

FINAL REPORT

Leptospirosis in Endangered Island Foxes and California Sea Lions: Outbreak Prediction and Prevention in a Changing World

Unforeseen Impacts of a Severe Oceanographic Anomaly on Pathogen Transmission in Marine Mammals

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List of Acronyms

AC	Antibody Concentration
AIC	Akaike Information Criterion
ANI	Año Nuevo Island
ARS	Agricultural Research Service
BUN	Blood Urea Nitrogen
CAHFS	California Animal Health and Food Safety Laboratory
CDC	Centers for Disease Control and Prevention
CI	Confidence Interval
CrI	Credible Interval
CSL	California Sea Lion
DNA	Deoxyribonucleic Acid
DoD	Department of Defense
ENSO	El Niño Southern Oscillation
FM	Feature Matching
GPS	Global Positioning System
HKY	Hasegawa, Kishino, and Yano (substitution model)
IACUC	Institutional Animal Care and Use Committee
IHC	Immunohistochemistry
IBDR	Infectious Bacterial Diseases Research Unit
LOOIC	Leave-One-Out Information Criterion
MAT	Microscopic Agglutination Test
MCMC	Markov Chain Monte Carlo
MML	Marine Mammal Laboratory
MMPA	Marine Mammal Protection Act
NADC	National Animal Disease Center
NES	Northern Elephant Seal
NMFS	National Marine Fisheries Service
NOAA	National Oceanic and Atmospheric Administration
NPS	National Park Service
NSF	National Science Foundation
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
POS	Positive
qPCR	Quantitative PCR
RNA	Ribonucleic Acid
RFLP	Restriction Fragment Length Polymorphism
SCL	San Clemente Island, California
SE	Standard Error
SERDP	Strategic Environmental Research and Development Program
SMI	San Miguel Island, California

SNI	San Nicolas Island, California
SNP	Single Nucleotide Polymorphism
SRI	Santa Rosa Island, California
SST	Sea Surface Temperature
TMMC	The Marine Mammal Center
UCLA	University of California, Los Angeles
UCSC	University of California, Santa Cruz
US	United States
UME	Unusual Mortality Event
USDA	United States Department of Agriculture
VNTR	Variable Number of Tandem Repeats
WGS	Whole Genome Sequencing

Keywords

Antibody titer kinetics, California sea lion (*Zalophus californianus*), capture-mark-resight analysis, climate change, cross-reactivity, cross-ecosystem transmission, cross-species transmission, demographic reconstruction, disease ecology, DNA enrichment, El Niño Southern Oscillation, epidemic, epizootic, extrinsic drivers, fadeout, force of infection, host susceptibility, infectious disease dynamics, intrinsic drivers, , *Leptospira interrogans* serovar Pomona, leptospirosis, marine heatwave, marine infectious disease, oceanographic anomalies, pathogen outbreak, phylogenetics, population ecology, quantitative serology, semiparametric models, seroprevalence, spatial models, susceptible reconstruction, transmission dynamics, transmission modeling, whole genome sequencing, wildlife pathogen surveillance, zoonosis

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1. Abstract

Introduction and Objectives:

Leptospirosis, the disease caused by pathogenic bacteria of the genus *Leptospira*, is a major health burden for humans and animals worldwide and a recognized risk for military personnel. *Leptospira* circulated endemically for decades in California sea lions (CSL: *Zalophus californianus*), then disappeared from 2013 to 2017 during a period of severe oceanographic anomalies. This project studied the ecology of *Leptospira* in CSL and their surrounding ecosystem, to understand how non-stationary environmental conditions contributed to the fadeout and re-emergence of the pathogen in this system. Our objectives were to learn: (1) what factors gave rise to the fadeout of *Leptospira* in the CSL population in 2013, (2) what conditions enabled the re-emergence of *Leptospira* in 2017, and (3) whether these perturbations had lasting impacts or whether CSL/*Leptospira* system dynamics returned to pre-fadeout patterns.

Technical Approach:

With our partners, we extended long-term studies of host demography, movement and *Leptospira* spread in CSL. We analyzed spatiotemporal patterns and genomic sequencing data to confirm and characterize the fadeout event. We compiled and analyzed time series of putative drivers of transmission that could mediate the impact of environmental perturbations. We searched for possible outside reservoirs that could have reintroduced the pathogen to CSL to enable re-emergence. Throughout, we developed mechanistic and statistical models to reveal underlying processes and integrate data streams.

Results:

We confirmed that *Leptospira* ceased circulating in CSL from 2013-2017, using data from diagnostic testing of ~3800 samples collected during this period, and analysis of pathogen genomic sequences. The fadeout event in 2013 was associated with unprecedented reductions in yearling CSL recruitment and normal migratory behavior, both driven by anomalously poor feeding conditions, which eliminated the susceptible ‘fuel’ needed to sustain the transmission chain. The fadeout period lasted for four years until the confluence of two events: oceanographic conditions returned to normal, and the pathogen was reintroduced to CSL. The resulting outbreak in 2017 was small, but 2018 brought one of the largest outbreaks on record; these patterns are explained by severe demographic impacts of the marine heat wave in 2015-16, which decimated the juvenile cohorts needed to drive an outbreak in 2017. Field surveillance and genomic sequencing revealed that terrestrial mesocarnivores in mainland California could be the reservoir that reintroduced *Leptospira* to CSL, since they carry a closely related strain of the pathogen.

Benefits:

Our project generated new knowledge and tools to support management of two wildlife species of concern in the California coastal ecosystem. By extending long-term field studies and developing mathematical and statistical models to analyze the data, we uncovered the causes of an unprecedented fadeout event, discovered a new community of hosts carrying *Leptospira* in proximity to humans and sensitive wildlife species, and generated evidence-based guidance for species managers and public health agencies. This work strengthens our ability to anticipate and manage consequences of future non-stationarities, and advances our understanding of species- and ecosystem-level impacts of global climate change.

2. Executive Summary

Introduction

From 2013-15 the northeastern Pacific Ocean experienced a dramatic warm-water anomaly, commonly referred to as ‘the Blob’, followed by an El Niño event in 2015-16. This period of severely anomalous conditions has been described as a ‘climate change stress test’, due to its unprecedented duration and intensity and the fact that such events are projected to become more common under future global change scenarios. This presents a compelling opportunity both to study the effects of a major climatic disruption to a key US ecosystem (the California Current System), and to learn about the potential impacts of future environmental non-stationarities on ecosystem function and related natural phenomena, including infectious disease risks. We observed unprecedented changes in the transmission dynamics of pathogenic *Leptospira* in California sea lions (CSL: *Zalophus californianus*) during this period (Figure E.1). Given our team’s long-term and multidisciplinary research on this system, we set out to investigate the fadeout and subsequent re-emergence of *Leptospira* in CSL as a case study of the effects of severe environmental anomalies on infectious disease ecology.

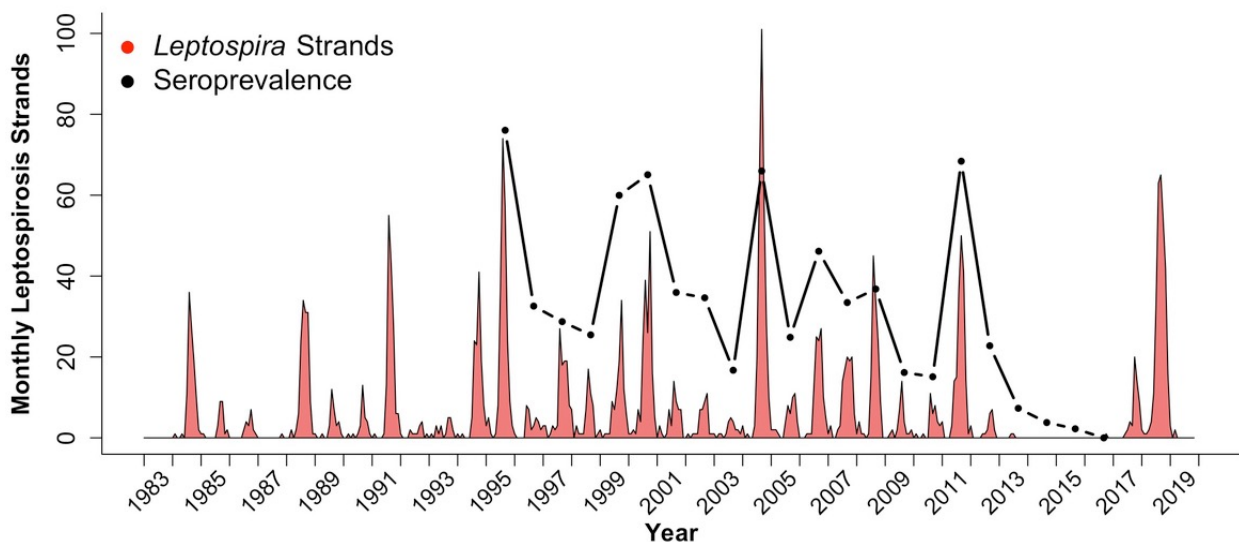


Figure E.1. Time series of *Leptospira* strands and seroprevalence in California sea lions. Number of CSL stranding each month at The Marine Mammal Center (TMMC) with leptospirosis (solid red) and annual seroprevalence of anti-*Leptospira* antibodies (reflecting past exposure to the pathogen) in animals stranding at TMMC (black line; results pending after 2016). Figure represents information available when our study was proposed.

Leptospirosis, the disease caused by pathogenic *Leptospira* species, is a major health burden for humans, domestic animals, and wildlife worldwide. It causes over 500,000 severe cases annually in humans and has long been recognized as a major threat to US military personnel. *Leptospira* species have a complex ecology that can involve multiple host species, environmental reservoirs, and climatic drivers. There are over 300 pathogenic serovars of *Leptospira*, which exhibit variable interactions with different host species, ranging from chronic and asymptomatic infection to acute and fatal disease. The strain identified in our system, *L. interrogans* serovar Pomona, has been associated with pigs, skunks, foxes and sea lions.

Leptospira causes systemic infection and colonizes the kidneys, transmitting via urine either directly or via contaminated water or soil. Transmission among sea lions is assumed to occur while they are hauled out on land, but preliminary data show that *L. interrogans* serovar Pomona can survive for short periods in seawater so transmission within dense aggregations in the water is possible.

California sea lions (CSL) are a gregarious pinniped, with a US stock of approximately 300,000 that migrates seasonally between rookery sites on San Miguel (SMI), San Nicolas (SNI) and San Clemente (SCL) Islands (Department of Defense-owned islands in the California Channel Island archipelago) to coastal haulout sites from central California to Washington. Our team members at the NOAA/NMFS Marine Mammal Laboratory (MML) have conducted a long-term study of CSL demography, with pup counts (>40 years) and a mark-resight program (31 years, before this study) based at SMI, which characterized how CSL recruitment and survivorship respond to environmental conditions including El Niño events and other oceanographic anomalies.

Our team is conducting a long-term study of *Leptospira* ecology in California sea lions (CSL: *Zalophus californianus*) and other protected species in the California Current ecosystem. Through our collaboration with The Marine Mammal Center (TMMC), we have amassed a remarkable long-term time series of leptospirosis incidence (35 years, before this study) and seroprevalence (22 years) from wild CSL that strand along the California coast (Figure E.1). For a 30-year period beginning in 1984, *Leptospira* caused outbreaks of varying intensity each fall, with major outbreaks causing mass stranding and mortality every 3-5 years. Based on pathogen genetics and ecological data, CSL were understood to be a maintenance host population with uninterrupted transmission of *Leptospira* during this period. Our team has studied the mechanisms of this persistence, including the role of chronic carrier individuals in the CSL population. In addition, since 2010 we have conducted an annual program of sampling wild-caught CSL at numerous sites throughout their migratory range, to corroborate the stranding data and chart the spatial variation in CSL-*Leptospira* dynamics. Under a separate project supported by SERDP (project RC-2635), we investigated the risks to other threatened and endemic species that share the coastal ecosystem, including Channel island foxes (*Urocyon littoralis*), and working to understand how intrinsic (e.g. host demography) and extrinsic (e.g. environmental variation) factors combine to shape the interannual variation of leptospirosis outbreak intensity in CSL since 1984.

In the context of these long-term studies and well-characterized system dynamics, the suspected disappearance of *Leptospira* from the CSL system in late 2013 was a major surprise. At first the low numbers of leptospirosis cases were interpreted as a period of low activity, presumably triggered by low CSL birth rates in 2013 and 2014 which meant fewer susceptible animals entering the population. As time passed, and other signatures of *Leptospira* infection decayed to zero, we eventually concluded that the pathogen had ceased circulating in the population. We reached this conclusion cautiously, because we were aware of no precedent for the spontaneous fadeout of a pathogen with a well-documented history of circulation spanning several decades, in a host population which – despite recent perturbations to recruitment – numbered in the hundreds of thousands. As the scale of the contemporaneous oceanographic anomalies became clear, we speculated whether other impacts on CSL natural history, from shifts in migratory and foraging behavior to decreased survival in older classes, may have played a role in interrupting *Leptospira* transmission.

In late 2017 the pathogen reappeared in the CSL population, causing a mid-sized outbreak late in the fall season. Retrospective sample analyses revealed an isolated case in a sea lion that stranded in fall 2016, presumably linked to a reintroduction that did not cause an observable outbreak. Given the inferred build-up of susceptible CSL during the years of little to no activity since the last major outbreak in 2011, it is an open question why the 2016 and 2017 pathogen introductions did not trigger major outbreaks. Regardless, once the pathogen re-established circulation in the CSL population we predicted a major outbreak for fall 2018, and the 2018 outbreak was one of the largest on record (Figure E.1).

These observations raised pressing questions. If *Leptospira* truly did cease circulating in the CSL population between 2013 and 2017, then how – and from where – did it re-emerge? Is there some other unknown animal reservoir in California that seeded the re-emergence, and if so does it pose risks to other sensitive species, or to humans? Why did the apparent reintroduction in 2016 fail to establish, and why was the outbreak in 2017 not larger? Will the *Leptospira* strains circulating after the fadeout period exhibit different dynamics from the long-term patterns observed in the decades leading up to 2013? How do changing ocean and climate conditions shape all these risks? We proposed an integrative program of field, laboratory and modeling research to identify the factors underlying the response of this host-pathogen system to major environmental non-stationarities, to support future species management of numerous protected, threatened and endemic species that use DoD lands and adjacent waters, and build fundamental understanding of how non-stationary environmental conditions influence pathogen transmission.

Objectives

The large-scale oceanographic anomalies from 2013 to 2016, coinciding with spontaneous fadeout and subsequent re-emergence of *Leptospira* in the CSL population, presented a unique opportunity to study how major environmental non-stationarities can affect pathogen circulation in a host species of interest. Our study objectives were to answer three major research questions:

- Q1. What factors gave rise to the fadeout of *Leptospira* in the CSL population in 2013, and what was the role of anomalous environmental conditions?
- Q2. What conditions enabled the re-emergence of *Leptospira* in the CSL population in 2017?
- Q3. Did the fadeout and environmental anomalies have lasting impacts on the CSL/*Leptospira* system, or have system dynamics returned to pre-fadeout patterns?

Technical Approach

Our project combined field, laboratory, and modeling research. The field and laboratory work served to extend our long-term studies of CSL demography and *Leptospira* incidence. Our partners at the National Marine Fisheries Service's Marine Mammal Laboratory (MML) have continued their program to brand and re-sight sea lions, to extend their demographic study of CSL to include the yearling cohort of 2019. We analyzed these data using capture-mark-resight models to estimate population size and age structure through time, and to estimate the spatial distribution of CSL across resighting sites. With our partners at The Marine Mammal Center (TMMC) we have extended our time series of leptospirosis incidence and seroprevalence in CSL that strand on the California coast, until 2019, using serum, urine, and kidney samples as described above, along with clinical records of CSL in rehabilitation after stranding. These efforts have extended long-term data sets of leptospirosis in CSL to 37 years, and CSL

demography to 33 years. To complement and ground-truth the information gathered from stranded CSL, we sampled free-ranging sea lions at San Miguel Island and Año Nuevo Island, California. Combining these efforts with earlier work supported by other funders led to a 10-year time series of *Leptospira* surveillance in wild sea lions on the California coast.

To characterize and confirm the fadeout event, we analyzed the spatiotemporal distribution of suspect and confirmed leptospirosis cases, and patterns of pathogen detection (by PCR) and seropositivity. We analyzed the quantitative titers of anti-*Leptospira* antibodies to test whether observed titers were consistent with the fadeout hypothesis. In all cases we considered the age distribution of results, with reference to whether CSL were born before or during the fadeout. We also analyzed whole-genome sequences of *Leptospira* to assess whether genotypes circulating in CSL after the fadeout were distinct from those found in CSL before the fadeout.

To examine how environmental perturbations could have driven the fadeout and re-emergence events, we constructed time series of factors that could influence *Leptospira* transmission dynamics, including:

- (i) supply of susceptible CSL, which we reconstructed for the period from 1984-2019, using a model to integrate long-term data on CSL demography and *Leptospira* incidence.
- (ii) CSL movement and mixing, which we reconstructed for the period from 1996-2019 by applying multistate mark-recapture models to thousands of resighting events for marked CSL each year, collected by MML.
- (iii) CSL condition, which we reconstructed using MML data on CSL pup weight gain each year from 1996-2019, and data on forage fish stocks from the literature.

Each of these putative drivers has known or postulated links to oceanographic conditions, and exhibited major fluctuations during the oceanographic anomalies in our period of study.

To search for unknown reservoir species, which could have sustained the circulation of *Leptospira* during fadeout and reintroduced it to CSL in 2017, we leveraged an on-going study that was surveying pathogens in wildlife species in the greater Los Angeles region. In addition, opportunistically, we investigated the case of a short-beaked common dolphin (*Delphinus delphis*) that stranded in southern California with signs of leptospirosis. The Los Angeles study collected carcasses of five focal species – coyotes (*Canis latrans*), raccoons (*Procyon lotor*), opossums (*Didelphis virginiana*), striped skunks (*Mephitis mephitis*) and fox squirrels (*Sciurus niger*) – and performed necropsies and laboratory diagnostics to detect pathogen status. Serum samples were analyzed to detect antibodies reflecting prior exposure to *Leptospira*, and urine and kidney samples were tested by PCR to detect current infections. For a subset of PCR-positive samples (two raccoons, one coyote, one striped skunk, and the dolphin), we employed newly-available molecular techniques to enrich the *Leptospira* DNA to a level that enabled whole-genome sequencing, allowing us to determine the genetic relationship of *Leptospira* from these hosts to the strains found in CSL – and hence to assess whether they could be the missing reservoir.

Throughout our work, we developed mathematical and statistical models to formalize insights from our studies, integrate lines of evidence, and learn about exposure risks. We emphasized quantitative analysis of serological data, since the presence and quantity of antibodies provide crucial insights into past infections. In particular, to confirm the fadeout event it was essential to reach a rigorous understanding of the low-titer seropositivity observed in some older CSL, to assess whether it was consistent with infection prior to the fadeout event. Conversely, the fadeout period created an opportunity to characterize the long-term decay of antibody titers in CSL, a crucial process that is ordinarily masked by on-going re-exposures. We

developed several novel models to analyze quantitative serology data, including Bayesian methods to estimate antibody decay rates from partial or censored information, to integrate all sources of uncertainty in analyses of the microscopic agglutination test (the standard serologic assay for *Leptospira*), and to estimate the time of infection of individual animals from longitudinal data collected in the field (drawing on other available datasets from our long-term studies in this ecosystem). These tools were applied to learn about the determinants of observed patterns, and enable robust inference about underlying dynamics of pathogen transmission and how this may change under environmental non-stationarities.

Results and Discussion

Epidemiological and genetic confirmation of fadeout and re-emergence

By extending and deepening our team's long-term surveillance of *Leptospira* in the CSL population, we assembled compelling multifactorial evidence of a 4-year cessation in leptospirosis circulation in CSL from early 2013 to mid-2017, followed by re-emergence of the pathogen in 2017. Our inability to detect infections from 2013-2017 was not for lack of effort: during this period, we assessed over 3000 stranded sea lions and captured and sampled over 1300 free-ranging sea lions; we tested ~1400 CSL by PCR to detect current infections and ~2400 CSL to detect antibodies against recent infections. The resulting data are conclusive: in CSL of all ages, infection prevalence dropped to zero during the fadeout (Figure E.2); in the youngest animals, seroprevalence also dropped to zero, while in older animals some seropositives were observed, but always at low antibody titers reflecting infections years prior. These epidemiological signs of fadeout were corroborated by genomic evidence, since the genotypes of *Leptospira* sampled from CSL after the re-emergence in 2017 were not descended from the genotypes that circulated before 2013 – clear evidence that the post-fadeout strains resulted from new introductions from some unknown reservoir. This is the first known example of spontaneous fadeout of an endemically circulating pathogen from a large, robust host population.

Fadeout was triggered by reduced susceptible supply and disrupted sea lion migration, both linked to malnutrition

Our analyses revealed that the fadeout in 2013 was associated with unprecedented simultaneous anomalies in susceptible supply and CSL migratory behavior. Using our long-term mark-resight dataset, we reconstructed the size of the yearling population and the proportion of yearlings that left the rookery islands to migrate north up the California coast, each year since 1996 (Figure E.3). While considerable interannual variation is evident in the pre-fadeout period, including major reductions in yearling population size during the 1997-1998 El Niño event, we found that conditions in 2013 brought the CSL population into uncharted territory. Specifically, both the size of the yearling population and the proportion leaving the rookery islands to migrate up the coast were anomalously low in that year. This resulted in a record low number of new susceptible CSL in the coastal region where *Leptospira* outbreaks usually start each summer. We believe this broke a key link in the transmission chain, when the fresh wave of susceptible yearlings encounters the older CSL on their southward return migration, enabling the dwindling *Leptospira* cases from the previous year to spark the new outbreak. Without the susceptible 'fuel' on hand, the spark didn't catch and the transmission chain was broken.

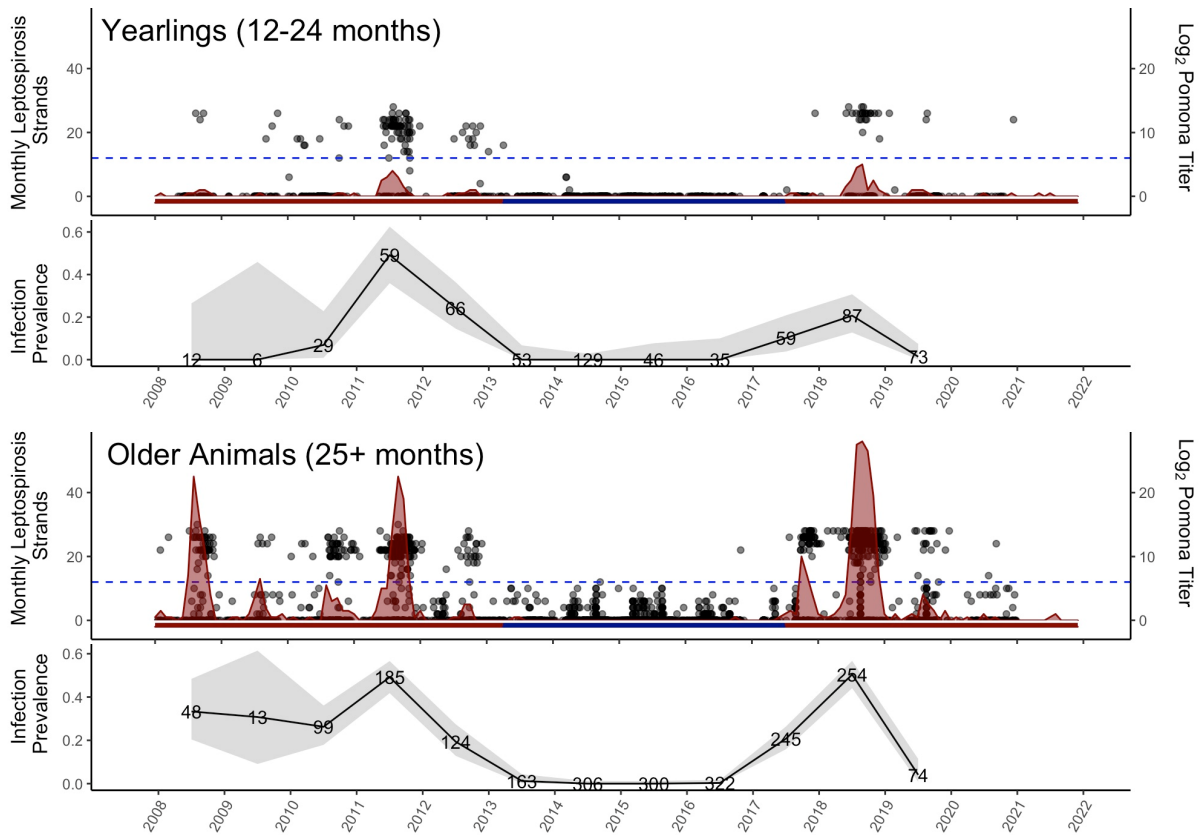


Figure E.2. Evidence of fadeout of *Leptospira* from CSL population from 2013 to 2017. Data on monthly CSL strandings due to leptospirosis (red fill), antibody titers against *Leptospira* (circles), and prevalence of infection with *Leptospira* (black lines), plotted for yearlings vs older CSL. Dashed horizontal lines show the titer threshold denoting recent exposure to *Leptospira*. Grey regions depict 95% confidence intervals on infection prevalence, and numbers show the number of samples tested that month.

Our analyses showed that the unprecedented impacts in 2013 were tied to very poor weight gain in the 2012 pup cohort, so many animals starved and the survivors were too weak and thin to migrate. This corresponds to an Unusual Mortality Event declared by NOAA in January 2013, which was attributed to poor feeding conditions suspected to be driven by unusual oceanographic conditions, though the marine heat wave had not yet started at this time.

*Re-emergence of *Leptospira* could only occur after oceanographic conditions returned to normal*

Once *Leptospira* had ceased circulating in CSL, in order for it to re-emerge it required the confluence of favorable conditions and reintroduction of the pathogen from some outside reservoir. In 2014, conditions were favorable with a large yearling population exhibiting normal migration behavior (Figure E.3), but *Leptospira* remained absent. We interpret this to mean that there was no reintroduction ‘spark’ that year, and furthermore the absence of *Leptospira* despite favorable conditions reinforces the conclusion that the pathogen faded out in 2013. In 2015 and 2016, the marine heat wave and subsequent El Niño event pushed the CSL population back into strongly unfavorable conditions. In 2016 we detected a single case of leptospirosis in a subadult CSL, which we interpret as a reintroduction that failed due to poor conditions. In 2017, yearling population size and migration patterns returned to normal and *Leptospira* re-emerged.

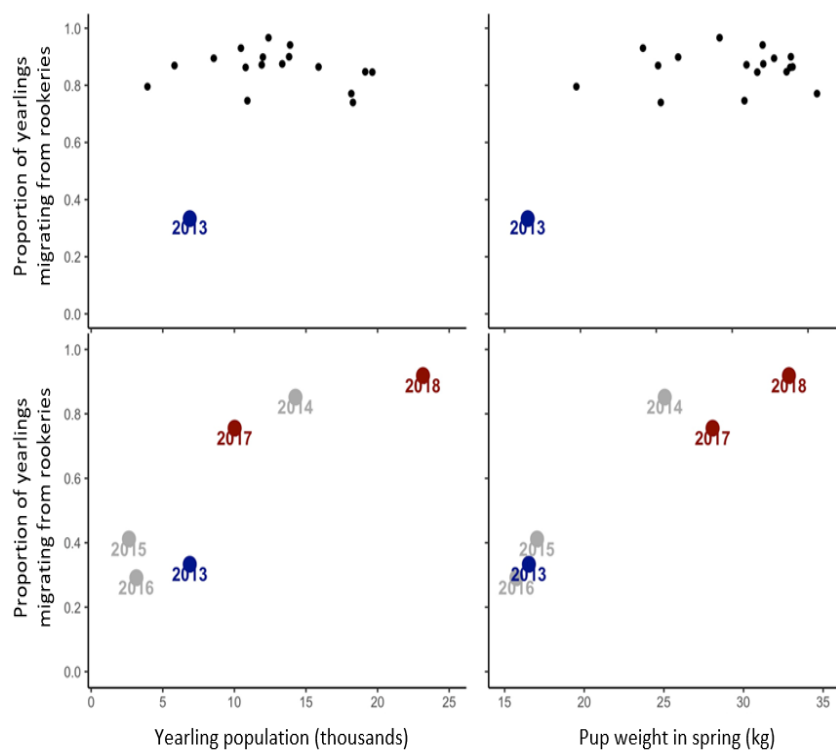


Figure E.3. Fadeout and re-emergence of *Leptospira* were linked with shifts in susceptible supply and migratory behavior, driven by trophic conditions.

Black points indicate conditions each year from 1996-2012. The year fadeout occurred (2013) is marked in blue; the years between fadeout and re-emergence are marked in grey, and re-emergence years are red. In 2013, both the yearling population size and proportion leaving the rookery islands to migrate north were anomalously low, due to malnutrition and low body weight. By 2017 conditions had returned to normal and by 2018 conditions were excellent for *Leptospira* transmission.

Leptospira dynamics after the fadeout showed understandable deviations from past patterns

When the re-emergence was detected in mid-2017, we initially anticipated a massive outbreak due to the accumulation of susceptibles over four years without *Leptospira* circulating. As it turned out, the 2017 outbreak was relatively minor, while the 2018 outbreak was one of the largest on record (Figure E.1). We analyzed the age-structured incidence data from each year, in combination with our age-structured reconstruction of the susceptible CSL population, to show that these patterns align with our understanding of *Leptospira* transmission ecology, built up over many years of study.

Compiling data from decades of outbreaks before the fadeout, we showed that *Leptospira* outbreaks in CSL typically begin in June and peak from August to October, with a systematic progression by age, from yearlings to adults, with 2-3 year-old juveniles fueling the major surge in cases (Figure E.4). The 2017 outbreak deviated from all these patterns: it started late, only reached high levels by October, and had minimal cases in yearlings or juveniles. Then the 2018 outbreak closely resembled the typical patterns from the pre-fadeout era. These patterns are explained by our reconstruction of the age-structured susceptible population on the coast. The marine heat wave decimated the 2015 and 2016 sea lion cohorts, so in 2017 there were almost no juveniles present. Since this the crucial age class for propelling outbreaks, the 2017 outbreak fizzled. But by 2018, there was a huge cohort of yearlings and a good number of 2-year-old juveniles (Figure E.4), and the CSL were well-nourished and migrating normally (Figure E.3), so a massive outbreak ensued. In 2019, a very small outbreak was observed, consistent with past patterns when the immunity following a large outbreak gives rise to a period of low incidence for several years, giving rise to multi-annual cycles.

