002	70 25	80 25	0.015
			70 25	70 30	0.014

All groups DF = 3, associated test statistic shown and 2-sided p-value.

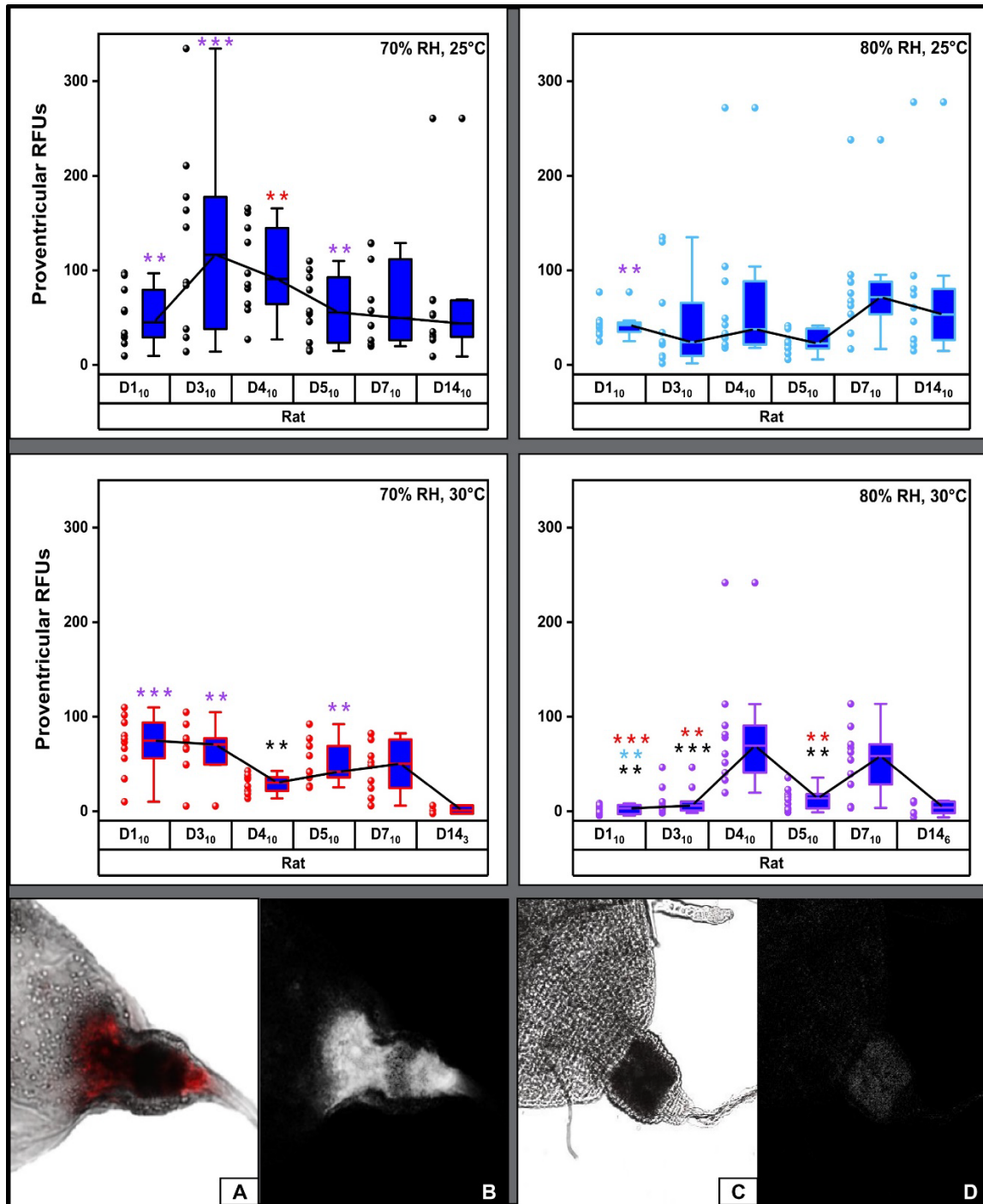


Figure 8 Increased RH and temperature caused a decrease in proventricular RFUs during EPT with a significant decrease occurring when both RH and temperature were increased in combination. Kruskal-Wallis H was performed on groups with different RH and temperature combinations for each day post-infection in fleas fed on rat blood. Analysis was followed by pairwise comparisons with a Bonferroni correction for multiple comparisons using Dunn's (1964) procedure to determine statistically significant groups. Asterisks are aligned above each day with statistically significant groups, the color of the asterisks and of the box plot border represent the two groups that are statistically different. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Box borders are associated with parameters, black (70%, 25°C), light blue indicates an increase in relative humidity (80%, 25°C), red indicates an increase in temperature (70%, 30°C), and purple indicates an increase in both relative humidity and temperature (80%, 30°C). Panels below the graphs are images representing fleas from a statistically significantly different group. A-B, day 4 p.i. at 70%, 25°C with a proventricular RFU of 160.97. C-D, day 4 p.i. at 70%, 30°C with a proventricular RFU of 26.59. Box plots illustrate the minimum and maximum values as whiskers and the box represents data that falls within 25-75% of all data with the median line. The line is connecting the median value for all sets of data.

Data points that lie outside but are in line with the box and whiskers are outliers. Data points to the left represent each data point for the set. Sample sizes for each group are subscripts on the day label along the X-axis.

Overall RFUs. *Host specific bloodmeal analysis (Rat):* In fleas fed with host specific

bloodmeal there was a statistically significant three-way interaction between day p.i., RH, and temperature $F(5,205)=2.326, p=0.044$. Investigating each day p.i. indicated

significant differences in multiple groups based on altered RH and temperature (Table 3).

The kinetics of infection days 1-3 are influenced by abiotic factors noted by a drop in RFUs with increased humidity but the opposite happening with temperature. Again, this indicates that abiotic factors can have individual impacts on the infection within fleas, but they can also cause significant changes when more than one are varied as seen with 80%, 30°C. Images illustrate the differences seen between groups on day 1 p.i. (Fig.9) that

follow the pattern seen in the box plots except for the group with increased temperature.

However, the image shows that most of the bacteria is localized to the proventriculus, and this group did have the highest proventricular RFUs of all other groups (Fig. 8). A similar

pattern was seen in CFUs when compared to box plots, however; some days (e.g. day 1 for 80%, 25°C) are different. This could be due to the large distribution in values seen in the box plot indicating individual variation of fleas which can lead to discrepancies

between samples. Fleas experienced increased mortality with higher temperatures for days 14 which caused low sample sizes for two of the groups. *Non-host specific*

bloodmeal: In fleas fed with mouse blood, there was not a statistically significant three-

way interaction, $F(5,205)=1.566, p=0.171$. In fleas fed with pdog blood, there was a

statistically significant three-way interaction $F(5,191)=2.817, p=0.018$. In fleas fed with

pig blood, there was a statistically significant three-way interaction, $F(5, 207)=3.101,$

$p=0.01$. Investigating each day p.i. indicated significant differences in multiple groups

based on altered RH and temperature (Table 3). Humidity and temperature led to deviations of RFUs from the standard parameters in all three of these bloodmeals (Fig. 10,11,12). Humidity and temperature influenced the localization of the bacteria, and bacterial growth in relation to day p.i. differently across bloodmeals. Nonetheless, these factors did have an impact on the kinetics of infection regardless of the bloodmeal source.

Table 3 Overall RFUs were statistically significantly different between groups of fleas fed on different bloodmeals on multiple days post-infection.

Overall RFUs					
Rat Bloodmeal					
Day p.i.	χ^2	p-value	Groups		p-value
Day 1	24.385	<0.001	70 25	80 30	0.004
			80 25	80 30	0.005
			70 30	80 30	0.000014
Day 3	13.782	0.003	70 25	80 30	0.010
			70 30	80 30	0.012
Day 4	13.860	0.003	70 25	70 30	0.018
			80 25	70 30	0.010
			70 30	80 30	0.017
Day 5	10.245	0.017	70 30	80 30	0.010
Mouse Bloodmeal					
Day 1	15.843	0.001	70 25	80 30	0.019
			80 25	80 30	0.017
			70 30	80 30	0.002
Day 4	9.476	0.024	70 25	80 25	0.050
			70 25	80 30	0.047
Day 7	11.280	0.010	70 25	80 25	0.008
PDog Bloodmeal					
Day 1	13.961	0.003	70 25	70 30	0.035
			80 25	70 30	0.050
			70 30	80 30	0.003
Day 4	14.452	0.002	70 30	80 30	0.001
Day 7	10.835	0.013	80 25	70 30	0.013
Pig Bloodmeal					
Day 1	21.290	<0.001	70 25	80 30	0.022
			80 25	80 30	0.000032
Day 4	17.080	<0.001	80 25	80 30	0.00235

All groups DF = 3, associated test statistic shown and 2-sided *p*-value.

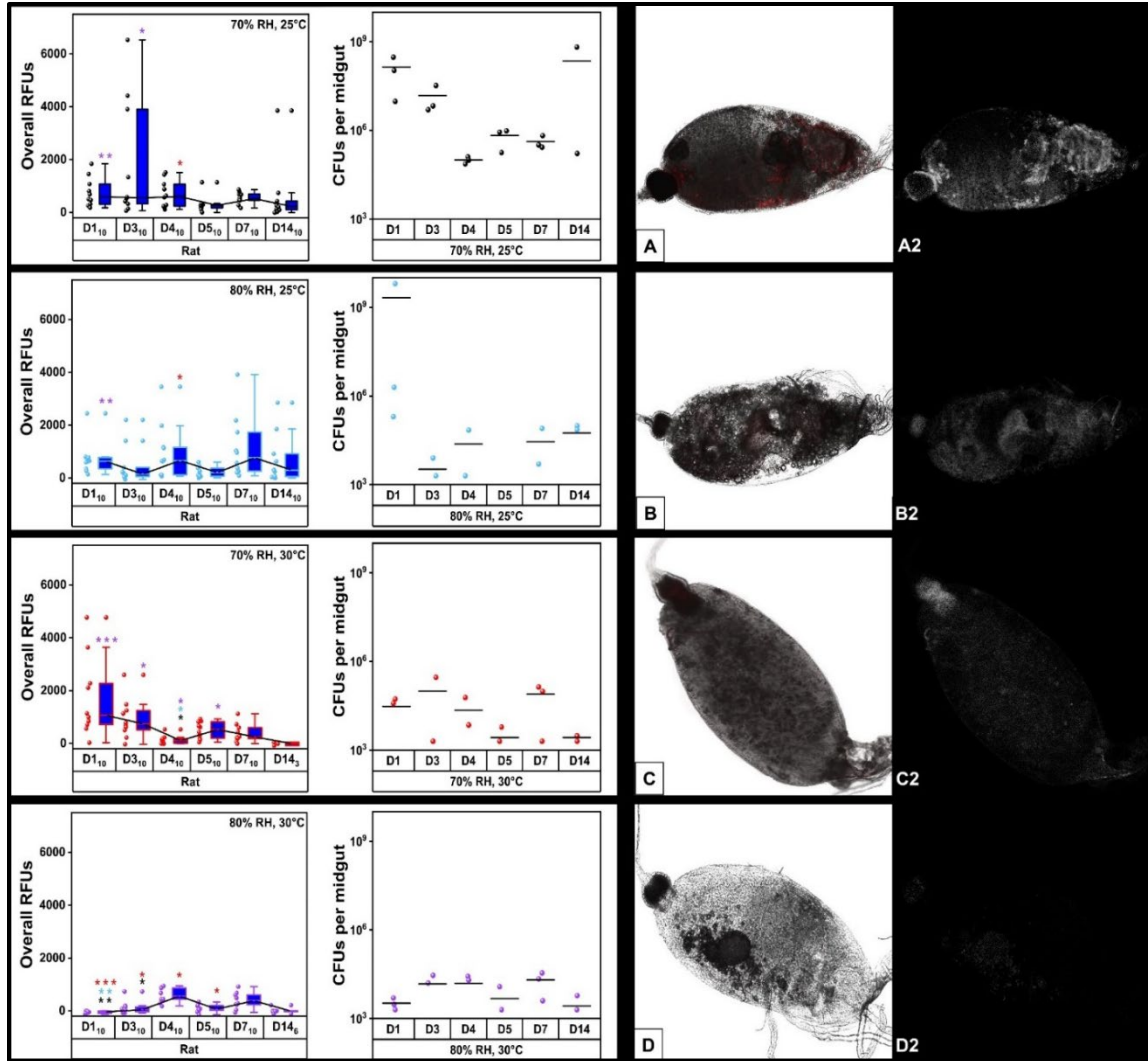


Figure 9 Humidity and temperature have direct impacts on the kinetics of infection in both an independent and dependent manner in *Xenopsylla cheopis* infected with host-specific bloodmeal. Kruskal-Wallis H was performed on groups with different RH and temperature combinations for each day post-infection in fleas fed on rat blood. Analysis was followed by pairwise comparisons with a Bonferroni correction for multiple comparisons using Dunn's (1964) procedure to determine statistically significant groups. From left to right, each row represents data from fleas associated with one of four combinations of RH and temperature. The far-left graph shows a box plot displaying the distribution of data for each day p.i., followed by a chart of CFU data for each day p.i. with each dot representing 3 midguts. Bars represent mean value for CFUs since each dot represents three midguts. Asterisks are aligned above each day with statistically significant groups, the color of the asterisks and of the box plot border represent the two groups that are statistically different. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Box borders are associated with parameters, black (70%, 25°C), light blue indicates an increase in relative humidity (80%, 25°C), red indicates an increase in temperature (70%, 30°C), and purple indicates an increase in both relative humidity and temperature (80%, 30°C). Box plots illustrate the minimum and maximum values as whiskers and the box represents data that falls within 25-75% of all data with the median line. The line is connecting the median value for all sets of data. Data points that lie outside but are in line with the box and whiskers are outliers. Data points to the left represent each data point for the set. Sample sizes for each group are subscripts on the day label along the X-axis. The last two images for each row are images representing a flea midgut from that RH and temperature combination on Day 1 p.i.. Panels show the RGB image next to the saturation channel of the converted RGB to HSB. For panels A-A2, 70%, 25°C with an overall RFU of 1839.34, B-B2, 80%, 25°C with an overall RFU of 799.82, C-C2, 70%, 30°C with an overall RFU of 714.18, and D-D2, 80%, 30°C with an overall RFU of 5.53.

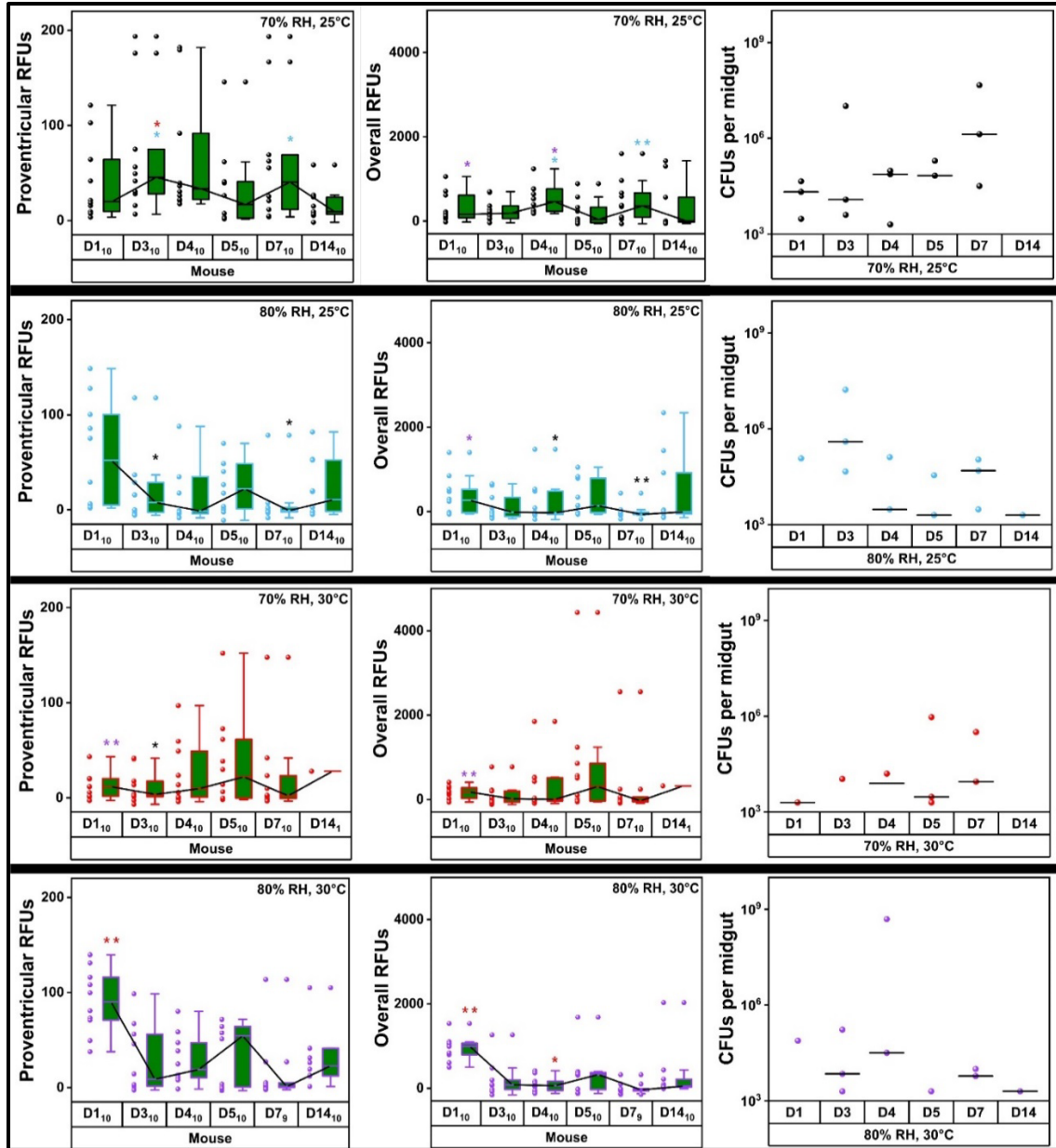


Figure 10 Humidity and temperature increased RFUs later p.i. with combined increases in humidity and temperature facilitating proventricular colonization in *Xenopsylla cheopis* infected with mouse blood. Kruskal-Wallis H was performed on groups with different RH and temperature combinations for each day post-infection in fleas fed on mouse blood. Analysis was followed by pairwise comparisons with a Bonferroni correction for multiple comparisons using Dunn's (1964) procedure to determine statistically significant groups. Each row represents a group based on RH and temperature starting with 70% RH, 25°C and shows proventricular RFUs, overall RFUs, and CFU data for all days p.i. Each dot for the CFU data represents 3 midguts. Bars represent mean value for CFUs since each dot represents three midguts. Each column represents one combination of RH and temperature, so each row shows data from left to right for, 70%, 25°C, 80%, 25°C, 70%, 30°C, and finally, 80%, 30°C. Asterisks are aligned above each day with statistically significant groups, the color of the asterisks and of the box plot border represent the two groups that are statistically different. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Box borders are associated with parameters, black (70%, 25°C), light blue indicates an increase in relative humidity (80%, 25°C), red indicates an increase in temperature (70%, 30°C), and purple indicates an increase in both relative humidity and temperature (80%, 30°C). Box plots illustrate the minimum and maximum values as whiskers and the box represents data that falls within 25-75% of all data with the median line. The line is connecting the median value for all sets of data. Data points that lie outside but are in line with the box and whiskers are outliers. Data points to the left represent each data point for the set. Sample sizes for each group are subscripts on the day label along the X-axis.

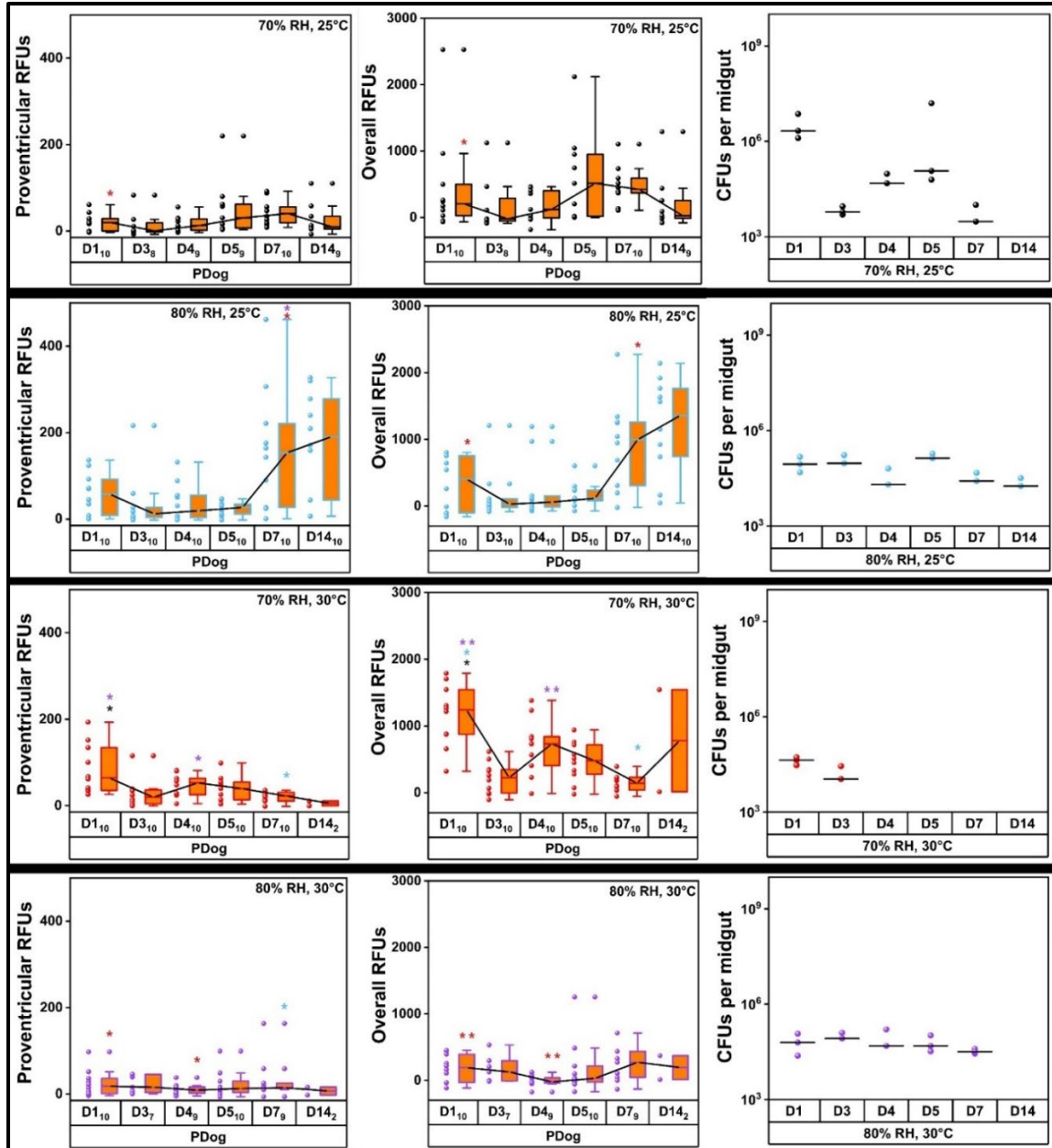


Figure 11 Increased humidity promoted bacterial growth and colonization later in the infection whereas increased temperature slightly enhanced infection continually in *Xenopsylla cheopis* infected with pdog blood. Kruskal-Wallis H was performed on groups with different RH and temperature combinations for each day post-infection in fleas fed on pdog blood. Analysis was followed by pairwise comparisons with a Bonferroni correction for multiple comparisons using Dunn's (1964) procedure to determine statistically significant groups. Each row represents a group based on RH and temperature starting with 70% RH, 25°C and shows proventricular RFUs, overall RFUs, and CFU data for all days p.i. Each dot for the CFU data represents 3 midguts. Bars represent mean value for CFUs since each dot represents three midguts. Each column represents one combination of RH and temperature, so each row shows data from left to right for, 70%, 25°C, 80%, 25°C, 70%, 30°C, and finally, 80%, 30°C. Asterisks are aligned above each day with statistically significant groups, the color of the asterisks and of the box plot border represent the two groups that are statistically different. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Box borders are associated with parameters, black (70%, 25°C), light blue indicates an increase in relative humidity (80%, 25°C), red indicates an increase in temperature (70%, 30°C), and purple indicates an increase in both relative humidity and temperature (80%, 30°C). Box plots illustrate the minimum and maximum values as whiskers and the box represents data that falls within 25-75% of all data with the median line. The line is connecting the median value for all sets of data. Data points that lie outside but are in line with the box and whiskers are outliers. Data points to the left represent each data point for the set. Sample sizes for each group are subscripts on the day label along the X-axis.