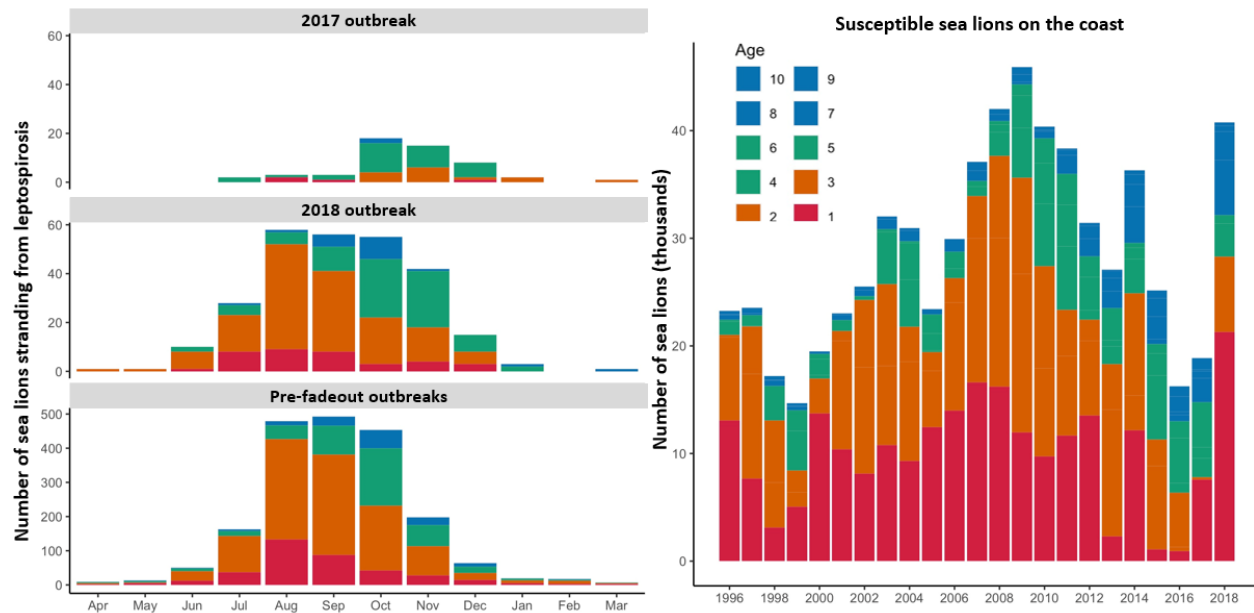


Figure E.4. Age structure and seasonal progression of *Leptospira* outbreaks and age-structured susceptible reconstruction. Left-hand panels show the progression of leptospirosis strands, by month, for the 2017 outbreak, 2018 outbreak, and composite pattern for all major outbreaks from 1984-2012. The right-hand panel shows our age-structured reconstruction of the susceptible CSL, by year. All panels show CSL age structure, colored by age class: yearlings (1 year old), juveniles (2-3 years old), subadults (4-6 years old), and adults (7+ years old).

*Mainland terrestrial wildlife could be the long-term reservoir for *Leptospira**

The final puzzle is where *Leptospira* was maintained during the period it was absent from sea lions, i.e. from what reservoir was it reintroduced? We sampled over 960 individual wild animals from mainland California, including over 700 from five target species in the greater Los Angeles region. We found evidence of widespread circulation of *Leptospira*, with seroprevalences ranging from 5% in opossums to 60% in fox squirrels, and infection prevalences from 0.8-15.2% in all species (except fox squirrels, where infection was not detected). Three species of mesocarnivore – coyotes, raccoons and striped skunks – showed serological signatures consistent with infection with *L. interrogans* serovar Pomona, the same serovar that infects CSL. However the serologic test used for *Leptospira* is notorious for cross-reactivity, so this is suggestive but not definitive evidence that these species can carry serovar Pomona.

For confirmation we needed to test genetic relatedness, and for *Leptospira* this traditionally requires a culture isolate. This is a major obstacle since the organism is hard to grow, and we tried for years and failed to get an isolate from these mainland hosts. In an exciting development, we formed a collaboration with a group pioneering a new DNA capture technique to obtain whole genome sequences of *Leptospira* without an isolate. We were able to sequence samples from four animals – two raccoons, one coyotes, and one striped skunk. Phylogenetic analysis confirmed that these animals were infected with *L. interrogans* serovar Pomona, and furthermore, that they were most closely related to (but not identical to) pre-fadeout isolates from CSL and a Channel Island fox (Figure E.5). This is a major breakthrough for our studies of *Leptospira* in the California coastal ecosystem, showing that these mainland mesocarnivore species carry a closely related strain, and may be the source of repeated observed

introductions of *Leptospira* into marine mammals, including the one that triggered re-emergence in 2017.

We applied the same technique to *Leptospira* DNA obtained from a short-beaked common dolphin, which revealed that this animal's infection was caused by a different species of pathogen, *Leptospira kirschneri*. This is a significant finding, as it is the first detection of a *Leptospira* infection in a cetacean in the Eastern Pacific Ocean, but the genetics indicate that it is not connected to the CSL/*Leptospira* system.

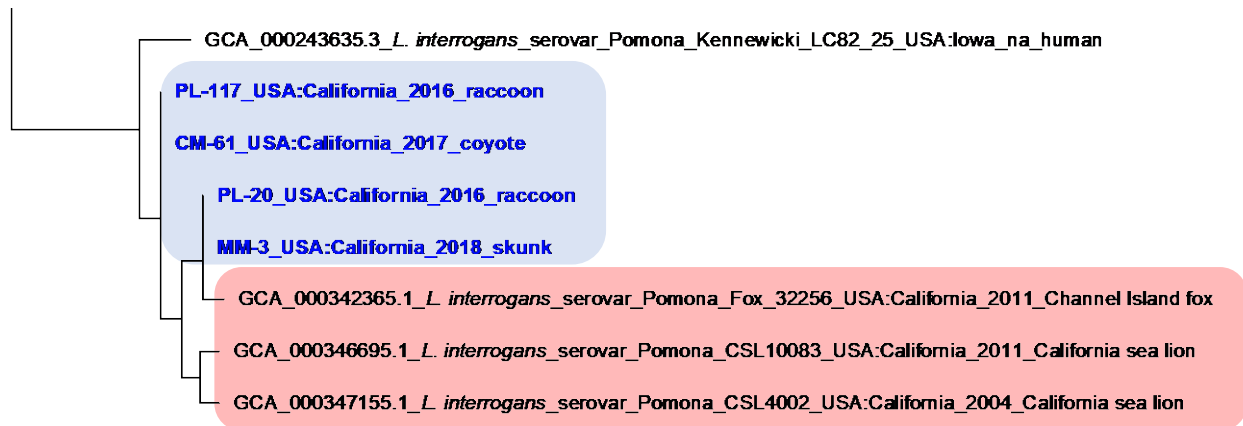


Figure E.5. Phylogeny of *L. interrogans* serovar Pomona strains, showing mainland mesocarnivore strains closely related to CSL and island fox strains. Excerpt of a maximum likelihood phylogeny of *L. interrogans* serovar Pomona genomes. *Leptospira* from four mainland mesocarnivores (blue background) form a single clade with isolates collected from two California sea lions and one Channel Island fox (pink background).

Implications for Future Research and Benefits

This project advanced our understanding of how natural host/pathogen systems respond to environmental perturbations, leveraging long-term datasets to reconstruct effects mediated by demography, trophic ecology, and migratory behavior, which together gave rise to the unprecedented phenomenon of pathogen fadeout from a large, robust wildlife host population. The implications of our work extend beyond the CSL/*Leptospira* system, encompassing valuable lessons for species management, public health, and the influence of climatic and oceanographic change on infectious disease dynamics. Additionally, our project extended priceless long-term data sets, discovered new communities of hosts carrying an important zoonotic pathogen, and developed new analytical tools and models that will provide lasting benefits for fundamental and applied research.

Our work has pushed the frontiers of disease ecology in marine ecosystems. The CSL/*Leptospira* system was already one of the best-characterized marine mammal disease systems in the world, bolstered by decades of time series data and sustained research effort. This study, by investigating the consequences of an ‘ecosystem stress test’, has expanded our understanding of the system to include new scientific domains and the recognition of transmission links to host communities on the mainland. Most research on climate change impacts on marine infectious disease has centered on ectotherms, including fish, coral, and

mollusks, which are dominated by direct effects of temperature and pH on the physiology of host and pathogen species. Our work shows that different conceptual frameworks are needed for marine mammals, whose physiology and cognitive capacity mean that environmental perturbations can have indirect effects on disease via changes in behavior, trophic ecology and demography. Nevertheless the consequences can be striking, as in the environmentally-driven fadeout of the pathogen in the CSL/*Leptospira* system after three decades of transmission. Our study adds to the growing list of major ecosystem impacts of the Eastern Pacific marine heatwave (“the Blob”) and associated anomalies. As marine heatwaves are becoming more frequent and intense on our changing planet, this is a valuable contribution to understanding their potential downstream impacts.

This study also yielded benefits for the study of *Leptospira*, a globally important zoonotic pathogen, and for disease ecology more generally. We broke new ground by applying newly-developed genomic techniques to study the ecology of *Leptospira* in wildlife hosts, demonstrating previously unknown connections between terrestrial and marine host communities. We developed new tools for disease ecologists, including a method to estimate time of infection from antibody titer kinetics using longitudinal field samples, and a method to integrate uncertainties from serological assays (particularly the microscopic agglutination test used for *Leptospira*) into population-level analyses. During the project period, our team also applied its skills to the national and global response to COVID-19, providing a number of impactful contributions to support efforts to mitigate the pandemic.

Further benefits of our project included concrete data products and guidance for species managers, stranding network partners, and public health agencies. With our partners at The Marine Mammal Center and the NMFS Marine Mammal Laboratory, we extended the mark-resight study of CSL demography to 33 years, the time series of *Leptospira* incidence in stranded CSL to 37 years, seroprevalence in CSL to 25 years, and sampling of free-ranging CSL to 10 years. These long-term studies play an irreplaceable role in understanding the responses of complex ecological systems to our changing world, and support management and conservation goals that cannot be addressed through short-term studies. We also led the first systematic effort to screen wildlife species in the Los Angeles region for zoonotic pathogens, and discovered widespread circulation of *Leptospira* in multiple species. Beyond its importance for the CSL/*Leptospira* system, this knowledge has direct utility for public health agencies and veterinarians, to guide differential diagnoses, health communication, and vaccination guidelines. We are sharing our findings with end users, including the NOAA/NMFS West Coast Marine Mammal Stranding Network, the Centers for Disease Control & Prevention, and the Los Angeles County Department of Public Health.

As our planet changes, marine and terrestrial ecosystems are buffeted by increasingly intense environmental fluctuations. By leveraging long-term investments in fieldwork and sample analysis, and developing novel quantitative methods to analyze and integrate data streams, our project has illuminated the mechanisms by which these perturbations can shape pathogen spread. We deepened our understanding of the ecology of *Leptospira* in California sea lions, a leading case study of infectious disease in marine mammals, and demonstrated a new connection between the coastal marine environment and the neighboring terrestrial communities. In aggregate, this work strengthens our ability to anticipate and manage consequences of future non-stationarities, and advances our understanding of species- and ecosystem-level impacts of global climate change.

3. Objectives

This project aimed to characterize and understand the impacts of severe oceanographic anomalies on pathogen transmission among marine mammals. Our study focused on the ecology of *Leptospira interrogans* serovar Pomona in a protected wildlife species, California sea lions (CSL: *Zalophus californianus*), where we had identified preliminary evidence that oceanographic changes had triggered macroscopic shifts in pathogen circulation. Specifically, after three decades of consistent annual outbreaks in the CSL population, *Leptospira* appeared to spontaneously fade out (i.e. cease causing infections) in 2013, and then re-emerge in 2017. This time period coincided with several major oceanographic anomalies. In addition to generating actionable knowledge on a federally protected species that frequents Department of Defense (DoD) lands, we aimed to learn general principles regarding disease transmission in marine systems, possible connections to terrestrial systems, and how these may be impacted by current and future environmental non-stationarities.

The project was funded as a supplement to project RC-2635, which in turn was funded under SERDP Statement of Need RCSON-16-01 ('Changes in pathogen exposure pathways under non-stationary conditions and their implications for wildlife and human exposure on Department of Defense lands'). This supplemental project was targeted at core priorities of this Statement of Need. It was rooted in understanding the ecology of *Leptospira* in its wildlife hosts and the resulting risks to sensitive wildlife species, which utilize DoD lands for rookery and haulout habitat. Our study offered a unique opportunity to understand and anticipate future challenges, including direct and indirect effects of our planet's changing oceans and climate, and the consequences of these changes for population dynamics of key wildlife hosts and patterns of pathogen circulation. Mathematical and statistical modeling was integrated throughout our project plan and was central to our scientific approach. We also aimed to build baseline knowledge and long-term data sets to elucidate the causes of mass stranding events and other demographic anomalies in CSL and other marine mammals, and to address crucial knowledge gaps regarding other potential reservoirs of *Leptospira* in terrestrial and marine ecosystems.

When our study began, our preliminary data indicated that *Leptospira* had ceased circulating in the CSL population from 2013 to 2017, after thirty years of consistent circulation and annual outbreaks. This fadeout period coincided with a series of major oceanographic anomalies, including an unprecedented marine heatwave in the Eastern Pacific Ocean and a subsequent El Nino event. The co-occurrence of these environmental perturbations with striking shifts in *Leptospira* dynamics in the CSL population presented a unique opportunity to study how a major environmental non-stationarity affects pathogen circulation in a host species of interest. Our study objectives were to answer three major research questions, with associated hypotheses:

Question 1. What factors gave rise to the fadeout of *Leptospira* in the CSL population in 2013, and what was the role of anomalous environmental conditions?

Hypothesis 1. *Leptospira* circulation in CSL was interrupted due to a combination of reduced susceptible supply and disruption of sea lion mixing patterns.

Question 2. What conditions enabled the re-emergence of *Leptospira* in the CSL population in 2017?

Hypothesis 2. Re-emergence of Leptospira in 2017 was facilitated by elevated susceptibility of the CSL population combined with a return to more typical oceanographic conditions.

Question 3. Did the fadeout and environmental anomalies have lasting impacts on the CSL/*Leptospira* system, or have system dynamics returned to pre-fadeout patterns?

Hypothesis 3. Leptospira transmission dynamics will return to pre-fadeout patterns, following predictable transient effects of the fadeout.

4. Background

4.1. Format of this report

The remaining sections of this report will share our project findings in the form of completed manuscripts, describing our contributions in three domains:

Investigation of the fadeout and re-emergence of *Leptospira* in CSL, addressing connections to environmental perturbations.

Section 5. Fadeout and re-emergence of an endemically circulating pathogen in a wildlife host. Manuscript in preparation.

Authors: Katherine Prager, Benny Borremans, Riley Mummah, Ana C. R. Gomez, Sarah K. Helman, Renee L. Galloway, David P. Alt, Qingzhong Wu, Sharon Melin, Tony Orr, Jeffery Harris, Julia Burco, Mike Brown, Jeff Laake, Frances M. D. Gulland, Denise J. Greig, James O. Lloyd-Smith

Investigation of alternative reservoirs for *Leptospira* in the California coastal ecosystem, which could have reintroduced the pathogen to CSL.

Section 6. Pathogenic *Leptospira* are widespread in the urban wildlife of southern California. Manuscript in second review at *Nature Scientific Communications*.

Authors: S.K. Helman, A.F.N. Tokuyama, R.O. Mummah, M.W. Gamble, C.E. Snedden, B. Borremans, A.C.R. Gomez, C. Cox, J. Nussbaum, I. Tweedt, D.A. Haake, R.L. Galloway, J. Monzón, S.P.D. Riley, J.A. Sikich, J. Brown, A. Friscia, J.W. Lynch, K.C. Prager, J.O. Lloyd-Smith

Section 7. Detection of *Leptospira kirschneri* in a short-beaked common dolphin (*Delphinus delphis*) stranded off the coast of southern California, USA. Manuscript in preparation.

Authors (alphabetical after first author): K.C. Prager, Kathleen M. Colegrove, Kerri Danil, Renee Galloway, Niesa Kettler, James O. Lloyd-Smith, Rinosh Mani, Ryelan F. McDonough, Jason W. Sahl, Nathan E. Stone, David M. Wagner, Elyse Wurster

Novel methods for analyzing quantitative antibody data, enabling estimation of decay kinetics to connect serological profiles to epidemiological processes.

- Section 8. Leveraging a natural fadeout event to estimate long-term antibody decay of an endemically circulating pathogen in a wildlife system. Manuscript in preparation.

Authors: Ana C. R Gomez, Dylan H Morris, Benny Borremans, Riley O Mummah, K C Prager, James O Lloyd-Smith

- Section 9. Inferring time of infection from field data using dynamic models of antibody decay. Manuscript in press at *Methods in Ecology and Evolution*.

Authors: Benny Borremans, Riley O Mummah, Angela H Guglielmino, Renee L Galloway, Niel Hens, K C Prager, James O Lloyd-Smith

4.1.1. General background

In this section we provide some general background on our study system. The study system centers on two focal species: the pathogen *Leptospira interrogans* serovar Pomona and the host species, CSL (Figure 4.1). CSL are federally protected under the Marine Mammal Protection Act and rely on DoD lands for their survival, giving strong incentive for successful species management. Leptospirosis, the disease caused by pathogenic bacteria of the genus *Leptospira*, causes over 500,000 severe cases annually in humans (Haake & Levett, 2015; World Health Organization, 2011) and has long been recognized as a major threat to US military personnel (Corwin et al., 1990; Johnston et al., 1983; Katz et al., 1997; Leggat, 2010; Mackenzie et al., 1966; McCrumb et al., 1957; Russell et al., 2003). Thus, there are substantial reasons, rooted in human health, species conservation, and land management, to understand the routes of *Leptospira* transmission in this system and how they are influenced by non-stationary factors.

Here we summarize pertinent information about the ecology of the pathogen and the major host species, and the history of *Leptospira* in CSL. This will be complemented by more study-specific background provided in the manuscripts.

4.2. *Leptospira* and leptospirosis

4.2.1. Ecology of *Leptospira*

Leptospira spp. have a complex ecology that can involve multiple host species, environmental reservoirs, and climatic drivers (Adler, 2015; Ellis, 2015; Ko et al., 2009). There are over 300 pathogenic serovars of *Leptospira*, which exhibit variable interactions with different host species, ranging from chronic and asymptomatic infection to acute and fatal disease (Ellis, 2015; Faine et al., 1999; Heath & Johnson, 1994; Ko et al., 2009). The serovar identified in our system, *L. interrogans* serovar Pomona, has been associated with pigs, skunks, foxes, and CSL (Burnstein & Baker, 1954; Campagnolo et al., 2000; Dierauf et al., 1985; Gerber et al., 1993; Gulland et al., 1996; Hathaway et al., 1984; Kingscote, 1986; Lloyd-Smith et al., 2007; Tabel & Karstad, 1967; Vedros et al., 1971). *Leptospira* infects the kidneys and transmits via urine, directly or via contamination of fresh water bodies or moist soil, where the organism can survive and remain infectious for weeks or months (Faine et al., 1999). Trophic transmission (by eating an infected host) has been postulated but not confirmed.

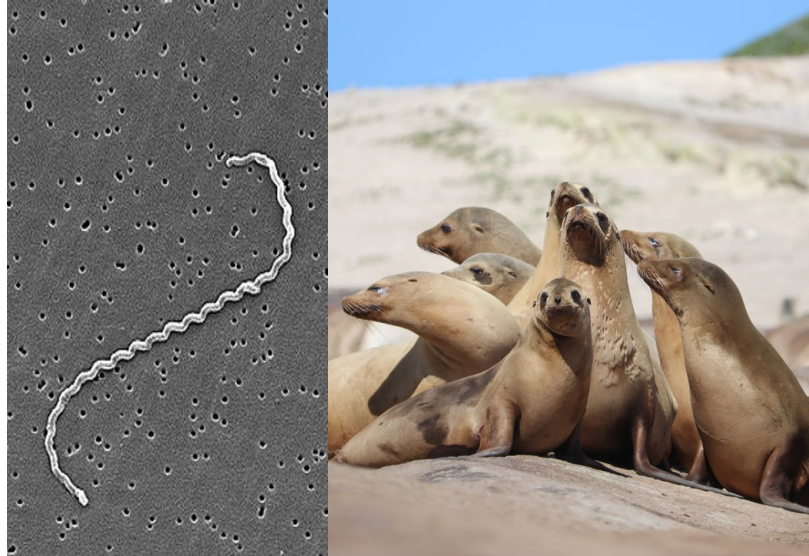


Figure 4.2. Focal species in the project: *Leptospira interrogans* and California sea lions (*Zalophus californianus*).

4.2.2. Laboratory procedures to study *Leptospira*

Biological samples and diagnostic assays.

Serum and urine are the standard samples used to test live animals for evidence of *Leptospira* infection. Serum is typically analyzed using the microscopic agglutination test (MAT), which detects anti-*Leptospira* antibodies that indicate past exposure (Faine et al., 1999; Lloyd-Smith et al., 2007). Serum chemistry analyses can be run to assess renal compromise caused by the infection. Current infection status and potential infectivity can be tested from urine samples, using real-time polymerase chain reaction (PCR) to detect *L. interrogans* DNA (Wu et al., 2014) or by attempting to culture leptospires to confirm live and infectious pathogen. If kidney tissue can be collected from a fresh carcass, then PCR, culture, immunohistochemistry (IHC), and histopathology can be used to assess infection, kidney pathology characteristic of *Leptospira* infection, and extent of kidney damage.

Classification and strain typing.

Historically, *Leptospira* was classified into serovars based on serological reactivity and clustered into serogroups based on antigenically-related serovars (Levett, 2001). However, MAT reactions exhibit cross-reactivity among serovars and are not a reliable indicator of the serovar responsible for a given infection. Reliable strain typing requires isolation through culture of live leptospires to conduct genetic analyses, including pulsed-field gel electrophoresis (PFGE) to determine the infecting serovar. This is a significant challenge, since pathogenic *Leptospira* are notoriously fastidious and slow-growing, making them difficult to culture from field samples. Consequently, serological classification of leptospires remains the dominant approach, despite its shortcomings (Faine et al., 1999; Levett, 2001).

Further delineation of strains within a serovar is not commonly attempted for *Leptospira*, though one pioneering study used variable number of tandem repeat (VNTR) analysis to delineate distinct strains of *L. interrogans* serovar Pomona (Zuerner & Alt, 2009). Before our

project, whole genome sequencing (WGS) and phylogenetics had not yet been used to examine the fine structure of *Leptospira* lineages within a serovar. Sequencing efforts were significantly constrained by the need to grow isolates in culture to obtain sufficient genetic material, which is challenging because *Leptospira* are slow-growing, fastidious spirochete bacteria.

Serology

The Microscopic Agglutination Test (MAT) is the standard serologic test for anti-*Leptospira* antibodies. The test involves mixing serum with live antigens of a chosen set of leptospiral serovars. Since there are over 300 pathogenic serovars of *Leptospira*, MAT analyses are conventionally run against panels of locally relevant serovars. These tests are read microscopically for agglutination to a given serovar at a specific titration, and the titer level for a given sample is the highest titration at which 50% agglutination occurs.

The MAT is not an ideal test partially because it exhibits strong cross-reactivity among serovars, and thus does not definitively identify the infecting serovar. Additionally, while this assay is inexpensive and demands little technology, it is time consuming and requires significant training as well as maintaining a collection of live antigens. A positive MAT test is also only a measure of past exposure and does not indicate a current infection because infected individuals can maintain positive antibody titers for long periods of time (Cumberland et al., 1999, 2001; Faine et al., 1999; Levett, 2001). Titer levels decline over time since exposure and can drop below the level defined as seropositive, so seronegativity does not necessarily imply that an animal has never been exposed to *Leptospira*. However, titer level may offer insights into the time of infection as the rate of titer decline, initial titer estimates, and potential antibody boosting by re-exposure become better understood.

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) assays have been developed for leptospirosis testing, and PCR can be run on any tissue or fluid that may contain leptospires, with urine and kidney tissue most commonly used. PCR tests do not require successful culture of *Leptospira* isolates. With carefully chosen primers, PCR can accurately identify the pathogenic *Leptospira* species causing an infection, but cannot distinguish finer classifications such as serovars (Wu et al., 2014). Quantitative real time PCR (qPCR) can determine the amount of leptospiral genetic material present in the substance tested. Positive PCR and qPCR results indicate the presence of leptospires in the tissue or fluid analyzed and thus also indicate a current or recent infection. However, because PCR and qPCR cannot distinguish between living and dead leptospires, and nonviable DNA may remain present for some time in host tissues, some caution is required when interpreting these results as active infections (Wu et al., 2014).

Immunohistochemistry (IHC)

Immunohistochemistry (IHC) is an assay that tests for antigens in infected tissues and is another available method to test for *Leptospira* infection (Szeredi & Haake, 2006). Two main benefits of IHC is the ability to visualize the distribution and location of leptospires in tissues and its ability to detect infections of *Leptospira* in formalin fixed tissues (thus enabling retrospective testing) (Szeredi & Haake, 2006).

Bacterial culture

Culture is the most specific test available for leptospiral infection, but its sensitivity is very low (Faine et al., 1999; Levett, 2001). Cultures can be attempted from blood, urine, or kidney samples, though leptospires are only found in blood for a short period of time following initial infection. Cultures of *Leptospira* are difficult to grow because leptospires grow very slowly and have complex environmental and nutrient requirements. Consequently, it is common that an individual with an active infection will have a negative culture result (Adler, 2015). However, cultures are the only current method of definitively strain typing leptospires and one of a few assays that indicate a current infection. Traditionally, isolates have been strain-typed using molecular techniques such as pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), or variable number of tandem repeat (VNTR) analysis. The techniques used have been advancing rapidly with several whole genome sequences of *Leptospira* species being completed since 2003 (Ahmed, et al., 2012; Ko et al., 2009; Zuerner & Bolin, 1997).

4.3. California sea lions (CSL)

CSL have a US stock of approximately 300,000 that migrates seasonally between rookery sites on DoD-owned islands – SMI, SNI, and SCL – in the California Channel Island archipelago (Figure 4.2) and coastal haulout sites from central California to Washington (Carretta et al., 2006). Since the passage of the Marine Mammal Protection Act in 1972, the US population of CSL has grown roughly six-fold, but is recently showing signs of density-dependent regulation and substantial drops in survival rates linked to oceanographic variability (DeLong et al., 2017; Laake et al., 2018).

Ecology of CSL

CSL are generalist predators whose diet and foraging behavior shift with ocean conditions; primary prey species include Pacific sardines, northern anchovies, Pacific hake, rockfish, and market squid (Melin et al., 2010; Stewart et al., 1991). Most foraging occurs in near-shore waters (<50 km from the coast), interspersed with time spent hauled out on coastal rocks and beaches, where they share space with other marine mammal species (Figure 4.3) (Lowry & Forney, 2005; Peterson & Bartholomew, 1967; Weise et al., 2006).

CSL pups are born in the summer, and remain on the rookery islands until the following spring. During this time, CSL mothers conduct foraging bouts lasting several days, and return to the rookeries to nurse their pups. Pup weight gain over this period is a marker of trophic conditions, and is essential to prepare the pups for migration northward up the coast. Pups typically wean and depart the rookery islands between March to May (Melin et al., 2010). Other age classes exhibit a seasonal migration, with yearling and juvenile animals and most females remaining on the California coast, and subadult and adult males venturing further north to Oregon, Washington and beyond.

Long-term demographic data for CSL are available from National Marine Fisheries Service (NMFS) population monitoring programs, which include over 40 years of population surveys. Furthermore, our team members at the NMFS Alaska Fisheries Science Center, Marine Mammal Laboratory (MML) have conducted the mark-resight study continuously since 1987, with several hundred CSL pups branded with unique numbers each year on SMI, and thousands

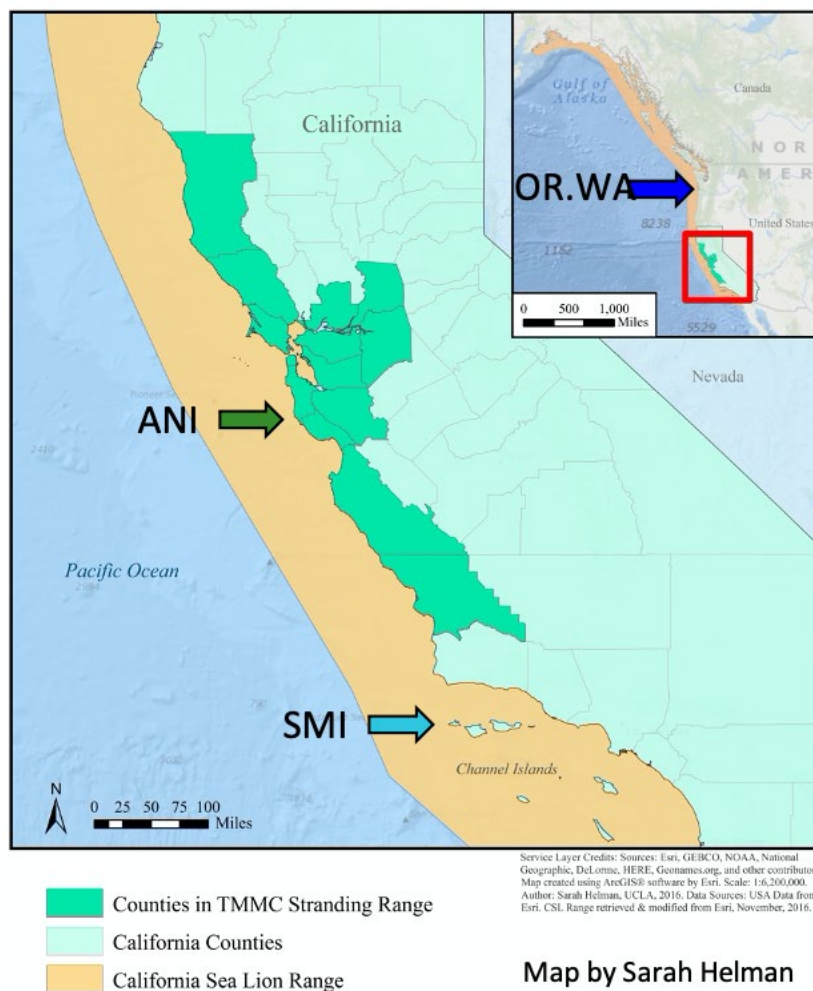


Figure 4.3. Map of CSL range, sampling sites, and stranding response range of The Marine Mammal Center (TMMC). The map includes the Marine Mammal Center stranding range (in bright green), the California Channel Islands of the coast of southern California, and the three locations where wild, free-ranging sea lions were sampled – ANI (Año Nuevo Island), SMI (San Miguel Island), OR.WA (Oregon and Washington - shown in the inset). The area of the coast colored in orange indicates the California sea lion range.

of resights obtained in yearly surveys at SMI and Año Nuevo Island (ANI). Analyses of these data using Cormack-Jolly-Seber models (Brownie & Robson, 1983) yield estimates of annual survivorship by sex and age class.

These analyses and on-going observations show that CSL demography is highly sensitive to non-stationary oceanographic conditions that have intensified in recent years (DeLong et al., 2017; Laake et al., 2018; Melin et al., 2010; Melin, Laake, et al., 2012; Melin, Orr, et al., 2012). While CSL pup counts at rookeries on SMI and SNI reflect marked population growth since 1970, there were sharp drops in recruitment in 1984, 1993-1994, 1999, and 2004 linked to El Niño conditions that lower the primary and secondary productivity of the California Current ecosystem, leading to decreased prey availability (survival of older age classes may also be



Figure 4.3. CSL sharing a beach with northern elephant seal (*Mirounga angustirostris*) and Northern fur seals (*Callorhinus ursinus*).

impacted) (Carretta et al., 2012; DeLong et al., 1991; Melin et al., 2010, 2012; Trillmich et al., 1991). Other oceanographic anomalies, such as the upwelling failures that occurred in 2009 and 2013, also impact CSL health and can cause dramatic reductions in pup survival (Melin et al., 2010). When our project was proposed, there was emerging recognition of on-going severe demographic impacts linked to abnormally warm sea surface temperatures (SST) in the Eastern Pacific Ocean beginning in late 2013. In fact, an Unusual Mortality Event (UME) was declared beginning in January 2013 (Fisheries, 2021), due to severe pup and yearling mortality linked to anomalous ocean conditions, and this UME remained in effect until 2016 (Figure 4.4).



Figure 4.4. Malnourished CSL pups in rehabilitation at TMMC in 2014.

Leptospirosis in CSL

L. interrogans serovar Pomona was first detected in CSL in 1970 and has circulated persistently in CSL since at least 1984 (Buhnerkempe et al., 2017; Clark et al., 1983; Colagross-Schouten et al., 2002; Prager et al., 2020). Through our collaboration with The Marine Mammal Center (TMMC), we have amassed long-term time series data on leptospirosis in CSL, tracking disease incidence and seroprevalence from wild CSL that strand on the California coast (Figure 4.5). Outbreaks occur on the California coast each year, in the fall, and vary markedly in intensity with major outbreaks causing mass stranding and mortality every 3-5 years (Dierauf et al., 1985; Gerber et al., 1993; Greig et al., 2005; Gulland et al., 1996; Lloyd-Smith et al., 2007). The factors causing these periodic major outbreaks were not understood, until our work under SERDP RC-2635 demonstrated that annual outbreak intensity was governed by a combination of population susceptibility and extrinsic oceanographic factors including sea surface temperature and the timing and intensity of coastal upwelling (Borremans et al, unpublished).

Several consistent patterns give further clues to the underlying ecology of *Leptospira* transmission. Documented stranding events caused by leptospirosis are concentrated in central and northern coastal California, but disease impacts are not observed on the rookery islands in southern California (Buhnerkempe et al., 2017; Greig et al., 2005). There are marked patterns by age. Pups on the rookery islands are not exposed, presumably because they are not mixing with the infected older age classes on the coast. Impacts are concentrated in younger animals (yearlings aged 1-2 years old, and especially juveniles aged 2-3 years old), though subadult and adult CSL can also be affected (Greig et al., 2005; Gulland et al., 1996; Lloyd-Smith et al., 2007). These patterns are indicative of an immunizing infection, where the most severe impacts are observed in an animal's first lifetime infection. Mixing among age classes is crucial to sustain *Leptospira* transmission from year to year, since infected older animals need to introduce the infection to the new cohort of susceptibles when they arrive on the coast each summer.

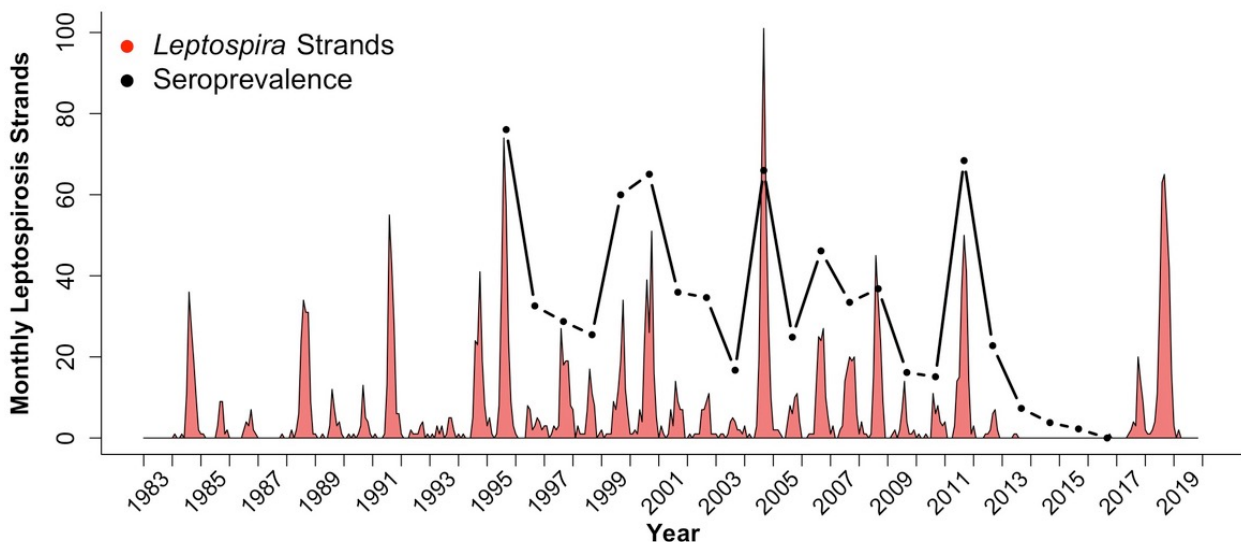


Figure 4.5. Time series of *Leptospira* strands and seroprevalence in California sea lions. Number of CSL stranding each month at The Marine Mammal Center (TMMC) with leptospirosis (solid red) and annual seroprevalence of anti-*Leptospira* antibodies (reflecting past exposure to the pathogen) in animals stranding at TMMC (black line; results pending after 2016). Figure represents information available when our study was proposed.

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5. Fadeout and re-emergence of an endemically circulating pathogen in a wildlife host

5.1. Abstract

Endemically circulating pathogens in wildlife can pose important risks to wildlife conservation, and to human and animal health. Current knowledge on the impact of global change on pathogen dynamics is limited, especially in wildlife, and the direction and magnitude of effects can differ depending on the host-pathogen system. Using data and samples collected from California sea lions (*Zalophus californianus*) for 36 years, and intensively between 2010-2019, we show that *Leptospira interrogans* serovar Pomona circulated endemically for three decades, then disappeared from the California sea lion population in early 2013 and re-emerged mid-2017. We provide multiple lines of evidence that shifts in host demography and seasonal movement patterns – likely driven by climatic perturbations – disrupted conditions favorable to *Leptospira* transmission and led to pathogen fadeout in the system. Subsequent climate-associated shifts back to favorable conditions, coupled with reintroduction from an unknown source, enabled re-emergence of *Leptospira* four years later. This is the first recorded example of spontaneous fadeout of an endemically circulating pathogen from a large, robust, host population. In a future where greater fluctuations in global climatic variables are predicted, and the impacts of zoonoses on wildlife, domestic animal and human populations are increasingly of concern, our study provides insights into how climatic and intrinsic host factors may interact to influence pathogen persistence in a natural system.

5.2. Introduction

In the absence of catastrophic environmental or demographic stochasticity, once circulation of an endemic pathogen is established in a robust host population, it persists (Lloyd-Smith et al., 2005). Transmission patterns and persistence are driven by pathogen characteristics and the frequency and type of contact between susceptible and infected individuals (Donnelly et al., 2003; Hagenaars et al., 2004; Swinton et al., 1998), as well as host demography such as population size, social organization, age structure, and the timing, frequency, and rate of births (Bartlett, 1957, 1960; Begon et al., 2003, 2009; Donnelly et al., 2003; Grenfell et al., 2002; Lloyd-Smith et al., 2005; Swinton et al., 1998). As seen in bats, demographic fluctuations such as large birth pulses can drive seasonal peaks in infection prevalence of viruses such as henipaviruses and paramyxoviruses, a secular increase in birth rates can shift the annual frequency of peaks as with measles in humans (Bartlett, 1957; Grenfell et al., 2002), and steady, consistent birth rates may lead to less variation. Environmental factors can also influence both the host and pathogen through several different mechanisms. Major environmental perturbations or fluctuations could cause catastrophic host declines, driving a population below the critical community size for pathogen persistence (Bartlett, 1957, 1960; Lloyd-Smith et al., 2005), or they could change transmission dynamics by altering pathogen survival in the environment or contact patterns within or between host populations due to shifts in behavior (Donnelly et al., 2003; Eby et al., 2023). To date, there are no known natural occurrences of the disappearance of pathogens that have demonstrated long-term endemic circulation (e.g. > 5-10 years). However there are two

documented cases of viruses – smallpox and rinderpest (Henderson, 1980; Mariner et al., 2005, 2012; Roeder et al., 2013) – that were eradicated through aggressive vaccination campaigns of susceptibles which drove $R_{\text{eff}} < 1$ (Henderson, 1980; Roeder et al., 2013).

We are experiencing a period of extreme climate events which can cause severe, and sometimes long-term ecosystem-level perturbations (Frölicher et al., 2018; Mazdiyasni & AghaKouchak, 2015; Suryan et al., 2021). For example, marine heatwaves are increasing in spatial extent, intensity, duration and frequency (Di Lorenzo & Mantua, 2016a; Frölicher et al., 2018; Hughes et al., 2018; Jacox et al., 2020; Laufkötter et al., 2020; Suryan et al., 2021). These heatwaves, El Niño events and other oceanographic anomalies can have major impacts on the species within an ecosystem such as poor recruitment and survival, elevated stress and reduced immunity, changes to foraging patterns and range shifts, all of which have been documented in marine mammals and, in theory, could impact pathogen transmission (Altizer et al., 2013; Burek et al., 2008; Cavole et al., 2016; DeLong et al., 1991, 2017a; DeRango et al., 2020; Eby et al., 2023; Feldman et al., 2017; Gulland et al., 2022; Hughes et al., 2018; Jacox et al., 2020; Laake et al., 2018a; Melin et al., 2008, 2010; Melin, Laake, et al., 2012; Melin, Orr, et al., 2012). Understanding the impact of such perturbations is critical, yet, despite efforts to determine the nature and magnitude of climate change's impact on pathogens, their hosts, and the host-pathogen relationship, deciphering this puzzle remains a major challenge (Altizer et al., 2013; Burge et al., 2013; Cohen et al., 2020; C. D. Harvell et al., 2002a; Lafferty, 2009; Lafferty & Mordecai, 2016; Rohr et al., 2011; Rohr & Cohen, 2020; Thomas, 2020). This is in part because there is no one consistent pattern: we see an increased risk or prevalence of infectious disease in cases such as hantavirus in bank voles, a decrease in others such as avian influenza in reservoir bird hosts (Brown & Rohani, 2012), and a host-dependent response for cases like chytrid fungus where its impact depends on whether the amphibian host is cold or warm weather adapted (Bosch et al., 2007; Clement et al., 2009; Cohen et al., 2020; Sonn et al., 2019). While major advances have been made over the last two decades, much remains unknown, especially in marine ecosystems (Altizer et al., 2013; Burge et al., 2013; C. D. Harvell et al., 2002b; D. Harvell et al., 2004; Lafferty, 2009; Omazic et al., 2019; Rohr et al., 2011, 2013; Rohr & Cohen, 2020). One major challenge in detecting the impact of climate change on infectious disease dynamics is the need for long-term datasets in conducting the necessary analyses (Burek et al., 2008; Gulland et al., 2022).

Leptospirosis is an infectious disease caused by pathogenic members of the genus *Leptospira* and causes significant morbidity and mortality worldwide in wildlife, domestic animals, and humans (Faine et al., 1999). Leptospirosis in California sea lions (*Zalophus californianus*) was first reported in 1970 during an epizootic that caused major California sea lion stranding and mortality along the eastern Pacific coast (McIlhattan et al., 1971; Vedros et al., 1971). The organism was isolated from sea lion kidney and urine, and identified as belonging to *L. interrogans* serovar Pomona, a serovar previously associated with pigs, cattle and wildlife (Delay et al., 1955; Roth, 1970). During almost three decades, beginning in 1984, *Leptospira* caused outbreaks in CSL of varying intensity, with major outbreaks causing mass stranding and mortality every 3-5 years (Dierauf et al., 1985; Gerber et al., 1993; Greig et al., 2005a; Gulland et al., 1996; Lloyd-Smith et al., 2007a) (Figure 5.1A). *Leptospira* phylogenetic, epidemiologic and ecological data indicate endemic circulation of *Leptospira* in the California sea lion population (Buhnerkempe et al., 2017; Gulland et al., 1996; Lloyd-Smith et al., 2007a; Prager et al., 2013a, 2015a; Zuerner & Alt, 2009a). We use a uniquely large, integrated, long-term dataset on California sea lion demography, movement and *Leptospira* dynamics, comprising thousands

of biological samples and associated data, collected throughout their range, and spanning almost 4 decades, to describe the unprecedented fadeout and subsequent re-emergence of an endemically circulating pathogen in a robust wildlife population.

5.3. Results

Analysis of high resolution clinical, molecular and serological data collected from stranded and wild-caught, free-ranging sea lions between 2010-19 (Figure 5.1A-C, Table 5.1) reveal a clear and conspicuous break in continuous circulation from early 2013 to mid-2017, indicating pathogen fadeout from the system. Apart from a few notable exceptions, during this period there were essentially no new *Leptospira* infections detected in California sea lions that either stranded and were treated at The Marine Mammal Center (TMMC; Sausalito, CA), or were captured and sampled in the wild (Tables 5.1 and 5.2). Three of the 3128 sea lions stranding during fadeout were diagnosed with leptospirosis, the disease caused by infection with pathogenic *Leptospira*: one in late 2016 was confirmed as infected via PCR and was a presumptive failed re-emergence, but two in 2013 were likely misdiagnosed (Table 5.2; IDs 13270, 10544, 10677). Of the 1405 sea lions tested (310 stranded, 1095. Wild-caught), two additional infected sea lions were detected, but they had stranded in 2013, soon after fadeout, showed no evidence of clinical disease, and were likely chronic carriers from infections that occurred prior to fadeout (Table 5.2, IDs 10502, 10769) (Buhnerkempe et al., 2017; Prager et al., 2013a, 2015a, 2020a). Age-structured analyses of quantitative serologic data from stranded and wild-caught, free ranging sea lions tested between 2008-2022, provide further support for pathogen fadeout. Microscopic agglutination test (MAT) serum antibody titers in animals born prior to fadeout were low and consistent with historic, pre-2013 exposure in all but the presumptive failed re-emergence animal, and there was no clear evidence of exposure in sea lions born and sampled during fadeout (Figure 5.1B; Tables 5.1 and 5.2). The few exceptions may be readily explained by one or a combination of the following: incorrect age classification, false positives and maternal antibodies (n=4; Table 5.2). The one notable exception is the presumed failed re-emergence which occurred in late 2016, roughly 4 years from the last cases of leptospirosis seen at TMMC, and was a 2-4 year old male sea lion that died of acute leptospirosis (Table 5.2; ID 13270). Despite this single case, the stranding, antibody and infection data, supported by robust sample sizes which are equivalent to or greater than those from years during which endemic circulation was evident, provide strong evidence that continuous transmission in the sea lion population had ceased.

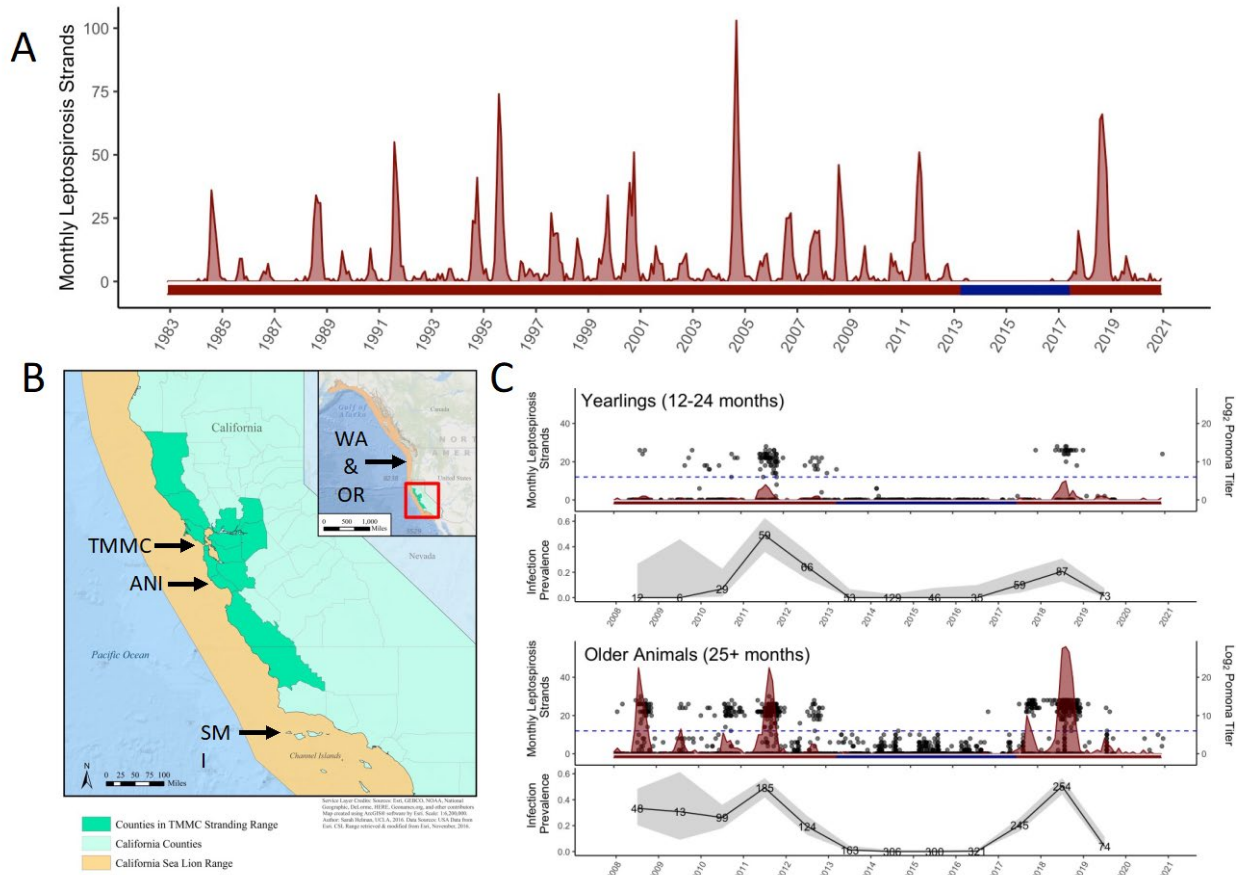


Figure 5.1. Evidence of *Leptospira* fadeout from the California sea lion population. **A.** Number of California sea lions stranding each month at The Marine Mammal Center (TMMC, Sausalito, CA) with leptospirosis from 1983 to 2021. The red and blue bar running along the bottom of the strand time series indicates the period of fadeout in blue. **B.** Map of the California sea lion range. Inset shows the full range in orange with the wild-caught sampling site in Washington and Oregon (WA & OR) indicated by the black arrow. The larger map is the area that falls within the red box in the inset. The TMMC stranding range is indicated in bright green, with the location of TMMC and wild captures on Año Nuevo Island (ANI) and San Miguel Island (SMI) indicated with black arrows. **C.** Number of yearling (top) and older – 25 months old and greater – (bottom) California sea lions stranding each month at TMMC with leptospirosis (solid pink) from 2008 to 2020, and log₂ serum anti-*Leptospira* antibody titers (grey circles) from stranded and wild-caught sea lions. Antibody titers are plotted as grey circles on the day of sampling; points are transparent and jittered, so darker circles represent multiple animals tested on that day. The horizontal dashed blue line shows the level below which antibody titers correspond to past infections, not current ones. Infection prevalence (black line) for each age group is plotted in the panels directly below the strand and seroprevalence time-series. Numbers represent total sample size tested and grey bands are the 95% binomial confidence interval surrounding the infection prevalence estimate.

Table 5.1. Summary of *Leptospira* surveillance in CSL, 2010-2019. Summary of all animals included in the study divided into Total, Strand and Wild. Total is the total number of microscopic agglutination test (MAT) and PCR tests run with positive samples in parentheses. Strand is the number of stranded animals with those diagnosed with leptospirosis in parentheses. Wild is the total number of wild-caught sea lions sampled. For both Strand and Wild, MAT and PCR indicate how many of those that stranded had samples tested by a particular test modality.

Source	Test	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
Total		685	457	376	449	1069	1607	775	558	734	758
	MAT	428	411	332	381	831	798	391			
	(POS)	(58)	(230)	(60)	(18)	(55)	(63)	(28)	66 (2)	0 (0)	0 (0)
		130	256	193					276		
	PCR (POS)	(28)	(123)	(40)	224 (2)	442 (0)	353 (0)	329 (1)	(41)	118 (7)	2 (1)
		597	351	233			1351		328	550	561
Strand (L)		(34)	(182)	(22)	297 (2)	701 (0)	(0)	472 (1)	(53)	(297)	(35)
	MAT	340	305	192	230	465	547				
	(POS)	(51)	(205)	(44)	(17)	(19)	(19)	219 (9)	2 (2)	0 (0)	0 (0)
	PCR (POS)	82 (21)	159 (95)	67 (13)	87 (2)	85 (0)	110 (0)	71 (1)	76 (35)	11 (5)	2 (1)
		88	106	143	152	368	256	303	230	184	197
	MAT			140		366	251	172			
Wild	(POS)	88 (7)	106 (25)	(16)	151 (1)	(36)	(44)	(19)	64 (0)	0 (0)	0 (0)
				126							
	PCR (POS)	48 (7)	97 (28)	(27)	137 (0)	357 (0)	243 (0)	258 (0)	200 (6)	107 (2)	0 (0)

Table 5.2. CSL exhibiting signs of possible *Leptospira* exposure during fadeout period. Possible exceptions to the assumption that *Leptospira* had ceased circulating between fadeout in early 2013 and re-emergence in 2017. Results of diagnostic tests to assess clinical disease (serum chemistry analyses), infection (PCR) and exposure (serum MAT) are presented with our interpretation and conclusion as to why our fadeout hypothesis still holds.

ID	Date ¹	Leptospirosis ²	Age	Sex	Disease: Serum Chemistry ³	Infection: PCR	Exposure: Serum MAT ⁴	Conclusion
10496	1/9/13	no	Yearling	Male	no (BUN = 35 mg/dl, creatinine = 0.6 mg/dl)	Negative urine	7	Past exposure
10502	1/22/13	no	Adult	Female	no (BUN = 36 mg/dl, creatinine = 0.5 mg/dl)	Positive urine (Negative kidney)	0	Chronic subclinical infection
10544	3/17/13	no	Yearling	Male	no (BUN = 37 mg/dl, creatinine = 0.2 mg/dl)	Not Tested	8	Past exposure
10663	6/18/13	yes	Subadult	Male	yes (BUN = 203 mg/dl, creatinine = 4.32 mg/dl)	Negative urine & kidney	4	Past exposure, renal compromise from non-leptospirosis related etiology ⁵
10677	7/24/13	yes	Subadult	Male	no (BUN = NA mg/dl, creatinine = NA mg/dl)	Not tested	NA	Insufficient data for diagnosis of leptospirosis
10769	11/18/13	no	Juvenile	Male	no (BUN = 19 mg/dl, creatinine = 0.55 mg/dl)	Positive urine (Negative kidney)	3	Chronic subclinical infection
1492	3/14/14	NA (wild-caught)	Yearling	Female	no (BUN = 20 mg/dl, creatinine = 0.51 mg/dl)	Negative urine	3	Past exposure or incorrect age classification
1448	3/16/14	NA (wild-caught)	Yearling	Male	no (BUN = 22 mg/dl, creatinine = 0.56 mg/dl)	Negative urine	3	Past exposure or incorrect age classification
686	4/2/14	NA (wild-caught)	Yearling	Female	no (BUN = 27 mg/dl, creatinine = 0.5 mg/dl)	Negative urine	1	Past exposure, incorrect age classification or false positive

ID	Date ¹	Leptospirosis ²	Age	Sex	Disease: Serum Chemistry ³	Infection: PCR	Exposure: Serum MAT ⁴	Conclusion
11711	2/17/15	no	Pup	Female	no (BUN = 55 mg/dl, creatinine = 0.19 mg/dl)	Not tested	1	Maternal antibodies or false positive
13270	10/28/16	yes	Juvenile	Male	yes (BUN = 224 mg/dl, creatinine = 2.36 mg/dl)	Positive urine & kidney	11	Leptospirosis (failed re-emergence)

¹Admission or Capture Date

²Diagnosis at TMMC of leptospirosis – the disease caused by infection with pathogenic species in the genus *Leptospira* - based on clinical assessment laboratory tests and necropsy findings.

³Evidence of disease based on abnormal serum chemistry results consistent with leptospirosis induced renal (kidney) compromise (Gulland et al., 1996)

⁴Log₂ Serum MAT anti-*Leptospira* antibody titer

⁵Amyloidosis of the kidneys noted on immunohistochemistry. This is a known cause of renal compromise and will result in elevated serum BUN and creatinine levels.

A phylogeny of *Leptospira* sequences isolated from 38 California sea lions, 3 northern elephant seals, 2 pigs (*Sus scrofa*) from Iowa, 10 Channel Island foxes (*Urocyon littoralis*) and 2 island spotted skunks (*Spilogale gracilis amphialus*) was built with an HKY + Γ + I substitution model and uncorrelated relaxed molecular clock. This phylogeny revealed that the endemically circulating strain had disappeared and was replaced by a similar, but genetically distinct strain (Figure 5.2). Analyses of the clade structure also provide evidence of a shared common ancestor at the root of the phylogeny and evidence of 3-4 separate introductions, just in the last decade, of *Leptospira* to California sea lions and northern elephant seals. Furthermore, a post-fadeout sea lion isolate (Figure 5.2, CSL13990) clustered with two isolates taken from pigs in Iowa in 1984, linking the sea lion isolate to a strain that was once in a terrestrial host. Taken together, these analyses suggest that an as-yet-undiscovered reservoir of *Leptospira*, perhaps in a terrestrial host along the California coast, has seeded multiple lineages in marine mammal and island communities.

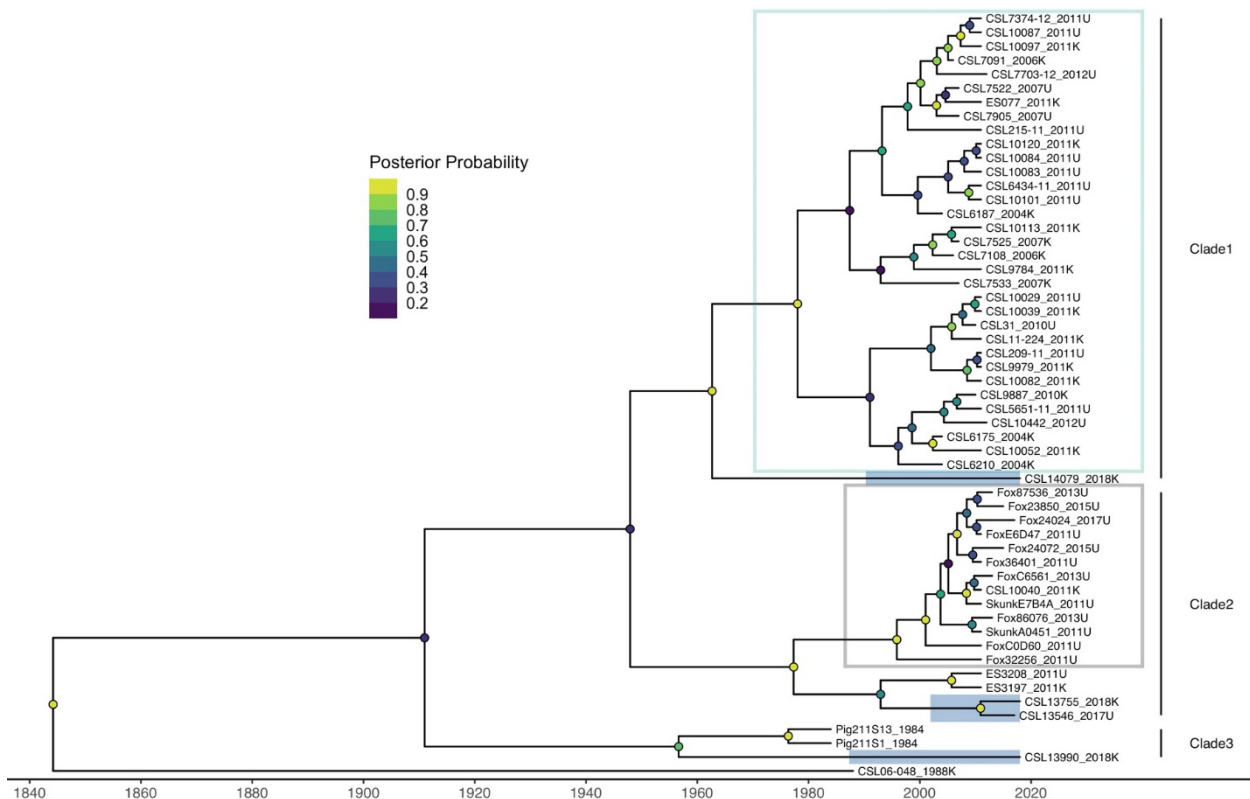


Figure 5.2. Time-calibrated maximum clade credibility tree of *Leptospira interrogans* serovar Pomona in marine and terrestrial mammals. Interior nodes are labelled with the posterior probability (lower values are purple, higher values are yellow). Tips are labeled with host species (CSL = California sea lions, ES = elephant seal), ID number, year, and tissues type (K = kidney, U = urine). California sea lion isolates sampled between 2000 and 2013 (pre-fadeout) are contained within the light blue box. The terrestrial island isolates are denoted by the grey box. Branches of post-fadeout CSL isolates are highlighted in blue.