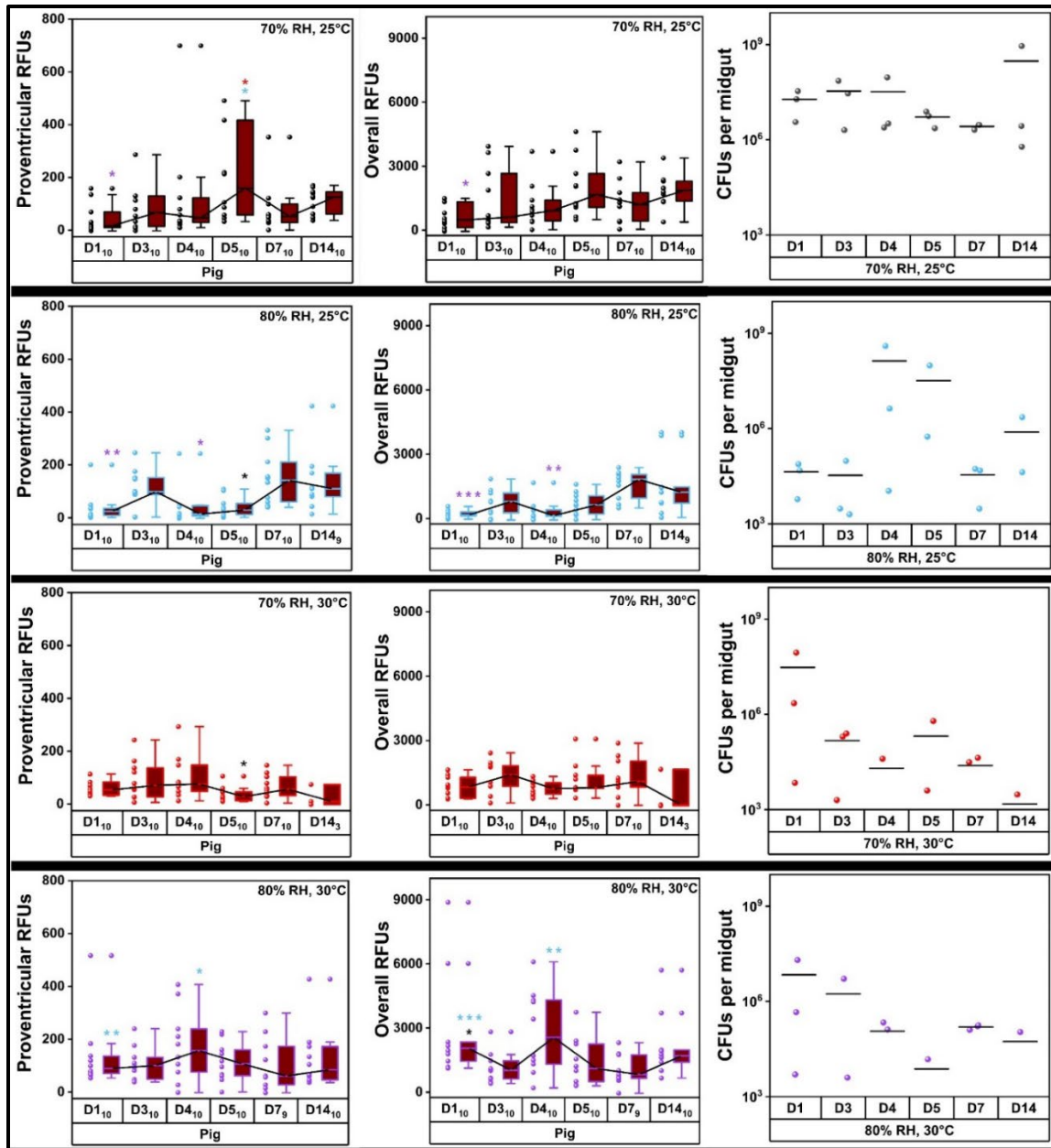


Figure 12 Increased humidity and temperature independently reduced infection but a combination of increased humidity and temperature led to comparable infection levels to standard parameters in *Xenopsylla cheopis* infected with pig blood. Kruskal-Wallis H was performed on groups with different RH and temperature combinations for each day post-infection in fleas fed on pig blood. Analysis was followed by pairwise comparisons with a Bonferroni correction for multiple comparisons using Dunn's (1964) procedure to determine statistically significant groups. Each row represents a group based on RH and temperature starting with 70% RH, 25°C and shows proventricular RFUs, overall RFUs, and CFU data for all days p.i. Each dot for the CFU data represents 3 midguts. Bars represent mean value for CFUs since each dot represents three midguts. Each column represents one combination of RH and temperature, so each row shows data from left to right for, 70%, 25°C, 80%, 25°C, 70%, 30°C, and finally, 80%, 30°C. Asterisks are aligned above each day with statistically significant groups, the color of the asterisks and of the box plot border represent the two groups that are statistically different. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Box borders are associated with parameters, black (70%, 25°C), light blue indicates an increase in relative humidity (80%, 25°C), red indicates an increase in temperature (70%, 30°C), and purple indicates an increase in both relative humidity and temperature (80%, 30°C). Box plots illustrate the minimum and maximum values as whiskers and the box represents data that falls within 25-75% of all data with the median line. The line is connecting the median value for all sets of data. Data points that lie outside but are in line with the box and whiskers are outliers. Data points to the left represent each data point for the set. Sample sizes for each group are subscripts on the day label along the X-axis.

Biofilm assay: The in vitro biofilm assay for our bacterial strain under the varying conditions showed that an increase in temperature and/or relative humidity did influence biofilm production (Fig. 13). There was an increase when compared to biofilm production at the standard maintenance conditions of 70% RH, 25°C for all three time points of 24 hrs, 48 hrs, and 72 hrs. However, it was prominent at 72 hrs after a decrease at 48hrs. For the first 48 hrs all three altered conditions had higher biofilm production than standard but the variance between them was reduced. At 72 hrs, biofilm production was increased at all four parameters. Analysis indicated significant differences between groups at each time point (Table 4).

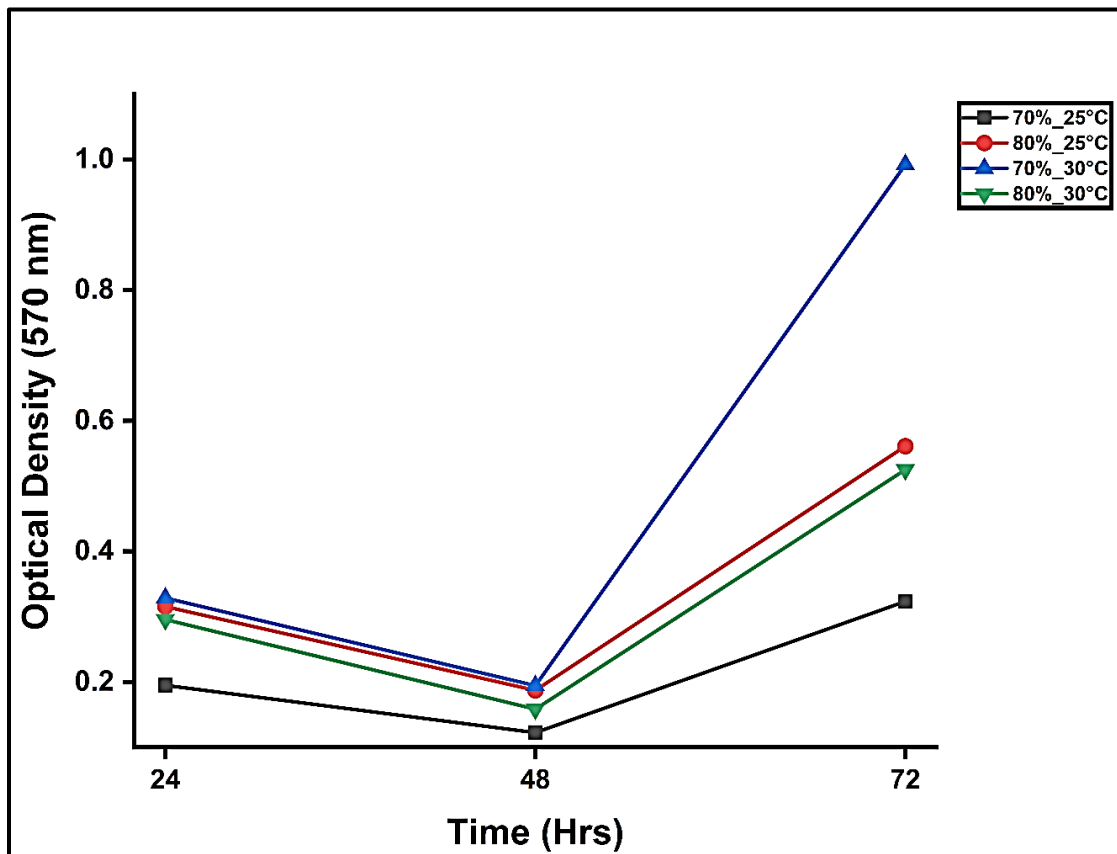


Figure 13 Increases in temperature and relative humidity elevate biofilm production in vitro by 72 hrs. Standard maintenance of 70% RH, 25°C is shown in black with the other three conditions represented with red (80%, 25°C), blue (70%, 30°C), and green (80%, 30°). Biofilm production was measured using the crystal violet assay in a 96-well plate with absorbance measured at 570 nm. The points at each time represent the mean for each group.

Table 4 Biofilm production was significantly different between groups based on humidity and temperature at each time point.

Time	χ^2	p-value	Groups		p-value
24 Hours	31.249	<0.001	70 25	80 30	0.022
			70 25	70 30	<0.001
			70 25	80 25	<0.001
48 Hours	29.857	<0.001	70 25	80 25	<0.001
			70 25	70 30	<0.001
			70 30	80 30	<0.001
72 Hours	65.851	<0.001	70 25	80 30	<0.001
			70 25	80 25	<0.001
			70 25	70 30	<0.001
			70 30	80 30	<0.001
			70 30	80 25	0.002

DF=3, N=96 for each group

Mass measurements in flea midguts: Flea midguts with one or more masses present were processed in image J to measure the area and determine the percentage of midgut area attributed to the masses. Only masses associated with bacteria were measured and used as an indicator for biofilm formation. Comparisons were made within each bloodmeal between days p.i. and conditions to determine how biofilm formation may be influenced by environmental variables in vivo.

Table 5 Independent samples median test with reported median value, test statistic, degrees of freedom, and p-value. Days with significantly different medians and the associated statistically significantly different groups are shown.

Rat Bloodmeal						
Day p.i.	Median	χ^2	p-value	Groups		p-value
Day 3	0.109	7.817	0.049	70 25	80 30	0.022
				70 25	70 30	0.028
				70 25	80 25	0.041
Mouse Bloodmeal						
Day 1	0.050	10.292	0.016	70 30	80 30	0.005
PDog Bloodmeal						
Day1	0.060	16.797	<0.001	70 25	70 30	<0.001
				70 30	80 30	<0.001
Day 4	0.040	10.091	0.018	80 25	80 30	0.027
				70 30	80 30	0.003
				70 25	70 30	0.039
Day 7	0.070	10.667	0.014	70 30	80 30	0.001
				80 25	70 30	0.007
Pig Bloodmeal						
Day 14	0.242	14.171	0.003	70 25	80 30	0.003
				70 25	80 25	<0.001

DF = 3 for all

Statistically significant differences were seen between groups maintained at different parameters on certain days p.i. in each bloodmeal. Interestingly, the days with significantly different medians across groups were associated with either EPT or biofilm-mediated and none on day 5 during the transitional timeframe. For host-specific bloodmeal, day 3 had significant differences in all groups when compared to standard maintenance parameters. Several groups were statistically significantly different when fed on prairie dog blood and all differences were associated with groups that had higher temperatures.

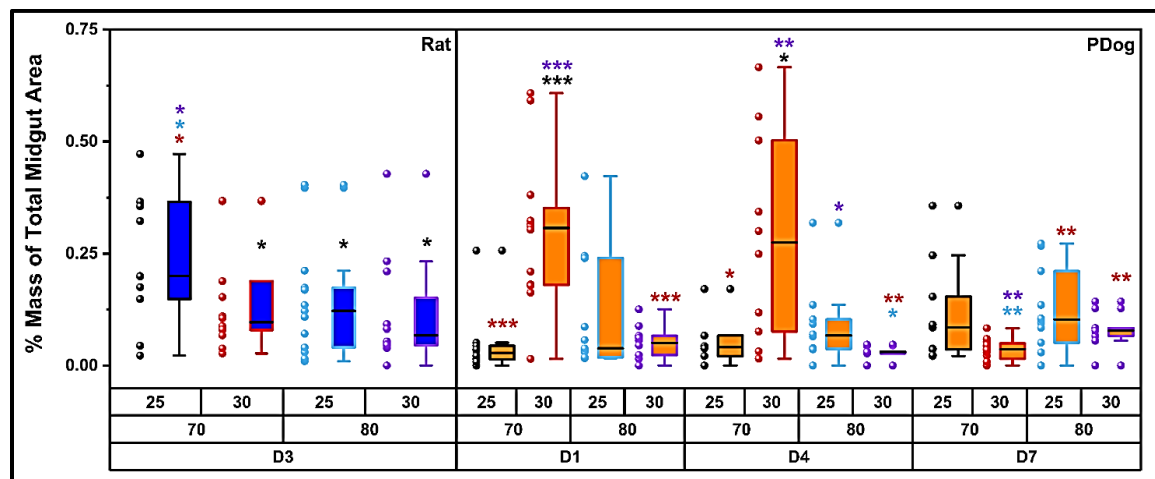


Figure 14 Increases in humidity and temperature cause a unidirectional decrease in midgut localized mass formation on day 3 in *Xenopsylla cheopis* infected with host specific blood, but large variations are seen throughout the infection in fleas fed on prairie dog blood. An independent samples median test indicated significant differences in the percentage of samples above or below median values (black lines in boxes). Asterisks are aligned above each day with statistically significant groups, the color of the asterisks and of the box plot border represent the two groups that are statistically different. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Asterisks are assigned based on the day post-infection so that all boxes in day 1 have asterisks indicating their differences to the other parameters for only day 1. Box borders are associated with parameters, black (70%, 25°C), light blue indicates an increase in relative humidity (80%, 25°C), red indicates an increase in temperature (70%, 30°C), and purple indicates an increase in both relative humidity and temperature (80%, 30°C). Box plots illustrate the minimum and maximum values as whiskers and the box represents data that falls within 25-75% of all data with the median line. The line connects the median value for all sets of data. Data points that lie outside but are in line with the box and whiskers are outliers. Data points to the left represent each data point for the set.

3.3 Transmission Efficiency of Fleas Infected with Different Host Bloodmeals and Maintained at Varying Temperatures and Relative Humidity Levels

To determine the potential impact of variation in microclimatic factors on enzootic or epizootic occurrences, we analyzed transmission efficiencies of fleas

maintained at the different parameters. Both phases of transmission were analyzed, EPT and biofilm-mediated. All variables were included in the experiment and subsequent analysis which included 4 host bloodmeals, days 1, 3, 4, 5, 7, and 14 p.i., 70% or 80% RH, and 25°C or 30°C.

Methods: Fleas were artificially fed with one of four host bloodmeals collected under the same methods previously stated. Fleas were starved for 5-7 days and fed an inoculated bloodmeal, containing between 5×10^8 to 1×10^9 *Y. pestis*/ml, through an artificial feeding system set up in the same way as previously described. The same strain used in previous experiments was used for transmission studies, KIM6+ (*pgm*+ *hms*+ *pla*+ pCD1-) attenuated strain carrying fluorescent plasmid tdTomato. Fleas fed for one hour, were observed for bloodmeal ingestion and only those that took a bloodmeal were included in the analysis. Fleas were separated into 8 groups of 10 and two groups were maintained at each of the environmental variable combinations (70%-25°C, 80%-25°C, 70%-30°C, 80%-30°C) with one group analyzed for transmission on day 3 (EPT) and one on day 7 (biofilm-mediated). Fleas were housed in the mesh containers until either day 3 or day 7 where they were allowed to feed on an artificial feeder. A non-infected bloodmeal was used, fleas were allowed to feed for one hour before being removed and euthanized. Blood from the feeder was collected for plating. The feeder was washed three times with each wash collected for plated and the skin was homogenized for plating. All samples were plated on Yersinia selective agar (YSA) and analyzed for CFUs after incubating for 48 hrs at 26°C. This was repeated 3 times for each group on day 3 and on day 7.

Statistical Analysis: We determined if there were differences in CFUs transmitted on day 3 or 7 between groups based on RH and temperature. The data were analyzed using the

Kruskal-Wallis H followed by a Bonferroni correction for multiple comparisons using Dunn's (1964) procedure in SPSS 27.

Results: Differences in the amount of CFUs transmitted occur across all groups for each bloodmeal at both day 3 and day 7, however; only day 7 had statistically significantly different groups for fleas fed on host-specific blood. An increase in RH led to significant differences in CFUs transmitted on day 7. Though not statistically significant, differences in CFUs transmitted are notable with increased RH for day 3 and increased temperature and RH for day 7 (Fig.16). Day 3 and day 7 were variable among groups fed on mouse bloodmeal but the pattern altered between day 3 and 7. On day 3, an increase in relative humidity led to an increase in CFUs for all 3 experiments but there was a decrease in this group for day 7. Standard parameters in fleas fed with pig blood led to higher transmission in comparison to the other groups. All groups fed pdog blood had lower CFUs than any of the other bloodmeals with the highest transmission occurring at day 3 in fleas maintained at higher temperatures (Fig.16).

Table 6 CFUs transmitted were statistically significantly different between groups of fleas fed on rat bloodmeal on day 7 post-infection.

Transmission CFUs					
Rat Bloodmeal					
Day p.i.	χ^2	p-value	Groups		p-value
Day 7	7.615	0.05	70 25	80 25	0.017
			80 25	80 30	0.024

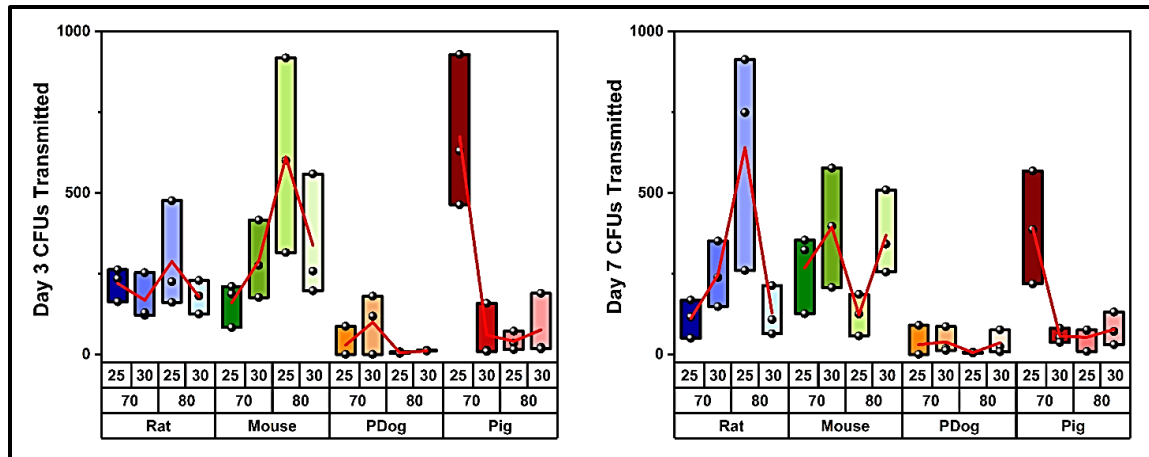


Figure 15 Humidity and temperature had significant impacts on the number of CFUs transmitted on day 3 and day 7 in all bloodmeals with only fleas infected on pig blood exhibiting a unidirectional shift in all altered parameters. The number of CFU transmitted by *X. cheopis* fleas on days 3 and 7 post-infection across groups based on RH and temperature. Colors are associated with bloodmeal, rat (blue), mouse (green), orange (pdog), and maroon (pig) and the different shades indicate changes in group associated parameters.

3.4 Discussion

In this work, we investigated the potential impact of abiotic factors, temperature, and humidity, in conjunction with biotic factors, host bloodmeal, on vector competence and subsequent transmission of *Y. pestis* by *X. cheopis* fleas. Our results indicate that temperature and humidity influence colonization, bacterial growth, and transmission both independently and jointly regardless of bloodmeal. Proventricular colonization early in the infection, during EPT, was altered by temperature and humidity with 80% RH, 25°C providing an optimal environment. This correlated to increased bacterial growth and transmission rate on day 7, suggesting an impact on biofilm-mediated transmission as well as EPT. Transmission by fleas infected with non-host specific blood was altered under the varying conditions, with the most notable in increased transmission efficiencies with higher humidity. Our data suggests that altered levels of humidity and temperature directly influence transmission efficiency as seen with increased transmission in fleas fed with mouse under different conditions. These data indicate synergistic impacts on the spread of plague under optimal environmental conditions in the flea habitat.

Both transmission models are regurgitative and rely on bacterial colonization of the proventriculus. Host bloodmeal has proven to be a source of variation in the extent and timing of proventricular colonization, in particular the solubility of the hemoglobin has been highlighted as the driving factor [24]. EPT has been categorized as insufficient for epizootics due to the need for fleas to be blocked to some extent. Fleas fed rat blood have shown to become blocked during EPT, which is not seen in mouse [20,22,26,27]. For standard environmental parameters, we did observe an increase in proventricular colonization during EPT in fleas fed on rat blood and an increase on day 7. Fleas fed on mouse blood exhibited a similar pattern, but infection was lower for each day analyzed, however; there was not a significant difference in proventricular RFUs between rat and mouse blood. In our study we included pdog blood which is expected to have relatively insoluble hemoglobin comparable to rat blood [23]. However, pdog blood had the lowest levels of proventricular colonization than the other three bloodmeals. Groups of fleas fed on rat and pdog blood were statistically significantly different from each other for proventricular RFUs on days 3 and 4 and for overall RFUs on day 3. Although pdog specific fleas such as *O. montana* exhibit high proventricular colonization when fed on rat blood, the same is not seen with rat specific fleas fed on pdog blood. The insolubility of the hemoglobin has shown to be an important factor, but the importance is still limited by the host-specificity representative of the natural system. This is further supported by the high proventricular colonization seen in fleas fed on pig blood. Moderate to high colonization was seen in host specific bloodmeal or in pig which has been shown to increase reproduction in fleas, indicating the presence of nutritional blood characteristics.

Our results support that bloodmeal is a source of variation in standard environmental conditions, however; the extent to which non-specific bloodmeal plays a role in the natural sylvatic cycle is likely very low. The microclimatic factors of the burrow directly influence flea survival and reproduction and according to our results, play an important role in the flea infection. In all bloodmeals, there were significant differences in proventricular and overall RFUs during EPT in fleas maintained at different environmental conditions. Plague occurrences have been linked to environmental conditions including an increase during the dry season and a decrease with increased temperatures, however; this is without investigation into the microclimate and varies geographically [18, 35]. In host-specific bloodmeal, an increase in RH decreases the proventricular colonization throughout the infection. The impact that precipitation has on the humidity of the burrow will depend on temperature but more importantly edaphic characteristics. These characteristics will impact the overall ability of the soil to allow water to flow through and/or retain water. This introduces distinct differences across large geographic ranges which could help explain the disparities seen across ecological studies. An increase in both temperature and humidity, greatly reduced proventricular colonization during EPT for host-specific bloodmeals. There were variable responses to changes in non-host specific bloodmeals, particularly in pdog blood with increases in bacterial loads later in the infection with increased humidity or temperature. Environmental variables alter infection but there is specificity in how the kinetics of the infection change based on bloodmeal. CFUs showed some variability in relation to boxplots, however, this is likely due to individual flea variation and differences in sample sizes.

The differences seen between groups during EPT were also seen when we analyzed the mass formation. For rat blood, there were significant differences seen from the standard parameters in all three conditions on day 3, the peak transmission for EPT. An increase in temperature significantly increased mass percent for both day 1 and day 3 in pdog blood, as well as RH which showed an increase in day 1, 3, and 7, though not as prominent. This indicates that ideal conditions for biofilm formation vary with bloodmeal and are not specific to the timeline associated with biofilm-mediated transmission. Altering conditions facilitate the formation of masses earlier in the infection. It is known that biofilm in the proventricular valve increases the number of transmitted bacteria due to the regurgitative mechanism. Additionally, the lack of adhesins in *Y.pestis*, makes it reliant on biofilm for increased colonization. This is influenced by host bloodmeal as supported with transmission studies indicating increased transmission and host mortality when fleas were infected with rat blood versus mouse blood [20, 21]. However, the difference in transmission efficiency between bloodmeals is reduced as long as partial or full blockage occurs. Environmental variables influencing biofilm formation could directly impact proventricular colonization and subsequent transmission without a temporal constraint. We saw distinct differences between host bloodmeals in the amount of CFUs transmitted on day 3 and day 7 p.i. Analysis to determine differences between groups within a single bloodmeal indicated significant differences in day 7 for host specific bloodmeal. All three altered groups had higher transmission than the group at standard parameters, with an increase in RH the highest. Mass formation for day 7 was not significantly different between groups. When looking at proventricular RFUs, there is greater variability in the standard group dropping the median value, whereas the other

three groups had reduced variation across samples and higher median values. We also see higher overall RFUs in these groups compared to the standard group. Although day 3 was not significantly different for transmission, it was for mass formation and exhibited the opposite relationship with the standard group higher than the others. This is also represented in the proventricular and overall RFU boxplots. We can see the same pattern between mass formation and increased proventricular and overall RFUs in groups fed pdog blood. The pattern between mass formation and RFUs is easily distinguishable in groups fed on mouse. It is less distinguishable in pig blood as there was less variation between groups throughout the infection, however; the noticeable drop in groups with increased RH is seen in the transmission analysis as well.

Flea vectors rely heavily on environmental and ecological relationships within the ecosystem for survival and reproduction. As such, these relationships have become a focus in investigations attempting to discover the mechanisms that drive sudden epizootic events in long-term enzootic cycles. This has led to understanding that host bloodmeal is important for vector competency but to what capacity is still unclear. EPT was implicated as the transmission model facilitating epizootic events, however; recent suggestions have been made on the role of EPT in developing immunity in mammalian host with high exposure rates [20, 21, 36, 37]. These populations still experience periodic epizootics, so this immunity is limited when exposed to high amounts of bacteria but in host-specific fleas already present, it is unclear on what drives the increase in proventricular colonization. For rat fleas fed with rat blood, we have seen significant increases in proventricular colonization and higher rates of blockage, but our results have indicated that is altered when environmental variables change. In addition, host specificity is

imperative to fully understand how the bloodmeal may influence overall vector competency and subsequent transmission. Prairie dog blood was not comparable to rat blood in our analysis. Microclimatic variables influence vector competency and transmission in both host-specific and non-host specific bloodmeals. These factors alter throughout the geographic range of natural sylvatic plague cycles lending to the inconsistencies seen across field studies in epizootic and enzootic cycles. Microclimatic variables directly influence the colonization of fleas by *Y.pestis* and host-specific vector competency.

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4 EFFECTS OF HOST BLOODMEAL, TEMPERATURE, AND HUMIDITY ON DIGESTION IN INFECTED FLEAS

Background: Acquisition of *Yersinia pestis* by flea vectors occurs via the bloodmeal, and the bacteria must survive the digestive process to colonize the flea. The bloodmeal has significant impacts on physiological processes such as metabolism and reproduction in hematophagous arthropods [1]. Due to the importance of the bloodmeal for acquisition of pathogens and survival of arthropod vectors, recent research has investigated the impact it may have on the vector competency of fleas transmitting *Y. pestis*. To date, it has been shown to influence the colonization within the foregut and subsequent transmission of *Y. pestis* by fleas [2-6].

In early studies, the digestion process in fleas was characterized into 3 stages: stage 1 is blood intake followed by hemolysis, stage 2 is the end of hemolysis and digestion of blood to hematin, and stage 3 is the darkening of the midgut signifying the start of excretion [7]. The digestion process is relatively quick as fleas do not have an expandable midgut like other hematophagous arthropods and are therefore not able to store protein [8]. Close associations with their host, correlates to the frequency in which they feed, size of meal, and reproduction[8]. *Xenopsylla cheopis* fleas feed frequently and take in an average of 0.15 mg of blood within 4-10 minutes [9]. As the majority of flea species do, they live off host and must occupy the environment in close proximity. For those that feed on burrowing rodents such as *X. cheopis* and *Oropsylla sp.*, they will occupy the host burrow to achieve this. The burrow habitat maintains different temperature and relative humidity levels than outside with minimal fluctuations, thus providing a stable environment for fleas [10, 11].

Digestion rates have been shown to differ between bloodmeal sources. The median blood digestion rate for *X. cheopis* fed on mouse blood was 12 hrs, 17 hrs when fed on golden hamster and humans, 21.5 hrs when fed on guinea pig, and 25 hrs when fed on brown rat [12]. Different bloodmeals will not only have varying digestion rates but will influence reproduction. For example, fleas that fed on human blood did not reproduce [12]. Certain blood components are necessary for oocyte development in *Xenopsylla* sp. When the blood cell fraction was removed and fleas were fed on plasma only, oocyte resorption was observed but when this component was provided, vitellogenesis was triggered [8]. The deletion of cholesterol or substitution of hemoglobin with albumin led to the depletion of yolk deposition [8]. In rabbit fleas, *Spilopsyllus* sp., albumin could be utilized but this species lives on host and was directly impacted by host hormone levels [8, 13]. The initial reproductive event after emerging from the cocoon, is reliant on bloodmeal. In *X. cheopis*, the bloodmeal triggers the development of ovaries and dissolution of the testicular plug [8, 9].

Temperature can also impact the rate of digestion. An increase in temperature is typically associated with shorter digestion times in several *Xenopsylla* sp. including *X. cheopis* [9, 14]. Bloodmeal digestion increases the production of digestive enzymes necessary for lipid digestion and increased temperatures facilitate enzymatic activity [15]. Four hrs after feeding, genes encoding serine proteases such as trypsin or trypsin-like enzymes are upregulated [15]. Increased digestion may indirectly influence vector competency of fleas if *Y. pestis* relies on bloodmeal components for colonization by potentially eliminating this resource. An increase in temperature was associated with a decline or elimination in proventricular blockage, however; the exact mechanism leading

to this reduction is unknown [16-18]. Considering host specificity of fleas, inhabitation of rodent burrows, bloodmeal impacts on flea reproduction and temperature influencing digestion, we wanted to investigate the interrelationship between bloodmeal, temperature, and humidity on digestion in uninfected and infected fleas. To the best of our knowledge, these factors have never been investigated collectively, nor have the potential impacts humidity may have on digestion. Often times, pathogen:host or pathogen:vector interactions are investigated linearly, effectively removing the ecological influences of the natural system. The inclusion of factors from the ecosystem in which the pathogen persists, both biotic and abiotic, are necessary to understand its' ecology and the potential risk it poses to mammalian species and as a zoonotic disease [19].

Hypothesis: Temperature and humidity positively influence digestion in both uninfected and infected fleas.

Methods: Fleas reared in the lab were designated into groups based on infection, RH, temperature, and bloodmeal. Fleas were maintained at one of the following RH, temperature combinations of 70%,25°C, 80% RH, 25°C, 70% RH, 30°C, or 80%, 30°C for 3 days prior to feeding. They were fed one of four bloodmeals (infected or uninfected) and held at the respective parameters until euthanized. According to previous research, *X. cheopis* that fed on different bloodmeals had durations of digestion from 12-25 hrs [9]. We analyzed protein and chymotrypsin-like serine protease levels in fleas every 3 hrs until 18 hrs post-feeding to capture peak digestion.

For protein analysis, we used the PierceTM BCA protein assay kit. For each time point, 3 fleas were washed in sterile PBS and each midgut was dissected into 10µl of sterile PBS for a total of 30µl per time point. We ran each sample in triplicate, using 10µl

of sample per well. We followed the microplate protocol with a standard curve on each plate. Blank wells were included in triplicate. The absorbance was measured at 562 nm, the blank wells were averaged, and the average was subtracted from all wells. A standard curve was plotted and used to calculate the protein concentration of samples.

The same groupings were used for RT-qPCR with 3 fleas per timepoint. Fleas were stored at -80°C until processing which occurred within one week of the experiment. Fleas were washed in ddH₂O and placed into sterile RNase-free PBS where the midguts were dissected out and transferred to 10µl fresh PBS per midgut. Any tissue outside of the alimentary canal was removed. Contents were then placed into an RNase-free 1.5mL tube with 200µl of TRIzol reagent and stored at -80°C until further processing. RNA was isolated using the Direct-Zol™ RNA miniprep kit from Zymo. We generated cDNA from 1 ng of isolated RNA using the MMLV reverse transcriptase from Promega. Power SYBR green PCR mastermix was used in 20µl volume reactions with the following conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 60°C for 1 minute. Reactions and analysis were carried out on the QuantStudio™ 3 system and software. We used the elongation factor 1 delta (*ef-1d*) as our housekeeping gene and uninfected fleas fed on rat blood and maintained at 70% RH and 25°C as our control to calculate $\Delta\Delta CT$. Previously published primers for the housekeeping gene and for chymotrypsin-like serine protease were used [20, 21]. Based on amplification plots, negative samples consistently fell above 36 cycles. Therefore, any samples that fell above 36 were considered negative (Fig. S1).

Statistical analysis: For the protein assay, data were not normally distributed across all groups. To compare protein levels of uninfected and infected bloodmeals under the

different parameters for each bloodmeal, we used a Mann Whitney U. To compare if protein levels were different between the four bloodmeals at the different parameters, we used a Kruskal-Wallis H followed by a Bonferroni correction for multiple comparisons using Dunn's (1964) procedure in SPSS 27.

4.1 Digestion in Uninfected and Infected Fleas Fed on Host-Specific Bloodmeal Under Different Abiotic Conditions

Previous investigations on digestion in fleas have primarily been based on the visualization of the bloodmeal over time or measurements of energy expenditure, such as the production of CO₂ [12, 22-25]. We utilized a protein assay to assess digestion and determine differences between uninfected and infected.

Results: In fleas fed on host-specific bloodmeal, infected fleas had lower protein levels early in the infection. Analyzing chymotrypsin-like serine protease showed increased levels in infection up to 6 hrs prior to declining (Fig. 1). A peak in protein is seen at 12 hrs which could be a lag due to the increased protease levels at 6 hrs (Fig. 1). The Δ CT levels were compared between uninfected and infected for fleas maintained at 70% RH and 25°C since there was no control. It is difficult to determine the biological significance of the compared protease levels and will need further evaluation. Comparing digestion in uninfected fleas across different parameters, we saw increased humidity leading to increased protein levels, increased temperatures decreased protein levels, and increasing both humidity and temperature decreased protein early but the remaining digestion was similar to that of standard parameters (Fig. 2). An increase in humidity increased protein in uninfected fleas but decreased protein in infected (Fig. 3). The opposite effect was seen with increased temperature only. Increased humidity and temperature increased infection early but decreased later in infection and the opposite

was seen in uninfected (Fig. 3). Protease levels followed a similar pattern to protein digestions. Increased humidity only and increased temperature only were both statistically significantly different than standard parameters (Table 1).

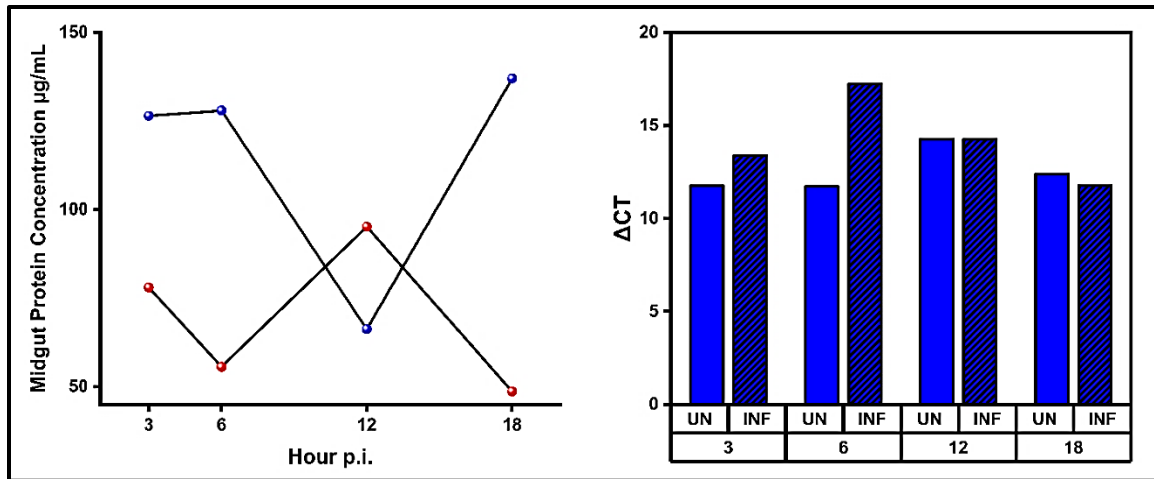


Figure 1 Fleas fed bloodmeal inoculated with *Y. pestis* have increased chymotrypsin-like serine protease levels within the first 6 hrs of infection prior to drastic and sustained reductions for the remainder of the infection. The right graph shows differences in levels of chymotrypsin-like serine proteases for fleas infected with rat blood (blue bars with black stripes) in relation to uninfected fleas fed rat blood, both maintained at 70% RH, 25°C. The left graph shows the protein levels in the midgut of uninfected and infected fleas maintained at 70% RH, 25°C. The blue symbols are uninfected rat, and red represents infected.

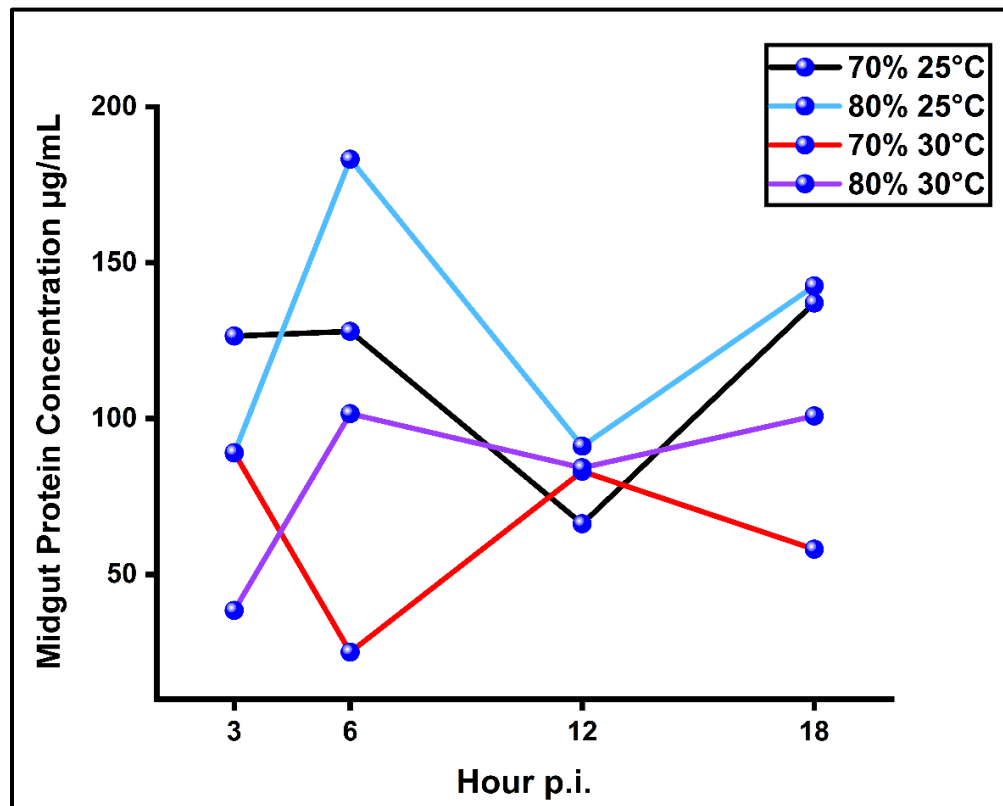


Figure 2 Digestion is influenced by relative humidity and temperature in fleas fed host-specific bloodmeal. The graph shows differences in protein levels in fleas fed with rat blood. The blue symbols represent rat bloodmeal and the lines are representative of

the different parameters the fleas were maintained in. Light blue indicates increased humidity, black is standard parameters of 70% RH, 25°C, red represents increased temperature, and purple represents increased temperature and humidity.

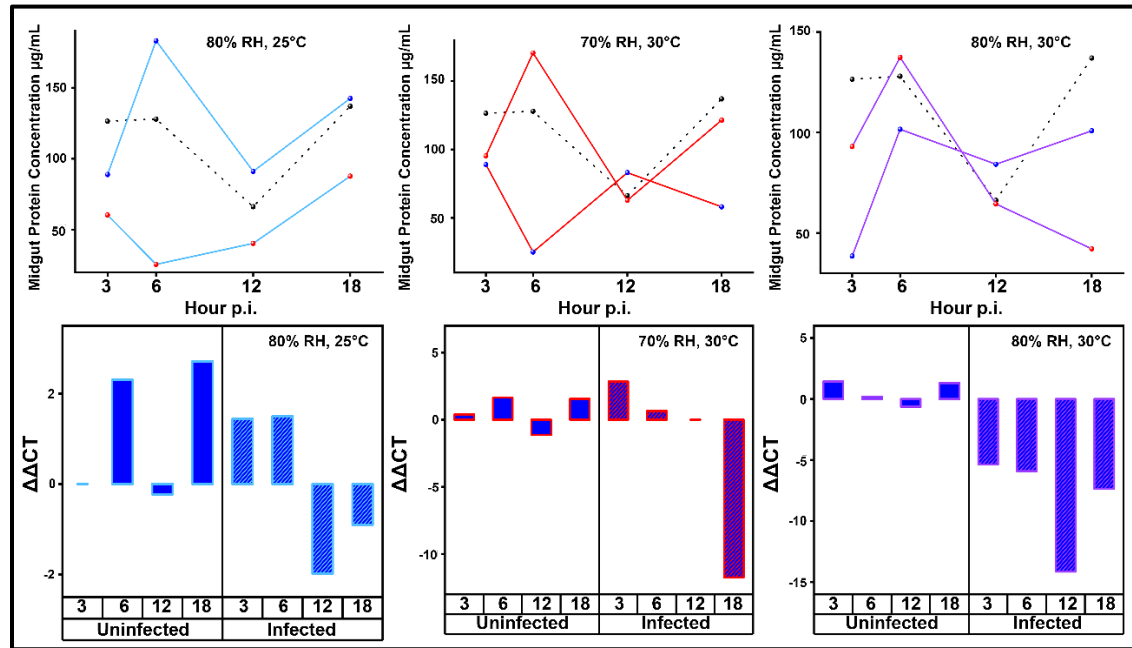


Figure 3 Increases in humidity and temperature influence bloodmeal digestion in uninfected and infected fleas fed on rat blood. The top graphs show the protein levels in the midgut of uninfected and infected fleas maintained at the indicated parameters labeled on the graphs. The y-axis was not standardized across groups so that the data could be fit optimally for each one. The x-axis is grouped based on infection and the hours post-infection for each sample. Blue symbols represent uninfected and red represent infected. The color of the line indicate which parameter was altered as with the outline of the bars in the top panel. Dotted lines represent uninfected fleas at standard parameters of 70% RH, 25°C. The bottom graphs show differences in levels of trypsin-like serine proteases for fleas maintained at the different parameters labeled on the tops graphs for 3 days prior to being fed with uninfected or infected rat blood. Bars with stripes indicate infected bloodmeal. The outlines of the bars represent the parameter that was altered in relation to standard. Light blue represents an increase in humidity, red is an increase in temperature, and purple is an increase in both humidity and temperature.

Table 1: Statistically significant differences in digestion between uninfected and infected fleas fed rat blood when humidity only or temperature only is increased.

Blood	RH Temp	Uninfected Mean Rank	Infected Mean Rank	U	2-tailed p-value
Rat	80%, 25°C	11.38	5.63	55	0.016
	70%, 30°C	5.88	11.13	11	0.027
	80%, 30°C	8	9	28	0.674

4.2 Digestion in Uninfected and Infected Fleas Fed Different Host Bloodmeals Under Varying Environmental Conditions

Previous studies have shown different digestion rates in *X. cheopis* fed on varying bloodmeals including mouse and rat. However, they have not compared uninfected to infected bloodmeals. Additionally, we have included prairie dog bloodmeal as it is important to consider in investigations into the sylvatic plague cycle. We also

investigated the impacts of abiotic conditions on digestion across the four bloodmeals in both uninfected and infected fleas. Understanding how temperature and humidity may impact digestion individually is important. However, increases in temperature can lead to increases in humidity within the habitat of the flea. Therefore, in order to fully understand the impacts these factors may have on the sylvatic cycle, we need to investigate how the interrelationships of these factors impact the flea:bacteria interaction.

Results: Digestion in uninfected groups varies across time points making it difficult to determine when full digestion has occurred, if at all. Particularly with increases at later time points, it is unclear if this is protein from other sources or the continued digestion of the bloodmeal. Considering the removal of tissues outside of the alimentary canal, the observed pattern across all bloodmeals, it is likely associated with the bloodmeal. The results imply there is an additional breakdown of any protein that may remain after hemolysis.

Overall rat blood had the lowest amounts of protein than any other bloodmeal. In fleas fed on mouse blood, there was a decrease in protein in infected fleas at standard temperatures (Fig. 4). Increased humidity led to a decrease in protein with more of a decrease in infected. Increases in temperature decreased protein in comparison to standard parameters but infected and uninfected were similar. This indicates that humidity may slow digestion when temperatures are higher which would typically lead to faster digestion. There was also a decrease in protein with increased humidity and temperature compared to standard parameters (Fig. 4). There were no statistically significant differences between any of the altered parameters compared to standard.

Table 2: Comparison of uninfected and infected protein levels for fleas fed each bloodmeal at 70% humidity and 25°C. The associated mean ranks for uninfected and infected, the U statistic and 2-tailed p-value are given.

Blood	Uninfected Mean Rank	Infected Mean Rank	U	2-tailed p-value
Rat	10.13	6.88	45	0.195
Mouse	10.38	6.63	47	0.115
PDog	9.75	7.25	42	0.294
Pig	10.25	6.75	46	0.141

Protein levels in fleas fed on uninfected pdog blood had clear declines with minimal variation in comparison to fleas fed on other bloodmeals (Fig. 5). All uninfected groups showed sharp declines in protein levels. Lower protein levels were seen in infected fleas under the remaining two groups with increased temperatures. Increased humidity and increased temperatures were statistically significantly different than standard parameters (Table 3). For pig, there was minimal difference between uninfected and infected groups at standard parameters (Fig. 6). The increased humidity group showed inverse levels between uninfected and infected early in the infection and the same was seen in the group with infected humidity and temperature except later in the infection (Fig. 6). Increased temperature maintained similar levels between uninfected and infected but were lower than standard parameters early in the infection (Fig. 6).

Comparison of digestion between bloodmeals for uninfected and infected across all parameters showed significant differences at higher humidity only, higher temperature only, and with the combined increase of humidity and temperature (Table 4). Only the significant groups are shown on the table, but all significant differences involve rat as one of the two bloodmeals. This could potentially indicate that host-specific bloodmeal has larger variations under altered environmental conditions.

Table 3: Statistically significant differences in digestion between uninfected and infected fleas fed pdog blood when humidity only or temperature only is increased.

Blood	RH Temp	Uninfected Mean Rank	Infected Mean Rank	U	2-tailed p-value
Mouse	80%, 25°C	9.13	7.88	37	0.60
	70%, 30°C	6.88	10.13	19	0.172
	80%, 30°C	7.13	9.88	21	0.248
PDog	80%, 25°C	11.13	5.88	53	0.027
	70%, 30°C	11	6	52	0.036
	80%, 30°C	9.38	7.63	39	0.462
Pig	80%, 25°C	9.75	7.25	42	0.294
	70%, 30°C	10.5	6.5	48	0.093
	80%, 30°C	10	7	44	0.208

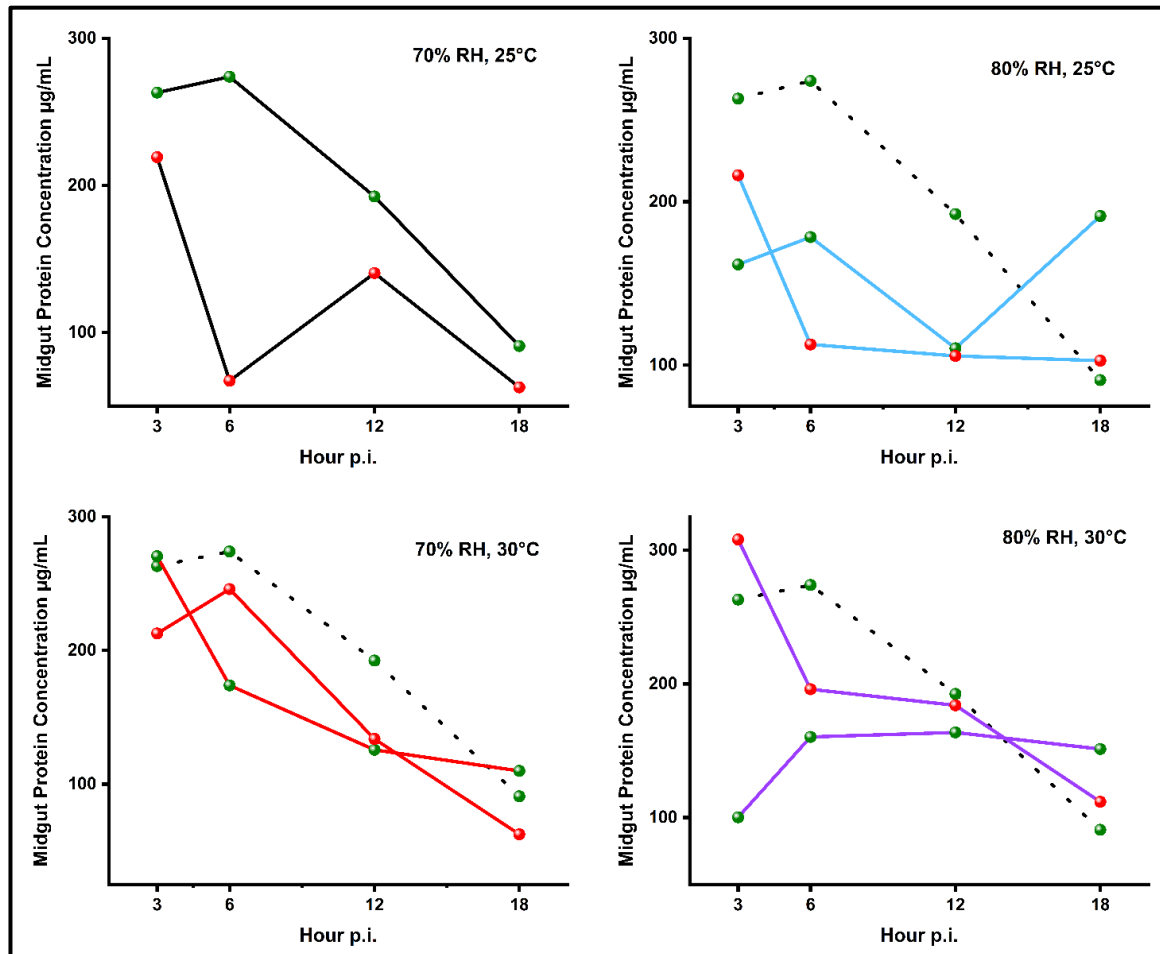


Figure 4 Infected fleas had decreased protein in standard parameters and increased humidity in fleas fed on mouse blood. The graphs show differences in levels of protein for fleas maintained at the different parameters labeled on the graphs for 3 days prior to being fed with uninfected or infected mouse blood. The dotted line represents uninfected fleas maintained at 70% RH and 25°C. The y-axis was not standardized across groups so that the data could be fit optimally for each one. Green symbols represent uninfected and red represent infected. The color of the line indicates which parameter was altered as with the outline of the bars in the top panel.