Fadeout in 2013 was associated with major anomalous shifts in sea lion demography and movement (Figure 5.3)(Thompson et al., 2018). Insufficient frequency of high-quality forage species in the diet of adult females, observed in 2012 and 2013, is associated with poor recruitment and growth of California sea lions during the first year of their life (Fennie et al., 2023; McClatchie et al., 2016; Thompson et al., 2018), and this was reflected in a 2013 yearling population that was abnormally small, both in population size, as well as individual mass (Figure 5.3 A and B). Climate and oceanographic factors such as sea surface temperature, oxygen saturation and upwelling index have been associated with the abundance, density and distribution of forage fish species in the eastern Pacific, however the relationships are complicated, and in many cases scientists struggle to find a clear predictive relationship (Howard et al., 2020; MacCall et al., 2016; Suca et al., 2022; Sydeman et al., 2020; Thayer et al., 2017). Despite the complexity of this relationship, it is clear that anomalous oceanographic conditions were occurring around the fadeout event. Low yearling weights, suggesting insufficient energetic reserves, perhaps in combination with continued poor prey availability (Fennie et al., 2023; McClatchie et al., 2016) and oceanographic anomalies (e.g. sea surface temperatures and upwelling), during a period when the young of the year are typically weaned and leave the rookeries, likely led to their failure to follow historic migratory and population mixing patterns. Contrary to typical movement patterns, in 2013 most yearlings (12-24 months old) remained on the rookery islands in the southern part of their range instead of moving to the coast and north, and those few yearlings that did leave the rookery, failed to head north along the coast (Figure 5.1B, Figure 5.3 A-C and B, and Figure 5.4). Endemic circulation in any population is driven by contact and transmission between susceptible and infected individuals (Anderson & May, 1992). In California sea lions, susceptible yearling and juvenile (2-5 years old) males are the most important drivers of *Leptospira* outbreak dynamics (Borremans et al., In prep). We hypothesize that age- and sex-specific movement patterns link infected older animals with susceptible yearlings and juveniles (Figure 5.4), likely through either chronic carriage (Buhnerkempe et al., 2017; Prager et al., 2013a, 2015a, 2020a), stuttering chains of transmission (Blumberg & Lloyd-Smith, 2013; Lloyd-Smith et al., 2009) that can persist long enough to spark an annual outbreak, or a combination of the two. We propose that oceanographic anomalies drove forage fish dependent changes in sea lion demography and movement, interrupting the seasonal transmission of *Leptospira* by altering typical age- and sex-specific transmission patterns and causing fadeout of a pathogen that had circulated endemically for at least 3 decades. This fadeout was an unexpected demonstration of the extreme outcomes possible when environmental conditions deviate too far from normal.

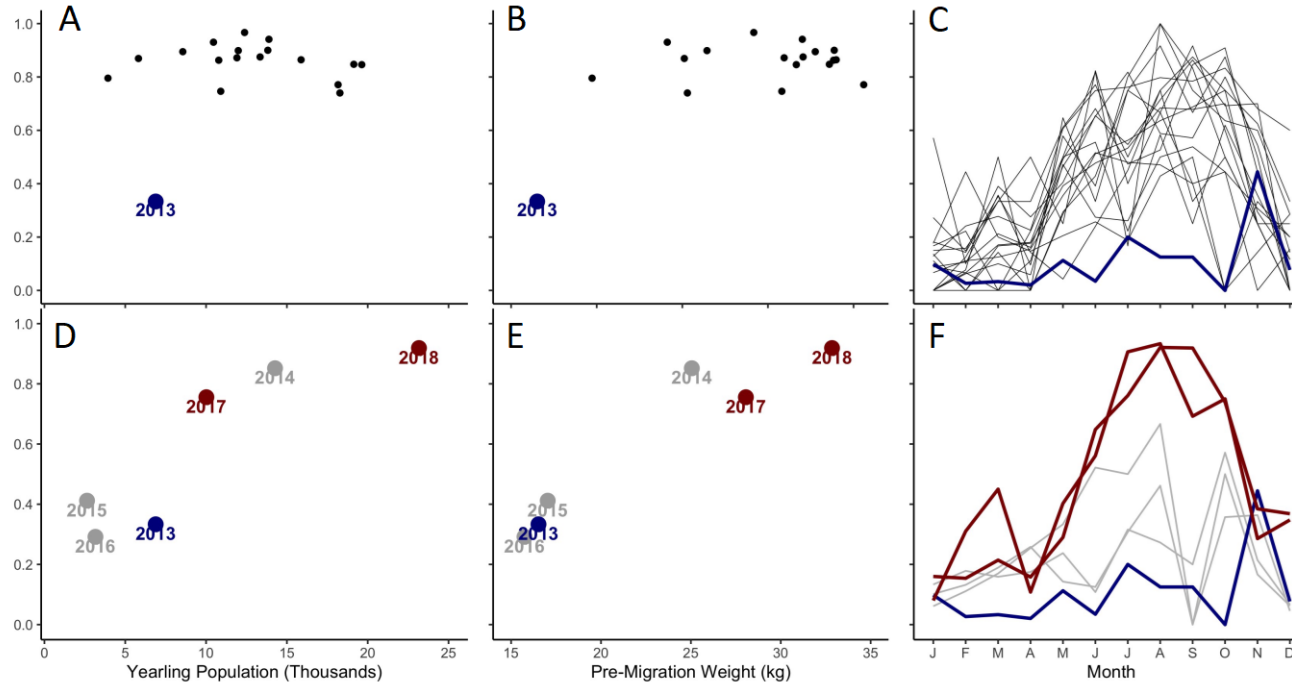


Figure 5.3. Fadeout and re-emergence of *Leptospira* in CSL were associated with simultaneous fluctuations in susceptible supply and migratory behavior. The proportion of the yearling CSL population migrating northward from the rookery islands in July is plotted versus the total yearling population (**A and D**) and the mean yearling male weight the February prior to migration (**B and E**). Pre- (**A and B**) and post-fadeout (**D and E**) data are separated for clarity. Black points indicate conditions from 1996-2012. The year fadeout occurred - 2013 - is marked in blue, the years between fadeout and re-emergence are marked in grey, and re-emergence years are red. In 2013, both the proportion of yearlings migrating north as well as the total yearling population were anomalously low, and the association between the low pre-migration weights (i.e. February weights) and lack of migration, suggests that these animals were too small or lacked sufficient energetic reserves to migrate. Conditions in 2016, the year that a single case of leptospirosis was observed at the Marine Mammal Center (TMMC), were similar to those seen in 2013, suggesting that they were inadequate to support re-emergence. But by 2017 conditions had returned to 'normal' and by 2018 they were excellent for *Leptospira* transmission in the sea lion population. The proportion of yearling California sea lions stranding above 36 degrees latitude further illustrate shifts in migratory behavior. Pre- (**C**) and post-fadeout (**F**) periods are separated for clarity. Grey lines in the top figure indicate years from 1996-2012, and the year that fadeout occurred - 2013 - is indicated in blue. Grey lines in the bottom figure indicate 2014-2016, red lines are for re-emergence years - 2017 and 2018 - and 2013 is again shown in blue. These stranding data provide further evidence that yearling sea lions were not following their typical fall pattern of migrating north in 2013, but that they had resumed their historic migratory behavior in 2017, when re-emergence occurred. Note: only male weights and movements are shown here for clarity, however female weights show the same association with proportion migrating north.



Figure 5.4. Annual yearling migration drives fall outbreaks. We propose that age- and sex-specific movement patterns drive *Leptospira* transmission and the observed annual outbreaks. At around 10-12 months old, sea lions are weaned and begin to migrate away from the rookery islands and north along the coast. There they likely encounter older infected sea lions that throw a ‘spark’ into this susceptible pool of yearlings and initiate the annual *Leptospira* outbreak observed to peak in the fall.

In 2017, four years after *Leptospira* disappeared from the sea lion population, a return to conditions conducive to California sea lion recruitment, growth and migration, coupled with *Leptospira* spillover from an unknown reservoir – an event governed by stochastic processes – resulted in re-emergence of a new *Leptospira* strain in the sea lion population (Figure 5.1). Data from the period between fadeout and re-emergence show the importance of both events in successful re-emergence. In 2014, yearlings were in good condition, the cohort was large and the majority left the rookeries (Figure 5.3 D-F), as is typical. Yet there was no outbreak, so presumably no spillover event. In 2016, there was an apparent spillover event (Figure 5.1 A and C; Table 5.2, ID 13270), but yearling condition was poor, there were few of them and the majority failed to leave the rookeries (Figure 5.3 D-F), so no outbreak occurred.

In 2017, the outbreak associated with re-emergence was relatively small, occurred later in the year than was typical historically, and was dominated by older age classes, with very few yearling and juvenile cases observed (Figure 5.5 A). Epidemiological theory predicts that after a sustained absence of endemic pathogen circulation, older age classes will be immune, therefore, after the observed 4-year fadeout event, a large outbreak would be predicted. However, reconstructing California sea lion demography we see that the 2014-15 marine heat wave and the 2015-16 El Niño (Thompson et al., 2018) decimated several cohorts, so that in 2017 there were essentially no juvenile (2-3 year old) sea lions (Figure 5.5 D), which are key drivers of outbreak intensity (Borremans et al., In prep). The role of these juveniles is evident in the temporal dynamics of outbreaks, there is a common pattern of outbreaks starting in yearlings and juveniles and then spreading to the older age classes, with the relative proportion of younger age classes declining after the outbreak peaks (Figure 5.5 B and C). By 2018, both the yearling cohort – another important driver of outbreak magnitude – and the juvenile cohort had grown sufficiently to cause one of the largest *Leptospira* outbreaks in California sea lions on record (Figure 5.1 A and Figure 5.5 D). This 2018 outbreak was also consistent with the historic temporal and demographic pattern of outbreaks seen during sustained endemic circulation (Figure 5.5 B and C). An abnormal initial outbreak followed by a more typical pattern is consistent with pandemic waves theory. Because re-emergence was in part governed by the stochastic process of spillover from an unknown reservoir, the timing and location of outbreak initiation were likely atypical. Coupled with a sea lion population that was missing key susceptible age classes, this explains the abnormal pattern and timing of the initial epidemic wave.

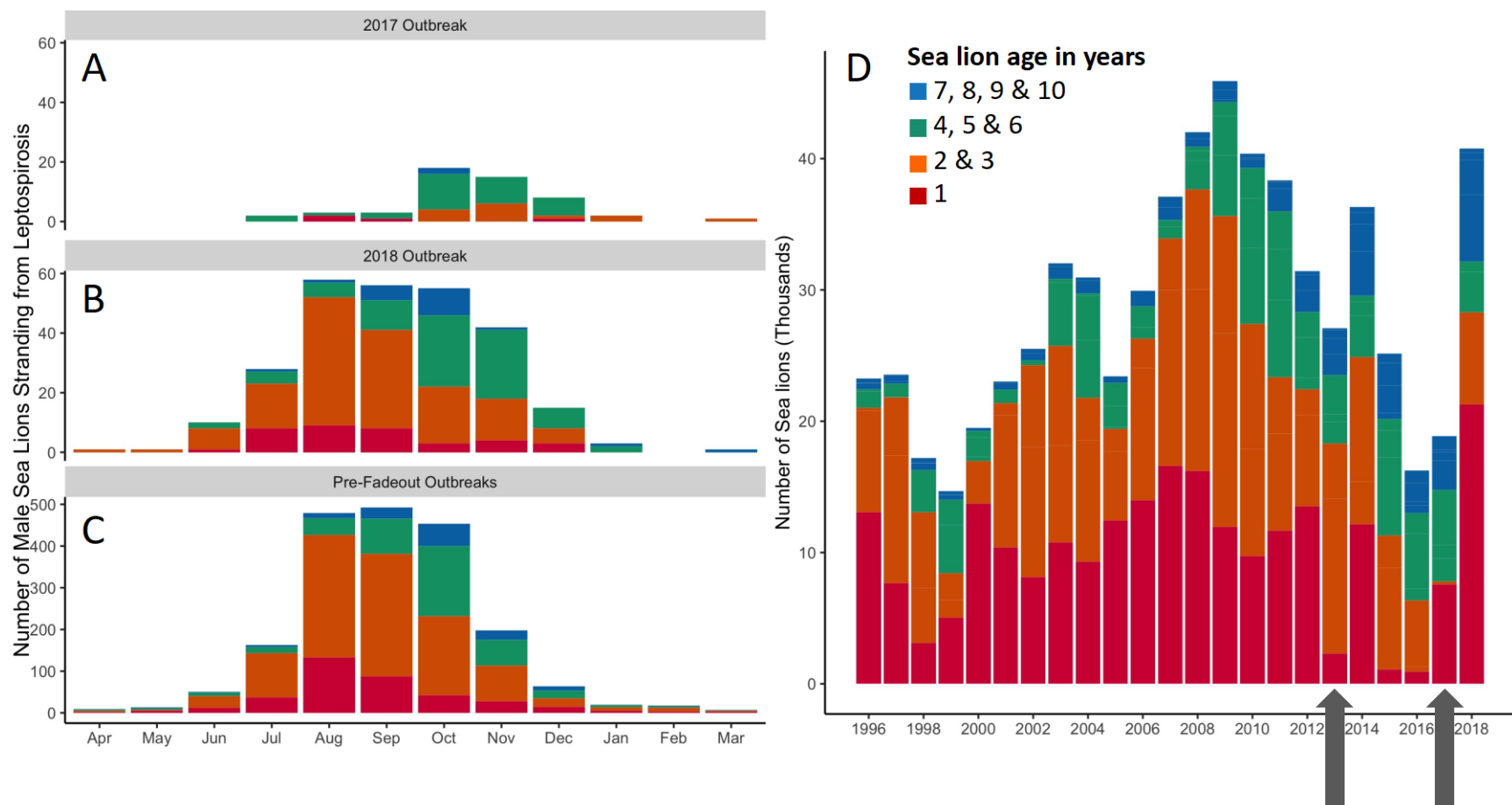


Figure 5.5. Age-structured outbreak curve and population reconstruction. Number of California sea lions stranding each month, by age class, during *Leptospira* re-emergence in 2017 (A) in 2018, the year following re-emergence (B), and for all pre-fadeout outbreaks combined (C). The age structure of the 2017 outbreak differs from those of 2018 and all pre-fadeout outbreaks; the leptospirosis strands are dominated by older age classes in 2017 and very few animals stranded. The population reconstruction (D) shows that this is because there were very few individuals in existence in the younger age classes in 2017. Note: only males are shown here for clarity, however females show the same patterns.

5.4. Discussion

Crucially, the disappearance of *Leptospira* may have been missed had the fadeout window been only a year. However, the large, 4-year window, facilitated by oceanographic anomalies such as the 2014-15 marine heat wave and the 2015-16 El Niño (Thompson et al., 2018), and their continued impact on sea lion demography, enabled detection of the fadeout event, re-emergence, and identification of their underlying causes. Reflecting on the long-term time-series of leptospirosis strands and the *Leptospira* phylogeny, there are at least two other periods in the system where fadeout may have gone undetected due to insufficient data or re-emergence in the year following disappearance. After the initial detected introduction in 1970, and sometime between 1988 and 2004. The first outbreak of leptospirosis in California sea lions occurred in 1970 (McIlhatten et al., 1971; Vedros et al., 1971) and between then and the early 1980s, there were no other reports of sea lion outbreaks. During this initial period before endemic circulation, the California sea lion population was roughly a quarter its current size (DeLong et al., 2017a; Laake et al., 2018a), this, coupled with herd immunity from the 1970 outbreak, may have played a factor in limiting endemic circulation. Then, by 1984, when presumably the demographic conditions were suitable for sustained transmission and spillover must have occurred, TMMC began detecting yearly seasonal outbreaks of leptospirosis in stranded California sea lions (Figure 5.1A). The second instance of likely fadeout and re-emergence is suspected because the 1988 isolate (CSL060-048_1988K), the only one in the phylogeny from before 2004, is genetically quite different from all other *Leptospira* isolates included (Figure 5.2). An alternative explanation is that this strain was not representative of the dominant, endemically circulating strain at the time, but was another example of occasional spillover from an external, presumably terrestrial, reservoir. Other examples include the post-fadeout sea lion isolates, two of the northern elephant seal isolates (ES3208_2011U and ES3197_2011K), and the sea lion isolate nested within Clade 2 of the phylogeny (CSL10040_2011K), the otherwise terrestrial clade.

This study adds to the long and growing list of ecosystem impacts of extreme climate anomalies (e.g. the Blob, marine heatwaves, El Niños) on the marine ecosystem and these events are becoming more frequent and intense (Cai et al., 2014; Di Lorenzo & Mantua, 2016b; Mazdiyasni & AghaKouchak, 2015; Suryan et al., 2021; Thompson et al., 2018). As a result, we may expect to see the recurrence of conditions which led to fadeout. While pathogen fadeout from the system may seem beneficial, there could be unexpected consequences – longer fadeout windows could lead to a greater buildup of susceptibles, and outbreaks at re-emergence that infect a larger proportion of the population, including older age classes. Older animals may experience more severe disease, it could influence reproductive success in older females due to *Leptospira* infection in utero (Smith, Brown, & DeLong, 1974; Smith, Brown, Skilling, et al., 1974), and older males could bring the pathogen farther north than is typical due to age- and sex-specific differences in movement patterns, potentially impacting vulnerable species such as Steller sea lions (*Eumotopias jubatus*) (Cameron et al., 2008) and northern sea otters (*Enhydra lutris*) (Knowles et al., 2020; White et al., 2017). Climate-related shifts in host movement patterns as we saw with yearlings in this study and have been reported in others, could impact transmission dynamics within the sea lion population in other ways, or could lead to increased sea lion to other marine host transmission. The impact of climate change on marine mammal disease is understudied, with many studies focusing on sessile organisms such as sea stars, urchins, oysters and corals, and centers on direct consequences of abiotic factors such as

temperature(Burge et al., 2013; Gulland et al., 2022; C. D. Harvell et al., 2002b; D. Harvell et al., 2004, 2009; Lafferty, 2017). For marine mammals, understanding behavior and trophic ecology are critical. There is a particular need to increase studies focusing on impacts on marine animals such as sea lions that are known to shift movement patterns in response to changes in their local environment(Gulland et al., 2022; Kuhn & Costa, 2014; McHuron et al., 2017; Melin et al., 2008; Ream et al., 2005; Weise et al., 2006). Improving our understanding of host movement and mixing patterns would vastly improve our ability to understand and predict climate change impacts on pathogen transmission both within a host population as well as to new populations outside of a host's historic range.

For directly transmitted pathogens or those with limited survival outside of the host, contact between infected and susceptible individuals drives transmission dynamics. This study further elucidates the critical role of the climate-movement interaction, and underscores the need for more detailed movement data between susceptible hosts within and between host populations. In addition, the pattern of fadeout and re-emergence reveals challenges us to reconsider or reframe how we define pathogen persistence by revealing a system in which there are multiple timescales and persistence hierarchies. At the population-level, in face of extreme environmental stochasticity, perhaps California sea lions are not competent reservoirs. However, on a larger time-scale and in an ecosystem for which spillover from an external reservoir occurs at some unknown rate, perhaps we can call a host population a reservoir if it can maintain endemic pathogen circulation for decades, or is it simply part of a larger metareservoir(Prager et al., 2012)? Moving forward, we hope to address these challenges by determining the key players in maintaining *Leptospira interrogans* serovar Pomona in the marine and coastal terrestrial ecosystem of the eastern Pacific Ocean.

5.5. Methods

5.5.1. Surveying leptospirosis in stranded CSL

The Marine Mammal Center collected data and samples from California sea lions that stranded along the central and northern coast of California (Figure 5.1 B; Table 5.1) and were admitted for rehabilitation between January 1, 2010 and December 31, 2021. A subset were diagnosed with leptospirosis using characteristic serum chemistry results, clinical signs, and post-mortem findings when appropriate (Colagross-Schouten et al., 2002; Greig et al., 2005b; Gulland et al., 1996). Clinical, morphometric, and demographic data as well as blood and urine were collected from all sea lions during the course of routine clinical care, and kidney samples were collected in those that died or were euthanized (as described in Prager et al. 2020). Samples were analyzed using MAT to assess prior exposure; PCR of urine or kidneys, or culture and *Leptospira* isolation from urine and kidney, to assess current infection; and serum chemistry analyses to assess clinical impact of leptospirosis (Table 5.1).

Serum chemistry analyses were performed on an ACE Clinical Chemistry System (Alfa Wassermann Inc., West Caldwell, New Jersey, USA). Serum MAT was performed at the California Animal Health and Food Safety (CAHFS) laboratory, Davis, California, or at the CDC, Atlanta, Georgia, using live cultured *Leptospira* spp. (reference strains) to measure serum anti-*Leptospira* antibody titers. Samples run at CAHFS were run against a 1 or 6 serovar panel and samples run at the CDC were run against a 2 or 19 serovar panel (as described in (Prager et

al., 2015b, 2020b)). We only report MAT titer results against *L. interrogans* serovar Pomona as historically this is the strain that elicits the highest MAT titer in the majority of California sea lions tested (Lloyd-Smith et al., 2007b), and it is the only serovar ever isolated from this host species over five decades of study (Prager et al., 2013b; Zuerner & Alt, 2009b). Serum samples were tested at doubling dilutions starting from 1:100, and agglutination was read using dark field microscopy. Endpoint titers were reported as the highest dilution that agglutinated at least 50% of the cells for the strain tested (Dikken & Kmety, 1978). Titer results were log transformed for ease of interpretation using the following formula: $\log_2(\text{titer}/100) + 1$, thus a titer of 1:100 = 1, 1:200 = 2, etc. Titers reported as <1:100 were set equal to 0 on both the log and regular scale. Throughout this section, “antibody titer” refers to this log transformed titer value. All animals with a detectable titer (i.e. $\geq 1:100$) were considered seropositive and assumed to have been infected with *Leptospira* at some point.

We assessed leptospiral DNA shedding in urine using real-time PCR (Wu et al., 2014). Because urine was collected under anesthesia, and anesthesia can pose a health risk to compromised animals, urine was collected only from sea lions undergoing anesthesia for other clinical purposes or during necropsy; anesthesia was never performed for the sole purpose of collecting a study sample. Individuals shedding leptospiral DNA were considered infected and infectious because the primary mode of transmission of *Leptospira* is by either direct or indirect contact with leptospire shed in the urine of infected individuals (Ellis, 2015).

All California sea lion samples were collected under authority of Marine Mammal Protection Act Permits No. 932-1905-00/MA-009526 and No. 932-1489-10 issued by the National Marine Fisheries Service (NMFS), NMFS Permit Numbers 17115-03, 16087-03, and 13430. The sample collection protocol was approved by the Institutional Animal Care and Use Committees (IACUC) of The Marine Mammal Center (Sausalito, CA; protocol # 2008-3), the University of California Los Angeles (ARC # 2012-035-12), and the Marine Mammal Laboratory (Alaska Northwest 2013-1 and 2013-5). The Marine Mammal Center and the Marine Mammal Laboratory adhere to the national standards of the US Public Health Service Policy on the Humane Care and Use of Laboratory Animals and the USDA Animal Welfare Act. UCLA is accredited by AAALAC International and adheres to the national standards of the US Public Health Service Policy on the Humane Care and Use of Laboratory Animals and the USDA Animal Welfare Act. Isoflurane gas was used to anesthetize all wild-caught, free-ranging sea lions for sampling, and from all stranded sea lions from which urine was collected.

5.5.2. Demography and movement of CSL

California sea lion mark-resight-recovery efforts were conducted from 2015 through 2018 (as described in (DeLong et al., 2017b)), extending our long-term time-series to 33 years (1987-2019). Briefly, approximately 300 California sea lion pups were weighed and branded on San Miguel Island each year in late September or early October (i.e. at roughly 4 months old). A subset of branded pups were reweighed in February of the following year (i.e. at roughly 8 months old), and mean weights were calculated by age and sex. Brand-resights of all age and sex classes were conducted on San Miguel Island and Año Nuevo Island at key timepoints throughout the year. These data were then analyzed using a Cormack-Jolly-Seber model to estimate age-specific survival. The modeling approach is as described in DeLong et al. (DeLong et al., 2017b), however the R (R Core Team, 2019) package markedTMB (Laake, 2020), a derivative of marked (Laake, 2013), was used rather than RMark (Laake, 2013), and a multi-state

model was used to estimate movement between locations. Survival estimates and pup counts were then used to numerically reconstruct the population by age and sex class as described previously (Laake et al., 2018b), with the exception that density-dependent effects were not included.

Figures were made in R (R Core Team, 2019) using the package ggplot2.

5.6. References

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6. Pathogenic *Leptospira* are widespread in the urban wildlife of southern California

6.1. Abstract

Leptospirosis, the most widespread zoonotic disease in the world, is broadly understudied in multi-host wildlife systems. Knowledge gaps regarding *Leptospira* circulation in wildlife, particularly in densely populated areas, contribute to frequent misdiagnoses in humans and domestic animals. We assessed *Leptospira* prevalence levels and risk factors in five target wildlife species across the greater Los Angeles region: striped skunks (*Mephitis mephitis*), raccoons (*Procyon lotor*), coyotes (*Canis latrans*), Virginia opossums (*Didelphis virginiana*), and fox squirrels (*Sciurus niger*). We sampled more than 960 individual animals, including over 700 from target species in the greater Los Angeles region, and an additional 266 sampled opportunistically from other California regions and species. In the five target species seroprevalences ranged from 5-60%, and infection prevalences ranged from 0.8-15.2% in all except fox squirrels (0%). *Leptospira* phylogenomics and patterns of serologic reactivity suggest that mainland terrestrial wildlife, particularly mesocarnivores, could be the source of repeated observed introductions of *Leptospira* into local marine and island ecosystems. Overall, we found evidence of widespread *Leptospira* exposure in wildlife across Los Angeles and surrounding regions. This indicates exposure risk for humans and domestic animals and highlights that this pathogen can circulate endemically in many wildlife species even in densely populated urban areas.

6.2. Introduction

Leptospirosis, the disease caused by pathogenic bacteria from the genus *Leptospira*, is reported to be the most widespread zoonotic disease in the world¹⁻³. *Leptospira* spp. are generalist pathogens capable of infecting a broad range of primarily mammalian hosts^{4,5}. They are considered emerging pathogens of concern⁶, with one million human cases and 60,000 deaths estimated globally each year^{7,8}, and many more cases likely undiagnosed⁹. Given the extensive global impact of *Leptospira* spp., it is surprising that their ecology and epidemiology remain understudied¹⁰. Though many pathogens, including all zoonoses, are capable of infecting multiple hosts, studying complex generalist pathogen systems remains a challenge, particularly in wildlife for which extensive, multi-host surveillance is required but resources and sample access are often limited^{11,12}. Studying *Leptospira* poses additional challenges since host-pathogen epidemiology differs widely across host species and strains, and multiple *Leptospira* strains can co-circulate in communities. Consequently, extensive knowledge gaps remain regarding the role many hosts play in *Leptospira* transmission and the risks they pose to other host species, including humans. Epidemiological patterns also vary across climates and locations, with greater attention paid to the high incidence of human leptospirosis observed in wet tropical regions, and less concern historically placed on urban transmission, especially in more developed countries^{1,8}. These complexities underscore the importance of broad surveillance efforts to inform veterinary and public health efforts, particularly in densely populated urban settings where the potential for human-wildlife contact is high and *Leptospira* transmission dynamics remain poorly understood.

Awareness of *Leptospira* genomic diversity has increased in recent years, with pathogenic *Leptospira* bacteria now categorized into 40 species^{9,13,14}. Pathogenic *Leptospira* strains are classified into over 250 serovars based on cell surface antigen similarities and serologic reactivity⁴, with related serovars traditionally grouped into serogroups, though neither serovar nor serogroup are reliable predictors of *Leptospira* species¹⁵. Transmission usually occurs after leptospires are shed in the urine of infected hosts, leading to environmental contamination that can indirectly infect susceptible hosts via mucous membranes or abraded skin^{16–19}. Following this contact, leptospires colonize the kidneys and other organs, which can cause pathology and associated clinical signs ranging from mild flu-like symptoms to fulminant multi-organ system failure and death^{17,20}. Disease severity varies across *Leptospira* serovars and host species, with some broad host-serovar associations. Historically, the literature has categorized *Leptospira* epidemiology into: (i) maintenance hosts (and host-adapted serovars) that are likely to exhibit asymptomatic and/or chronic infections and maintain circulation of the pathogen, or (ii) accidental hosts (and non-host-adapted serovars) that exhibit symptomatic infections and do not maintain pathogen circulation¹. These dichotomous classifications have been challenged in recent years, and there is clear evidence that clinical manifestations range widely irrespective of population-level circulation of the pathogen^{6,21,22}. Evidence of infection is typically determined from urine or kidney samples using either polymerase chain reaction (PCR) to detect *Leptospira* DNA or culturing to detect live infectious leptospires. The most widely used diagnostic test for *Leptospira* spp. is the microscopic agglutination test (MAT)^{4,23}, which tests serum for anti-*Leptospira* antibodies to assess past exposure. Serum MAT panels typically include serovars known or suspected to circulate in the geographic region of interest.

Despite increasing reports of *Leptospira* in urban areas, including dramatic outbreaks linked to flooding²⁴, significant knowledge gaps remain regarding the prevalence and transmission risk across a variety of potential hosts in urban environments. Urbanization is increasing at a global scale^{25,26} and can influence wildlife pathogen dynamics through many mechanisms (e.g. altered community structure and contact rates²⁷), with high densities of humans and domestic animals providing increased opportunity for cross-species contacts and possible spillover of infection²⁸. Unhoused individuals in crowded urban areas may be at increased risk for zoonotic disease transmission due to poor sanitation and intensified contact with urban wildlife species, including in high-income countries where urban homelessness is a growing public health crisis²⁹. Since changes to transmission risk are pathogen- and host-specific, investigations into pathogen dynamics and host diversity are critical for urban disease management and risk assessment. This is especially true for *Leptospira*, as most studies in cities have focused on rodents in high-density urban centers^{7,30–33}, with much less attention placed on other urban-adapted wildlife that may influence the ecology of this important zoonosis^{1,34,35}.

In the United States, many *Leptospira* infections in humans, domestic dogs, horses, and livestock are associated with spillover from wildlife^{36–38}. Increasing reports of leptospirosis in domestic dogs^{39,40} and humans in some locations (e.g. Hawaii and California^{41–43}) further highlight the need for expanded surveillance in wildlife. One recent study concluded that *Leptospira* exposure is common in a variety of wildlife across the country, and called for more studies investigating the relationship between serology and shedding to better understand the risk this poses to the health of humans, domestic pets, and livestock⁴⁴. In California, a wildlife survey in the 1970s reported *Leptospira* exposure or active infections in multiple species, including coyotes (*Canis latrans*), raccoons (*Procyon lotor*), and striped skunks (*Mephitis mephitis*)⁴⁵, and other surveys have detected *Leptospira* antibodies in black bears (*Ursus americanus*)⁴⁶ and feral

pigs (*Sus scrofa*)⁴⁷. A recent survey in northern California reported *Leptospira* in multiple mesocarnivore and rodent species³⁵, with both skunks and raccoons identified as potential reservoir hosts for *Leptospira interrogans* serovar Pomona⁴⁸ – a serovar with a long history of circulation in terrestrial mammals on the California Channel Islands and California sea lions (*Zalophus californianus*) along the California coast^{21,22,49–53}.

In contrast with northern and coastal regions, there has been little surveillance for *Leptospira* in inland southern California. The greater Los Angeles area features a range of landscapes, from natural and agricultural land to a dense urban center, as well as many wildlife species that are potential *Leptospira* carriers. There are scattered reports of *Leptospira* exposure in some local animals, including dogs^{54,55}, deer (*Odocoileus hemionus*)⁵⁶, mountain lions (*Puma concolor*) and bobcats (*Lynx rufus*)⁵⁷, but prior studies have had limited host, geographic, and temporal ranges. The paucity of region-specific surveillance data in greater Los Angeles, the second largest metropolis in the United States⁵⁸, represents a critical public health gap, and more comprehensive wildlife screening is needed to assess zoonotic spillover risk from the full range of susceptible wildlife hosts.