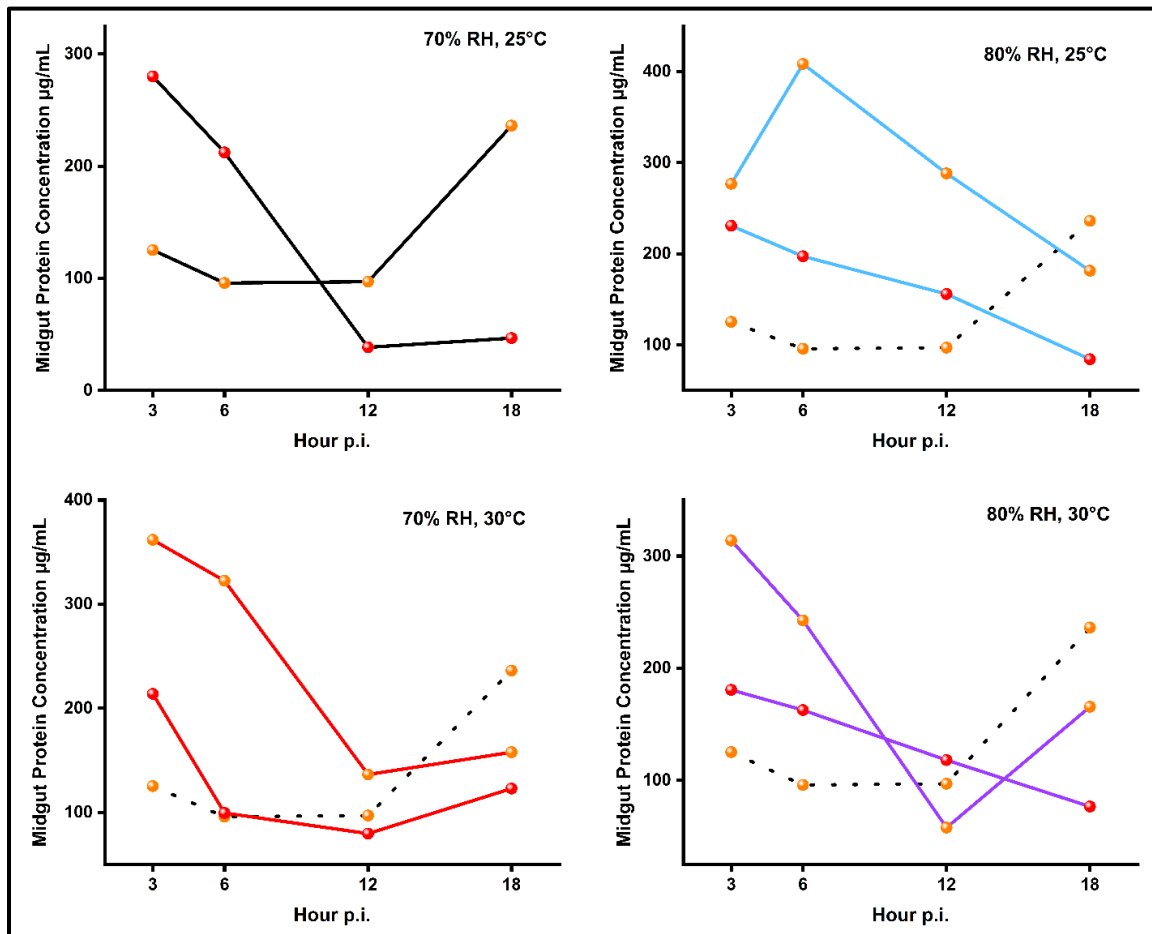


Figure 5 Protein levels were decreased in infected fleas fed on pdog blood across all parameters. The graphs show differences in levels of protein for fleas maintained at the different parameters labeled on the graphs for 3 days prior to being fed with uninfected or infected pdog blood. The dotted line represents uninfected fleas maintained at 70% RH and 25°C. The y-axis was not standardized across groups so that the data could be fit optimally for each one. Orange symbols represent uninfected and red represents infected. The color of the line indicates which parameter was altered as with the outline of the bars in the top panel.

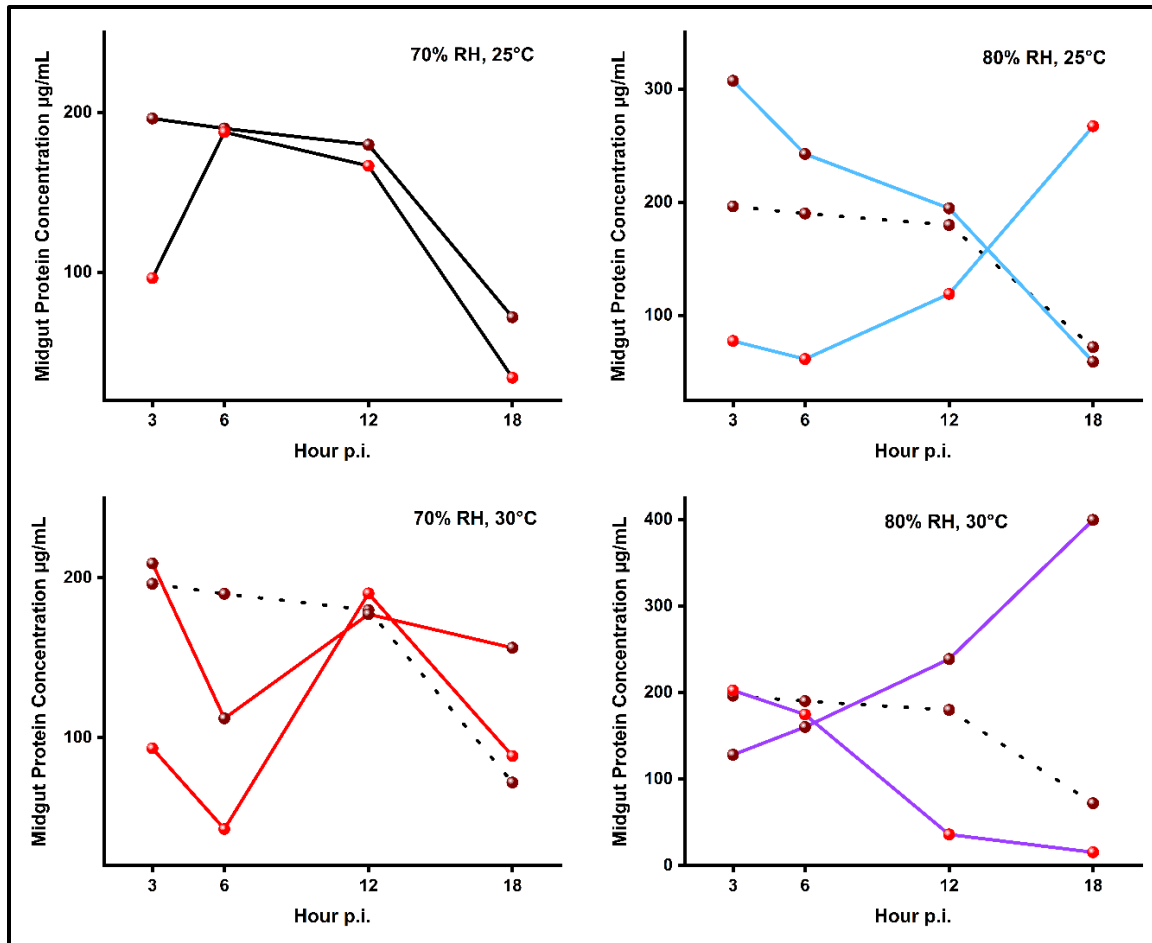


Figure 6 Protein levels had inverse levels early in the infection with increased humidity and late in the infection with increased humidity and temperatures in fleas infected with pig blood. The graphs show differences in levels of protein for fleas maintained at the different parameters labeled on the graphs for 3 days prior to being fed with uninfected or infected pig blood. The dotted line represents uninfected fleas maintained at 70% RH and 25°C. The y-axis was not standardized across groups so that the data could be fit optimally for each one. Maroon symbols represent uninfected and red represent infected. The color of the line indicate which parameter was altered as with the outline of the bars in the top panel.

Table 4: Significant differences in protein levels between uninfected and infected fleas fed on different host bloodmeals maintained under different parameters. Analysis was done with Kruskal-Wallis H with a Bonferroni correction. The test statistic and p-value are given for distributions across groups based on infection and the humidity and temperature combination, followed by the specific bloodmeals that were significantly different and the associated 2-tailed p-value.

Comparison of Bloodmeals					
Uninfected					
Parameters	χ^2	p-value	Bloodmeals		p-value
80% RH, 25°C	13.287	0.004	Rat	PDog	0.003
70% RH, 30°C	10.048	0.018	Rat	PDog	0.011
80% RH, 30°C	10.616	0.014	Rat	Pig	0.011
Infected					
80% RH, 25°C	14.202	0.003	Rat	Mouse	0.016
			Rat	PDog	0.003
80% RH, 30°C	10.048	0.018	Rat	Mouse	0.011

4.3 Discussion

With this work, we wanted to evaluate if the interrelationship of abiotic factors and bloodmeal would impact blood digestion in uninfected and infected fleas. Considering the flea habitat, it suggests that these abiotic factors work in tandem and are likely to have a combinatorial impact on fleas and potentially the flea:bacteria interface. With standard parameters of 70% relative humidity and 25°C, digestion in infected fleas was distinctly different from uninfected fleas though not statistically significantly different. There was less variation in protein values across the time points and interestingly, reduced protease levels occurred after the initial 6 hrs. The steady protein levels in comparison to uninfected in addition to reduced protease implies that somehow, the presence of *Y. pestis* inhibits digestion. The digestive process makes the flea midgut a harsh environment in which *Y. pestis* has to initially survive to be able to colonize the flea [15, 27-29]. Slowing this process down could increase survival rate, allow for masses to form sooner, or utilize the components of the blood to adhere/clump together as no adhesins have been found in *Y. pestis* that are essential for colonization of the flea [15]. An earlier study suggested that during the digestion process, a large portion of the bacteria die off but as digestion begins to slow, the bacteria then begins replicating [30]. The potential mechanism that the bacteria may be using to slow this process is unknown and requires further investigation.

Protease levels varied for some timepoints from the protein levels. Additional proteases that are not being captured by the assay are likely present which would continue to break down proteins if our targeted protease declined in expression. Additionally, several samples were not detected in the assay. We used 3 midguts for each

sample, which provided low yields in some samples. In order to get enough RNA for cDNA conversion, we had to condense the RNA in the sample and reanalyze the samples on the nanodrop. This may have reduced the quality of the samples. Increasing sample sizes to 5 or 10 midguts would provide enough yield for cDNA conversion and increase replicates. Several proteases are in the midgut of insects and another possible solution would be to look for expression of multiple proteases in our assay.

Protein levels showed a double peak in most analyses and will also need further investigation. Patterns indicated a bimodal peak in several of the groups. The initial peak is likely associated with hemolysis (stage 1), and the second peak associated with digestion of blood to hematin (stage 2) where any remaining protein from the hemolysis will be broken down. This would need to be evaluated to confirm this as a possible source. Increases in humidity reduced protein levels for all bloodmeals except pig. Increases in temperature showed quicker digestion for infected fleas, however; when temperature was increased with humidity, infected digestion was similar to uninfected. Interestingly, looking at host-specific bloodmeal, infection under all parameters except increased temperature only led to reduced protein levels suggesting slower digestion. It is currently unclear how *Y. pestis* colonizes the proventriculus quickly after bloodmeal intake or is able to form clumps in the midgut prior to expulsion early in the infection. It is possible that components of the bloodmeal are used to form clumps or adhere to the cuticular spines of the proventriculus. It would be an advantage in this case to slow down digestion to allow for this to occur but also to mediate the harsh environment that occurs during bloodmeal digestion. Tracheal respiration in fleas is achieved through the opening of spiracles in the trachea that lead to the abdomen. The opening and closing of these

spiracles is related to water loss and in times of increased temperature, reproduction, and digestion, these spiracles are open permanently or rhythmically. During the peak of digestion, these spiracles would remain open or rhythmically open and close resulting in water loss and production of lactic acid [31]. Reducing the amount of time these spiracles are open would slow digestion, reduce lactic acid formation, and reduce water loss. This is a potential mechanism explaining the differences we see between uninfected and infected and under different abiotic conditions.

Supplemental Figures:

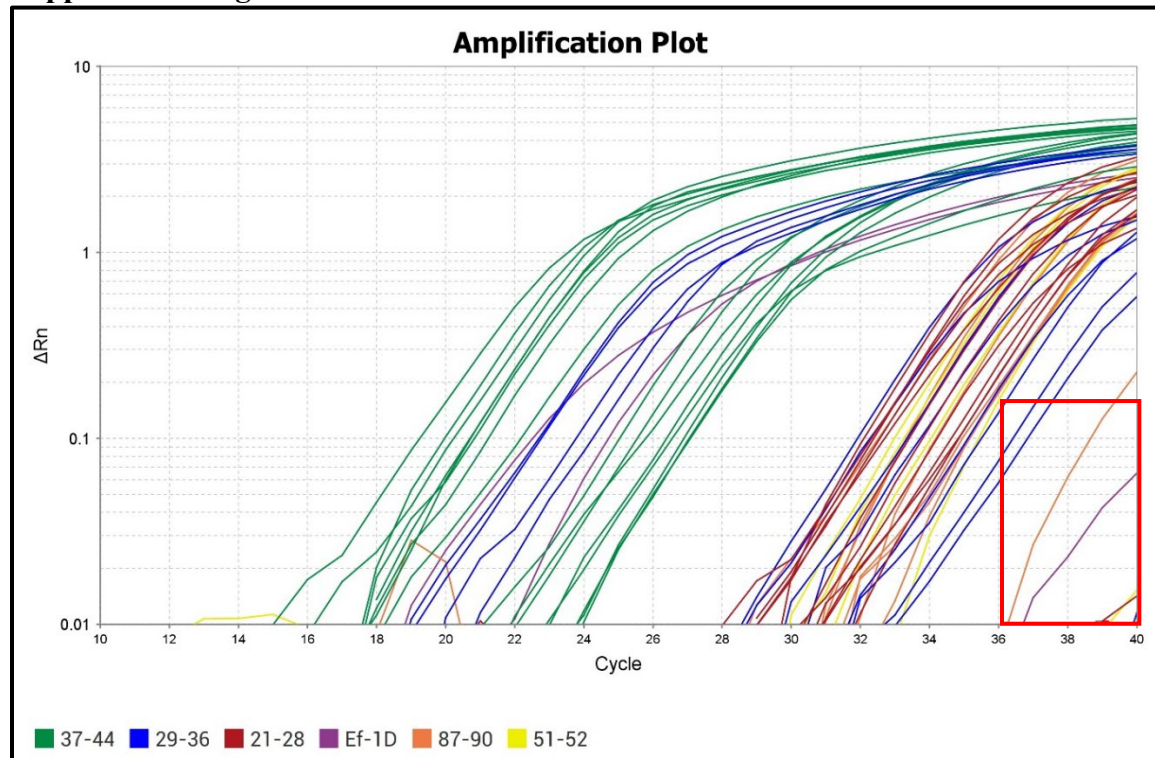


Figure S1: Negative samples fell beyond the 36th cycle in qPCR analysis of flea midguts, shown in the red box. Any sample with a CT of 36 or higher was considered negative.

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5 TRANSOVARIAL TRANSMISSION OF *YERSINIA PESTIS* BY ORIENTAL RAT FLEAS, *XENOPSYLLA CHEOPIS*

Background: *Yersinia pestis* is the causative agent of plague, which is classified as a prioritized re-emerging zoonotic disease within the United States. Worldwide plague foci persist due to enzootic sylvatic cycles consisting of burrowing rodents and their respective flea vectors, which are an essential component for transmission [1-7].

Numerous fleas species have been implicated as potential vectors; however, within the United States, the prairie dog and ground squirrel sylvatic cycles are the primary source of long term maintenance through established natural foci [8, 9]. Fleas can transmit *Y. pestis* as early as day 1 post-infection and up to 28 days post-infection (p.i.).

Transmission on days 7-28 p.i. occurs through mechanisms dependent on biofilm formation for colonization and adherence. For infection to establish within the flea, intake of a bloodmeal from a bacteremic mammal is necessary to prevent complete expulsion of the bacteria with feces [10, 11]. Shortly after feeding, bacteria in the midgut begin to form aggregates that contain large quantities of bacteria. These aggregates can migrate to the proventriculus leading to partial or full blockage of the valve resulting in a regurgitative inoculation of the mammal during intake of subsequent bloodmeals. This colonization is necessary for efficient transmission and has long been observed early in the infection, within the first few days [12-19]. However, advances in investigative tools have allowed researchers to evaluate early colonization of the proventriculus to a much greater extent. Recent work has shown colonization as early as one hour post bloodmeal [20]. A soft mass forms in the anterior portion of the proventriculus early and is subsequently flushed into the midgut with the intake of another bloodmeal. This flushed

mass is referred to as a cast. This process repeats, eventually leading to a hard enough cast that is unable to be flushed to the midgut, causing partial or full blockage of the proventriculus. The mechanisms that facilitate the initial adherence remain unclear.

Digestive enzymes in the midgut lead to rapid hemolysis and liquification of the bloodmeal within 6 hrs of intake with full digestion occurring in less than 24 hrs [21, 22]. Rapid digestion of the bloodmeal is believed to facilitate the quick formation of aggregates within the midgut but is also a characteristic of the flea species. On-host fleas have been reported to feed as many as 3-5 times per day, whereas off-host fleas such as *X. cheopis*, have been reported to feed approximately 1-2 times per day [22, 23]. They occupy the burrows of rat hosts, providing consistent access resulting in frequent small bloodmeals with quick digestion providing continual nutrition. Studies on the European rat flea indicated that host-specific bloodmeal has a significant impact on the initial reproductive event for newly emerged fleas [24]. For a portion of these fleas, reproductive processes may already be underway including open testes (no testicular plug) or the presence of yolk in oocytes when previous generations were fed on rats. In other species, newly emerged fleas have testicular plugs or underdeveloped ovaries and follicular plugs which dissolve after the first bloodmeal [22]. Abiotic factors, such as temperature and humidity, have extensive impacts on the poikilothermic flea, impacting digestive, reproductive, and developmental processes. Temperature can alter the mating behavior of fleas, impact flea development, and rates of digestion [22,25]. Temperature and humidity can impact reproductive success, survival, and development of all four life stages, egg, larval, pupae, and adult [22,26].

Microscopy has been utilized since the initial investigations into fleas as vectors and led to the observation of digestive phases, reproductive structures, and the localization of bloodmeal and bacteria within the flea. When the flea was discovered as a vector of plague as early as 1897, one hypothesis on how transmission occurred was by colonization of the salivary gland [27]. Through experiments to examine this possibility, hundreds of flea alimentary canals were observed using microscopy or culture to verify infection [27, 28]. It was at this time that the determination was made that *Y. pestis* was confined to the alimentary canal. With major advances in microscopy, it is once again becoming a common tool in investigating localization of bacteria in fleas, particularly in the proventriculus and midgut [17, 18, 20, 29, 30]. These studies have shown early colonization of the proventriculus through the formation of casts, as well as described PIER, a post-infection esophageal reflux that occurs in association with specific bloodmeals leading to bacteria in the proventriculus and esophagus [17, 18]. These studies provided visualization of biofilm formation containing high quantities of bacteria and the localization of this biofilm throughout the infection [29, 31]. Although visualization is useful in determining localization to understand the kinetics of infection, bacterial quantification was still lacking. For ongoing investigations, a method was developed for quantifying bacteria by analyzing images obtained from microscopic observations. By conjugating bacteria with a plasmid emitting fluorescent signal outside of the autofluorescent range of the proventricular spines, RGB (red, green, blue) images could be converted to HSB (hue, saturation, brightness) images. The fluorescent signal is compressed into a gray value and could be measured in the saturation channel. The saturation channel detects all ranges of a single color so that a gray value can be assigned

to bacteria even if it is quenched. The region of interest (ROI) was identified prior to measurement so that area could be included in the calculations for relative fluorescent units (RFUs) for proventriculus alone or proventriculus and midgut together. A recent publication has shown a similar method developed in another lab that utilized a fluorescent signal that also falls outside of the autofluorescent range but not to a degree in which autofluorescence is eliminated [20]. To account for this autofluorescence, a calculation was created to quantify the number of bacteria and area colonized within the proventriculus. The ability to quantify microscopic images provides an opportunity to investigate the kinetics of infection by determining not only localization but how the bacteria quantities are changing within localized areas within the alimentary canal. In addition, ranges of bacterial loads can be detected which were likely missed in early investigations.

Even with advances increasing the ability to research the bacteria:flea interactions within the lab and continual field based studies on sylvatic cycles, research has yet to establish cause and effect for ecological maintenance of *Y. pestis* or the occurrence of epizootics. To date, field studies have shown low-levels of bacteria in host populations and cyclic mass mortality events, or epizootics, where bacteria is increased to high levels and spread rapidly, resulting in large population losses within a short time frame. It is unclear what facilitates either one of these events. Mathematical modeling and current ecological hypotheses on long-term maintenance of *Y. pestis* in host populations or routes of reintroduction, are unable to provide satisfactory explanations. We have an interest in bridging these two areas of focus in an attempt to address knowledge gaps within the sylvatic cycle. Due to the ectothermic flea vector and *Y. pestis*' response to

environmental factors, the impact of environmental factors on the acquisition, colonization, and transmission of *Y. pestis* within and by the flea are investigated. During the utilization of microscopic analysis and quantification, bacteria was found outside of the alimentary canal. It was observed primarily in reproductive organs and Malpighian tubules. None was observed in the salivary glands, supporting aspects of early investigations [27, 28]. The presence of bacteria outside of the alimentary canal led to the evaluation of the possibility of transovarial transmission of *Y. pestis*. Transovarial transmission is seen in fleas infected with *Rickettsia mooseri*, where F₁ generation fleas successfully transmitted the pathogen to 18% of rats they parasitized [32]. It has been historically and widely accepted that this route of transmission does not occur in plague infected fleas. Within this work, the localization of *Y. pestis* within the flea was explored and the potential for transovarial transmission of *Y. pestis* was reexamined.

Hypothesis: *Y. pestis* escapes the midgut during infection of *Xenopsylla cheopis* and remains viable in tissues outside of the alimentary canal.

5.1 The Presence of *Y. pestis* in Tissues Outside of the Alimentary Canal Post-infection in *X. cheopis*

Methods: Infection: Laboratory reared *X. cheopis* that were unfed for at least 5 days but no more than 7 days, were infected with *Y. pestis* strain KIM 6+ carrying ptd Tomato (Excitation: 554, Emission 581) [34]. An artificial feeder holding rat blood inoculated with 5×10^8 to 1×10^9 *Y. pestis* and maintained at 37°C was used and fleas fed for 1 hour. When fleas were removed from the feeder, they were observed to determine intake of the bloodmeal and only those that fed were included in the study.

Analysis of infection: Fleas were euthanized on days 1, 3 and 7 post-infection and midguts were isolated on sterile slides. Midguts were fixed with 4% paraformaldehyde, rinsed 3 times with PBS for a minimum of 30 minutes each time prior to mounting. Midguts were then imaged using a Leica SP8 confocal microscope with image settings of 1024x1024 pixels, numerical aperture of 0.4, refractive index of 1, pinhole of 1 AU and emission of 580nm for all images. Image J was used to analyze the number of fluorescent bacteria present and to visualize the localization of the bacteria (Figure S1). Images were converted from RGB to an HSB stack, the ROI selected, and the saturation channel was used to measure area, mean gray value, and integrated density. The saturation channel detects all variations of the color associated with the fluorescence in the sample, allowing for detection of bacteria both associated and not associated with dense biofilms. Background was removed by measuring uninfected samples fed on rat blood and analyzed Day 1 post-feeding (N=10). The average of all negative samples was calculated for overall mean (1.998) and proventricular mean (2.84) to determine background values. Integrated density (ID) was measured through Image J and final analysis was $ID = ID - (Area \times Background)$, reported in relative fluorescent units (RFUs).

Egg collection: Initially, infected fleas were maintained in a 50mL conical tube with strips of filter paper to provide a vertical surface and to absorb feces. A 70µm cell strainer was used as the top of the tube and parafilm was used to secure it. To collect eggs, the tube was placed into the freezer to anesthetize the fleas. The contents of the tube were emptied onto a large glass petri dish maintained on a cold plate and the larvae/eggs were removed. The length of the eggs is 477-504 µm with a width of 297-333 µm and whitish in color [34]. *X. cheopis* have a steady reproduction at 1-2 eggs per clutch, 3.6 to

5.6 eggs per female per day which decreases as they age [22]. To increase successful collection of eggs, fleas were then placed in modified containers for ease of sifting and to help maintain humidity. Containers had 300-micron mesh on the lid with 550-micron mesh under the bedding with a lower chamber that could be easily removed, allowing for sifting without the need to anesthetize fleas (Fig.S2). The bedding material was larger than 550 microns to facilitate separation of material from eggs or larvae that fell through, decreasing contact between eggs and larvae with infected feces and fleas. Fleas were maintained in these modified containers in the insect chambers and sifting for eggs or larvae occurred every 1-3 days p.i.. If eggs adhered to a piece of bedding, it would prevent them from falling to the lower chamber. Therefore, some larvae were collected as they fell through once hatched. Fleas were maintained on uninfected rat blood every 7 days. To aseptically collect the eggs and larvae, a sterilized cotton applicator was moistened with ddH₂O and used to pick up eggs or larvae. They were then placed onto a sterilized slide, washed in sterile PBS three times, observed between washing to ensure no materials or feces were in contact, and mounted in 35% glycerol to match the refractive index of the tissue. Samples were imaged on the Leica SP8 confocal microscope. In addition to imaging, Z-stacks were done to create a 3D rendition of the egg to view the bacteria inside of the egg. A subset of collected eggs were not imaged and instead, transferred to an agar plate. After washing three times in sterile PBS, a new sterile cotton application moistened with ddH₂O was used to transfer the egg to an agar plate. A new sterile needle was used to puncture the egg, releasing the contents onto the agar. To plate larvae, euthanized larvae were washed three times with sterile PBS, sectioned in sterile PBS and plated.

Development of eggs or larvae: Eggs were also isolated, washed in sterile PBS, and placed into a sterile well of a 12-well plate with sterile sawdust or into a sterile flea chamber to continue development into the larval stage. Collected larvae were placed in sterile sawdust containing sterile larvae food within a 12-well plate to continue development to the pupal stage. This method had limited success so further isolations of eggs and larvae were placed into a sterile flea chamber with mesh on the top and bottom containing sterile sawdust and larvae food to increase the successful development of eggs. These samples were imaged or plated on agar after development.

PCR and genome annotation: Bacteria that were isolated from eggs were grown overnight and DNA was isolated using a Zymo *Quick-DNA*® Microprep kit. Conventional PCR targeting *cafI*, *ymt*, and *pla* was carried out using the primers and parameters in Figure S2. All positive PCRs were sent for sequencing to verify the bacteria was *Y. pestis* and sequenced nucleotides were aligned using MAFFT [35]. For further analysis of the bacteria, four isolates and the original strain, KIM6+ (*pgm*+ *hms*+ *pla*+ pCD1-) attenuated strain carrying fluorescent plasmid tdTomato, were sent to the University of Missouri Genomics Core for Illumina sequencing. The three egg isolates were collected 3 days p.i. (Egg A), 7 days p.i. (Egg B), 9 days p.i. (Egg C), and one isolated strain from a transmission study on fleas infected with bacteria isolated from eggs. Illumina reads were sent to the University of Missouri Bioinformatics core, where MultiQC reports were generated and reads were trimmed (Table S1) [36]. All subsequent genome analysis was performed by the investigators. Multiple platforms were utilized including uploading sequencing data to the Galaxy web platform using the public server usegalaxy.org and with programs run on a Linux operating system [37]. Reference guided

assembly was used, beginning with mapping reads to a reference genome using BWA to create contigs[38]. DupRemover was used to remove duplicate overlapping contigs [39]. Scaffolding was done using RagTag [40]. Alignment using LASTZ showed noticeable differences in alignment between the KIM6+ strain used in this study and the *Y. pestis* Reference Genome A112 [41]. MAUVE was used to reorder contigs of the draft assembly to the reference genome [42, 43] (Figure S3). Finally, the genome was annotated using PROKKA [44]. Each genome was aligned with the KIM6+ strain using LASTZ. Visualization and comparison of genomes was done using Proksee and BRIG and features added were obtained through NCBI genbank [45, 46].

Reinfection of fleas with isolated bacteria from eggs: Isolated bacteria was grown and used to infect fleas to evaluate the colonization and transmission of the bacteria in the fleas. Fleas were imaged using confocal microscopy and analyzed using Image J using the same methods and parameters used for all other samples. Fleas were maintained in two groups. One group was fed on an artificial feeder with an uninfected rat bloodmeal to test for transmission on day 3 p.i. and the second group was tested on day 7 p.i.. The bloodmeal was removed from the feeder, skin was removed and homogenized, and the feeder was washed three times. The bloodmeal, homogenized skin, and all three washes were plated to determine bacterial presence, indicating transmission of bacteria from the flea during feeding.

Results: Kinetics of infection show larger amounts of bacteria in fleas on Day 3 than Day 1 or Day 7 (Figure 1). On Day 7, bacteria in the proventriculus did not decrease to the same extent as overall bacteria indicating the possible exclusion of bacteria from the midgut but an ability to continue to colonize the proventriculus.

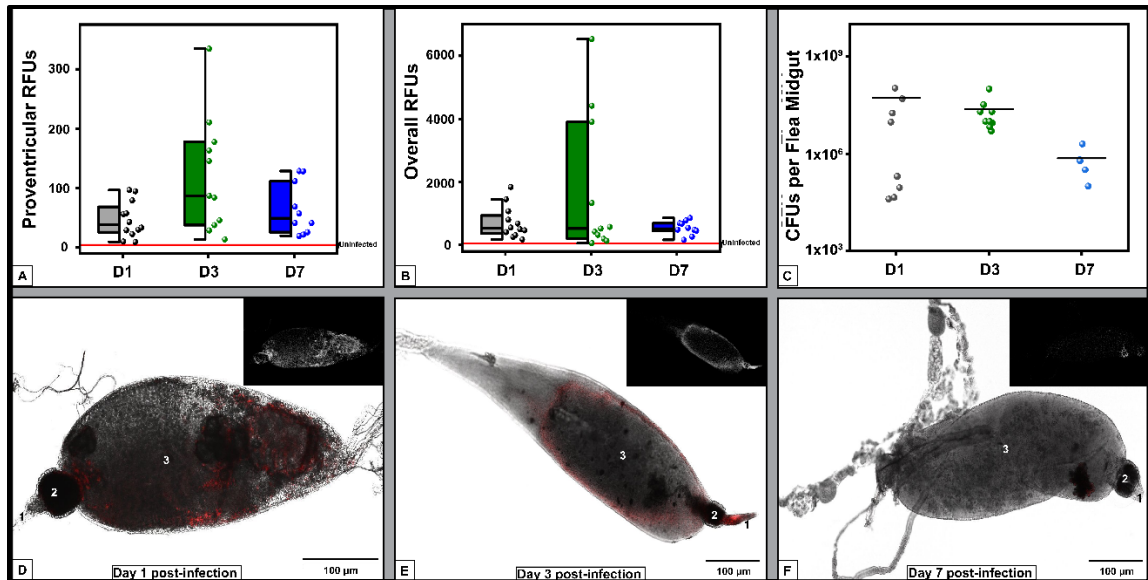


Figure 1 Bacterial loads are higher in fleas early in infection, days 1 and 3 post-infection. Integrated density of the proventriculus (A) and overall (B), in fleas infected on inoculated rat blood with 5×10^8 to 1×10^9 *Yersinia pestis*. Fleas were maintained at 25°C and 70% humidity prior to euthanasia on Days 1, 3 and 7 post-infection. Flea midguts were removed and prepped for imaging using a Leica SP8 confocal microscope and analyzed with Image J. A subset of infected fleas was used for CFU counts (C). Pools of 3 fleas were dissected to remove midguts which were homogenized and serially diluted prior to plating on YSA. Representative images of Day 1 (D), Day 3 (E), and Day 7 (F) are shown with the associated saturation channel from the analysis in Image J. Each image is numbered to show the esophagus (1), proventriculus (2) and midgut (3).

Bacteria was observed in tissues outside of the flea midgut, particularly the eggs and the testes of fleas from various times post-infection (Figure 2). Bacteria was also observed in the Malpighian tubules (Figure S4). The bacteria were seen in flea testes 15 weeks post-infection in fleas fed on varying bloodmeals as well (Figures 2, S4).

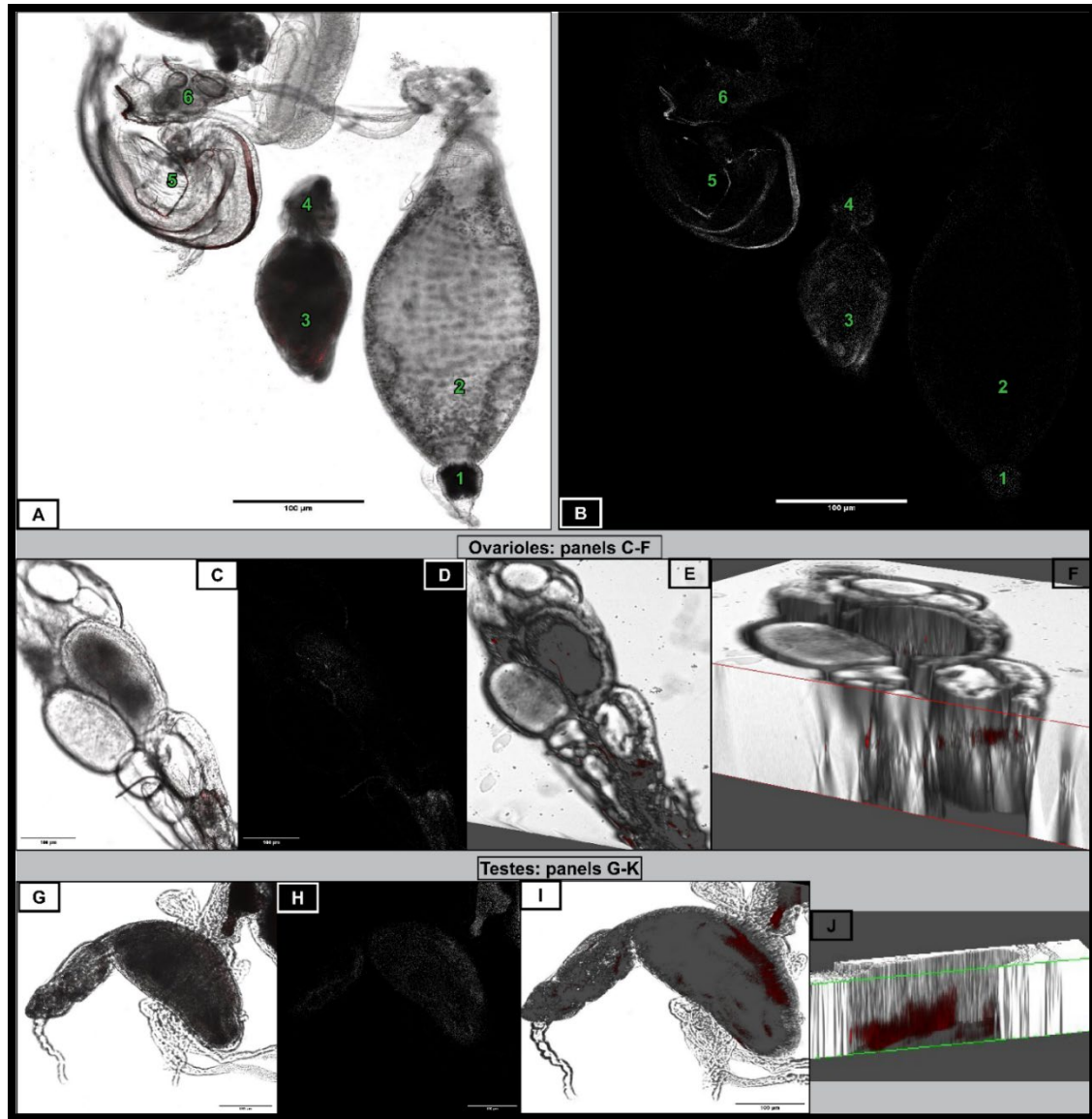


Figure 2 Bacteria is present in tissues other than the midgut during infection, particularly reproductive organs. Panel A is an RGB image of a flea infected with inoculated mouse blood 15 weeks post-infection and panel B represents the converted RGB image to HSB and shows the saturation channel. Anatomy is labeled as followed, 1. Proventriculus, 2. Midgut, 3. Testes, 4. Epididymis, 5. Aedeagus, and 6. Rectal papillae. Panel C shows bacteria in the developing eggs and ovarioles of a flea infected with inoculated prairie dog blood Day 7 post-infection in an RGB image and panel D shows the bacteria in the saturation channel in Image J. Panels E and F are a 3D rendition of the eggs to show the bacteria within the tissues. Panels G and H are of testes showing an RGB and the respective saturation channel. Panel I is a 3D rendition of the image with an overlay to show the bacteria in the testes and epididymis. Panel J is a side view of the 3D rendition to show the bacteria within the tissues.

To determine if live bacteria were in the eggs, eggs from infected fleas were collected, and placed them on heart-infusion agar (HIA) then ruptured using a sterile needle. The resulting three colonies were pink, which is characteristic of KIM6+ carrying high -level expression of ptd Tomato (Figure S2). To further validate that the bacteria

was *Yersinia*, eggs were subsequently collected and plated on Yersinia selective agar (YSA). The resulting growth consisted of very small colonies that could be observed using a dissecting microscope (Figure S2) but were difficult to count. Out of 16 eggs plated on YSA, 12 resulted in bacterial growth. Due to the small colony size, presumably from the selective and differential nature of YSA, all subsequently collected eggs were plated on HIA, resulting in 5 of 16 eggs having 1-3 normal sized pink colonies form suggestive of *Y. pestis* growth (Figure S2). Bacteria presence within the egg was confirmed by measuring integrated density with Image J and creating z-stacks in the LASZ software for 3-D visualization of the tissue (Figure 3). Fourteen eggs were imaged, of which 10 had bacteria. The number of days post-infection when they were collected varied with the collected eggs from 1.5 days to 20 days. Fleas were given uninfected maintenance bloodmeals every 3 or 7 days. Considering bloodmeal intake initiates copulation and subsequent oviposition, these data suggest the possible escape of *Y. pestis* from the midgut or the introduction of the bacteria into the spermatheca (Fig. S4)[47].

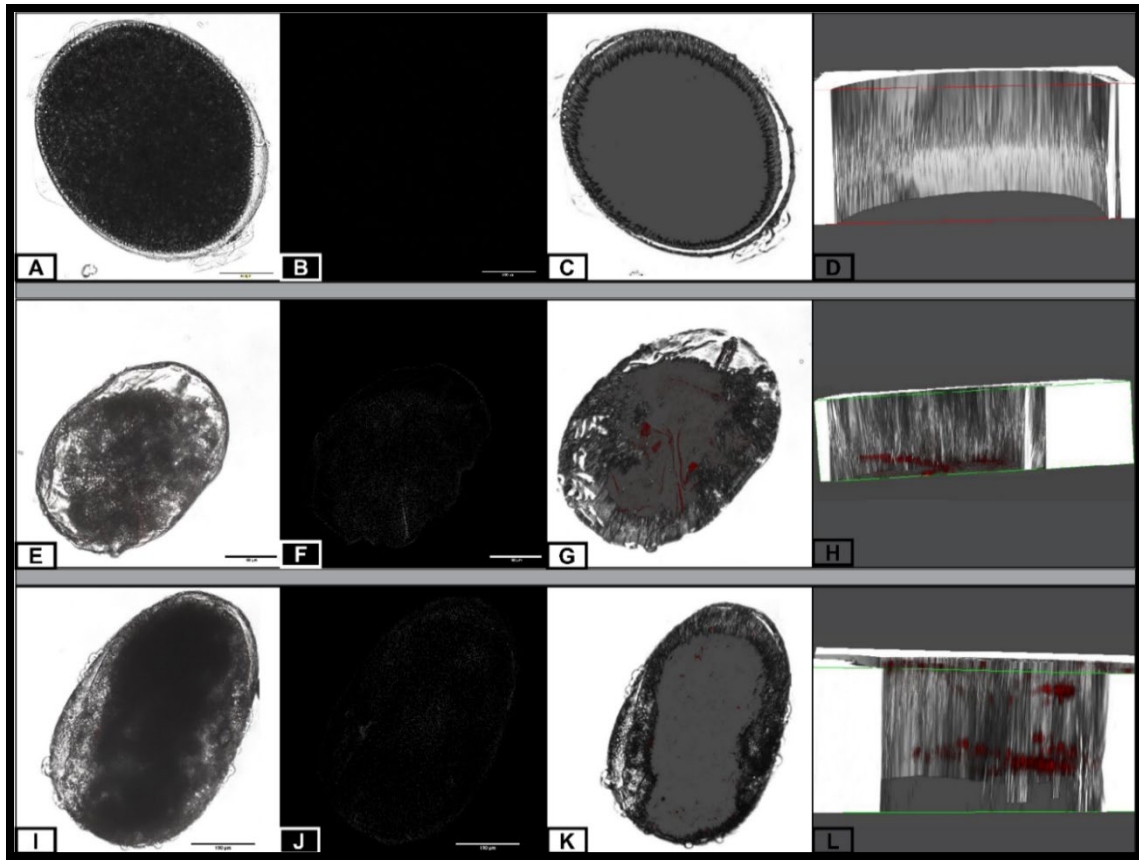


Figure 3 *Yersinia pestis* is transovarially transmitted in fleas. The first column (A, E, I) are RGB images. The second column (B, F, J) are converted RGB images to HSB and the respective saturation channel. The third column (C, G, K) are the 3-dimensional projections of Zstacks. The last column (D, H, L) is a cross section of the 3-dimensional projection to illustrate the bacteria within the tissue. Images A, B, C, D are of an uninfected egg. Images E, F, G, H are of an infected egg 7 days post-infection. Images I, J, K, L are of an infected egg 7 days post-infection.

Of the larvae collected, 5 of 8 were positive for bacteria in imaging. Two of the eggs collected were nearly mature and contained a fully developed larvae; both of these were positive when imaged. Two larvae were plated on YSA and had growth contained within the tissue or had bacterial growth in the tissue and in the surrounding area with 31 or 115 CFU (Figure S5). Two larvae were plated on HIA and 1 had growth with 1 pink colony, and the other had no growth. One egg that was isolated developed into a larva, was allowed to feed on sterilized larval food and then was collected and imaged 7 days post-hatching and 20 days post-infection. Eggs from uninfected fleas were collected and isolated, placed into chambers with sterile sawdust but no larval food to ensure unfed

larvae to compare to unfed infected larvae. Fed uninfected larvae were collected directly from modified flea chambers housing uninfected fleas. Larvae with midgut contents were used to compare to fed infected larvae for imaging (Figure S5).

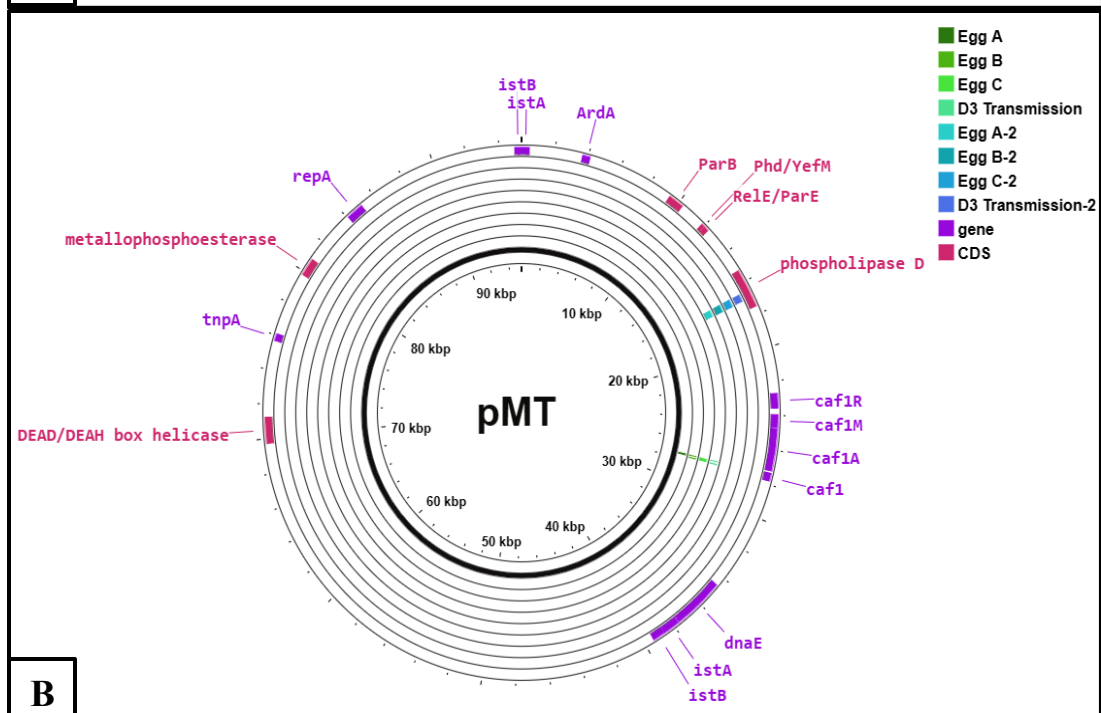
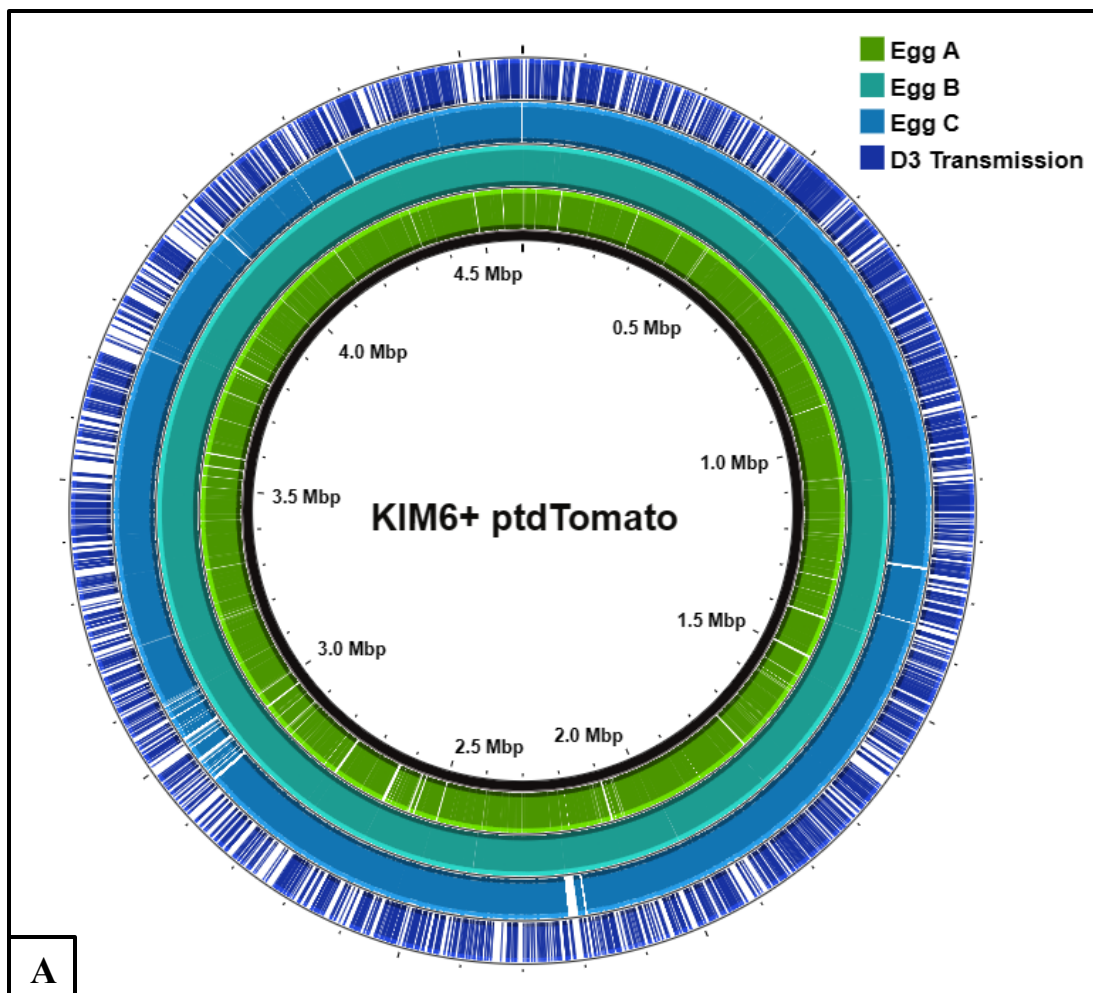
Additional eggs and larvae that were isolated and placed into sterile sawdust with sterile larvae food were left to develop into pupae or adults. A total of 7 samples progressed to the pupae stage, 3 were imaged with confocal and four were plated. The 3 samples imaged varied in their development and the further development increased the autofluorescence due to the increased development of chitin (Figure S6). However, areas that were not associated with chitin did show possible indications of bacteria. The 4 samples that were plated were all positive for bacterial growth with CFU counts of 51, 242, 273, and 274 (Figure S6). A total of 3 samples progressed from collected larvae to the adult stage, 2 were imaged with confocal and one was plated (Figure S7). The larvae were collected from the bottom chamber of the sterile chamber, nonetheless it should be noted that we cannot guarantee the larvae did not consume infected feces during the short time from hatching to our collection. However, once collected they were maintained in a sterile environment with sterilized larvae food indicating the potential for bacteria obtained either in vivo or during larval stages can remain through further developmental stages. The sample that was plated had two types of bacterial growth. A white colony with a distinct pink colony grew on HIA. The pink colony was targeted and streaked for isolation but due to the way the colonies were growing, it was not possible to isolate completely on the first isolation. The initial streak increased the number of pink colonies and overnights were grown to plate serial dilutions. The white bacteria were much more

abundant, but we were able to pick an isolated pink colony from higher dilutions for isolation.