We conducted the first in-depth surveillance of *Leptospira interrogans* in wildlife in the greater Los Angeles area to shed light on the prevalence and circulation of this multi-host pathogen across a densely populated and complex urban landscape. We aimed to assess *Leptospira* exposure and active infections in common wildlife, harnessing novel tools for phylogenomic analysis of infecting *Leptospira* strains to additionally explore potential transmission links among host species. Serologic reactivity profiles across species provided additional insights into multi-host *Leptospira* ecology, and facilitated additional explorations into predictors of exposure and infections in local wildlife. Improved knowledge of *Leptospira* epidemiology in a greater range of susceptible urban host species, and the diversity of *Leptospira* strains they carry, will provide valuable context for wildlife management agencies and clinicians assessing illness in humans and domestic animals across the region.

6.3. Methods

6.3.1. Study Animals

No live animals were captured or handled explicitly for the purposes of this study, and all wildlife sampling was carried out in accordance with relevant guidelines and regulations. This study focused primarily on wildlife from the greater Los Angeles region in southern California, with most results coming from opportunistically collected carcasses. Opportunistic carcass and sample collection was approved by the California Department of Fish and Wildlife (scientific collecting permits SC-13267 & SC-13700) and took place from September 2015 to June 2020. A small number of mountain lion (n=11) and coyote (n=19) samples were previously collected as part of National Park Service mountain lion and coyote studies, with capture and handling procedures permitted through the California Department of Fish and Wildlife (scientific collection permit SC-0005636) and the National Park Service Institutional Animal Care and Use Committee. Three additional serum samples were included from live-trapped striped skunks, with capture and handling procedures permitted for T. Stankowich through the California Department of Fish and Wildlife (scientific collection permit SC-006837) and the California State University Long Beach Institutional Animal Care and Use Committee (protocol #334). For the purposes of this study, the greater Los Angeles region refers to Los Angeles County and

neighboring counties: Orange, Riverside, San Bernardino, and Ventura. Sample collection focused on our ‘target species’, five common mammals in the Los Angeles region: striped skunks, raccoons, coyotes, Virginia opossums (*Didelphis virginiana*) and fox squirrels (*Sciurus niger*; Table 6.1, Figure 6.1). Collaborating wildlife agencies donated carcasses or existing samples, with the majority coming from animals killed by vehicle collisions or planned wildlife removal, or animals euthanized by animal control or rehabilitation agencies due to illness or injury. Carcasses were necropsied immediately or frozen at -20°C and thawed in a refrigerator prior to necropsy. Animal measurements and demographic information were collected at the time of necropsy, with age class (adult or juvenile) determined using a combination of animal size and tooth wear⁵⁹.

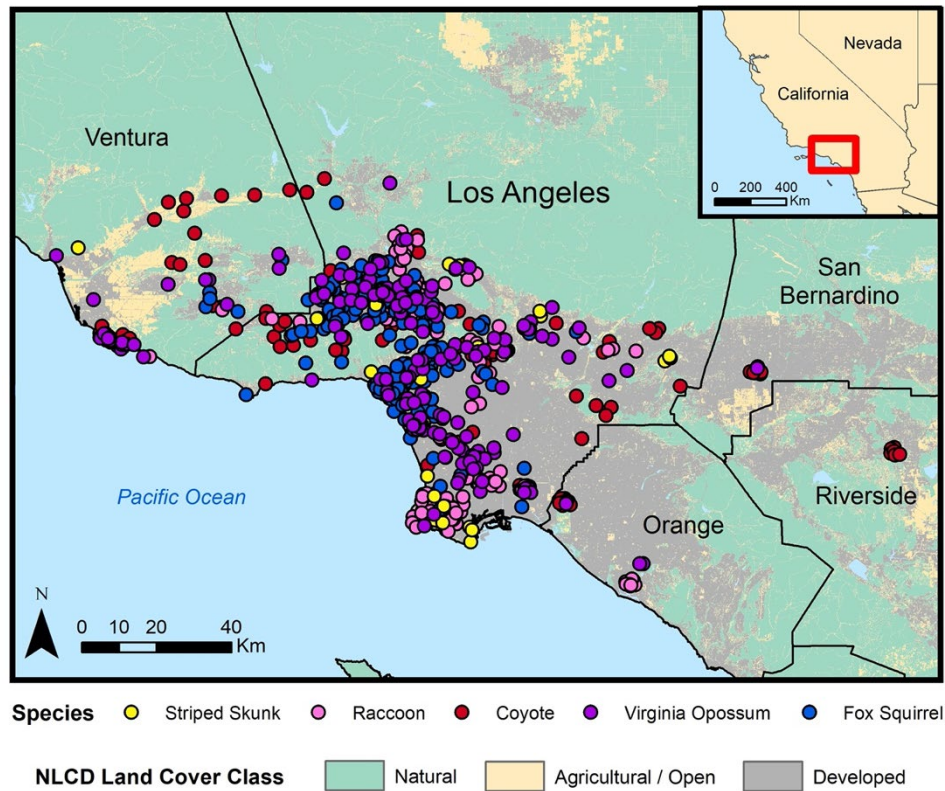


Figure 6.1: Distribution of *Leptospira* sample locations for the five target wildlife species in the greater Los Angeles region (2015-2020). Land cover data were obtained from the National Land Cover Database (2019).

Additional Species & Regions

On an opportunistic basis, we also tested samples provided by other agencies that included non-target regions of California and non-target species, including ground squirrels (*Otospermophilus beecheyi*), desert cottontails (*Sylvilagus audubonii*), feral pigs, bobcats, mountain lions, gray foxes (*Urocyon cinereoargenteus*), and red foxes (*Vulpes vulpes*). A total of 266 additional samples were collected from 11 species. This included non-target species in

Los Angeles County (n=74) and Ventura County (n=24), and all species from the following additional counties: Napa (n=15), Monterey (n=86), San Luis Obispo (n=61), Santa Barbara (n=6; Table 6.4).

Table 6.1: Descriptive characteristics for our five target wildlife species. Within-group percentages are proportions of column totals.

	Coyote (N=137)	Fox Squirrel (N=187)	Raccoon (N=172)	Striped Skunk (N=40)	Virginia Opossum (N=171)	Total (N=707)
Age Class						
Adult	100 (73.0%)	154 (82.4%)	136 (79.1%)	22 (55.0%)	133 (77.8%)	545 (77.1%)
Juvenile	32 (23.4%)	26 (13.9%)	32 (18.6%)	13 (32.5%)	27 (15.8%)	130 (18.4%)
Unknown	5 (3.6%)	7 (3.7%)	4 (2.3%)	5 (12.5%)	11 (6.4%)	32 (4.5%)
Sex						
Female	63 (46.0%)	84 (44.9%)	83 (48.3%)	16 (40.0%)	87 (50.9%)	333 (47.1%)
Male	67 (48.9%)	79 (42.2%)	80 (46.5%)	16 (40.0%)	71 (41.5%)	313 (44.3%)
Unknown	7 (5.1%)	24 (12.8%)	9 (5.2%)	8 (20.0%)	13 (7.6%)	61 (8.6%)
County						
Los Angeles	82 (59.9%)	182 (97.3%)	152 (88.4%)	38 (95.0%)	147 (86.0%)	601 (85.0%)
Orange	15 (10.9%)	0 (0%)	9 (5.2%)	0 (0%)	3 (1.8%)	27 (3.8%)
Riverside	8 (5.8%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	8 (1.1%)
San Bernardino	10 (7.3%)	0 (0%)	0 (0%)	0 (0%)	1 (0.6%)	11 (1.6%)
Ventura	22 (16.1%)	5 (2.7%)	11 (6.4%)	2 (5.0%)	20 (11.7%)	60 (8.5%)
Season						
Dry	60 (43.8%)	81 (43.3%)	53 (30.8%)	27 (67.5%)	73 (42.7%)	294 (41.6%)
Wet	77 (56.2%)	106 (56.7%)	119 (69.2%)	13 (32.5%)	98 (57.3%)	413 (58.4%)

6.3.2. Sample Collection

Serum and urine samples collected at external agencies were analyzed and included when available. From fresh carcasses processed at UCLA, intracardiac blood was collected into serum separation tubes, then kept in a cooler with an ice pack until centrifugation (1350 x g for 10-15 minutes). Kidney samples were collected from all necropsies, and urine was collected when available using cystocentesis. The largest possible kidney sample that would fit in a 58 ml Whirl-Pak® was excised and homogenized in the sealed Whirl-Pak® using manual pressure (approximate size: entire kidney from smaller mammals such as squirrels or half a kidney from larger mammals such as coyotes). Serum and urine samples were transferred into cryovials prior

to storage, and all cryovials and Whirl-Paks® were stored at -20°C or -80°C prior to testing (-80°C preferred and utilized when possible).

6.3.3. *Leptospira* PCR Analysis

The presence of pathogenic *Leptospira* DNA was determined in urine and homogenized kidney samples. For all species except mountain lions, samples collected from 2015-2017 were analyzed at the Hollings Marine Laboratory (Charleston, South Carolina, USA) using a quantitative polymerase chain reaction (qPCR) assay targeting the *lipL32* gene as detailed in Wu et al. (2014)⁶⁰. Samples collected after 2017 were analyzed at Colorado State University Veterinary Diagnostic Laboratory (Denver, Colorado, USA) using the VetMAX™ qPCR Master Mix kit and the primers specified by Wu et al. (2014)⁶⁰. Minor modifications to the protocol resulted in slightly higher sensitivity in the later samples, but results across laboratories were broadly consistent⁶¹. Samples that had a cycle threshold value less than 37 were considered PCR-positive and evidence of active infections. Samples from mountain lions were analyzed at the California Animal Health and Food Safety Laboratory (Davis, California, USA) using their standard protocol⁶².

6.3.4. Genetic Sequencing & Phylogenetic Analyses

DNA capture and enrichment methods

To facilitate robust genomic level species identification and phylogenomic analyses, four *Leptospira*-positive DNA samples (CM-61, MM-3, PL-20, and PL-117) were subjected to pan pathogenic *Leptospira* DNA capture and enrichment as described in detail elsewhere⁶³. Briefly, the sample DNAs were diluted separately to ~4ng/μL in a volume of 40μL, sonicated to an average size of 225bp using a Q800R2 sonicator (QSonica, Newtown, CT, USA), and then short-read next-generation libraries were prepared using Agilent Sure-Select methodology. The libraries were then subjected to one (MM-3 and PL-20) or two (CM-61 and PL-117) rounds of DNA capture and enrichment and then sequenced on an Illumina MiSeq instrument using a MiSeq v3 600 cycle kit (2x300bp reads).

Read classifications

To estimate the percentage of *Leptospira* reads in the enriched sequences, reads were mapped against the standard Kraken database with Kraken v2.1.2⁶⁴.

Read mapping and phylogenomics

Single nucleotide polymorphisms (SNPs) were identified among the four enriched genomes and >340 publicly available *L. interrogans* genomes (GenBank accession numbers provided in Figures 6.6 and 6.7) by aligning reads against reference genome *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (GCA_000007685.1) using minimap2 v2.22⁶⁵ and calling SNPs from the BAM file with GATK v4.2.2⁶⁶ using a depth of coverage ≥3x and a read proportion of 0.9. SNPs that fell within duplicated regions, based on a reference self-alignment with MUMmer v3.1⁶⁷, were filtered from downstream analyses. All of these methods were wrapped by NASP v1.2.1⁶⁸. Maximum likelihood phylogenies were then inferred on the concatenated SNP alignments using IQ-TREE v2.2.0.3 with the "-fast" option, default parameters⁶⁹, and the integrated ModelFinder method⁷⁰; the phylogenies were midpoint rooted.

To determine breadth of coverage across the reference, reads were aligned against the reference genome with minimap2 and the per base depth of coverage was calculated with Samtools v1.6⁷¹.

Pomona reference phylogeny and WG-FAST placement of enriched samples

We then utilized the Whole Genome Focused Array SNP Typing (*WG-FAST*) tool, a publicly available pipeline designed for phylogenetically typing samples with only partial SNP profiles. Reads were simulated from genome assemblies with ART vMountRainier⁷² using the command “-p -na -ss MSv3 -l 250 -f 75 -m 300 -s 30”. Based on the position of the enriched reads in a complete *L. interrogans* phylogeny, simulated reads were aligned against *L. interrogans* serovar Pomona str. Pomona (GCA_000216355.3) with minimap2 v2.24⁶⁵ and SNPs were called with GATK v4.2.6.1⁶⁶. A midpoint rooted maximum-likelihood phylogeny was inferred from a concatenated SNP alignment (529 positions out of a core genome size of 4,125,494 nts) with RAxML-NG v. 1.1.0⁷³. Enriched reads were inserted into this reference phylogeny with *WG-FAST* v1.2⁷⁴ using default settings.

6.3.5. *Leptospira* Serology

Past exposure to *Leptospira* was assessed using serum microscopic agglutination testing (MAT). In this test, dark-field microscopy is used to assess the presence of anti-*Leptospira* antibodies in serum by evaluating agglutination (i.e. clumping) when samples are combined with live cultures of *Leptospira* species²³. Serum samples are tested at doubling dilutions, beginning at 1:100 and continuing until endpoint, with the reported endpoint titers representing the highest dilution that achieved a 50% agglutination using the reference strain being tested. An antibody titer of 1:100 or higher to any serovar was considered positive for *Leptospira* exposure. Samples from 2015-2017 were analyzed at the California Animal Health and Food Safety Laboratory (CAHFS; Davis, California, USA) against a panel of 6 serovars that are common in the United States^{44,75}: Bratislava, Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, and Pomona (Table 6.5). The CAHFS lab uses *L. interrogans* serovar Copenhageni strain M20 as a representative member of the Icterohaemorrhagiae serogroup, and reports results as Icterohaemorrhagiae; it will henceforth be referred to as Icterohaemorrhagiae since they are used interchangeably on MAT testing⁷⁶. Samples from 2018-2020 were analyzed at the Centers for Disease Control and Prevention (CDC; Atlanta, Georgia, USA) using an expanded panel of 20 serovars: Alexi, Australis, Autumnalis, Ballum, Bataviae, Borinca, Bratislava, Canicola, Celledoni, Cynopteri, Djasiman, Georgia, Grippotyphosa, Icterohaemorrhagiae, Javanica, Mankarso, Pomona, Pyrogenes, Tarassovi, and Wolffi (Table 6.5). To assess consistency between the two laboratories, we compared the subset of wildlife samples analyzed at both laboratories (n=469), demonstrating 98.3% agreement in seropositivity and minor quantitative differences between titers. Serovar cross-reactivity patterns were visualized using heatmaps of serovar titers from all seropositive animals using package ‘Heatmap’ in R version 4.3.0⁷⁷.

6.3.6. Analysis of Surveillance Data

Prevalence was estimated for *Leptospira* exposure and infections. For *Leptospira* serology, individuals could be reactive to multiple serovars, so seroprevalence was calculated for each host species in two ways: proportion positive against any serovar, and proportion positive against each specific serovar. All 95% binomial confidence intervals were estimated using

package ‘PropCIs’ in *R*. Additional analyses were done in *R*, and maps were created using ArcGIS version 10.8.2⁷⁸.

Land cover analysis was conducted as detailed in Adducci II et al. (2020)⁷⁹ using data from the National Land Cover Database⁸⁰. Sample localities occurred across a diverse landscape gradient, ranging from natural vegetation and open space to the urban core of Los Angeles (Figure 6.1). To account for land cover variation within home ranges, we extracted 2019 land cover data (30m x 30m resolution) from home range buffers around each georeferenced sampling locality using the ‘raster’ and ‘rgdal’ packages in *R*. Buffer size varied by species based on home range estimates previously reported in the literature: 5 km² for coyotes⁷⁹, 2 km² for raccoons and striped skunks⁸¹, 1 km² for opossums⁸², and 0.5 km² for fox squirrels⁸³. As detailed in Adducci II et al. (2020)⁷⁹, we grouped land cover classifications into three composite categories: urban/suburban (land with 20-100% impervious surface cover), agricultural/open (land used for pastures, crops and open development with <20% impervious surface cover), and natural (shrubland, forest, grassland and wetland). We then calculated the relative proportions of these three land categories for each individual home range buffer, and used package ‘ggtern’ in *R* to make ternary plots showing *Leptospira* exposure (i.e. presence of antibodies) relative to land categories.

To explore potential predictors of *Leptospira* exposure (as indicated by an antibody titer of 1:100 or higher to any serovar), logistic regressions were conducted with the following covariates considered: age class, sex, season (wet Nov-April vs. dry May-Oct)³⁵, and composite land classification (i.e. percentage of the home range that was developed vs. agricultural/open vs. natural). Since the correlation was high between developed and agricultural/open land (Spearman’s $\rho = -0.81$), and developed and natural land (Spearman’s $\rho = -0.90$), the developed category was excluded from these models.

Antibody titers have been identified as significant predictors of *Leptospira* shedding and PCR status (i.e. active infections) in California sea lions^{22,84}. We therefore explored this association in raccoons, the only species with a sufficient number of paired antibody-PCR samples available (n=81). Since these data exhibited complete separation (i.e. a clear distinction between the two outcomes), we applied a Firth’s bias-reduced logistic regression, a penalized maximum likelihood approach that is effective in the presence of data separation⁸⁵, using the ‘logistf’ package in *R* to assess the association between antibody titers and PCR data.

6.4. Results

6.4.1. Broad Patterns of *Leptospira* Exposure and Infection

We detected evidence of *Leptospira* exposure in all five target species sampled in the greater Los Angeles region (Figure 6.2, Table 6.2). Overall seroprevalence in each species was calculated as the proportion of samples that were positive against any serovar. Fox squirrels had the highest seroprevalence at 60.6% (Table 6.2), though most maximum titers in this species were low (Table 6.3). Seroprevalence was moderate in mesocarnivores (32.6% in raccoons, 28.6% in striped skunks, 25.9% in coyotes), and low (5.2%) in opossums.

Of the 582 target animals tested by PCR in the greater Los Angeles region, 24 (4%) were PCR positive, with active infections detected in all species except fox squirrels (Table 6.2, Figure 6.2). Infection prevalence ranged from 0.8% in opossums to 15% in skunks, with coyotes and

raccoons intermediate at 3.7% and 8.6%, respectively. Infection prevalence was consistently lower than corresponding seroprevalence levels for each species, with no active infections detected in fox squirrels despite seroprevalence being highest in this species (Table 6.2).

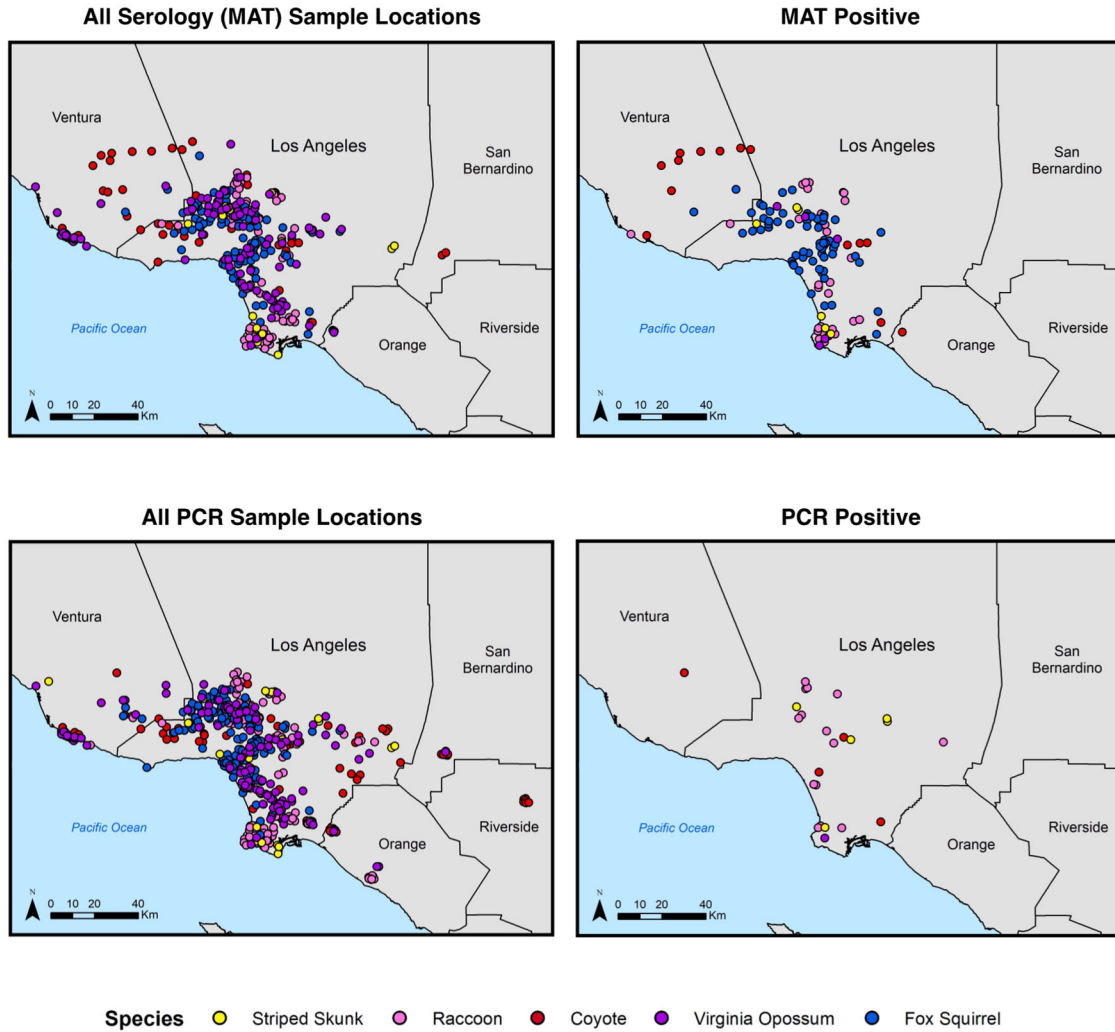


Figure 6.2: Locations of sample collection and associated *Leptospira* exposure and infection results for the five target wildlife species. Host sample locations are indicated in the left-hand panels, with the locations of animals with positive results shown in maps on the right. Samples tested by MAT for serum antibodies are shown in the top row, and those tested by PCR for pathogenic *Leptospira* DNA are shown on the bottom row.

Table 6.2: *Leptospira* exposure and infection by species. *Leptospira* antibody (MAT) and DNA (PCR) results in the five target host species sampled in the greater Los Angeles region. Antibody results include seropositives to all serovars tested.

Common Name	Scientific Name	<i>Leptospira</i> Exposure (MAT)			<i>Leptospira</i> Infections (PCR)		
		<i>POS</i>	<i>n</i>	% <i>POS</i> (95% <i>CI</i>)	<i>POS</i>	<i>n</i>	% <i>POS</i> (95% <i>CI</i>)
Striped Skunk	<i>Mephitis mephitis</i>	6	21	28.6 (11.3-52.2)	5	33	15.2 (5.1-31.9)
Raccoon	<i>Procyon lotor</i>	31	95	32.6 (23.4-43.0)	14	162	8.6 (4.8-14.1)
Coyote	<i>Canis latrans</i>	14	54	25.9 (15.0-39.7)	4	108	3.7 (1.0-9.2)
Virginia Opossum	<i>Didelphis virginiana</i>	5	97	5.2 (1.7-11.6)	1	131	0.8 (0.0-4.2)
Fox Squirrel	<i>Sciurus niger</i>	66	109	60.6 (50.7-69.8)	0	148	0 (0.0-2.5)
Total		122	376	32.4 (27.7-37.4)	24	582	4.1 (2.7-6.1)

Table 6.3: Maximum antibody titers for our five target wildlife species. Maximum antibody titers and corresponding serovars for each individual from the five target species sampled in the greater Los Angeles region, reported for our five primary serovars (Bratislava, Canicola, Grippotyphosa, Icterohaemorrhagiae, and Pomona). Serovars that are not shown were never the maximum titer for that host species. In cases where there were ties for maximum titer, both serovars were counted in the table.

Common Name	Scientific Name	Serovar	100	200	400	800	1600	3200	≥6400	#Pos / Total	Percentage (95% CI)
Striped Skunk	<i>Mephitis mephitis</i>	Pomona	1		1		1		2	5/5	100 (47.8-100)
Raccoon	<i>Procyon lotor</i>	Bratislava	1			1				2/27	7.41 (0.9-24.3)
		Icterohaemorrhagiae	1			1				2/27	7.41 (0.9-24.3)
		Pomona	5	3	5	3	2	2	3	23/27	85.19 (66.3-95.8)
Coyote	<i>Canis latrans</i>	Bratislava	1	1						2/7	28.57 (3.7-71)
		Icterohaemorrhagiae		1						1/7	14.29 (0.4-57.9)
		Pomona		1	1		1		1	4/7	57.14 (18.4-90.1)
Virginia Opossum	<i>Didelphis virginiana</i>	Icterohaemorrhagiae	1							1/2	50 (1.3-98.7)
		Pomona							1	1/2	50 (1.3-98.7)
Fox Squirrel	<i>Sciurus niger</i>	Bratislava		2	5	3	1			11/38	28.95 (15.4-45.9)
		Icterohaemorrhagiae	19	2	2	3		1		27/38	71.05 (54.1-84.6)

6.4.2. Phylogenetic Typing of *Leptospira* Infections

We used DNA capture and enrichment to enable sequencing of *Leptospira* DNA present in DNA extracts obtained from kidney or urine samples from four of our PCR-positive animals: one coyote (CM-61), one striped skunk (MM-3), and two raccoons (PL-20 and PL-117). Two samples underwent two rounds of enrichment and had very high proportions of sequencing reads assigned to *Leptospira*: 90.52% for PL-117 (1,615,997 of 1,785,218 total reads) and 84.71% for CM-61 (1,194,246/1,409,734). The other two samples underwent just one round of enrichment and had lower proportions of reads assigned to *Leptospira*: 11.05% for PL-20 (141,648/1,281,984) and 6.98% for MM-3 (115,700/1,658,612).

We used several approaches to phylogenetically characterize the *Leptospira* strains infecting these four animals based on the genomic sequence data we generated using targeted DNA capture and enrichment. First, we aligned the sequence reads from these four samples against reference genome *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, which revealed coverages of 82.67% for PL-117 and 84.07% for CM-61, which both underwent two rounds of enrichment, and 53.17% for PL-20 and 54.61% for MM-3, which both underwent just one round of enrichment. We then constructed a phylogeny using the core genome shared by all four samples and the >340 publicly available *L. interrogans* genomes, which corresponded to just 19.27% (891,916/4,627,366) of the reference genome. Within this phylogeny (Figure 6.6), the four enriched samples formed a single clade that was embedded within a larger clade containing published sequences from *L. interrogans* serovar Pomona isolates; the three most closely related isolates from outside our current study were collected within the last two decades in coastal California from two California sea lions (CSL4002 and CSL10083) and one Channel Island fox (*Urocyon littoralis*; Fox 32256). We then repeated this approach excluding the less-enriched samples PL-20 and MM-3, which increased the shared core genome among the analyzed samples/isolates to 41.53% (1,921,606/4,627,366) of the reference genome; the phylogenetic patterns remained unchanged (Figure 6.7). Finally, we used *WG-FAST* to map the *Leptospira* sequence reads from all four samples onto a reference phylogeny developed independently using only the published whole genome sequences from isolates, such that our new enriched genomes did not influence the tree topology. Again, the key patterns were preserved: our four new sequences were placed clearly within *L. interrogans* serovar Pomona and were clustered together along with recent isolates from California sea lions and a Channel Island fox (Figure 6.3).

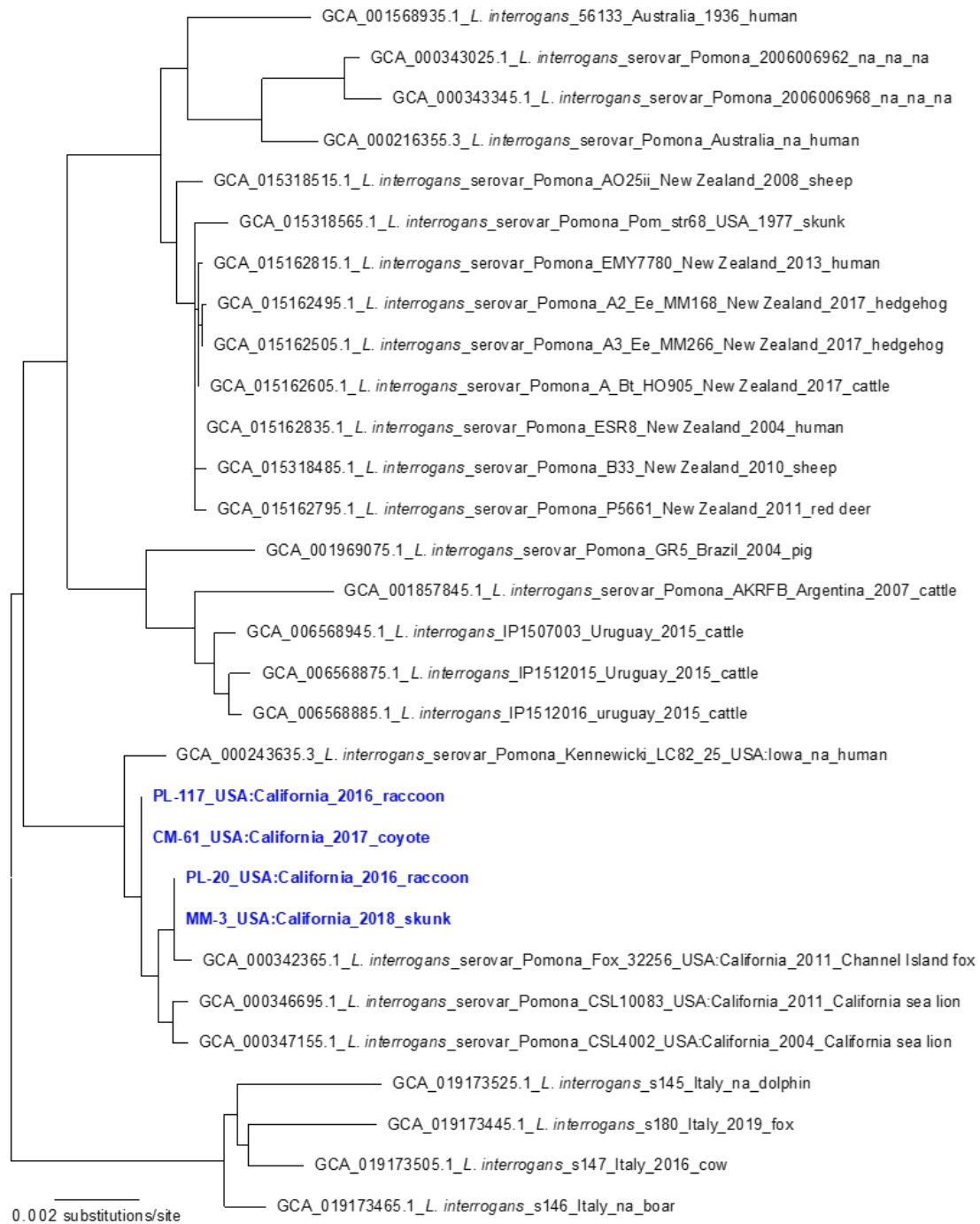


Figure 6.3. *L. interrogans* serovar *Pomona* phylogeny including mainland hosts. A maximum likelihood phylogeny of 26 *L. interrogans* serovar *Pomona* genomes together with enriched *Leptospira* genomic DNA from four PCR-positive animals in this study (highlighted with blue text). The tree was inferred from a concatenated SNP alignment (529 positions out of a core genome size of 4,125,494nts) with RAxML-NG v. 1.1.0. *Leptospira* from these four animals cluster in a single clade also containing isolates collected from two California sea lions sampled in 2011 and one Channel Island fox sampled in 2004.