Conventional PCR carried out on isolated bacteria amplified *cafI*, *ymt*, and *pla* (Figures S2 and S7). The amplified DNA was sent to the genomics core for sequencing and subsequent BLASTn indicated all amplifications were *Y. pestis* (Figures S2 and S7). Although the bacteria were positively sequenced as *Y. pestis*, most of the isolated bacteria from eggs had a slower growth pattern than the original strain used to infect fleas (Figure S8). The isolate from the Day 3 transmission study did have similar growth to KIM6+. Multiple genomes were downloaded from NCBI genbank to validate the tomato insertion. These included the *Y. pestis* reference genome A1122 (NCBI:txid 1035377), KIM5 (Accession: PRJNA240083), KIM10+ (NCBI:txid 187410), and medievalis strain Harbin 35 (NCBI:txid 547048) which were blasted against our KIM6+ strain (Figure S9). We annotated our KIM6+ genome with PROKKA using A1122 genome as a reference in the program. The locations of the majority of genes were different than in the reference genome and upon further evaluation, locations varied between the reference genomes as well, in some cases to a great extent. This made it difficult to confidently assign features from an annotation file to the circular genomes to evaluate the gaps. In addition, we have multiple gaps within the genomes that need further evaluation and analysis. Therefore, for preliminary comparisons, we aligned all four isolates to the KIM6+ genome and identified large gaps of 500 to 1500 bps that were present in all four genomes. We then blasted this portion of the KIM6+ genome for identification to begin to elucidate why the growth is attenuated. It should be noted that this is preliminary and extensive analysis must be done with each genome, including PCR to confirm annotations as well as fill in

the gaps. We are considering supplementing the Illumina reads with additional platforms as well. In the same context, we used features from our annotated KIM6+ strain and KIM10+, to verify the present of *cafI*, *ymt*, and *pla*. We also selected a subset of genes shown to be important to infection in the flea to get a preliminary idea on their overall retention [17, 29, 31, 48-62]. We chose the KIM10+ strain as it had 99.8% identity with ours. All isolates had the *cafI*, *ymt*, *pla* in their genomes which were aligned to the plasmid sequences from the reference genome A1122 (Fig.4). Based on the visualization of the genomes, it appears that the subset of genes we selected are in all genomes. The day 3 transmission isolate has gaps aligning to *bamB*, and *oxyR*, and the nucleotide sequences for these genes did not align to the day 3 genome. Considering the discrepancies in annotation, we looked for large gaps that spanned all four genomes. Of the few genes associated with these large gaps, *bssS* which is associated with biofilm regulation, and *pspB*, *pspC* envelope stress response membrane proteins are of interest.

Two of the genomes were contaminated by *Methylobacterium* which is a common contaminant of DNA isolation kits. Reads were mapped to the reference genome of *Methylobacterium* in order to remove contigs that aligned with this bacterium before proceeding with the assembly. This further supports the need for continued and extensive evaluation of the genomes. Pairwise sequence identity percentages when aligned to KIM6+ were 89.7% for day 3 transmission, 95.1% for EggA, 98% for EggB, and 97.2% for EggC. Further genomic analysis on SNPs or possible insertions will also be done.



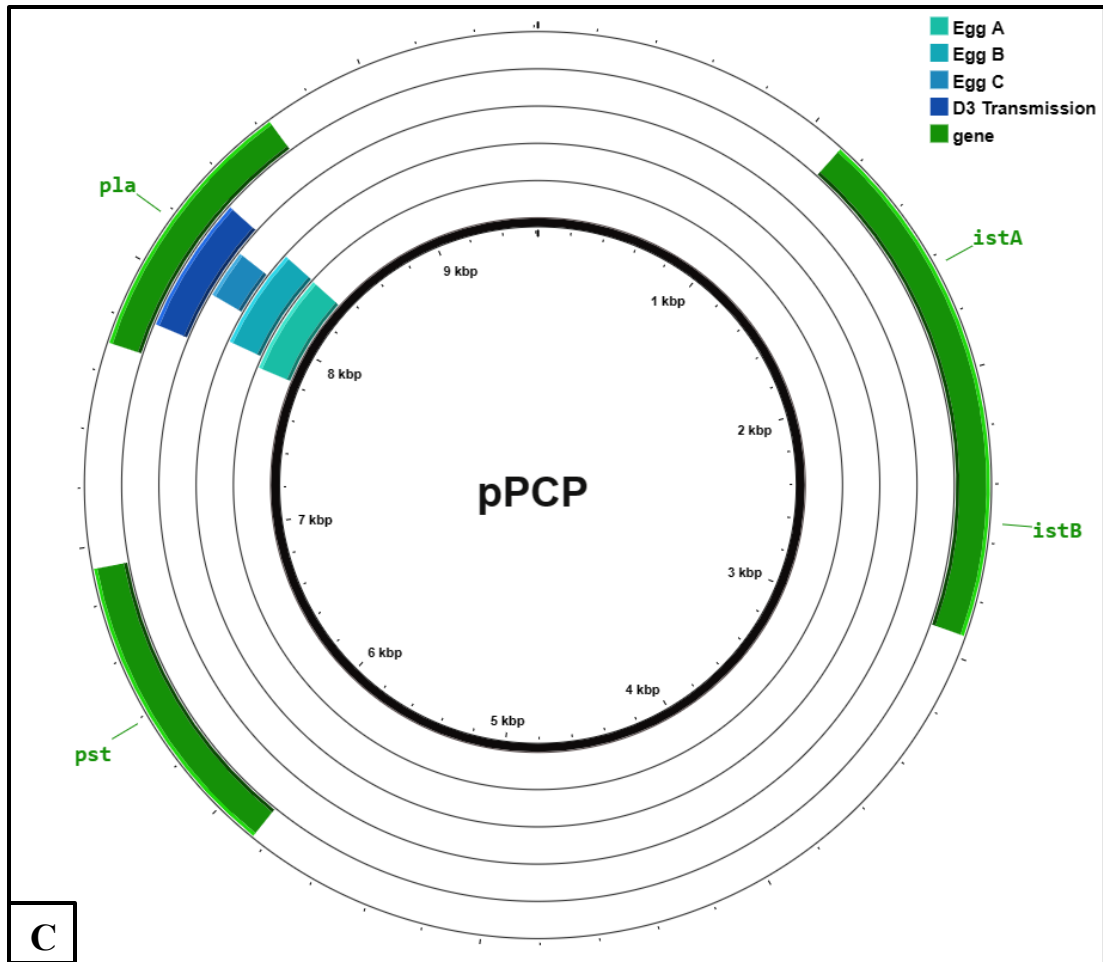


Figure 4 Bacterial isolates retained identifying genes for *Y. pestis* with high sequence similarity to KIM6+. Bacteria isolated from fleas infected with KIM6+ was used to infect fleas and analyzed for transmission. Isolates from eggs and from day 3 transmission were blasted against the original KIM6+ strain. Panel A shows the alignment of all isolates to the KIM6+ strain. Panels B and C are genome visualizations of *pla*, *cafI*, and *ymt* used to identify the strains as *Y. pestis* and the respective plasmids.

Colonization of bacteria isolated from eggs varied from the original strain used (Figure 5). The number of bacteria present in the proventriculus and overall was consistent with the lower range seen in fleas infected with the original strain. Interestingly, the highest number of bacteria in fleas infected with the isolated bacteria from eggs was on day 3, which has been suggested to be the most efficient transmission period for EPT. Fleas were able to transmit the bacteria on both days 3 and days 7 but at lower amounts than those infected with the original strain (Figure 5).

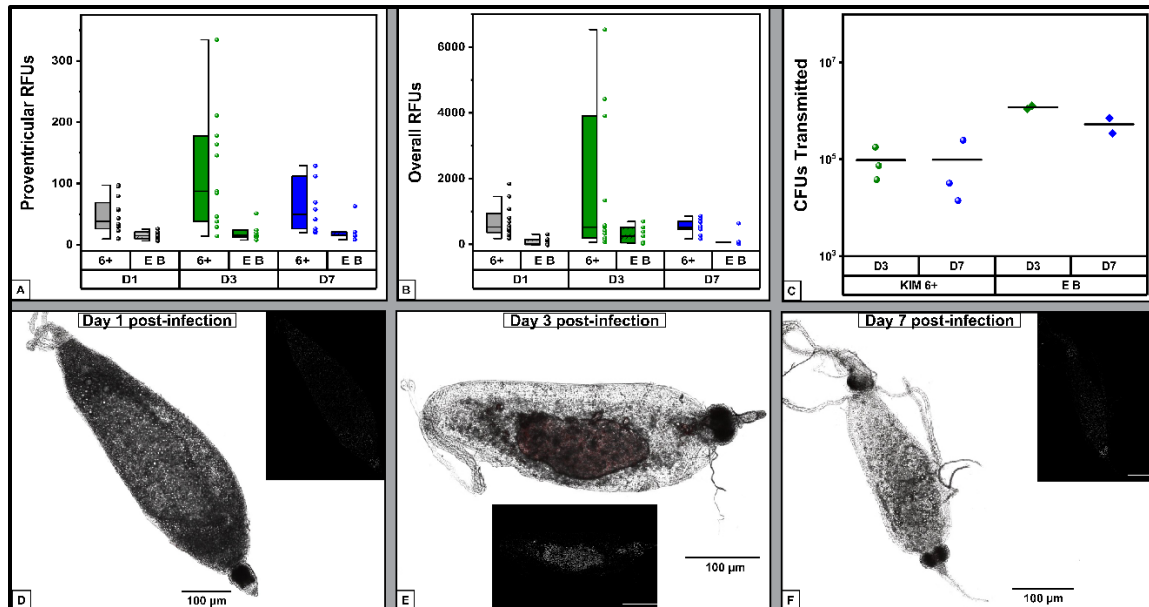


Figure 5 Transovarial transmission may play a role in the maintenance of sylvatic plague. Integrated density of the proventriculus (A) and overall (B) in fleas infected with inoculated rat blood with 5×10^8 to 1×10^9 of bacteria isolated from eggs. Fleas were maintained at 25°C and 70% humidity prior to euthanasia on Days 1, 3 and 7. Flea midguts were removed and prepped for imaging using a Leica SP8 confocal microscope and analyzed with Image J. A subset of infected fleas (N=10 for each time point) was used to determine if the bacteria could be transmitted and the resulting CFU counts are shown (C). Representative images of Day 1 (D), Day 3 (E), and Day 7 (F) are shown with the associated saturation channel from the analysis in Image J.

5.2 Discussion

In this work, evidence has been provided that transovarial transmission may occur in fleas infected with *Y. pestis*. These initial findings provide visual, quantitative, and genomic support for this phenomenon. Research is ongoing and will continue to expand in the near future in order to address necessary aspects, particularly the genomic evaluation of isolated bacteria from eggs and each of the life stages. Transovarial transmission occurs in fleas infected with *Rickettsia mooseri* [32]. *Y. pestis* has been identified in tissues outside of the midgut, in particular the reproductive tissues, ovarioles, eggs, testes, and spermatheca (Figure S4). This phenomenon may support several of the hypotheses associated with long-term maintenance due to the environmental and ecological influence of reproduction in fleas. The rate of oviposition is influenced by host and environmental factors, seen with an increase in pupae when temperature rises from 22°C up to 30°C [22]. A reduction in overall fecundity can also

occur when temperatures are at or higher than 32°C [22]. Previous studies have also indicated that bloodmeal may influence overall reproduction as seen with an increase in pupae when *X. cheopis* were fed on golden hamster versus laboratory rats or mice [63]. Porcine blood also led to a large increase in egg production in cat fleas [64]. Differences in overall colonization and bacterial loads in fleas fed on other bloodmeals has been observed, but overall reproductive rates in association with this factor have not been investigated. Bacteria was seen in eggs and tissues in fleas infected on all four bloodmeals used including rat, mouse, pig, and prairie dog.

Transovarial transmission would be a contributor to long-term maintenance of the bacteria within the mammalian population. To further support possible long-term maintenance, bacteria was observed in the testes up to 15 weeks post-infection (Figure 1). Additionally, if transovarial transmission is directly influenced by ecological and environmental factors, it could contribute to the explanation of sporadic epizootics. However, epizootics would only be possible if the bacteria were able to transition through each life stage of the developing flea. Preliminary evidence from this research shows that larvae without access to the feces of infected fleas were positive for bacteria via plating and microscopy (Figure S5). A small sample size was able to progress to the pupal and adult stages from either collected eggs or collected larvae. Samples developing fully into pupae or adults from eggs supported the ability of the bacteria to transition through all life stages; however, further investigations are needed due to the small sample size. Larvae were collected from the chambers that developed further but the possible contact with infected feces cannot be excluded, meaning only bacteria transitioning from the larval stage can be verified. Nonetheless, it still supports the potential transition between

life stages. Further investigations need to explore the colonization and localization of bacteria in the larvae as early literature indicates that they expel gut contents prior to development of a cocoon for transition to the pupal stage [22]. The results suggest that the bacteria migrated into developing tissues outside of the midgut. Transmission of the bacteria is another integral part of investigating this as a mechanism for long-term maintenance. During investigations with *R. mooseri*, the F₁ fleas were able to transmit the pathogen to rats [32]. Longer infection times increased the likelihood of both transovarial transmission and transmission by the F₁ fleas [32]. Research has been initiated using the same system to increase overall sample size. Transmission to the mammal and subsequent pathology studies are also planned.

Genomic analyses were able to further validate the presence of bacteria within eggs with the confirmation of three identifying genes for *Y. pestis*. The visualization of the genomes blasted against the original strain provides some insightful information, but it is incomplete. The gaps in the genome could be due to complete loss or “contaminants” picked up during the transition between environments. The use of YSA was not continued when plating eggs due to the harshness of the media. Further investigations could use a serial dilution of the antibiotics within the media to use a stepwise approach in isolating the bacteria. The ptdTomato was evident in all isolates providing an additional mechanism to use for isolation of the bacteria. In addition, conventional PCR can be utilized to further analyze the gaps, and to validate or correct the percentage similarity between the genomes. Initial investigations were limited to genes associated with flea infections or genes related to environmental responses due to the initial question into this mechanism’s role in the sylvatic cycle. However, extensive analyses can be

performed to fully identify the genomic changes. This will be especially important when transmission studies involving mammalian hosts are conducted. The addition of strains isolated from larvae, pupae and adults will give a fully comprehensive view into the genomic changes.

A major knowledge gap in the long-term maintenance of *Y. pestis* within sylvatic cycles is how the bacteria is maintained and/or introduced into the mammalian populations resulting in epizootics. The idea of transovarial transmission as a mechanism for long-term maintenance has been dismissed due to early studies stating the confinement of *Y. pestis* to the midgut of the flea. With advanced microscopy, the localization and colonization of the bacteria was observed and analyzed using unique methods, quantifications, and 3-D renderings of flea tissues. While we acknowledge that the genomic information is currently incomplete, there is substantial evidence that *Y. pestis* was isolated from multiple life stages developed in succession from the eggs laid by infected fleas. Considering the interrelationship between reproduction, host bloodmeal, and digestion rates with temperature and humidity, transovarial transmission could be contributing to both enzootic and epizootic cycles.

Acknowledgements: We would like to thank the University of Missouri Genomics Technology Core, Bioinformatics and Analytics Core, and the Advanced Light Microscopy Core for technical support.

5.3 Supplemental Figures:

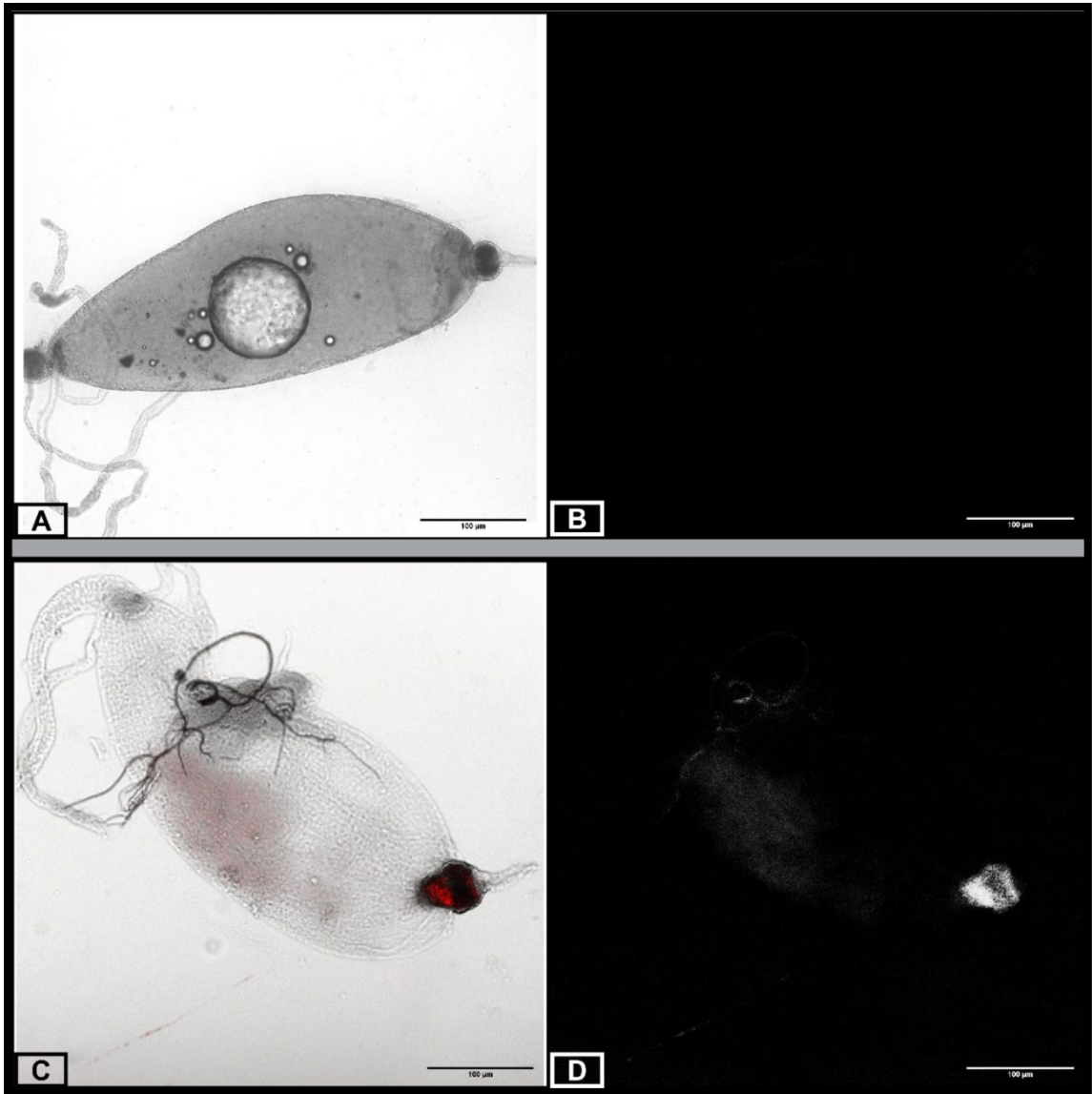
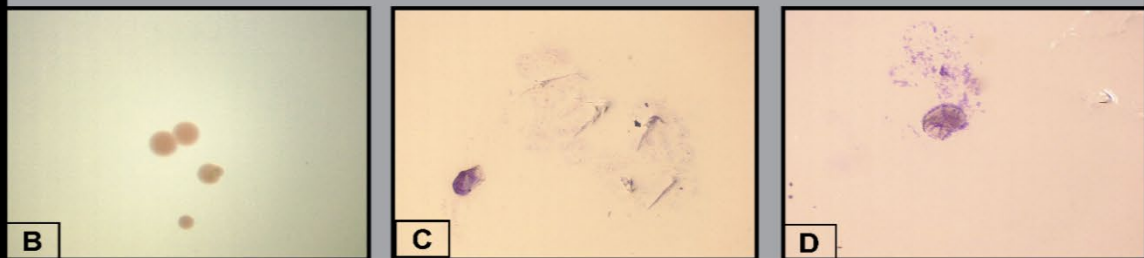
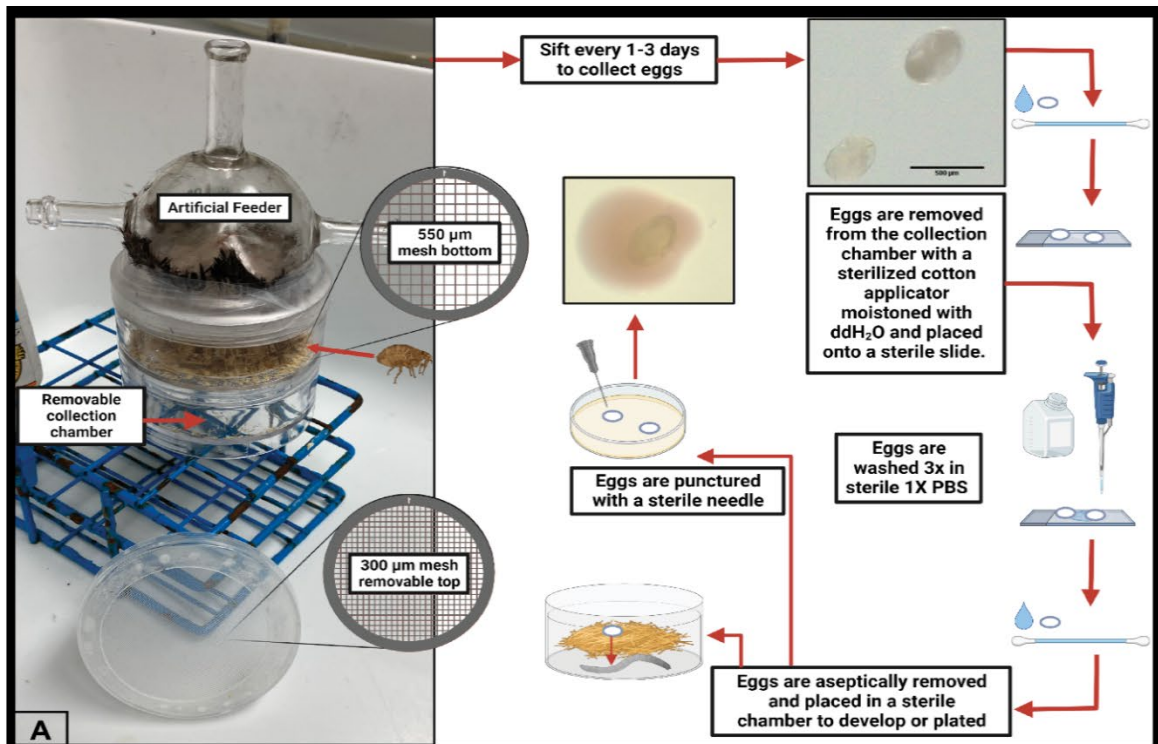
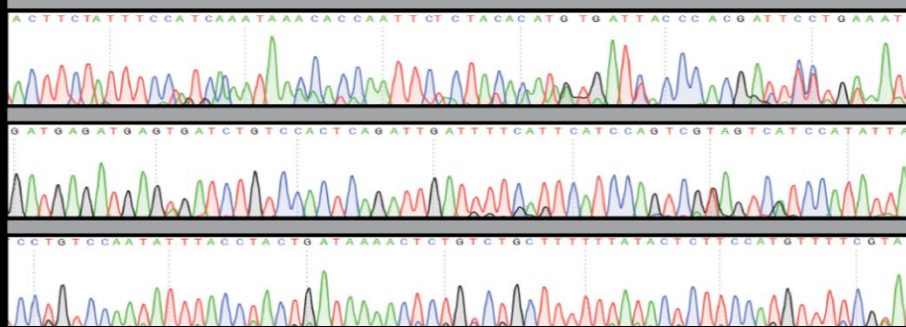
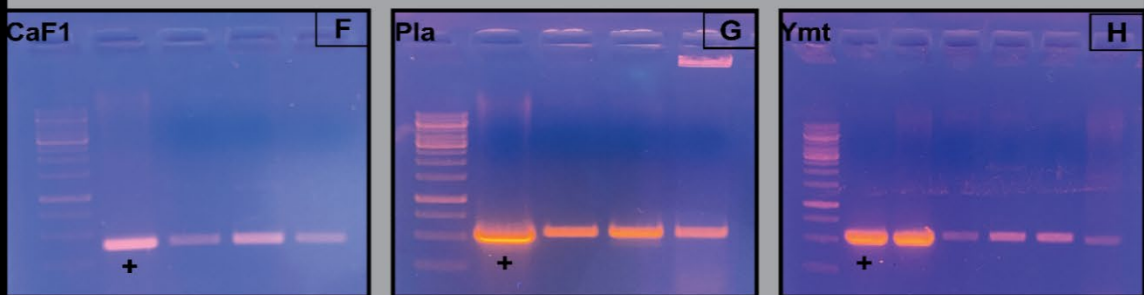


Figure S1 Panel A is an uninfected flea fed on rat blood day 3 post-feeding imaged on the Leica SP8. The RGB image was converted to an HSB stack. Panel B is the same image in the saturation channel used to measure mean gray value and integrated density. Measurements were: overall Area = 26.439, Mean gray value = 1.505, Integrated density = 39.780 (Final ID = **-13.05**), Proventriculus: Area = 1.202, Mean gray value = 3.468, Integrated density = 4.169 (Final ID = **0.756**). Panels C and D are of an infected flea fed on rat blood day 3 post-infection. Measurements were: overall: Area = 61.502, Mean gray value = 10.222, Integrated density = 628.650 (Final ID = **505.77**), Proventriculus: Area = 1.542, Mean gray value = 66.508, Integrated density = 101.365 (Final ID = **96.986**). Both fleas were maintained at 25°C and 70% humidity.



Target Gene	Forward	Reverse	Parameters	30 cycles
CaF1	5' ACTCTTGTGAACCCAGCCCG 3'	5' GGTTACGGTTACAGCATCAGTG 3'	98°C 30s	
			98°C 10s	
Pla	5' CGGGATGCTGAGTGGAAAGT 3'	5' TATCATGTGCCCCGAACCCAG 3'	80°C 30s	
			72°C 45s	
Ymt	5' TAGGTCCGTTCTGCTCCAGA 3'	5' TTCTGCTTATGGCTCCCAGC 3'	72°C 7 m	



CaF1	E-value	% Identity
	2.00E-35	78.46%

Pla	E-value	% Identity
	0.00E+00	92.78%

Ymt	E-value	% Identity
	0.00E+00	98.45%

Figure S2 Panel A is a diagram illustrating egg collection with the modified egg chamber and how eggs were aseptically removed, transferred, and washed after collection. (Created with BioRender.com). Panel B shows pink growth on an HIA agar plate one week after plating, surrounding an individual egg that was punctured. Panels C and D show bacterial growth on YSA of individual eggs that are observed under a dissecting scope. The number of colonies formed around punctured eggs varied from 2-21 (CFUs – 2, 6, 8, 11, 13, 14, 21). Panel E has the primers listed for the target genes and the parameters used in conventional PCR to determine if the bacteria isolated from eggs was *Yersinia pestis*. Panels F-H show agarose gels with PCR positive samples. The lanes are 1kb DNA ladder, positive control, egg 7 days post-infection, egg 3 days post-infection and an egg 9 days post-infection. Panel H also includes the bacteria from transmission studies with fleas infected with bacteria isolated from eggs. Below the panels are representative samples for each target gene with the chromatograph and results from NCBI nucleotide BLAST.

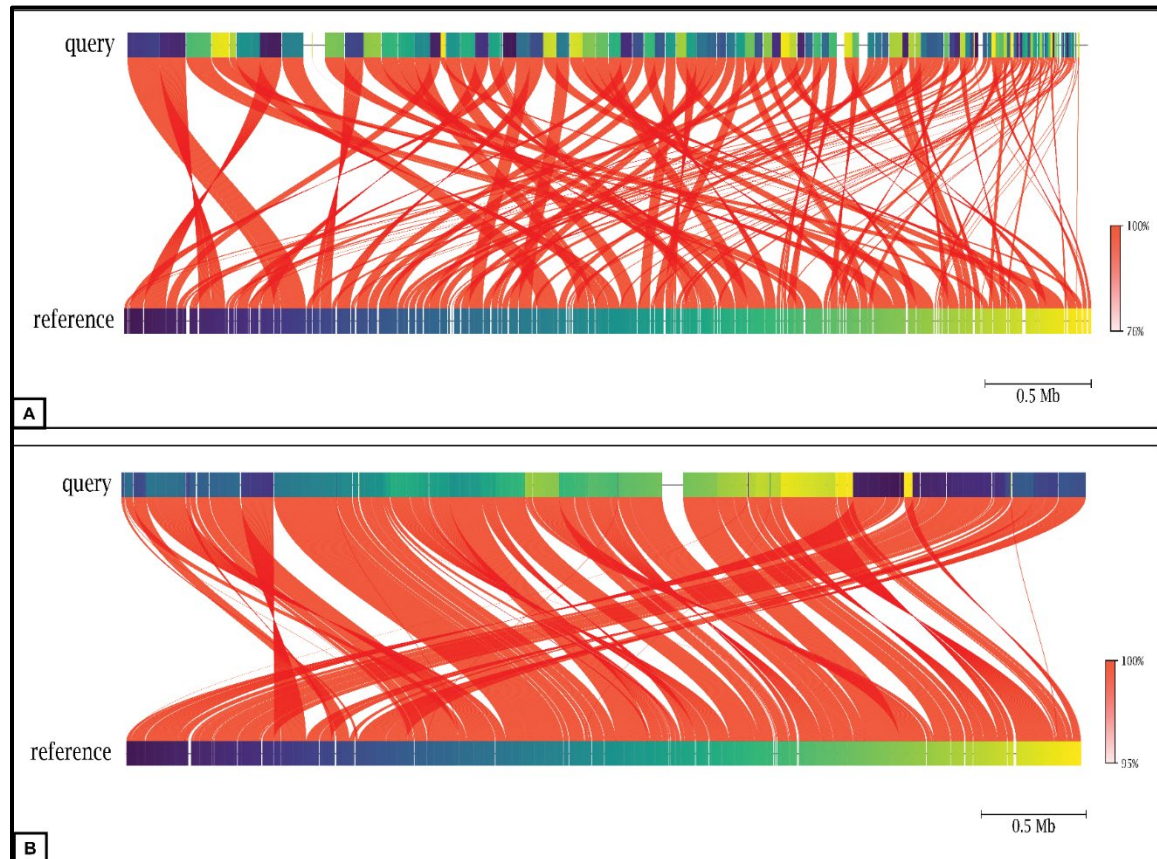


Figure S3 Alignment of the original assembled genome of KIM6+ pTom to *Y. pestis* reference genome is shown in panel A. Improvement of the genome assembly was done by reordering contigs to the reference genome in MAUVE. The reordered contigs are aligned to the reference genome to illustrate improvement of the assembly in panel B.

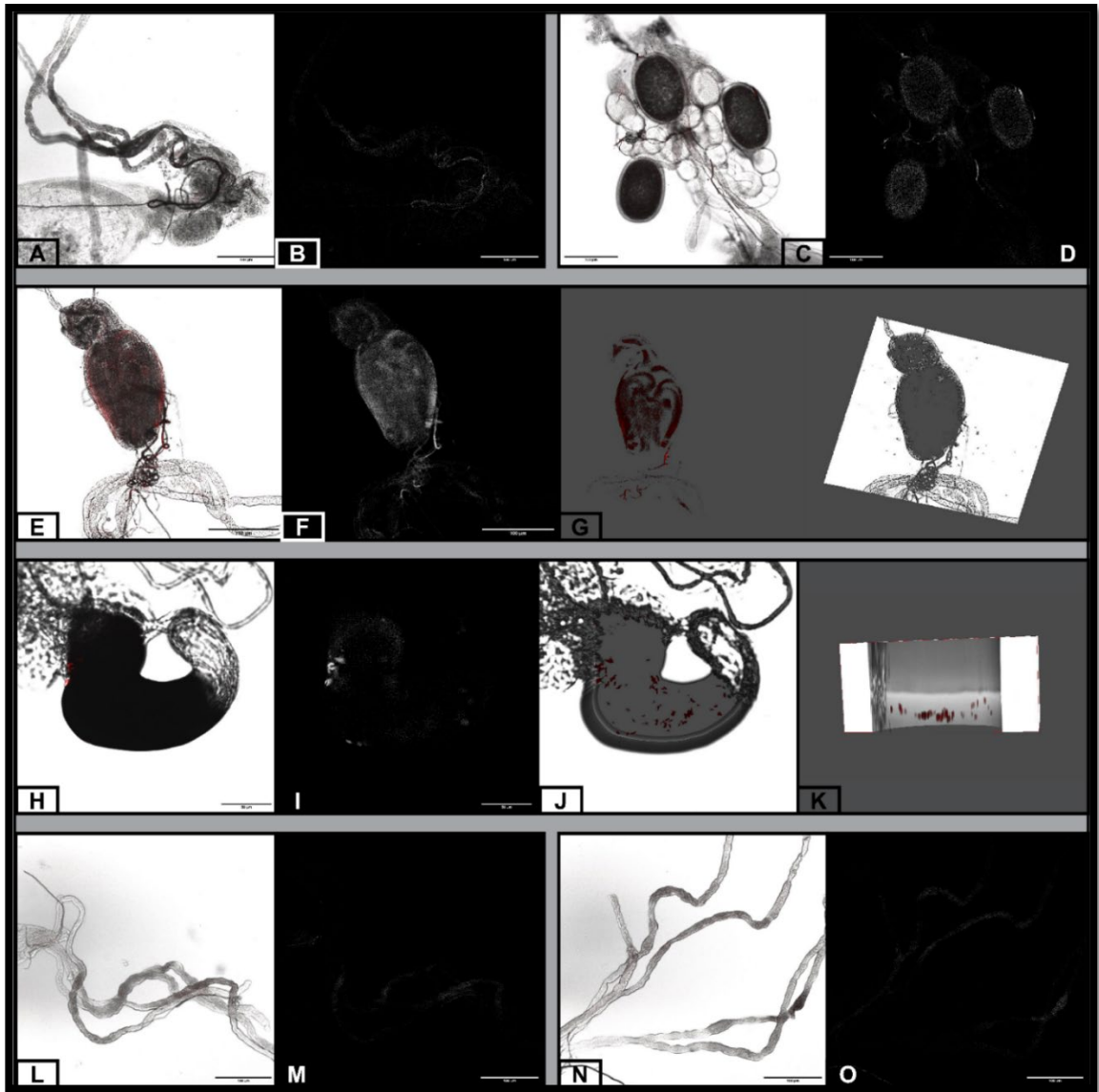


Figure S4 Representative images of various anatomical regions showing bacterial presence via confocal imaging and subsequent analysis in Image J. RGB images (A, C, E, H, L, N) with associated conversion to HSB and the saturation channel (B, D, F, I, M, O) are shown. Panels G, J, and K are 3-D renderings from Z-stacks of the associated tissues. Panels A-B show the developing ovarioles and eggs of a flea infected with inoculated rat blood 5 days post-infection and panels C and D show a flea infected under the same conditions 15 weeks post-infection. Panels E-G illustrate testes in a flea infected with inoculated mouse blood 5 days post-infection. Panels H-K illustrate the spermatheca in a flea infected with inoculated pigs blood 5 days post-infection. Panels L and M are Malpighian tubules from a flea infected with inoculated mouse blood 5 days post-infection and panels N and O show a flea infected with inoculated mouse blood 10 days post-infection.

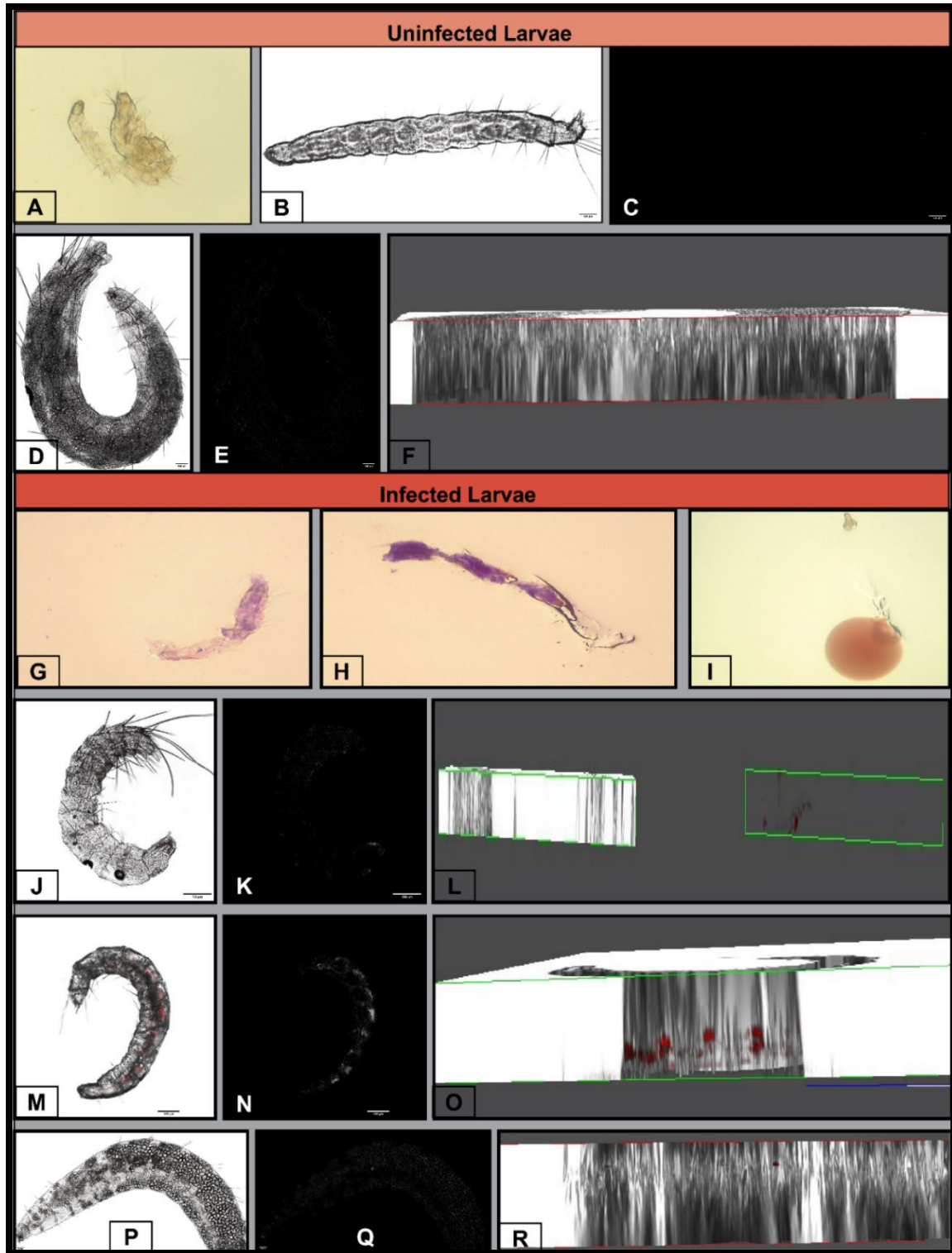


Figure S5 Panel A is a larva collected from uninfected fleas and plated on HIA. Panels B and C show a negative larva collected from uninfected fleas and never fed, Mean gray value = 1.723, ID = 106.221. Panels D through F show a negative larva collected from uninfected fleas with a Z-stack to observe inside the tissues as well (Panel F), Mean gray value = 3.583, ID = 455.409. Panels G and H show infected larvae plated on YSA and Panel I show an infected larva plated on HIA. Panels J-L are of a larva removed directly from an egg and is therefore unfed, Mean gray value = 3.724, ID = 124.333. Panel L shows the channels instead of overlay for easier viewing. Panels M-O are of a larva collected from a tube and was placed in sterile sawdust with sterile larvae food, Mean gray value = 9.028, ID = 255.957. Panels P-Q are of a larva hatched from a collected egg placed in sterile sawdust with sterile food and allowed to

develop for 7 days. Mean gray value = 8.863, ID = 1570.627. A representative RGB image, a converted RGB image to HSB and the respective saturation channel are shown for both larvae and a 3D rendition from a Z-stack is shown for the positive larva.

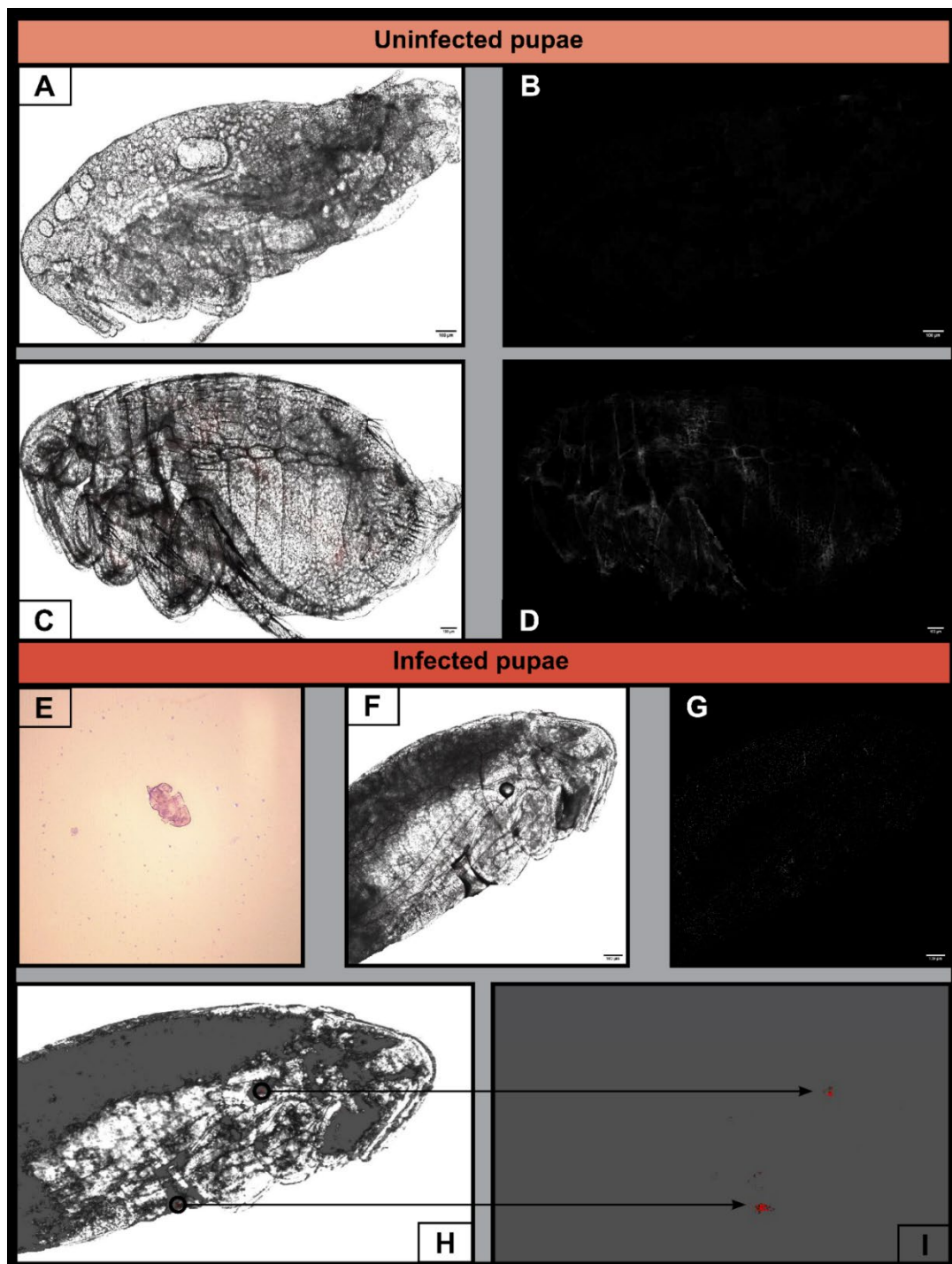


Figure S6 Panels A-D show two uninfected pupae at varying stages of development. Early developmental stages have minimal autofluorescence (Panels A, B); however, further development exhibits autofluorescence (Panels C, D). Panel E illustrates an infected pupae plated on HIA with small colonies surrounding the pupae. F and G show the representative RGB and Saturation channels for an infected pupae with the overlay of the Z-stack in panel H. Two internal sections of the pupae have possible bacteria that would not be

associated with chitin. The channel displaying only the bacteria are shown in Panel I with connecting arrows to the associated region in the pupae. Mean gray value = 3.253, ID = 468.295.

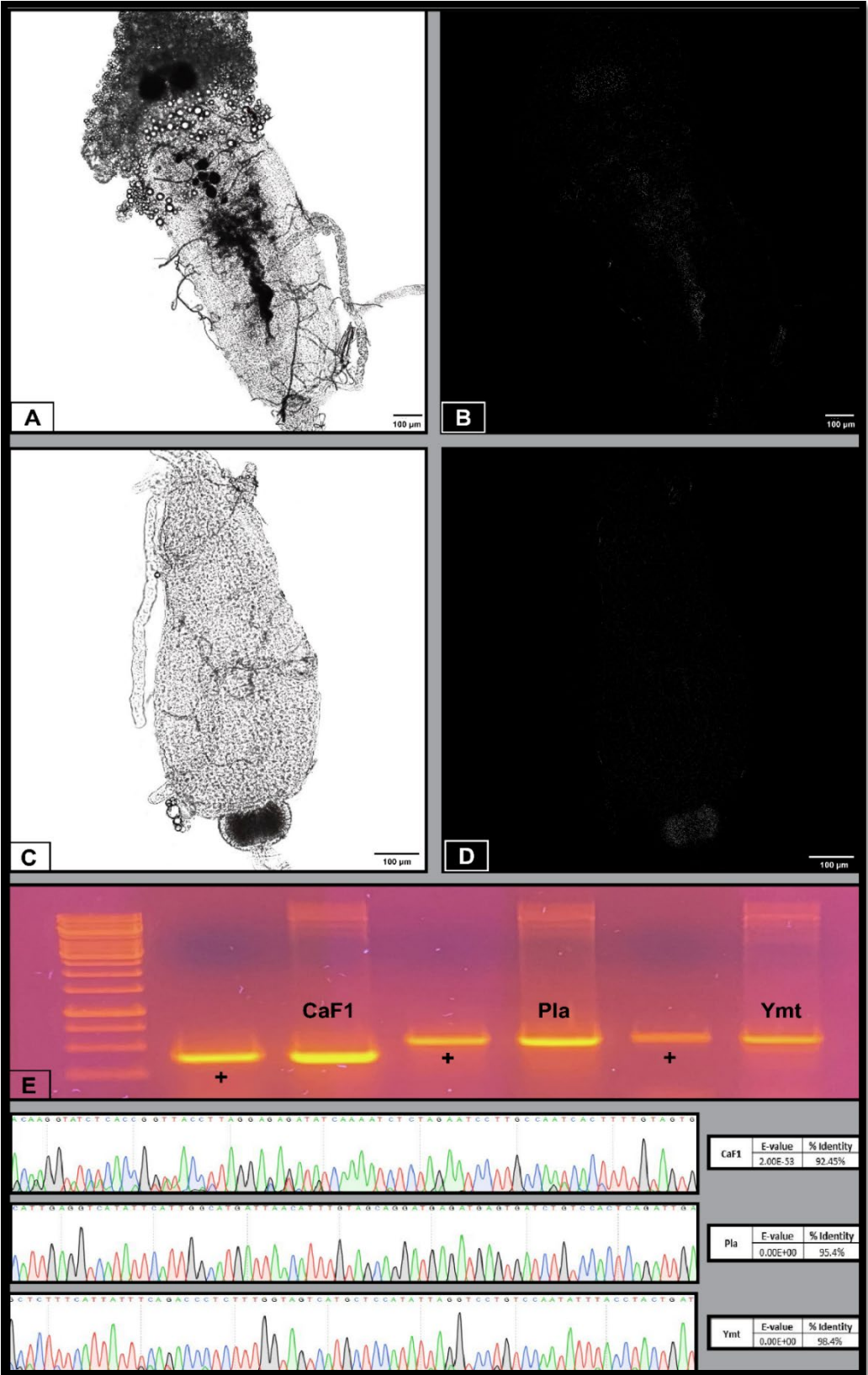


Figure S7: Panels A-D show midguts from two adults that developed from larvae collected from the sterile chamber which held infected fleas. Images A and B are of a female and images C and D are of a male. The larvae were collected from the bottom chamber of the sterile container, placed into a separate sterile container with sterile larval food to continue development. The adults were euthanized, dissected, and imaged for confocal microscopy or plated. Panel E shows the agarose gel with 1kb DNA ladder and PCR positive samples with each targeted gene having a positive control. Below panel E are representative samples for each target gene with the chromatograph and results from NCBI nucleotide BLAST.