6.4.3. Serological Reactivity Patterns

We analyzed patterns of serological reactivity to gain further insight into the *Leptospira* strains circulating in the Los Angeles region. We first investigated the five main serovars against which all samples were tested (serovars Bratislava, Canicola, Grippotyphosa, Icterohaemorrhagiae, and Pomona), and evaluated seroprevalence and titer magnitudes against each serovar (Figure 6.8, Table 6.6). In skunks, raccoons, and coyotes, the maximum MAT titers in each individual were most frequently against serovar Pomona (100%, 85%, and 57%, respectively; Table 6.3); among individuals with higher titers (1:1600 or above), the maximum titer was always against serovar Pomona. Maximum MAT titer is an imperfect indicator of the infecting serovar, due to well-known challenges with MAT cross-reactivity^{74,86}, but in our study the relationship was supported by the two animals that had both serologic and phylogenomic analyses conducted: the coyote (CM-61) and skunk (MM-3) samples both had highest titers to serovar Pomona (1:102400 and 1:12800, respectively), and both were phylogenomically clustered with isolates of *L. interrogans* serovar Pomona (Figure 6.3). Fox squirrels exhibited a pattern that was distinct from the other hosts, with highest titers most often to serovar Icterohaemorrhagiae (71%), which was not highly reactive in any of the other host species. Of the two opossums that exhibited reactions against these 5 serovars, one individual (the only opossum in this study with an active infection) had a maximum titer to serovar Pomona (1:12800).

To gain insight into possible sources of reactivity against other serovars, we visualized individual seroreactivity profiles using heatmaps as a way to make sense of the multidimensional data from MAT panels (Figure 6.4, Figure 6.9). The mesocarnivores show strongest reactivity against serovar Pomona. Reactions to other serovars tend to co-occur with strong reactions to serovar Pomona, consistent with the titers against other serovars being cross-reactions to an infection caused by serovar Pomona. A small number of individuals deviate from this pattern (e.g. one raccoon with high titer against serovar Bratislava and low titer against serovar Pomona, possibly indicating an isolated infection with another strain of *Leptospira*). Fox squirrels show strongest reactivity to serovar Icterohaemorrhagiae (Figure 6.4, Figure 6.9), with other reactivities tending to co-occur.

We then considered MAT results for all 21 tested serovars in target species from all regions (Table 6.6). Broad reactivity patterns in non-target regions were similar to patterns seen in greater Los Angeles with serovar Pomona highlighted in mesocarnivores (Figure 6.10). Serovar Pomona exhibited the highest seroprevalence in raccoons (40.7%), and was one of the highest in skunks (26.9%) and coyotes (18.9%). Mesocarnivore results from the CDC 20-serovar panel showed Pomona as dominant with strong cross-reactions to Autumnalis and Djasiman, with titers against those serovars higher than Pomona in some cases. Across all seropositive coyotes, maximum titers were most commonly observed to serovars Autumnalis (n=9/22) and Pomona (n=6/22), with 55% (n=12/22) and 64% (n=14/22) of coyotes positive to serovars Autumnalis and Pomona respectively. In coyotes with paired titers (n=15), Pomona titers were most strongly correlated with titers against serovars Autumnalis (Spearman's $\rho = 0.87$) and Djasiman (Spearman's $\rho = 0.83$). In aggregate, the data from raccoons, coyotes and skunks are consistent with most infections being caused by serovar Pomona. In opossums, the broader dataset revealed several individuals with low titers against serovar Hardjo, making it the most frequently positive serovar (Table 6.6), though Pomona was the highest titer observed in the one opossum with an active infection. Aside from the low-titer reactions against Hardjo that more than doubled the overall seroprevalence of opossums, the majority of our conclusions in target

species did not change with consideration of the expanded serovar panel: *Icterohaemorrhagiae* is the dominant serovar in fox squirrels and *Pomona* is dominant in the other target species.

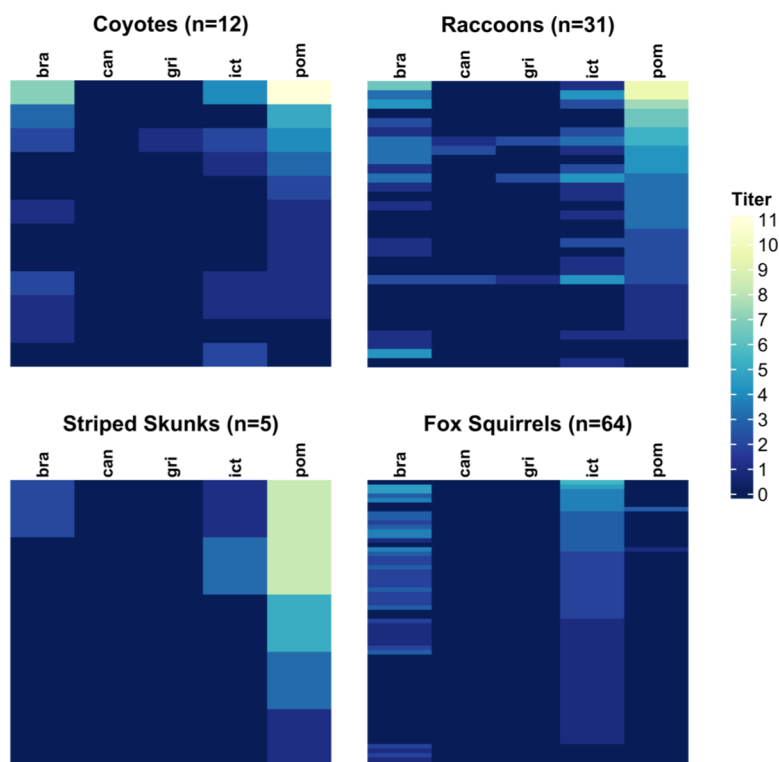


Figure 6.4. Serovar reactivity profiles for target species in the greater Los Angeles region. Data from all seropositive individuals are shown for the five serovars tested at both laboratories: Bratislava (‘bra’), Canicola (‘can’), Grippytyphosa (‘gri’), *Icterohaemorrhagiae* (‘ict’), and *Pomona* (‘pom’). Each row of colored bars represents MAT titers for an individual animal, with rows ordered by titer values for the serovar with the highest maximum titers in each species (serovar *Pomona* for coyotes, raccoons and skunks; serovar *Icterohaemorrhagiae* in fox squirrels). The colorbar legend shows antibody titer on a log₂ scale (1:100 equivalent to 1, 1:200 equivalent to 2, 1:400 equivalent to 3, etc.).

6.4.4. Associations Between Serology and PCR Results

To analyze the association between active infections and the maximum antibody titer against any serovar, we used paired PCR and MAT results, for which only raccoons had a sufficient sample size (n=81). Of those 81 animals, 29 were seropositive, and 86% of seropositive animals (n=25/29) had maximum titers to serovar *Pomona*. We found that maximum titer level was a significant predictor of active infection in raccoons (Firth’s logistic regression; p-value = 2.09×10^{-9}), which aligns with previous studies showing that maximum *Leptospira* MAT titers are effective predictors of infection in California sea lions^{22,84}. Individual raccoons with titers above 1:1600 were highly likely to be PCR-positive, and therefore likely to present a transmission risk because of their potential to be actively shedding *Leptospira* (Figure 6.5). Intriguingly, among host individuals of other target species that had paired PCR and MAT results, 80% (n=4/5)

of individuals with titers above 1:1600 were PCR-positive (1/1 coyotes, 2/2 skunks, 1/1 opossums and 0/1 fox squirrels).

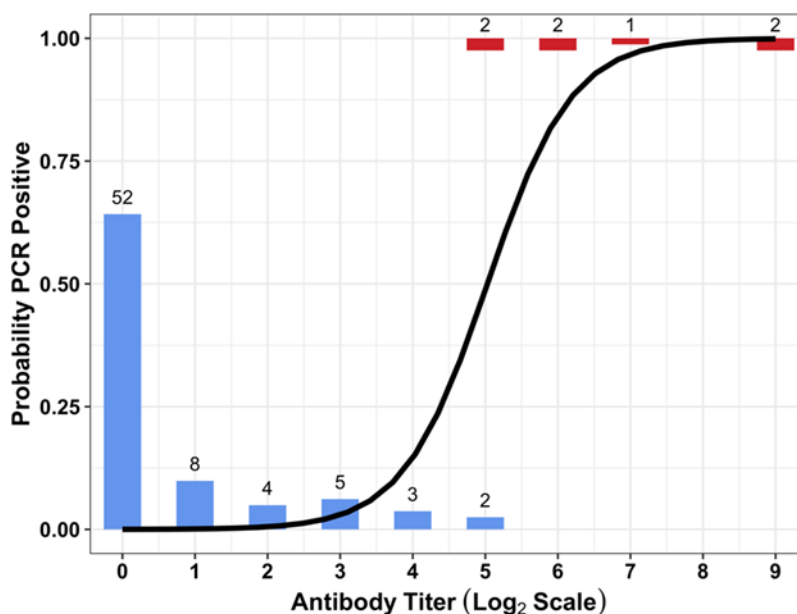


Figure 6.5. The predicted probability that raccoons are PCR-positive relative to maximum antibody titer to any serovar. The number of individuals with maximum MAT titer at a given level are represented by height of the bars and the corresponding number at the top of the bar. Animals with PCR negative results are shown in blue, and PCR positive individuals are shown in red. Antibody titer (x-axis) is shown on a log₂ scale (1:100 equivalent to 1, 1:200 equivalent to 2, 1:400 equivalent to 3, etc.). The solid black line shows the best-fitting solution of a Firth's logistic regression, which predicts that individuals with MAT titers greater than 1:1600 (5 on the log scale) are at least 80% likely to be PCR positive.

6.4.5. Spatial Patterns of *Leptospira* Exposure

We detected *Leptospira* exposure (i.e. MAT-positive individuals) throughout the sampled ranges of each host species (Figure 6.2). When we evaluated sample location relative to the composite land cover classes, we found indications that different species use the landscape in different ways. For instance, fox squirrel and opossum samples were clustered around areas with higher levels of human development, providing evidence for increased use of urban and suburban regions in these non-native species (Figure 6.11). In contrast, coyotes and raccoons were found across all land classes. When we evaluated *Leptospira* exposure data in light of these land classes, no clear patterns emerged to distinguish the locations of positive and negative samples (Figure 6.2, Figure 6.11), indicating that *Leptospira* circulates throughout the sampled range of each host species.

To probe how *Leptospira* exposure patterns might be influenced by land cover, season, and demographic factors, we used logistic regressions to assess possible correlates for each species. This was done in all species except skunks, which were excluded due to small overall sample size (n=23). Of the covariates explored here (age class, sex, season, and land classification), none exhibited significant correlations with *Leptospira* exposure in any of the target species (all p-values

> 0.09 in univariate analyses). This lack of correlation can be visualized as a lack of clustering of positive results in ternary plots (Figure 6.11), supporting that *Leptospira* is distributed throughout the sampled range of these species.

6.4.6. Additional Species & Regions

Overall prevalence levels in the non-target dataset were comparable to the target dataset: 4.4% (n=4/91) of animals were PCR-positive (2 mountain lions, 1 feral pig and 1 bobcat) and antibodies were detected in 30.6% (n=64/209), with species-specific seroprevalence levels ranging from 0% to 61% (Table 6.4). Feral pigs had overall seroprevalence of 27% (n=15/55) and were most frequently reactive to serovars Bratislava, Autumnalis, Djasiman, and Pomona (Tables 6.4 & 6.6). Of the bobcats tested, 46% (n=5/11) were seropositive (Table 6.4), with titers to Bratislava and Pomona most frequently positive (Table 6.6). Of the non-target species in the greater Los Angeles region, 10% (n=3/30) of desert cottontails were seropositive, with the three seropositive individuals exhibiting titers against serovars Georgia, Icterohaemorrhagiae, and Pomona, respectively (Table 6.6). Two mountain lions from the greater Los Angeles region were PCR-positive (18.2%; n=2/11), with both animals showing clinical pathology consistent with leptospirosis (nephritis) upon necropsy.

6.5. Discussion

We conducted a large-scale survey of *Leptospira* in mainland terrestrial mammals in California, focusing on the understudied region of southern California. We had two goals: 1) to identify *Leptospira* prevalence and potential risk factors in the greater Los Angeles region, and 2) to inform our knowledge of broader multi-host circulation in coastal California wildlife. We identified *Leptospira* exposure in all target species (skunks, raccoons, coyotes, opossums, and fox squirrels), and detected active infections in all target species except fox squirrels. Widespread evidence of exposure, which was not correlated with specific landscape factors, highlights that *Leptospira* spp. are endemic and circulating throughout the ranges of these wildlife species in this major metropolitan area. Our findings extend the evidence that *Leptospira* are widespread in many wildlife species throughout California^{35,45} and potentially link transmission in mainland mammals to pathogen circulation in island and marine mammal species.

In our five target species, we detected low to moderate levels of *Leptospira* infection (0-15%) and markedly higher seroprevalence levels (5-60%). Skunks, raccoons and coyotes all exhibited moderate levels of infection (3.7-15.2%), whereas infection prevalence was low in opossums (0.8%) and fox squirrels (0%). Only one opossum was PCR positive, consistent with prior surveillance of opossums elsewhere in California, which have found no⁸⁷ or low *Leptospira* infection prevalence³⁵. Fox squirrels had the highest seroprevalence of all target hosts (60%) despite no infections being detected in this species. This could be due to a shorter duration of carriage, longer duration of titer decay (and hence seropositivity), or potentially an alternate route of transmission (e.g. sexual) and associated tissue distribution which could explain the lack of detection in the urinary tract. In addition to the widespread exposure detected in our target species, we detected exposure in all non-target species except gray foxes, and we provide the first evidence that desert cottontails are exposed to *Leptospira* in California.

In our target species, we detected lower infection prevalence and broadly comparable seroprevalence levels to those reported in more northern regions of California³⁵ (Supplementary Table S4). This may reflect true regional differences, though comparisons across studies and laboratories should be considered carefully. Notably, the prior study used a PCR cycle threshold of 45 to define positivity³⁵, resulting in higher sensitivity and lower specificity (i.e. a lower risk of false negatives, but a higher risk of false positives) than the cycle threshold of 37 used in this study. This could explain the slightly lower infection prevalence in our study. Regional differences in *Leptospira* infection incidence could also arise from environmental differences between northern and southern California, given that wetter environmental conditions facilitate the bacteria's survival and transmission. For example, higher rainfall has been associated with higher *Leptospira* incidence in domestic dogs⁸⁸, including in northern California⁸⁹. Expanded analyses with broader sampling ranges and consistent protocols could elucidate possible environmental or seasonal patterns of *Leptospira* in California wildlife.

We used two broad approaches to characterize the *Leptospira* strains circulating in our system. For four individual animals (two raccoons, one coyote, and one skunk), we sequenced PCR-positive samples and used phylogenomic analysis to determine that they were infected with *L. interrogans* strains that exhibit little genetic distance from one another, and cluster within clades of *L. interrogans* serovar Pomona. For the broader set of individuals and species in our study, we analyzed patterns of seroreactivity to assess possible circulating strains, though we emphasize that serology cannot conclusively identify the *Leptospira* strain causing an infection⁸⁶. Over half of the seropositive animals in this study were reactive to multiple serovars (67%; n=124/186). Consideration of individual-level reactivity profiles indicated that most of these are likely cross-reactions since they co-occur with stronger reactions against other serovars (though infection by multiple strains of *Leptospira* is possible, as reported in cattle¹⁵). Trying to infer the infecting strain from serological data is challenging: this is sometimes assumed to be the serovar with the highest MAT titer, but this approach will fail if the infecting serovar is not on the test panel, or in cases of 'paradoxical reactions' where another serovar elicits a higher MAT titer than the true infecting serovar. In this study, coyotes, raccoons, and skunks typically had maximum antibody titers against serovar Pomona, which is consistent with our phylogenomic results, and aligns with findings from northern California where serovar Pomona predominated in mesocarnivores³⁵. Conversely, squirrels showed minimal seroreactivity to serovar Pomona and typically had low maximum titers against serovar Icterohaemorrhagiae, which was not highly reactive in other species. In our two individual animals with both serology and genomic data (one raccoon and one coyote), we confirmed that the maximum titer against Pomona was indeed associated with infection by *L. interrogans* serovar Pomona. In a subset of samples tested against a broader array of serovars, some coyotes exhibited highest titers to serovar Autumnalis, though titers against serovar Pomona were also high, and titers against Pomona and Autumnalis were strongly correlated. We note that similar patterns have been reported in other canids (domestic dogs⁹¹ and Channel Island foxes⁶¹), including island foxes with culture-confirmed serovar Pomona infections that caused peak titers against serovar Autumnalis⁶¹. Though further genomic data are needed to identify all distinct strains circulating in Los Angeles wildlife, based on current evidence we propose that there are at least two: a strain of *L. interrogans* serovar Pomona circulating in mesocarnivores (and perhaps other unsampled species), and another strain (with serologic reactivity against serovar Icterohaemorrhagiae) circulating in fox squirrels.

Our finding of widespread *Leptospira* circulation, with clear indications that serovar Pomona may be predominant in multiple host species, has potential implications for ongoing research in marine and terrestrial island mammals in California^{21,49–53,61,90}. Phylogenetic analyses of *Leptospira* genomes isolated from California sea lions, northern elephant seals (*Mirounga angustirostris*), Channel Island foxes and island spotted skunks (*Spilogale gracilis amphiala*) show evidence of repeated introductions of new strains of *L. interrogans* serovar Pomona into the broader coastal ecosystem⁵². The source of the introductions to the marine ecosystem is unknown, but mainland terrestrial mammals are one possibility. Our data are consistent with this hypothesis: our phylogenomic analysis of four PCR-positive samples from mainland mesocarnivores showed that they clustered with *L. interrogans* serovar Pomona genomes, and were nested closest to recent *L. interrogans* serovar Pomona isolates from two California sea lions and one Channel Island fox. More genomic data from mainland terrestrial mammals are needed to confirm the timing and direction of transmission links between these mainland and marine ecosystems⁹¹.

Information on wildlife disease occurrence is crucial to assessing wildlife and human health risks, but collecting samples to support broad-scale wildlife surveillance can be very challenging. New approaches to analyzing existing data and samples can provide valuable tools to scale up and make the most of surveillance efforts. Our work highlights the new insights that can be gained from emerging laboratory techniques like targeted DNA capture and enrichment⁶³, which facilitate whole genome-based phylogenomic analyses in the absence of the cultured isolates that *Leptospira* genomics previously required. Novel insights can also be gained from more accessible samples when surveillance efforts are limited. For example, the relationship between *Leptospira* antibody titers and infection status, which has previously not been well characterized in wildlife⁴⁴, may provide a proxy for determining infection prevalence from readily accessible serum samples. We showed that antibody titers are strongly predictive of infection status in raccoons, and similar results in California sea lions^{22,84} indicate a potentially robust general pattern. This could be a useful wildlife screening tool in situations where PCR results are not available or feasible to obtain, but more investigation is needed into the generality of this relationship given the known potential for *Leptospira* to exhibit species- or host-specific patterns. Consequently, using antibody titers as a proxy for infection status may not apply in some host species, such as squirrels, where antibody responses are variable^{92,93}, or opossums where some individuals fail to mount an antibody response and seronegative shedding is possible^{35,94}.

Baseline knowledge about the prevalence of zoonotic infections in urban wildlife has important utility for public health efforts. Diagnosing early cases of leptospirosis in humans and domestic animals can be challenging due to non-specific clinical signs. Raising clinical awareness about epidemiological risk factors (e.g. the prevalence in sympatric host species) is therefore critical for facilitating accurate diagnostics and disease risk assessments⁹. Broad-scale surveillance efforts also contribute to knowledge of circulating variants, which can be used to rapidly identify or exclude potential sources of transmission. For example, in 2021 there was a leptospirosis outbreak in Los Angeles dogs caused by *L. interrogans* serovar Canicola⁵⁵. Very few individuals in our study showed any reactivity at all against serovar Canicola. This corroborates the conclusion that this outbreak did not originate from local wildlife⁵⁵, highlighting the importance of longitudinal wildlife surveillance in determining (or ruling out) potential sources of outbreaks caused by multi-host pathogens.

This study has some limitations which suggest directions for additional surveillance efforts and future research. Since only five wildlife species could be sampled extensively, there

could be unobserved host species contributing to *Leptospira* persistence and transmission in greater Los Angeles. Identifying these cryptic contributors, sometimes referred to as ‘epidemiological dark matter’¹², remains a frontier in disease ecology and emphasizes the need for ongoing research to understand multi-host pathogen dynamics. We were primarily dependent on collaborating agencies for salvaged and opportunistically collected samples. This led to limited sample sizes with a degree of spatial clustering around collaborator facilities, which potentially reduced our ability to detect spatial patterns. Samples may also have been biased towards more developed areas, with higher road concentrations potentially increasing traffic-related deaths, and higher human densities increasing the likelihood of sick or dead animals being reported. Additionally, the composite land classification used here may have masked finer-scale spatial associations, and our use of circular home range buffers could overlook behavioral patterns in habitat use. Further investigations utilizing other metrics of urbanization (e.g. population density) or individual home ranges and land use patterns could reveal spatial relationships undetected in our analyses.

This study provides the first in-depth look at *Leptospira* ecology in terrestrial wildlife across the greater Los Angeles area. Expanded knowledge of this pathogen in southern California, including comparisons of prevalence levels and serological patterns across host species, provides key insights into multi-host pathogen dynamics and the potential for cross-species transmission, including from wildlife to humans and their pets. Evidence of *Leptospira* circulation in Los Angeles wildlife has been lacking, contributing to the perception that the pathogen does not pose a major risk in the area. Our study found evidence consistent with endemic circulation of at least two distinct strains of *Leptospira* among our five target species, and applied novel *Leptospira* DNA capture and enrichment techniques that yielded preliminary evidence linking mainland mammal transmission to nearby island and marine systems. High levels of exposure and wide geographic distribution indicate that *Leptospira* are ubiquitous across the region, with active infection rates substantial enough to warrant concern and to recommend that domestic dogs in the Los Angeles metropolitan area be vaccinated against this disease. Better understanding of *Leptospira* ecology and transmission dynamics in wildlife is critical to the management of this widely circulating pathogen, highlighting the need for more systematic, broad-scale research efforts to monitor this pathogen in wildlife, domestic animals, and humans.

6.6. Supplemental material

Table 6.4: *Leptospira* test results from non-target species and regions.

	Bobcat (N=12)	Coyote (N=20)	Desert Cottontail (N=38)	Feral Pig (N=55)	Gray Fox (N=9)	Ground Squirrel (N=38)	Mountain Lion (N=11)	Raccoon (N=41)	Red Fox (N=5)	Striped Skunk (N=5)	Virginia Opossum (N=32)	Total (N=266)
MAT												
Negative	6 (50.0%)	13 (65.0%)	27 (71.1%)	40 (72.7%)	4 (44.4%)	2 (5.3%)	1 (9.1%)	16 (39.0%)	3 (60.0%)	3 (60.0%)	30 (93.8%)	145 (54.5%)
Positive	5 (41.7%)	7 (35.0%)	3 (7.9%)	15 (27.3%)	0 (0%)	2 (5.3%)	1 (9.1%)	25 (61.0%)	2 (40.0%)	2 (40.0%)	2 (6.3%)	64 (24.1%)
Not Tested	1 (8.3%)	1 (5.0%)	8 (21.1%)	0 (0%)	5 (55.6%)	34 (89.5%)	9 (81.8%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	57 (21.7%)
PCR												
Negative	1 (8.3%)	1 (5.0%)	27 (71.1%)	7 (12.7%)	6 (66.7%)	36 (94.7%)	9 (81.8%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	87 (32.7%)
Positive	1 (8.3%)	0 (0%)	0 (0%)	1 (1.8%)	0 (0%)	0 (0%)	2 (18.2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4 (1.1%)
Not Tested	10 (83.3%)	19 (95.0%)	11 (28.9%)	47 (85.5%)	3 (33.3%)	2 (5.3%)	0 (0%)	41 (100%)	5 (100%)	5 (100%)	32 (100%)	175 (65.8%)
Age Class												
Adult	9 (75.0%)	14 (70.0%)	36 (94.7%)	36 (65.5%)	6 (66.7%)	27 (71.1%)	10 (90.9%)	39 (95.1%)	4 (80.0%)	5 (100%)	31 (96.9%)	217 (81.6%)
Juvenile	2 (16.7%)	4 (20.0%)	0 (0%)	19 (34.5%)	2 (22.2%)	11 (28.9%)	1 (9.1%)	2 (4.9%)	1 (20.0%)	0 (0%)	0 (0%)	42 (15.8%)
Unknown	1 (8.3%)	2 (10.0%)	2 (5.3%)	0 (0%)	1 (11.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (3.1%)	7 (2.6%)
Sex												
Female	2 (16.7%)	8 (40.0%)	16 (42.1%)	27 (49.1%)	1 (11.1%)	13 (34.2%)	6 (54.5%)	3 (7.3%)	2 (40.0%)	0 (0%)	7 (21.9%)	85 (32.0%)
Male	6 (50.0%)	9 (45.0%)	10 (26.3%)	26 (47.3%)	5 (55.6%)	24 (63.2%)	5 (45.5%)	7 (17.1%)	2 (40.0%)	3 (60.0%)	15 (46.9%)	112 (42.1%)
Unknown	4 (33.3%)	3 (15.0%)	12 (31.6%)	2 (3.6%)	3 (33.3%)	1 (2.6%)	0 (0%)	31 (75.6%)	1 (20.0%)	2 (40.0%)	10 (31.3%)	69 (25.9%)
County												
Los Angeles	2 (16.7%)	0 (0%)	24 (63.2%)	0 (0%)	5 (55.6%)	36 (94.7%)	7 (63.6%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	74 (27.8%)
Monterey	5 (41.7%)	7 (35.0%)	0 (0%)	0 (0%)	2 (22.2%)	1 (2.6%)	0 (0%)	40 (97.6%)	1 (20.0%)	2 (40.0%)	28 (87.5%)	86 (32.3%)
Napa	0 (0%)	0 (0%)	0 (0%)	15 (27.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	15 (5.6%)
San Luis Obispo	0 (0%)	9 (45.0%)	0 (0%)	40 (72.7%)	0 (0%)	0 (0%)	0 (0%)	1 (2.4%)	4 (80.0%)	3 (60.0%)	4 (12.5%)	61 (22.9%)
Santa Barbara	1 (8.3%)	4 (20.0%)	0 (0%)	0 (0%)	1 (11.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	6 (2.3%)
Ventura	4 (33.3%)	0 (0%)	14 (36.8%)	0 (0%)	1 (11.1%)	1 (2.6%)	4 (36.4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	24 (9.0%)
Season												
Dry	3 (25.0%)	9 (45.0%)	16 (42.1%)	17 (30.9%)	3 (33.3%)	28 (73.7%)	8 (72.7%)	13 (31.7%)	2 (40.0%)	0 (0%)	6 (18.8%)	105 (39.5%)
Wet	9 (75.0%)	10 (50.0%)	22 (57.9%)	29 (52.7%)	6 (66.7%)	10 (26.3%)	3 (27.3%)	28 (68.3%)	3 (60.0%)	5 (100%)	26 (81.3%)	151 (56.8%)
Unknown	0 (0%)	1 (5.0%)	0 (0%)	9 (16.4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	10 (3.8%)

Table 6.5: Table of reference serovars and strains used in microscopic agglutination testing at the United States Centers for Disease Control and Prevention (CDC) and the California Animal Health and Food Safety Lab System (CAHFS).

Laboratory	Species	Serogroup	Serovar	Strain
CDC	<i>L. interrogans</i>	Australis	Australis	Ballico
	<i>L. interrogans</i>	Australis	Bratislava	Jez-Bratislava
	<i>L. interrogans</i>	Autumnalis	Autumnalis	Akiyami A
	<i>L. borgpetersenii</i>	Ballum	Ballum	Mus 127
	<i>L. interrogans</i>	Bataviae	Bataviae	Van Tienen
	<i>L. interrogans</i>	Canicola	Canicola	Ruebush
	<i>L. weillii</i>	Celledoni	Celledoni	Celledoni
	<i>L. kirschneri</i>	Cynopteri	Cynopteri	3522 C
	<i>L. interrogans</i>	Djasiman	Djasiman	Djasiman
	<i>L. kirschneri</i>	Grippotyphosa	Grippotyphosa	Moskva V
	<i>L. santarosai</i>	Hebdomadis	Borincana	HS 622
	<i>L. interrogans</i>	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
	<i>L. interrogans</i>	Icterohaemorrhagiae	Mankarso	Mankarso
	<i>L. interrogans</i>	Javanica	Javanica	Veldrat Bataviae 46
	<i>L. santarosai</i>	Mini	Georgia	LT 117
	<i>L. interrogans</i>	Pomona	Pomona	Pomona
	<i>L. interrogans</i>	Pyrogenes	Pyrogenes	Salinem
	<i>L. interrogans</i>	Pyrogenes	Alexi	HS 616
	<i>L. interrogans</i>	Sejroe	Wolffi	3705
	<i>L. borgpetersenii</i>	Tarassovi	Tarassovi	Perepelitsin
CAHFS	<i>L. interrogans</i>	Australis	Bratislava	Jez-Bratislava
	<i>L. interrogans</i>	Canicola	Canicola	Hond Utrecht IV
	<i>L. interrogans</i>	Grippotyphosa	Grippotyphosa	Andaman
	<i>L. interrogans</i>	Sejroe	Hardjo	Hardjoprajitno
	<i>L. interrogans</i>	Icterohaemorrhagiae	Copenhageni	M20
	<i>L. interrogans</i>	Pomona	Pomona	Pomona

Table 6.6: *Leptospira* serovars and antibody titer levels for all California wildlife samples. Total number of animals tested by host species and serovar (n), the number of animals that exhibited a maximum titer at each titer level, the total number of animals that tested positive to each serovar (N), and the row-wise seropositivity (%) positive) with binomial confidence intervals (95% CI). Serovars with no positive results for a given host species are not shown.

Common Name	Scientific Name	n	Serovar	100	200	400	800	1600	3200	>=6400	N	Prevalence (%)
Bobcat	<i>Lynx rufus</i>	7	Autumnalis	0	0	0	0	0	1	0	1	14.29 (0.4-57.9)
		7	Bataviae	1	0	0	0	0	0	0	1	14.29 (0.4-57.9)
		11	Bratislava	2	1	0	1	0	0	0	4	36.36 (10.9-69.2)
		11	Canicola	0	0	0	0	1	0	0	1	9.09 (0.2-41.3)
		7	Cynopteri	1	0	0	0	0	0	0	1	14.29 (0.4-57.9)
		7	Djasiman	0	0	0	0	0	1	0	1	14.29 (0.4-57.9)
		11	Grippotyphosa	0	0	0	0	1	0	0	1	9.09 (0.2-41.3)
		11	Icterohaemorrhagiae	0	1	0	0	0	1	0	2	18.18 (2.3-51.8)
		7	Mankarso	0	0	0	0	1	0	0	1	14.29 (0.4-57.9)
		11	Pomona	0	2	0	0	0	1	0	3	27.27 (6-61)
		7	Pyrogenes	0	0	0	0	1	0	0	1	14.29 (0.4-57.9)
		7	Wolfii	1	0	0	0	0	0	0	1	14.29 (0.4-57.9)
Coyote	<i>Canis latrans</i>	46	Australis	0	1	0	0	0	0	0	1	2.17 (0.1-11.5)
		46	Autumnalis	3	4	2	1	0	2	0	12	26.09 (14.3-41.1)
		74	Bratislava	5	3	1	1	0	0	2	12	16.22 (8.7-26.6)
		74	Canicola	0	1	0	0	0	0	0	1	1.35 (0-7.3)
		46	Cynopteri	1	0	0	0	0	0	0	1	2.17 (0.1-11.5)
		46	Djasiman	2	3	1	2	0	2	0	10	21.74 (10.9-36.4)
		74	Grippotyphosa	2	2	1	0	0	0	0	5	6.76 (2.2-15.1)
		38	Hardjo	1	1	0	0	0	0	0	2	5.26 (0.6-17.7)
		74	Icterohaemorrhagiae	4	3	1	1	0	0	1	10	13.51 (6.7-23.5)
		46	Mankarso	1	3	1	1	0	0	0	6	13.04 (4.9-26.3)
		74	Pomona	5	1	3	1	2	0	2	14	18.92 (10.7-29.7)
		46	Tarassovi	1	0	0	0	0	0	0	1	2.17 (0.1-11.5)
Desert Cottontail	<i>Sylvilagus audubonii</i>	27	Georgia	0	0	0	1	0	0	0	1	3.7 (0.1-19)
		30	Icterohaemorrhagiae	1	0	0	0	0	0	0	1	3.33 (0.1-17.2)
		30	Pomona	0	1	0	0	0	0	0	1	3.33 (0.1-17.2)
Feral Pigs	<i>Sus scrofa</i>	55	Autumnalis	4	1	3	0	0	0	0	9	16.36 (7.8-28.8)
		55	Bratislava	4	4	2	0	0	0	0	10	18.18 (9.1-30.9)
		55	Canicola	1	0	0	0	0	0	0	1	1.82 (0-9.7)
		55	Cynopteri	1	1	0	0	0	0	0	2	3.64 (0.4-12.5)
		55	Djasiman	3	1	4	0	0	0	0	8	14.55 (6.5-26.7)
		55	Georgia	0	1	0	0	0	0	0	1	1.82 (0-9.7)
		55	Icterohaemorrhagiae	2	0	1	0	0	0	0	3	5.45 (1.1-15.1)
		55	Mankarso	1	0	1	0	0	0	0	2	3.64 (0.4-12.5)
		55	Pomona	2	0	1	0	1	1	2	7	12.73 (5.3-24.5)
		55	Pyrogenes	0	1	0	0	0	0	0	1	1.82 (0-9.7)
		55	Tarassovi	1	0	0	0	0	0	0	1	1.82 (0-9.7)
Fox Squirrel	<i>Sciurus niger</i>	69	Australis	2	0	0	0	0	0	0	2	2.9 (0.4-10.1)
		69	Autumnalis	2	2	0	0	0	0	0	4	5.8 (1.6-14.2)
		110	Bratislava	9	15	9	4	2	0	0	39	35.45 (26.6-45.1)
		69	Celledoni	1	0	0	0	0	0	0	1	1.45 (0-7.8)
		51	Hardjo	8	16	7	2	1	0	0	34	66.67 (52.1-79.2)
		110	Icterohaemorrhagiae	29	15	9	5	1	1	0	60	54.55 (44.8-64.1)
		69	Javanica	1	0	0	0	0	0	0	1	1.45 (0-7.8)
		69	Mankarso	1	1	1	0	0	0	0	3	4.35 (0.9-12.2)
		110	Pomona	1	0	1	0	0	0	0	2	1.82 (0.2-6.4)
Ground Squirrel	<i>Otospermophilus beecheyi</i>	69	Pyrogenes	2	0	0	0	0	0	0	2	2.9 (0.4-10.1)
		4	Bratislava	2	0	0	0	0	0	0	2	50 (6.8-93.2)
		4	Hardjo	0	1	0	0	0	0	0	1	25 (0.6-80.6)
Raccoon	<i>Procyon lotor</i>	16	Autumnalis	2	0	1	0	0	0	0	3	18.75 (4-45.6)
		135	Bratislava	11	3	9	4	3	3	2	35	26.12 (18.9-34.4)
		135	Canicola	2	4	2	2	1	0	0	11	8.21 (4.2-14.2)
		16	Celledoni	1	0	0	0	0	0	0	1	6.25 (0.2-30.2)
		16	Cynopteri	1	0	0	0	0	0	0	1	6.25 (0.2-30.2)
		16	Djasiman	1	1	1	0	0	0	0	3	18.75 (4-45.6)
		135	Grippotyphosa	1	5	4	1	0	0	0	11	8.21 (4.2-14.2)
		122	Hardjo	11	5	2	4	3	0	0	25	20.66 (13.8-29)
		135	Icterohaemorrhagiae	13	9	5	5	1	2	1	36	26.87 (19.6-35.2)
		16	Mankarso	0	0	1	0	0	0	0	1	6.25 (0.2-30.2)
		135	Pomona	6	10	8	9	7	3	12	55	40.74 (32.2-49.5)
		16	Pyrogenes	1	0	0	0	0	0	0	1	6.25 (0.2-30.2)
		16	Tarassovi	0	0	1	0	0	0	0	1	6.25 (0.2-30.2)
Red Fox	<i>Vulpes vulpes</i>	4	Autumnalis	0	0	1	0	0	0	0	1	25 (0.6-80.6)
		4	Djasiman	0	1	0	0	0	0	0	1	25 (0.6-80.6)
		5	Icterohaemorrhagiae	1	0	0	0	0	0	0	1	20 (0.5-71.6)
		4	Mankarso	1	0	0	0	0	0	0	1	25 (0.6-80.6)
		5	Pomona	0	1	0	0	0	0	0	1	20 (0.5-71.6)
Striped Skunk	<i>Mephitis mephitis</i>	4	Pyrogenes	1	0	0	0	0	0	0	1	25 (0.6-80.6)
		8	Autumnalis	2	0	0	1	0	0	0	3	37.5 (8.5-75.5)
		26	Bratislava	0	1	0	0	0	0	0	1	3.85 (0.1-19.6)
		8	Djasiman	1	0	1	0	0	0	0	2	25 (3.2-65.1)
		26	Icterohaemorrhagiae	2	0	1	0	0	0	0	3	11.54 (2.4-30.2)
		8	Mankarso	1	0	0	0	0	0	0	1	12.5 (0.3-52.7)
Virginia Opossum	<i>Didelphis virginiana</i>	26	Pomona	2	0	2	0	1	0	2	7	26.92 (11.6-47.8)
		129	Bratislava	1	0	0	0	0	0	0	1	0.78 (0-4.2)
		75	Hardjo	7	0	0	0	0	0	0	7	9.33 (3.8-18.3)
		129	Icterohaemorrhagiae	2	0	0	0	0	0	0	2	1.55 (0.2-5.5)
		129	Pomona	0	0	0	0	0	0	1	1	0.78 (0-4.2)

Table 6.7: Comparison of *Leptospira* exposure and infection detected in this study centered in southern California, and by Straub and Foley (2020) centered in more northern regions of California. *Leptospira* antibody (MAT) and DNA (PCR) results are shown for our five target species.

	Common Name	Scientific Name	<i>Leptospira</i> Exposure (MAT)			<i>Leptospira</i> Infections (PCR)		
			<i>POS</i>	<i>n</i>	% <i>POS</i> (95% <i>CI</i>)	<i>POS</i>	<i>n</i>	% <i>POS</i> (95% <i>CI</i>)
This Study	Striped Skunk	<i>Mephitis mephitis</i>	6	21	28.6 (11.3-52.2)	5	33	15.2 (5.1-31.9)
	Raccoon	<i>Procyon lotor</i>	31	95	32.6 (23.4-43.0)	14	162	8.6 (4.8-14.1)
	Coyote	<i>Canis latrans</i>	14	54	25.9 (15.0-39.7)	4	108	3.7 (1.0-9.2)
	Virginia Opossum	<i>Didelphis virginiana</i>	5	97	5.2 (1.7-11.6)	1	131	0.8 (0.0-4.2)
	Fox Squirrel	<i>Sciurus niger</i>	66	109	60.6 (50.7-69.8)	0	148	0 (0.0-2.5)
Straub and Foley, 2020	Striped Skunk	<i>Mephitis mephitis</i>	78	206	37.9 (31.5-44.7)	40	141	28.4 (21.6-36.3)
	Raccoon	<i>Procyon lotor</i>	52	119	43.7 (35.1-52.7)	23	87	26.4 (18.3-36.6)
	Coyote	<i>Canis latrans</i>	6	20	30.0 (14.5-51.9)	2	2	100.0 (34.2-100.0)
	Virginia Opossum	<i>Didelphis virginiana</i>	2	32	6.3 (1.7-20.1)	1	6	16.7 (3.0-56.4)
	Fox Squirrel	<i>Sciurus niger</i>	15	36	41.7 (27.1-57.8)	4	31	12.9 (5.1-28.9)

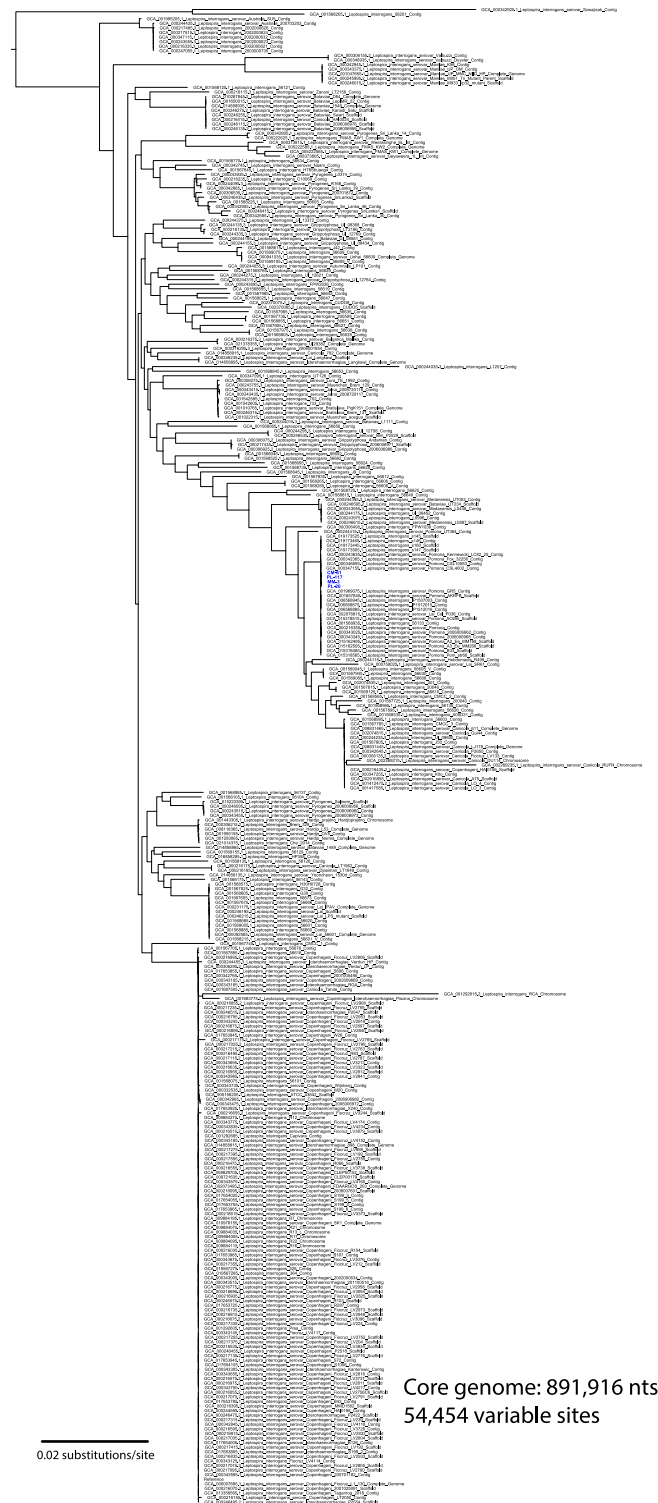


Figure 6.6. Maximum likelihood phylogeny of over 340 *L. interrogans* genomes (low coverage) together with enriched *Leptospira* genomic DNA from four PCR-positive animals in this study (highlighted with blue text) generated from one or two rounds of DNA capture and enrichment. Reads were mapped against *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (GCA_000007685.1) and single nucleotide polymorphisms (SNPs) were called with GATK. The tree was inferred by IQ-TREE using the core genome shared by all four enriched samples and the publicly available genomes, corresponding to 19.27% [891,916 of 4,627,366 nucleotides (nts)] of the reference genome.

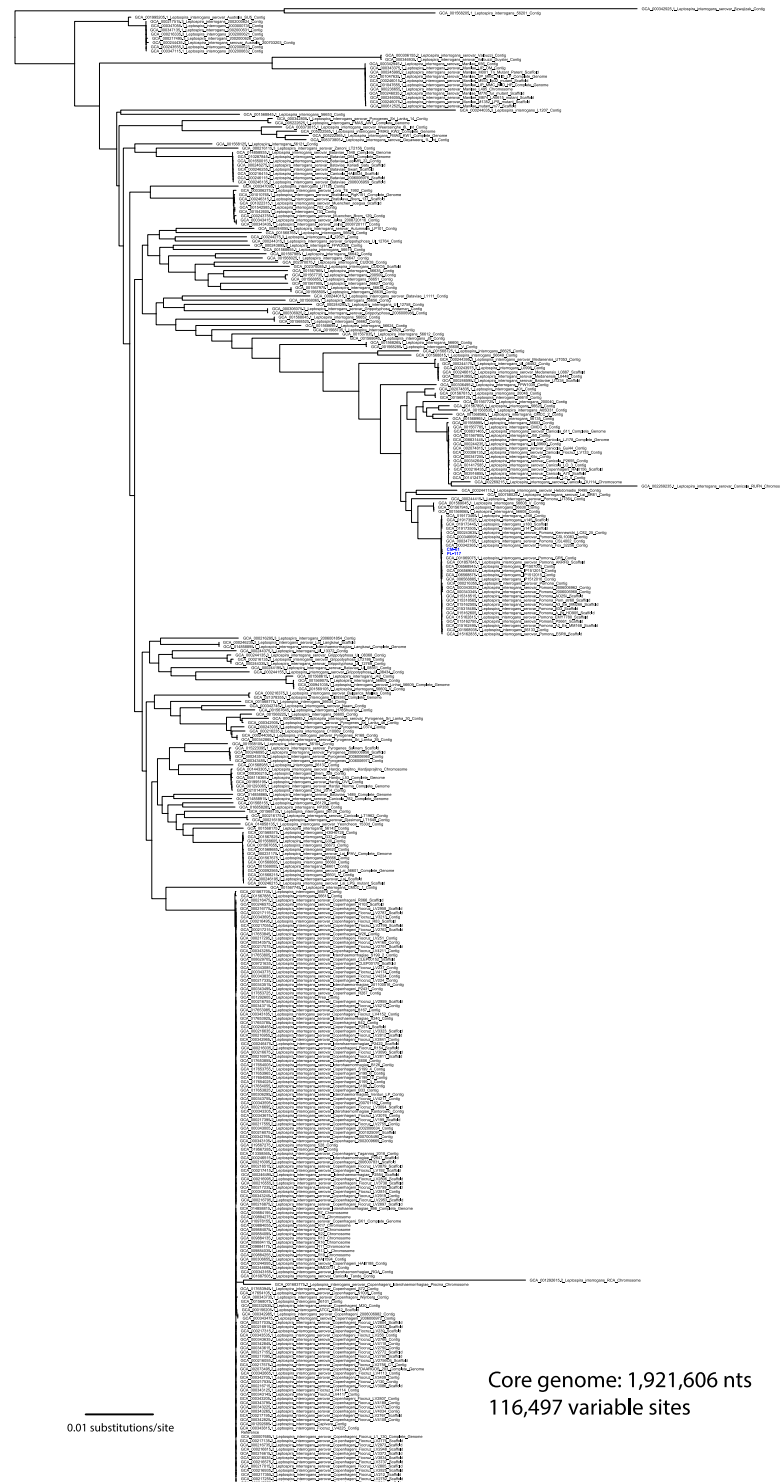


Figure 6.7. Maximum likelihood phylogeny of over 340 *L. interrogans* genomes (high coverage) together with enriched *Leptospira* genomic DNA from two PCR-positive animals in this study (highlighted with blue text) generated from two rounds of DNA capture and enrichment. Reads were mapped against *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (GCA_000007685.1) and single nucleotide polymorphisms (SNPs) were called with GATK. The tree was inferred by IQ-TREE using the core genome shared by two enriched samples and the publicly available genomes, corresponding to 41.53% [1,921,606 of 4,627,366 nucleotides (nts)] of the reference genome.

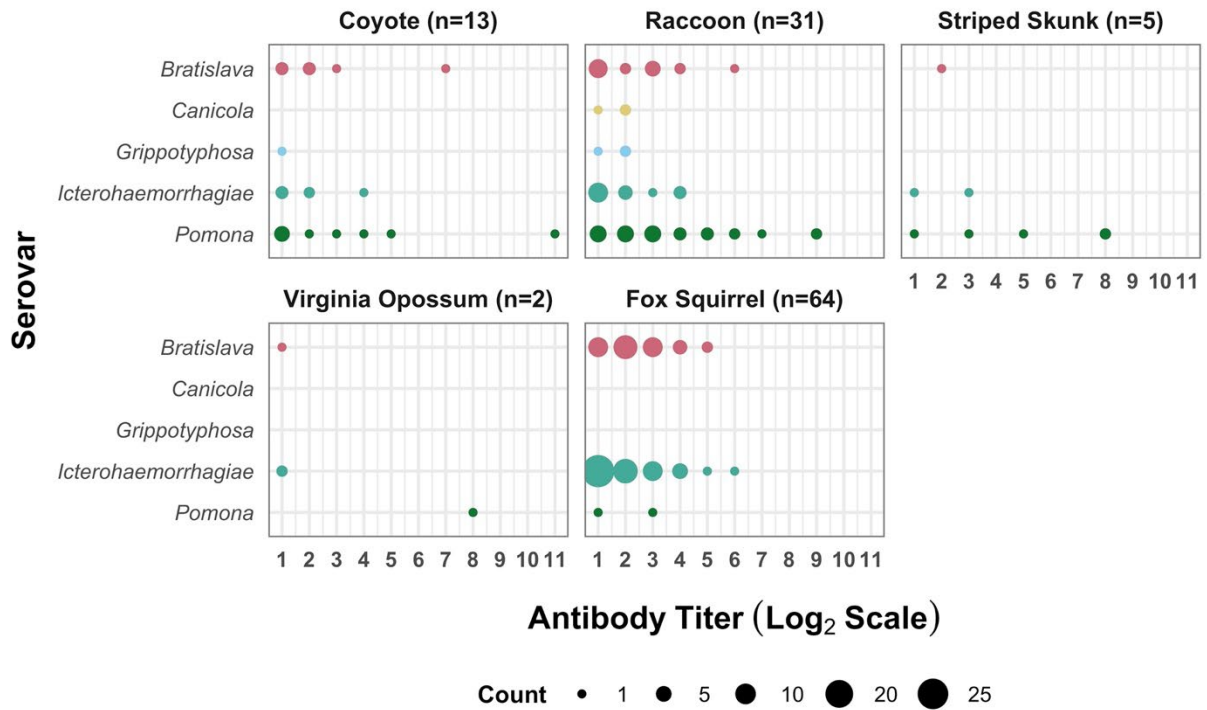


Figure 6.8. Samples positive for antibodies shown by host species, serovar, and titer level. Positive antibody results (MAT) for our five main serovars. Antibody titer (x-axis) is shown on a log₂ scale (1:100 equivalent to 1, 1:200 equivalent to 2, 1:400 equivalent to 3, etc.).

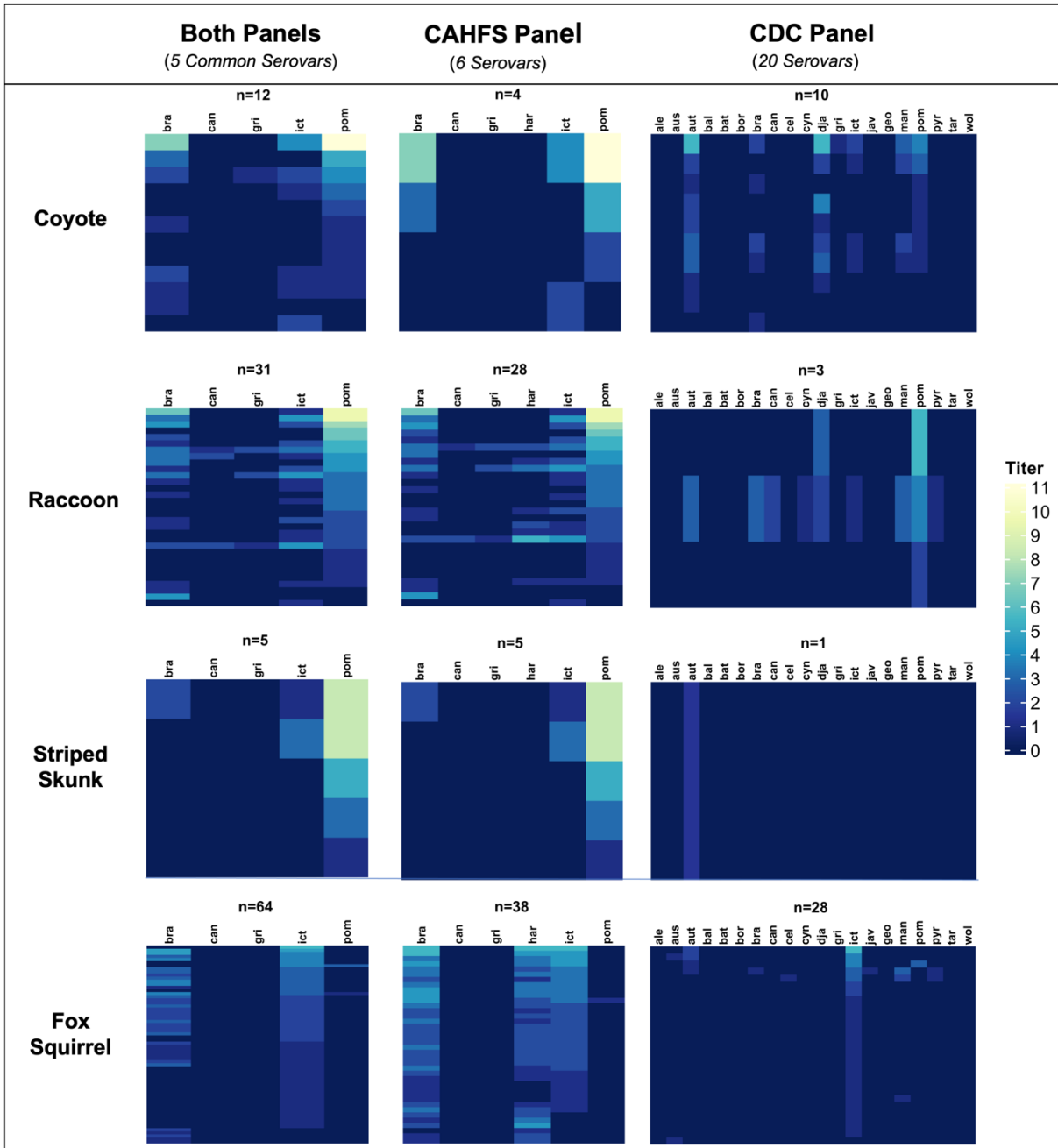


Figure 6.9. Serovar reactivity profiles for target species in the greater Los Angeles region, displayed by laboratory. All seropositive data are displayed for the serovars tested at both laboratories (left), CAHFS (middle) and CDC (right). Each row of colored bars represents MAT titers for an individual animal, with rows ordered by titer values for the serovar with the highest maximum titers in each species (serovar Pomona for coyotes, raccoons and skunks; serovar Icterohaemorrhagiae in fox squirrels). The colorbar legend shows antibody titer on a log₂ scale (1:100 equivalent to 1, 1:200 equivalent to 2, 1:400 equivalent to 3, etc.). Serovar results shown: Alexi ('ale'), Australis ('aus'), Autumnalis ('aut'), Ballum ('bal'), Bataviae ('bat'), Borincana ('bor'), Bratislava ('bra'), Canicola ('can'), Celledoni ('cel'), Cynopteri ('cyn'), Djasiman ('dja'), Grippotyphosa ('gri'), Hardjo ('har'), Icterohaemorrhagiae ('ict'), Javanica ('jav'), Georgia ('geo'), Mankarso ('man'), Pomona ('pom'), Pyrogenes ('pyr'), Tarassovi ('tar'), Wolffi ('wol').

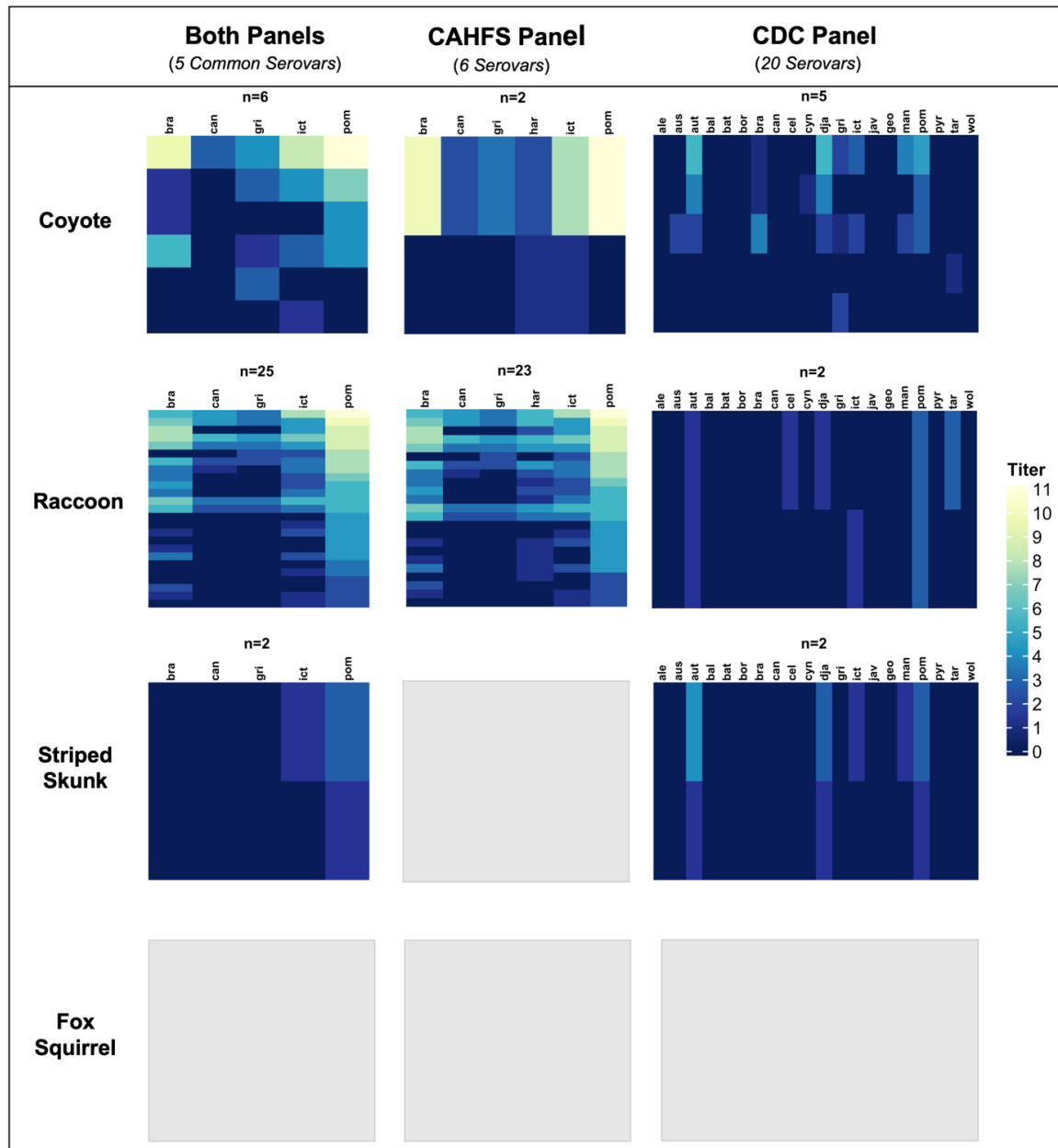


Figure 6.10. Serovar reactivity profiles for target species outside the Los Angeles region, displayed by laboratory. All seropositive data are displayed for the serovars tested at both laboratories (left), CAHFS (middle) and CDC (right). Gray boxes represent a lack of data. Each row of colored bars represents MAT titers for an individual animal, with rows ordered by titer values for the serovar with the highest maximum titers in each species (serovar *Pomona* for coyotes, raccoons and skunks; serovar *Icterohaemorrhagiae* in fox squirrels). The colorbar legend shows antibody titer on a log₂ scale (1:100 equivalent to 1, 1:200 equivalent to 2, 1:400 equivalent to 3, etc.). Serovar results shown: Alexi ('ale'), Australis ('aus'), Autumnalis ('aut'), Ballum ('bal'), Bataviae ('bat'), Borincana ('bor'), Bratislava ('bra'), Canicola ('can'), Celledoni ('cel'), Cynopteri ('cyn'), Djasiman ('dja'), Grippotyphosa ('gri'), Hardjo ('har'), Icterohaemorrhagiae ('ict'), Javanica ('jav'), Georgia ('geo'), Mankarso ('man'), *Pomona* ('pom'), *Pyrogenes* ('pyr'), Tarassovi ('tar'), Wolffi ('wol').