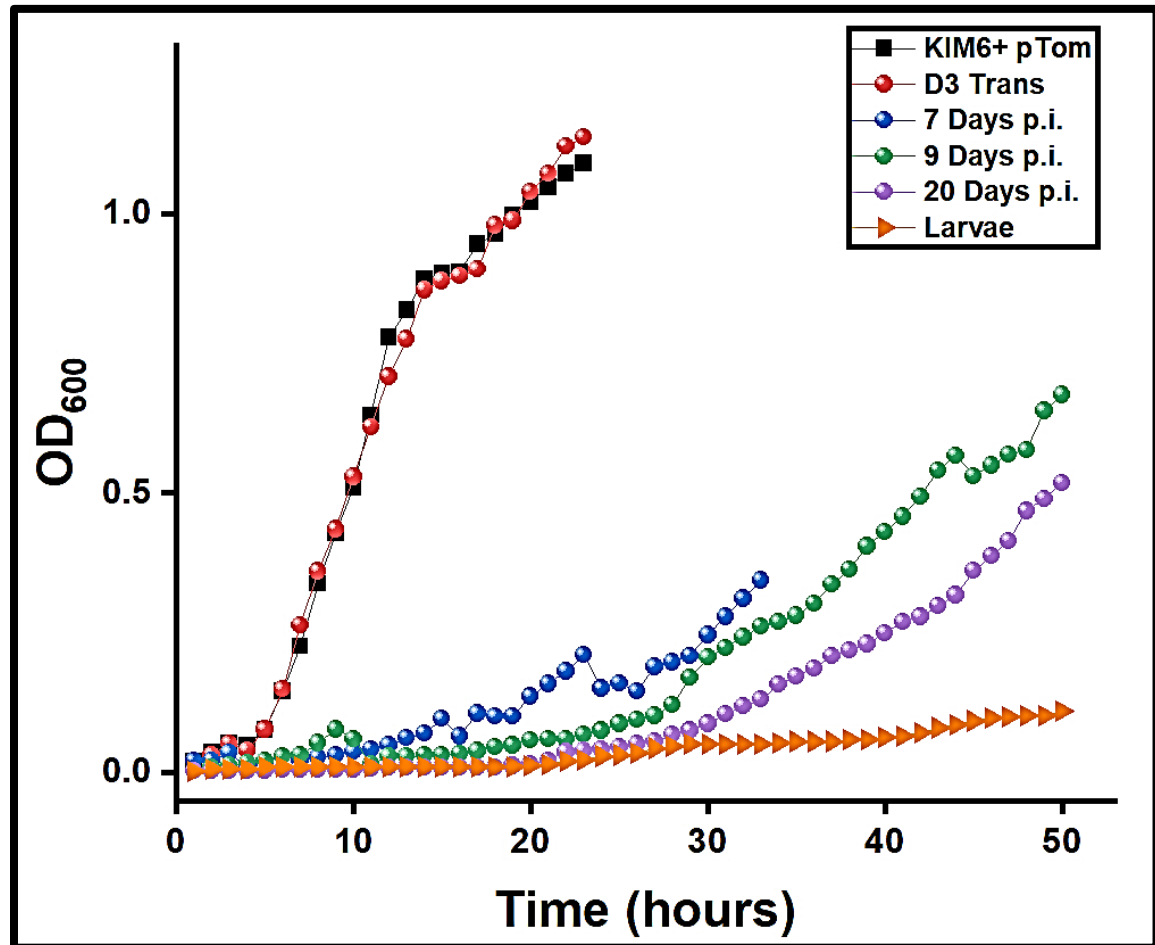


Figure S8 Growth curves of bacteria isolated from eggs collected from infected fleas at various days post-infection. Bacteria isolated from larvae allowed to develop after egg isolation from infected fleas are shown (orange, triangle). Fleas infected with isolated bacteria from eggs were analyzed for transmission and bacteria isolated from day 3 post-infection transmission was analyzed for growth (red, circles). The strain used to infect fleas, KIM6+ pTom, is also represented (black squares).

Sample	% >Q30	GC Content	% PF	% Adapter	Error Rate
KIM6+ pTom	90.9%	48.2%	99.3%	1%	0.3%
Egg Isolate	90%	70.9%	98.7%	1%	0.54%
D3_Trans	91.2%	60.3%	99%	0.6%	0.27%

Table S1 Results from the MultiQC report on the Illumina reads from sequencing the original strain (Kim6+ pTom), an egg isolate, and an isolate from Day 3 of a transmission study (D3_Trans). For the transmission study, an isolate from eggs was used to infect fleas to determine if the bacteria could colonize and subsequently be transmitted. The bacteria sequenced was from Day 3 post-infection. Infected fleas were fed on an artificial feeder and the skin, blood, and wash from the feeder was plated to determine if fleas transmitted bacteria during feeding. An isolate from plated samples was used to submit for sequencing. The table provides information on the percentage of bases with a quality score of 30 or higher (%>Q30), the GC content, the clarity of signal of bases sequenced (%PF), percentage of contaminating adapter-dimers (% Adapter), and the percentage of bases called incorrectly at any one cycle (Error Rate).

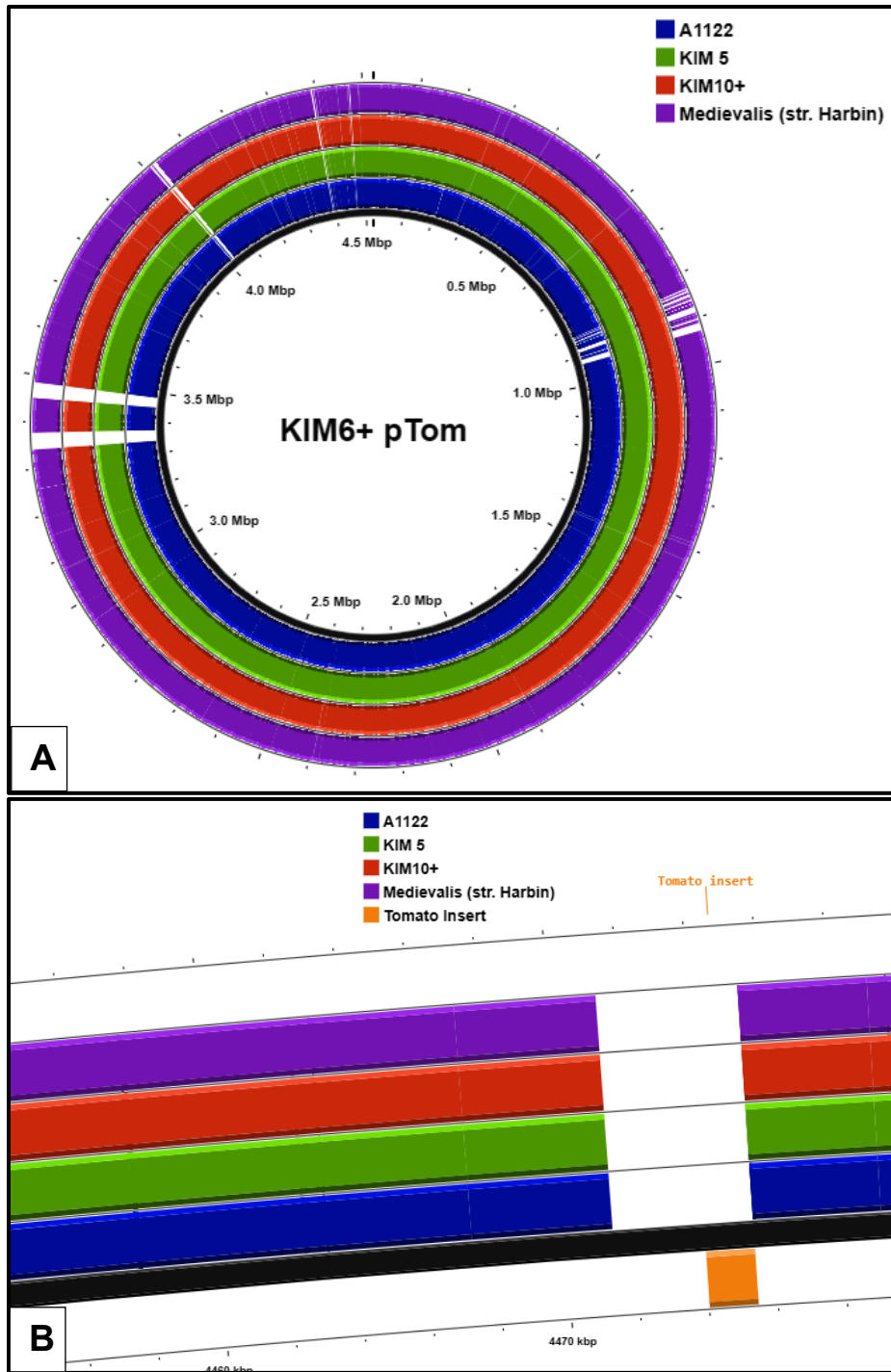


Figure S9 *Yersinia pestis* reference genome and three additional *Y. pestis* genomes blasted against KIM6+ carrying plasmid tomato (A). Panel B shows the insert from the tomato plasmid (orange block) within KIM6+ (black ring) but not in the other genomes (all colored rings).

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6 DISCUSSION

6.1 Association of Edaphic Factors and Plague Occurrences in Prairie Dog Colonies

The burrow of the rodent host is a microclimate which varies from the macroclimate outside of the burrow. For fleas of burrowing rodents, the microclimate is arguably the primary and most impactful environmental factor on the physiological, metabolic, and developmental processes of the flea. As such, it will have direct impacts on the flea: pathogen: host interface. Though individual burrows retain stable temperatures and humidity in relation to outside conditions, these parameters vary between burrows and across geographic ranges [1]. These differences could help explain why epizootics occur in individual towns but may not impact the entire metapopulation of prairie dogs. The abiotic conditions within the burrow are influenced by seasons to some extent, however; they are heavily influenced by depth of burrow, organic material, soil taxonomy, soil processes, water qualities of the soil, and rodent host populations [2, 3].

Our initial investigations extrapolated data from established soil sensors across an extensive geographic range and correlated those data to documented plague occurrences in prairie dogs. These data consisted of soil moisture and temperature which are defined by edaphic characteristics and taxonomy. The multi-state model indicated that increases in soil moisture at 8-inch depths and an increase in soil temperature at 4-inch depths increased the likelihood of plague. Water infiltration rate is impacted by soil characteristics close to the surface. As depth increases, pore size typically becomes smaller thus slowing the infiltration. For soils that have increased water infiltration, moisture at 8-inch depths is a good indicator that surface water is able to infiltrate through the soil indicating edaphic characteristics supporting water infiltration through soil layers. Soil temperature is subject to changes in the ambient temperature and will

have larger fluctuations than temperature at increased depths. From the surface to 20-inches deep, the soil will fluctuate based on the daily ambient temperature [4]. This measurement is a better representation of the ambient temperature indicating that summer months or geographic regions with higher temperatures are positively associated with plague occurrences. At increased depths the temperature will stabilize within soil layers and within burrows [4-6]. This analysis grouped all locations across a large geographic range together, removing the local specificities of geographically distant colonies. To capture geographically distinct factors, individual state models were created and showed increased significance on positive association between edaphic measurements and the likelihood of plague occurrences at varying soil depths. This highlighted that local factors potentially influence disease spread providing a possible explanation for variances in repetitive plague epizootics across the prairie dogs' geographic range. In Dallam County, TX epizootics were reported in 10 of the past 21 years and none were reported in Phillips County, KS though both areas have multiple prairie dog populations. The landscape differs in percent slope, soil taxonomy, mean annual precipitation and temperature, available water supply and calcium carbonate percentages in the soil. These factors can lead to different uses of the landscape and variation in vegetative composition supporting different species within the ecosystem. The prairie dog populations can be reduced or increased based on type of vegetation, competition for resources, and soil suitability for burrow construction. Increased population density has been associated with areas of repeated epizootics due to disease spread. Flea development is quicker with higher temperatures and humidity levels; therefore, these factors can have a direct impact on flea populations. On the plains in TX, the soil is calcareous loamy with 80% calcium

carbonates, 0-3% slopes, changing soil composition with layers to include clay and gravelly loam indicating increased water infiltration and ideal soil for burrow construction. With increased temperatures, it is likely the humidity in the burrows is maintained at high levels facilitating increased flea development. These local factors support increases in both host and vector density. Taken together, localized characteristics support repeated epizootics, again highlighting the importance of analyzing localized regions to a greater extent.

The extensive data collection and surveillance previously conducted on the KRB provided an opportunity to investigate the influence of chemical and physical soil characteristics at different soil layers. Metapopulations of prairie dogs occur within this area with a combination of colonies without epizootics and those experiencing repeated epizootics. Multiple soil characteristics that influence the permeability, infiltration, and ability to retain water were highly associated with plague occurrences. To refine the data collected for soil moisture and temperature, we installed soil sensors directly on a prairie dog colony in the KRB. This is the initiation of a long-term research study. Preliminary data spanning only a few months has shown differences in temperature data, though not significant, from the extrapolated data. We currently do not know how impactful these small differences will be in our model but over time, we will have the ability to conduct a more in-depth analysis. Most importantly, the installation of soil sensors allowed us to capture localized weather events that were missed on the sensor in which the data was originally extrapolated. This long-term study is novel for sylvatic plague research in the United States and will determine precise microclimatic parameters for accurate analyses, thus replacing macroclimatic data used for correlation studies.

6.2 The Impact of Environmental Factors on the Vector Competency of the Flea

Since the early 1900s, transmission of plague by fleas has been described as the process of biofilm formation in the midgut moving to the proventriculus causing a proventricular blockage as early as one-week post-infection. This biofilm contains high amounts of bacteria which are regurgitated into the host mammal when the flea attempts to take in a bloodmeal causing turbulence to break off pieces of the biofilm blocking the proventricular valve. Biofilm-formation in this case is hms-dependent, temporally and genetically defining the model. Due to the timeline of this established transmission paradigm, a disparity developed between this model and epizootics observed causing mass mortality in sylvatic cycles within a matter of days. An initial bloodmeal must be taken in from a bacteremic mammal to reach the infective dose necessary to colonize the flea midgut and avoid expulsion with feces. This knowledge further confounded the issue on how biofilm mediated transmission was possible during an epizootic event. Additional investigations revealed large variations in blocking rates between flea species as well as impacts of host bloodmeal on the timing and overall colonization of the proventriculus, which is imperative for regurgitative transmission. When early-phase transmission, EPT, was described, the early colonization of the proventriculus in conjunction with the ability of fleas to transmit days 1-4 post-infection, aligned well with the timing of epizootics. Therefore, EPT was implicated as the transmission model behind epizootics while biofilm-mediated was implicated as playing a role in long-term maintenance of *Y.pestis* in the sylvatic cycle through enzootic periods.

While the time frame is logical, the quantity of bacteria transmitted during EPT brought this model into question on its capability of facilitating epizootic events. Recent

literature has suggested that EPT promotes immunity within rodent hosts by repeatedly exposing hosts to low amounts of bacteria [7,8]. Surviving prairie dogs have been observed and testing for antibodies showed increased serum titers against *Y. pestis* [9]. This could support the various mortality rates seen in prairie dogs throughout their geographic range which correlate to suspected exposure rates based on endemicity of plague. It is important to note that immunity to plague in rodent species is not fully understood as most studies have shown a heterogeneity in disease development, survival, antibody production, and length of time for antibody production post-exposure [10-13]. Studies in black rats have shown increased chances of survival with previous exposures [14,15]. In prairie dogs from areas without plague, they were highly susceptible versus prairie dogs from areas with repeated plague epizootics [10]. However, in the case of low-level exposure, it would take high levels of bacteria from the flea to then cause mortality in the mammal which associates back to biofilm-mediated transmission. At the same time, it brings back into question how the infection reaches that level in the flea without taking in a large quantity of bacteria initially or how a bacteremic mammal occurs within this population for the flea to access. It is possible that an infected secondary host could introduce the pathogen but whether they occupy areas around colonies will vary across the geographic range. In addition, their susceptibility to the pathogen will be a factor and there is contradictory information on if prairie dog fleas opportunistically feed on these hosts [9,16,17]. Possible explanations of either a significant proportion of the flea population within the burrow system being partially or fully blocked simultaneously or higher amounts of bacteria transmitted during EPT are also counterintuitive.

Early proventricular colonization has been shown to occur, but the extent of the colonization and efficiency of transmission is varying. Lab experiments utilizing different flea species and bloodmeal combinations make it difficult to discern the results. To date, neither transmission mechanism has satisfactorily addressed the facilitation of either epizootic or enzootic events. Until we understand the biotic and abiotic parameters that impact transmission, these knowledge gaps will persist. Our research examines ecological factors that can influence the colonization of the flea, kinetics of infection, and subsequent transmission. Our previous analysis showed that increased temperature and moisture of the soil was positively associated with increased plague occurrences, suggesting an increase in humidity within the burrow facilitated bacterial survival in the flea and subsequent transmission. Therefore, we investigated the interrelationship of host bloodmeal, temperature and humidity on the flea:pathogen interface to represent variable microclimatic and ecological factors of the fleas' habitat.

We did not provide fleas with maintenance bloodmeals as previous research done in our lab indicated large changes in colonization and localization of the bacteria and we wanted to analyze these factors directly without additional variables. Future investigations can include additional variables that would be associated with the natural system, including post-infection bloodmeals. The number of blood feedings varies for flea species and digestion rates. *X. cheopis* would feed every day if a host was available [18]. Introducing this variable would determine how subsequent bloodmeals alter the infection in relation to number of bacteria and if proventricular colonization is enhanced. If the goal is to analyze the natural setting in which a flea would feed each day, it will be important to determine transmission efficiency more frequently than day 3 or day 7 as is

typically done. A more in-depth analysis could use the subsequent bloodmeals to understand fluctuations in host availability and the impacts on long-term infection in the flea. There were significant differences in colonization based on host bloodmeal with higher proventricular colonization observed in host-specific and nutrient rich bloodmeals. However, 94% of fleas maintained at 70% humidity and 25°C had proventricular colonization to some degree, regardless of bloodmeal. The levels of proventricular colonization change as the infection progresses without subsequent bloodmeals as a potential source for moving the bacteria out of the proventriculus. Humidity and temperature influenced colonization, but the extent and day post-infection was dependent on bloodmeal. For host-specific bloodmeal, all samples had proventricular colonization with standard parameters, or those that we maintain fleas at within the lab. Depending on the prairie dog colony location, our increased temperature and humidity may be what is typical of the burrow in these systems and what we term as ideal or standard for lab parameters facilitate higher infection when compared to the natural system. We altered our temperatures by 5°C as we did not see monthly changes larger than this in the soil data. Multi-month transitions may extend beyond 5°C, e.g., November to January but due to the short time frame of epizootics, we did not consider long-term changes for our temperature analysis.

When increased temperatures and humidity were used, early proventricular and overall colonization was greatly reduced. Fleas fed on non-host specific bloodmeal also showed changes in proventricular and overall colonization throughout the infection indicating the direct influence abiotic factors have on the pathogen:flea interface regardless of bloodmeal. Another interesting note is the variability seen between fleas.

For host-specific bloodmeal, there were outliers for day 3. This is likely to occur based on sex of the flea and the amount of blood each individual flea consumed. What is interesting is the amount of variability seen once the abiotic parameters were altered. More outliers are seen when humidity and temperature increase. This is particularly prominent in fleas fed mouse blood but in general, variation between fleas appears to be influenced by bloodmeal. Depending on the transmission efficiency and the number of fleas with either higher amounts of bacteria or lower amounts of bacteria, this variation potentially influences the epizootic and enzootic fluctuations occurring in populations.

CFU data does not follow the same patterns as RFUs for most of the analyses. Several reasons could be attributed to this but no one reason explains all the variation within groups. Groups are based on different bloodmeals and abiotic parameters which resulted in kinetically different infections; therefore, the reasons for disparities between CFUs and RFUs are likely group specific. For some of the differences, the pattern of CFUs correlates to the sample's distribution for RFUs indicating that the variation seen with individual fleas may be impacting the CFU data. CFU data is from a pooling of 3 fleas whereas the RFU data shows individual points for 10 fleas causing differences, particularly with increased variations between individual fleas. The calculation for RFUs accounts for the area of the region of interest, allowing comparisons to be made between samples but CFU measurements do not. The RFUs are also measured in the saturation channel which picks up any range of red to detect bacteria in tissues or biofilms that may be quenched, however; we cannot definitively confirm that the fluorescent signal is not dampened to any extent. Increasing the sample size for CFUs will allow us to analyze

these discrepancies further. In addition, further analysis on the potential reduction in expression of fluorescence as the bacteria replicates should be conducted.

For host-specific bloodmeals, higher RFUs were seen on day 3 under standard parameters which correlated well with the transmission, except when humidity was increased. When humidity only increased, RFUs were lower but transmission was higher, though not significantly. On day 7 post-infection, increased transmission was seen again but was statistically significantly different from standard parameters. Further research is needed to determine why RFUs would be lower, but transmission would be increased with high humidity. Reasons could relate to differences seen between RFUs and CFUs. If biofilm is increased or there is a higher association with the proventriculus, a reduction in detection may be occurring even when using the saturation channel in Image J. Though it did not appear as though we were losing signal, without further analysis on expression and plasmid retention in the strain, the possibility should be considered. The ptdTomato plasmid was altered by replacing the lac promoter with a *cysZK* promoter encoding an inner membrane protein involved in the uptake of sulfate [19]. This is part of a highly conserved gene cluster in bacteria and in *Escherichia coli* it has been shown to be pH dependent [20,21]. The sulfate concentration would impact expression in addition to pH, indicating potential environmental factors within the flea that alter expression of the fluorescent plasmid. Other potential mechanisms, independent of analyses or methodologies, relate to how the bacteria associates with the biofilm. Lower levels of RFUs could indicate that the biofilm was less developed with higher humidity. Higher transmission would suggest that a less developed biofilm may be better for regurgitative transmission. Although literature indicates that proventricular blockage

has higher amounts of bacteria associated with biofilms, transmission is variable. How the bacteria are released from the biofilm once it enters the mammal or whether it is viable is unknown. Transmission efficiencies are also not known for loose biofilms with easily dissociated bacteria versus biofilms with bacteria thoroughly embedded into PNAG. Higher humidity may facilitate a looser biofilm with fewer bacteria that can easily dissociate and are viable, leading to increased transmission efficiency without higher RFUs.

Mouse bloodmeal is often used in experiments investigating proventricular colonization and transmission, as well as for comparing transmission efficiencies between bloodmeals. Fleas fed mouse blood transmitted fewer CFUs under standard parameters supporting findings from previous publications, however; altered parameters led to increased transmission [7,8,22]. Fleas fed on mouse blood transmitted more efficiently than those fed on rat when all parameters were changed, indicating that what naturally occurs within the system may not be captured in the lab. To fully understand vector competency, experiments in the lab need to be more representative of the natural system. Although difficult due to rearing issues, flea: host specificity is important and secondary host bloodmeals should be relevant to the system under investigation. The geographic range of prairie dogs includes multiple systems with different ecological and edaphic components which should be taken into consideration in laboratory experiments. Considering that proventricular colonization occurs shortly after infection, transmission studies should correlate to flea feeding. This research highlights the importance of looking at the interrelationship of factors associated with the sylvatic cycle.

Digestion rates have previously been investigated by visually inspecting the bloodmeal under a microscope [23, 24]. Energy expenditure has also been used to determine digestion rates by measuring CO₂ as an indirect measure for metabolism and digestion. We utilized novel methods to measure protein in the midgut of fleas in the first 18-hrs post-feeding. Neither the protein assay nor the qPCR was able to make direct determinants of the rate of digestion. Increases in sample sizes are needed and the addition of gene expression assays. Nonetheless, our preliminary analysis was the first to investigate the impact of humidity on digestion which showed a significant impact. Tracheal respiration in fleas is increased during digestion with the spiracles opening repeatedly or staying open [25]. When this occurs, the water loss in fleas increases. An increase in humidity could alleviate this water loss and support more efficient digestion which may explain the increase in protein in uninfected fleas in comparison to standard parameters. The infection with *Y. pestis* at increased humidity resulted in lower protein levels indicating inhibition of digestion, though the possible mechanisms behind this are unknown leading to the need for further investigation. Digestion creates a harsh environment in the midgut of the flea which can cause death of the bacteria. Slowing digestion may increase overall survival and provide an opportunity for the bacteria to utilize the components of the bloodmeal for colonization and adhesion to the proventricular spines.

6.3 Transovarial Transmission of *Yersinia pestis* by *Xenopsylla Cheopis*

One large gap in knowledge regarding the sylvatic cycle is the introduction of bacteria into the sylvatic cycle to initiate an epizootic. There is conflicting research on if *Y. pestis* persists during enzootic cycles in flea populations or not. Several sources have

been hypothesized including secondary host species in close proximity to rodent host populations introducing infected fleas or long-term survival of *Y. pestis* within the soil [26]. Long-term survival in the flea has also been noted [27]. Our research has investigated factors associated with the ecology of the sylvatic cycle and how they may influence the flea: pathogen interface. These factors could influence this initial introduction of high levels of bacteria. Particularly if fleas are already infected but at low levels, external parameters will influence the internal environment of the flea, potentially increasing growth of the bacteria. Fleas can acquire the pathogen even at low levels, but no transmission occurs under a certain threshold [28].

During our investigations we found that contrary to the established paradigm, *Y. pestis* can exit the midgut and colonize other tissues, particularly reproductive tissues. Using aseptic techniques, we were able to isolate eggs to investigate bacterial presence through imaging, growth, and sequencing. Obtaining z-stacks allowed us to create 3-D models to look inside of the tissues to identify the bacteria. The bacteria were sustained through different life stages indicating a possible mechanism for long-term persistence with cyclic epizootics. If low levels of bacteria are present but will only increase to transmissible levels under certain ecological conditions, it could explain the disparity seen when defining these cycles to transmission models. In addition, it could help explain why some colonies experience sporadic epizootics across multiple years. The pupae stage of a flea can arrest development when environmental conditions are not ideal for continued development or survival [18,29]. Female fleas can store sperm in the spermatheca which we illustrated to have bacteria and we observed bacteria in the testes [29]. The presence of bacteria in eggs from infected fleas and the continual maintenance

through life stages illustrates a novel mechanism in long-term maintenance of *Y. pestis* between epizootics that has never before been described or considered.

In summary, our research showed associations between temperature, humidity, and bloodmeal with proventricular colonization, localization, and transmission indicating ecological variables may influence disease spread. The possibility of transovarial transmission could explain the long-term maintenance of *Y. pestis* in these sylvatic cycles. If ecological factors are driving the proliferation of bacteria in the flea, knowledge gaps on how high levels of bacteria are introduced to initiate an epizootic may be addressed. In addition, it will remove the constraints associated with trying to determine which transmission model is responsible for epizootics. Ecological factors such as temperature, humidity, and host bloodmeal can influence biofilm mechanisms independent of the hmsHFRS operon. The direct influence on bacterial loads within the ectothermic vector will not be restricted to a day or time-frame post-infection.

Understanding the associations between edaphic factors and plague occurrences will help identify areas that are suited for repeated epizootics. In conjunction, we will be able to determine how the microclimatic features will change under the influence of certain macroclimatic factors or rodent host population fluctuations. This knowledge could be used to regulate the timing and overall application of pulicides, reducing the repeated use and subsequent potential for insecticide resistance in fleas. Preventative measures, including pulicide application, and rodent population control could be optimized to reduce zoonotic potential.

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