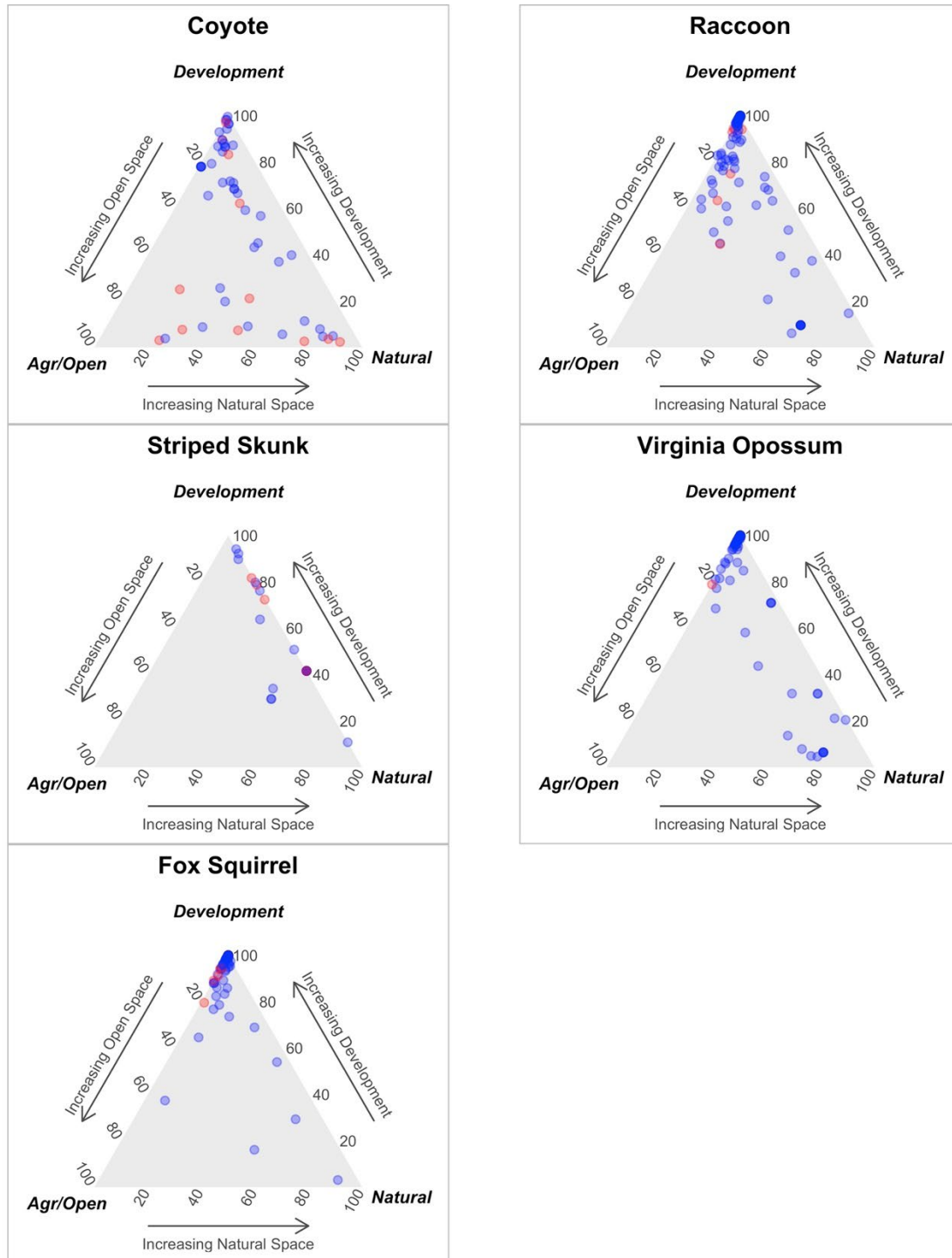


Figure 6.11. *Leptospira* exposure relative to land classification for target species in the greater Los Angeles region. Positive (red) and negative (blue) anti-*Leptospira* antibody (MAT) results are plotted relative to the proportion of natural, agricultural/open and developed land calculated within the home range buffer of each individual.

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7. Detection of *Leptospira kirschneri* in a short-beaked common dolphin (*Delphinus delphis*) stranded off the coast of southern California, USA

7.1. Introduction

Leptospirosis, the disease caused by infection with pathogenic species within the genus *Leptospira*, is a globally important zoonosis (Faine et al., 1999). There are 38 pathogenic *Leptospira* species and hundreds of known pathogenic serovars, each with slightly different characteristics, host affinity/tropisms, and host-pathogen interactions (Ellis, 2015; Faine et al., 1999; Sykes et al., 2022; Vincent et al., 2019; Wunder et al., 2018; Zuerner, 2015). Broadly, *Leptospira* can infect all mammals, and infection has been detected by culture, hamster inoculation and polymerase chain reaction (PCR) in a range of frogs, snakes and turtles (Biscola et al., 2011; Diesch et al., 1966; Ellis, 2015; Glosser et al., 1974; Gravekamp et al., 1991; Pérez-Flores et al., 2021; Rockwell et al., 2019; Rodamilans et al., 2020; Verma et al., 2022). The full range of host-pathogen interactions for *Leptospira* is still being uncovered, with significant challenges arising from the diversity of pathogen *Leptospira* strains, logistic hurdles in collecting samples from possible hosts, weaknesses in available tools for diagnosis and strain identification, and the broad spectrum of clinical presentations that accompany infection.

Knowledge of infection and transmission of *Leptospira* is based mostly on studies of terrestrial mammals. In mammalian hosts, infections can be completely subclinical, or they can present with clinical signs ranging from flu-like symptoms (fever, muscle aches, headache) to pulmonary manifestations, reproductive failure, liver or renal failure, and finally death (Adler, 2015a; Ellis, 2015; Faine et al., 1999; Zuerner, 2015). In infected hosts, leptospires ultimately colonize the kidneys and are shed in urine, and shedding can continue for months to years in some individuals or host species (Ellis, 2015). Leptospires may also colonize other sites including reproductive tissues, providing an additional route of transmission (Ellis, 2015), which may be especially important in obligate aquatic marine species. Clinical disease in infected hosts is due to the damage caused by leptospires during initial systemic infection and eventual tissue colonization, as well as the host inflammatory response against the pathogen (Faine et al., 1999). Gross and histopathologic lesions detected in cases of leptospirosis can vary depending on host and infecting species, and reflect the infectious and immune responses (Adler, 2015b). However, renal lesions are typically seen and are characterized by acute tubular nephritis and lymphocytic, plasmacytic nephritis or glomerulonephritis (Ellis, 2015; Haake & Levett, 2015; Haake & Zückert, 2015; Zuerner, 2015). Infectious leptospires are shed in urine, and the most common routes of transmission are direct contact with urine or indirect contact with contaminated soil or water; intact skin is a strong barrier to infection, but damaged skin and host mucous membranes are important routes of infection (Gostic et al., 2019). Vertical transmission can occur, and sexual contact or contact with infectious aborted tissues can also lead to transmission (Ellis, 2015). Leptospire survival in the environment varies by *Leptospira* species and genotype, as well as by environmental conditions, with survival ranging from hours to as long as months (Bierque et al., 2020; Casanovas-Massana et al., 2018; Khairani-Bejo et al., 2004). Some of the shortest reported survival times were recorded for seawater (Khairani-Bejo et al., 2004) and salts may be inhibitory for pathogenic *Leptospira* in the absence of nutrients (Trueba et al., 2004).

In marine mammals, reports of *Leptospira* have focused predominantly on pinnipeds, with isolated reports of infections in cetaceans. *Leptospira interrogans* serovar Pomona has been circulating endemically in California sea lions (*Zalophus californianus*), with seasonal outbreaks occurring yearly since at least 1984 (Greig et al., 2005; Gulland et al., 1996; Lloyd-Smith et al., 2007; Prager et al., 2020; Zuerner et al., 2009; Zuerner & Alt, 2009). *Leptospira* in other pinnipeds has also been documented, especially in the eastern Pacific Ocean, with seropositivity or infection reported in Northern fur seals (*Callorhinus ursinus*), Northern elephant seals (*Mirounga angustirostris*), Pacific harbor seals (*Phoca vitulina*) and Steller sea lions (*Eumetopias jubatus*) on the west coast of the United States (northeastern Pacific Ocean), South American sea lions (*Otaria byronia*) on the coast of Chile, and manatees (*Trichechus inunguis*) in the Peruvian Amazon (Cameron et al., 2008; Colegrove et al., 2005; Delaney et al., 2014; Delgado et al., 2015; Greig et al., 2014; Gulland et al., 1996; Lloyd-Smith et al., 2007; Prager et al., 2020; Sepúlveda et al., 2015; Smith et al., 1974, 1977; Stamper et al., 1998). In contrast, reports of *Leptospira* infection in cetaceans are quite rare, and *Leptospira* infection or seropositivity has never been detected in a cetacean host in the northeastern Pacific Ocean. Globally, only four cetacean species have been shown definitively to be infected with *Leptospira*: two isolates with 98 and 99% similarity to *L. interrogans* serovar *Copenhageni* were cultured from kidneys of a Fraser's dolphin (*Lagenodelphis hosei*) and a melon-headed whale (*Peponocephala electra*) from the Philippines in 2017 (Obusan et al., 2019); an isolate that, based on sequence results, is suspected to be *L. interrogans* was cultured from the kidney of a newborn Southern Right Whale (*Eubalaena australis*) calf that stranded dead in Argentina in 2010 (Grune Löffler et al., 2015); and an isolate of *L. interrogans* serovar Pomona was cultured from a bottlenose dolphin (*Tursiops truncatus*) that stranded along the coast of Sardinia Italy in 2016 (Piredda et al., 2020). Serologic evidence of *Leptospira* infection (current or historic) and/or detection using a PCR primer that targets both pathogenic and non-pathogenic *Leptospira* spp. has been reported in an additional nine cetaceans (Obusan et al., 2019).

Here we add to the limited body of knowledge regarding *Leptospira* in cetaceans. We report the first detection of *Leptospira* in a short-beaked common dolphin (*Delphinus delphis delphis*), and the first detection in any cetacean in the northeastern Pacific Ocean. We identify the species as *L. kirschneri* and show that infection was associated with tubulointerstitial nephritis, which was the most significant lesion reported in this animal and was the likely cause of death. Given the extremely limited body of knowledge regarding *Leptospira* in cetaceans and the marine ecosystem in general, this provides critical new data on host range. This report raises pertinent questions about the ecology of *Leptospira* in the marine environment, including how the pathogen transmits between cetaceans and whether these obligate marine hosts play a more significant role in *Leptospira* circulation than is currently recognized.

7.2. Case Presentation

On 28 June 2021, a female, juvenile short-beaked common dolphin stranded alive along the coast of southern California (Ponto Beach, Carlsbad, San Diego County) and subsequently died. The carcass was refrigerated at 4°C until necropsied on 30 June 2021. The necropsy examination included gross evaluation and collection of tissues from the adrenal glands, blubber, brain (cerebrum and cerebellum), colon, diaphragm, duodenum, esophagus, heart, kidneys, liver, lungs, lymph nodes, ovaries, pancreas, skeletal muscle, skin, spinal cord, spleen, teeth, thymus,

urinary bladder, umbilical arteries, and uterus. Grossly within the kidney there was multifocal cortical pallor within reniculi (Figure 7.1). Samples were placed in 10% neutral buffered formalin, processed routinely for paraffin embedding, sectioned at 5 μ m, stained with hematoxylin and eosin (H&E), and examined microscopically.



Figure 7.1. Gross necropsy of short beaked common dolphin infected with *Leptospira*. Top image shows cross-section of the abdominal cavity. Bottom image shows a kidney, with multifocal cortical pallor within the reniculi.

Multifocally, in the cortical interstitium of the kidney, there were few, small to moderately sized aggregates of lymphocytes and plasma cells that surrounded the tubules. Also observed in the renal tissue were occasional tubules with areas of inflammation, that were mildly to moderately dilated and contained pale eosinophilic granular debris or rare necrotic sloughed cells. The most significant lesion noted was mild to moderate multifocal tubulointerstitial nephritis suggestive of leptospirosis. Skeletal muscle changes were observed and may have been

due to stranding related trauma, and mild zymogen depletion in the pancreas was suggestive of recent inanition, possibly due to leptospirosis.

Immunohistochemistry (IHC) of paraffin-embedded kidney sections was performed using a streptavidin-biotin method and a *Leptospira*-specific polyclonal antibody (National Veterinary Services Laboratory, Ames, Iowa, USA) directed against *L. interrogans* serovars Bratislava, Canicola, Copenhageni (Icterohaemorrhagiae), Hardjo, and Pomona, and *L. kirschneri* serovar Grippotyphosa (Colegrove et al., 2005). Multiple renal tubules, both near and far from areas of inflammation contained wispy, IHC-positive antigenic staining in the tubular lumens. We conducted a multi-gene targeted real time polymerase chain reaction (PCR) approach on fresh frozen kidney to detect and differentiate *L. interrogans*, *L. kirschneri*, *L. borgpetersenii* and *L. noguchii*, as described by Ferreira et al., (2014) with the following modification - PerfeCTa Tough Mix Low Rox master mix and appropriate thermocycling conditions for this master mix were used. We also conducted *Leptospira* PCR using an Lfb primer targeting the *lfb* gene, and Lfb sequencing. These methods indicated that the infecting organism most closely resembled *L. kirschneri*.

To enable genomic level species identification and phylogenetic analyses, the extracted *Leptospira* positive kidney DNA was subjected to pan pathogenic *Leptospira* DNA capture and enrichment as described in detail elsewhere (Stone et al., 2023). Briefly, the sample DNA was diluted to 4ng/ μ L in a volume of 40 μ L, sonicated to an average size of 290bp using a Q800R2 sonicator (QSonica, Newtown, CT, USA), and a short-read next-generation library was prepared using Agilent Sure-Select methodology. The library was subjected to two rounds of DNA capture and enrichment and then sequenced on an Illumina MiSeq using a MiSeq Reagent Nano Kit v2 500 cycle kit (2x250).

7.3. Bioinformatic Analysis

To estimate the percentage of *Leptospira* reads in the enriched sequences, reads were mapped against the standard Kraken database with Kraken v2.1.2 (Wood et al., 2019). Reads assigned as *Leptospira* were then extracted and assembled using SPAdes v3.13.0 (Bankevich et al., 2012) with default settings. The BLH0012 assembly was placed into a genus dendrogram with Mashtree v1.2.0 (Katz et al., 2019) to confirm species membership. The large scale Blast Score Ratio (LS-BSR) tool v1.2.3 was used to identify a set of 131 probes that had a blast score ratio (BSR) value (Rasko et al., 2005) of ≥ 0.8 in 35 *L. kirschneri* genomes and < 0.4 in other *Leptospira* genomes (n=620). Reads from the short beaked common dolphin *Leptospira* enriched genome (BLH0012) were mapped against these probes and the breadth of coverage was calculated at 3x depth. Single nucleotide polymorphisms (SNPs) were identified among the BLH0012 enriched genome and 41 publicly available *L. kirschneri* genomes (GenBank accession numbers annotated in Figure 7.2) by aligning reads simulated by ART vMountRainier (Huang et al., 2012) against *L. kirschneri* serovar Valbuzzi str. 200702274 (GCA_000244515.3) with minimap2 v2.22 (Li, 2018) and calling SNPs from the BAM file with GATK v4.2.2 (McKenna et al., 2010) using a depth of coverage $\geq 5x$ and a read proportion of 0.9. All of these methods were wrapped by NASP v1.20 (Sahl et al., 2016). A maximum likelihood phylogeny was inferred on the concatenated SNP alignments using IQ-TREE v2.2.0.3 with default parameters (Minh et al., 2020), and the integrated ModelFinder method (Kalyaanamoorthy et al., 2017); the phylogeny was rooted with *Leptospira santarosai* strain LT821 (GenBank assembly

accession: GCA_000313175.2). To explore the possibility that the *Leptospira* lineage present in this sample has been described previously but perhaps without genomic level resolution, we queried our assembly against the *Leptospira* pubMLST database (Jolley et al., 2018), which contained 217, 925 *Leptospira* allele sequences on the date of access.

7.4. Genetic Results

Post enrichment, 81.45% of the BLH0012 sequencing reads assigned to *Leptospira* (1,323,517/1,624,860). The whole genome dendrogram, which included representative genomes of 63 *Leptospira* species from the P1, P2, S1 and S2 clades (Vincent et al., 2019), three *L. interrogans* serovar Pomona genomes derived from isolates obtained from California sea lions and a Channel Island fox (*Urocyon littoralis*), and the enriched *Leptospira* genome assembly from sample BLH0012 placed it closest to *L. kirschneri* (Figure 7.2). Of 131 *L. kirschneri* specific 120bp RNA probes that were included in our enrichment system, 87 were identified in the BLH0012 enrichment with $\geq 3\times$ coverage, providing further supporting evidence that this unknown *Leptospira* is most similar to *L. kirschneri* and falls within the phylogenetic clade of that species.

To more definitively assess that relationship, we built a SNP phylogeny among the BLH0012 *Leptospira* sequences and 41 *L. kirschneri* genomes; this analysis placed the dolphin sequence the *L. kirschneri* genomes, confirming the species identification although the long branch length (7893 unique SNPs) suggests it is a divergent lineage (Figure 7.3). Finally, our query of the enriched assembly against all *Leptospira* loci in pubMLST revealed the closest allelic matches were to *L. kirschneri*, however there was not a perfect match to any known *Leptospira* alleles at any locus; 6/7 loci were identified from *Leptospira* MLST schemes 1 (*tpiA* was absent) (Boonsilp et al., 2013) and 3 (*rrs* was absent) (Ahmed et al., 2006) and 7/7 loci were identified from scheme 2 (Varni et al., 2014). Overall, our analyses of the *Leptospira* genome enriched from dolphin sample BLH0012 suggest it is a divergent lineage of *L. kirschneri*, previously undescribed.

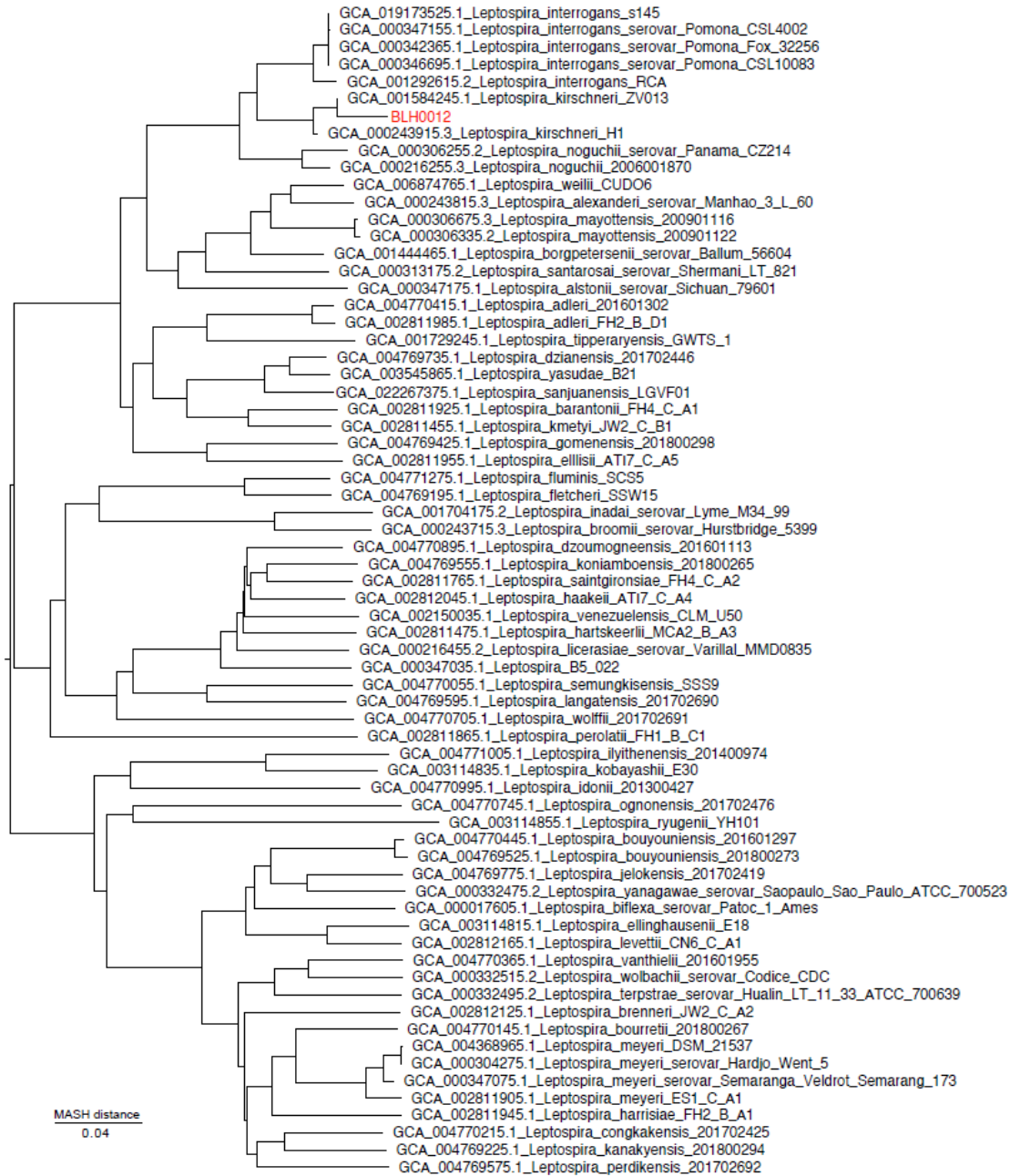


Figure 7.2. Whole-genome dendrogram of *Leptospira* genomes, showing dolphin sequence within clade of *L. kirschneri* isolates. The dendrogram includes representative genomes of 63 *Leptospira* species from the P1, P2, S1 and S2 clades, three *L. interrogans* serovar Pomona genomes derived from isolates obtained from two CSL and a Channel Island fox, and the enriched *Leptospira* genome assembly from sample BLH0012.

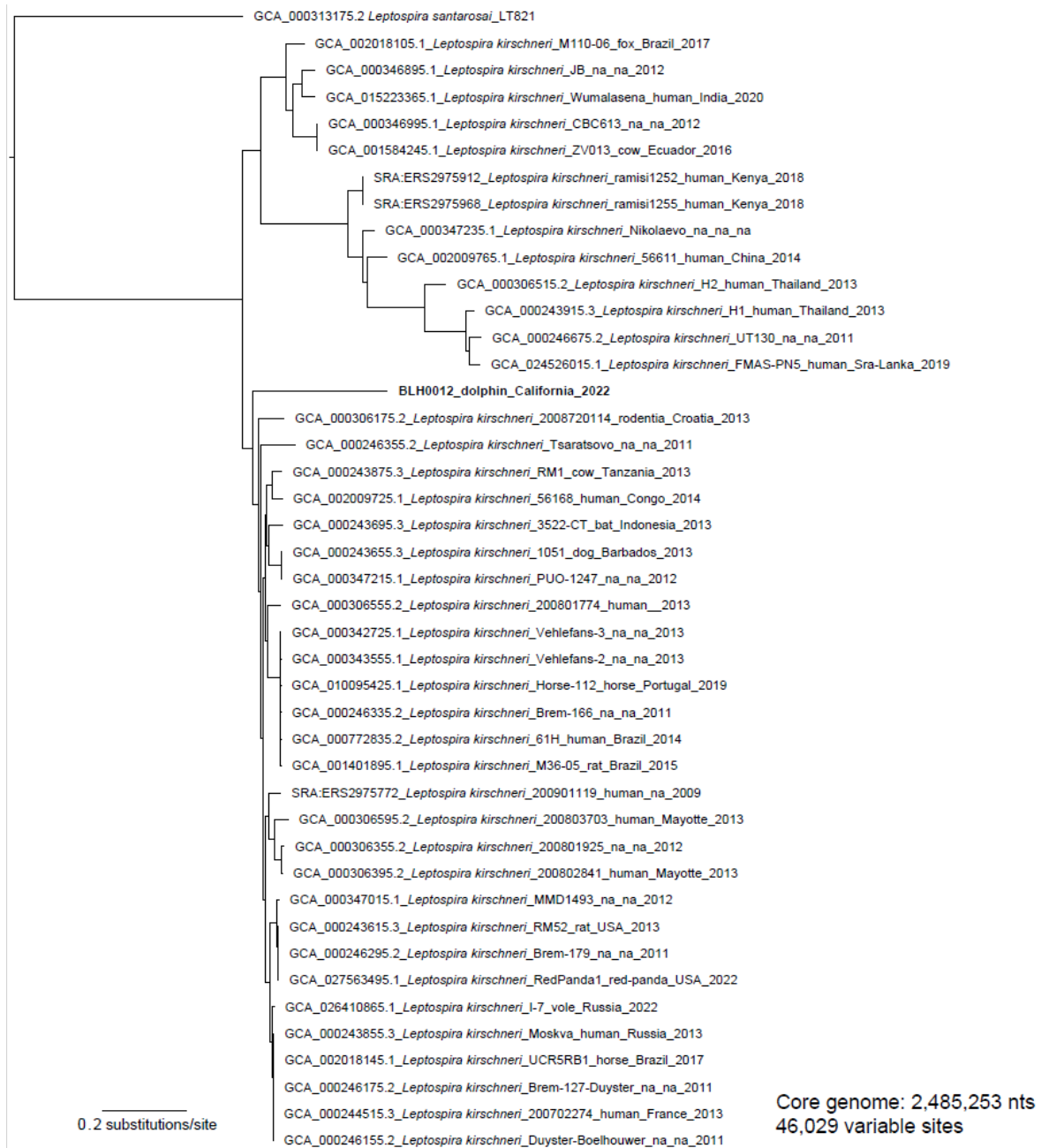


Figure 7.3. Phylogeny of *L. kirschneri* genomes, showing that dolphin sequence is a divergent lineage of *L. kirschneri*. SNP phylogeny including BLH0012 *Leptospira* sequences and 41 *L. kirschneri* genomes. The dolphin sequence falls among the *L. kirschneri* genomes, with long branch length (7893 unique SNPs) suggesting that it is a divergent lineage.

7.5. Discussion and Conclusions

We report the first detection of *Leptospira* infection in a juvenile short-beaked common dolphin, which is the first detection of *Leptospira* in any cetacean from the northeastern Pacific Ocean. Renal lesions identified on histopathology were consistent with clinical leptospirosis in other host species, including marine mammals (Cameron et al., 2008; Colegrove et al., 2005; Ellis, 2015; Gulland et al., 1996; Haake & Levett, 2015; Haake & Zückert, 2015; Knowles et al., 2020; Smith et al., 1977; Stamper et al., 1998; Zuerner, 2015), and were the most significant lesions detected, suggesting leptospirosis as the most likely cause of death. We identified the isolate as a divergent lineage of *L. kirschneri*, a species that has been detected only once before in the northeastern Pacific, or in any marine host, in a single northern elephant seal in 2004 (*Mirounga angustirostris*; (Cameron et al., 2008). While antibody reactivity against *L. kirschneri* serovar Grippotyphosa has been detected via microscopic agglutination testing (MAT) in a number of marine mammals from the northeastern Pacific, including Pacific harbor seals (*Phoca vitulina*), California sea lions, and northern elephant seals (Colegrove et al., 2005; Delaney et al., 2014; Greig et al., 2014; Stamper et al., 1998), titers against other *Leptospira* species and serovars were typically also seen in the same individuals. The MAT cannot be used to definitively identify the infecting species or serovar as *Leptospira* antibody cross-reaction is common and not all *Leptospira* species are included in MAT panels. Exposure to unknown species of *Leptospira* has also been detected via MAT in sea otters (*Enhydra lutris*; Goldstein et al., 2011; Hanni et al., 2003; Knowles et al., 2020; White et al., 2017).

Infection with *L. kirschneri* distinguishes this observation from other observations of *Leptospira* infections in marine mammals of the northeast Pacific Ocean. *Leptospira interrogans* serovar Pomona has caused yearly, seasonal outbreaks in California sea lions for decades, with infections ranging from subclinical to deadly (Greig et al., 2005; Gulland et al., 1996; Lloyd-Smith et al., 2007; Prager et al., 2020; Zuerner et al., 2009; Zuerner & Alt, 2009). Confirmed infections with *L. interrogans* serovar Pomona have also been detected by culture in northern elephant seals (Delaney et al., 2014) and northern fur seals (*Callorhinus ursinus*; Smith et al., 1974, 1977), and by PCR in a Steller sea lion (*Eumetopias jubatus*; Cameron et al., 2008). The different species of pathogen means there is no direct connection between these observations of *Leptospira* in marine mammals in the northeastern Pacific, but it is important to note that sampling and testing have been limited. Further *Leptospira* surveillance of marine mammals, including short-beaked common dolphins, is needed to identify other hosts carrying closely related strains of *L. kirschneri*, which would indicate potential intra- or interspecies transmission linkages. It is also possible that the dolphin was infected by cross-ecosystem spillover transmission from a terrestrial host (Borremans et al., 2019); *L. kirschneri* has been detected in a range of terrestrial species, including rodents, horses, cattle, dogs, humans and wild boar (Allan et al., 2015; Azhari et al., 2018; Cilia, Bertelloni, et al., 2020; Guedes et al., 2021; Guyader et al., 2020; Halliday et al., 2013; Harkin & Hays, 2016; Iverson et al., 2021; Jaeger et al., 2019; Masuzawa et al., 2006; Morgan et al., 2002; Philip et al., 2020; Salaun et al., 2006; Schmidt et al., 2021; Soares et al., 2020).

Confirmed infection of a cetacean raises interesting questions about the mechanism of transmission, given the obligate marine lifestyle of cetaceans (in contrast to pinnipeds, which spend time on land) and the commonly accepted view that *Leptospira* are quickly killed by salt water. If there is intraspecies transmission among short beaked common dolphins, it could be occurring via vertical or sexual transmission, preventing exposure to salt water (though sexual

transmission is unlikely for the juvenile individual in this study). Expanded surveillance and testing in this species would be needed to assess whether infection prevalence, age distribution, and tissue distribution are consistent with this possibility. If transmission occurred via the environment, whether from another marine host (of the same or different species) or cross-ecosystem spillover from a terrestrial host, the pathogen would need to survive for some period in sea water. *Leptospira* are generally understood to survive poorly in salt water (Bierque et al., 2020; Khairani-Bejo et al., 2004; Trueba et al., 2004). Some researchers have reported halophilic or halotolerant pathogenic *Leptospira* species; yet in all of these cases isolates were cultured in nutrient rich media of varying salinity which was often less than that of sea water (Cilia, Fratini, et al., 2020; Grune Löffler et al., 2015; Obusan et al., 2019). In addition, work by Trueba et al., (2004) suggests that the inhibitory impacts of salinity are most important under starvation conditions (i.e. what would be experienced in the ocean), hence in the absence of nutrient rich media, the reported halotolerant and halophilic species are unlikely to survive long. Finally, (Saito et al., 2014) showed that isolates of the pathogenic species *L. kmetyi* were killed within 12 hours in 3% NaCl solution (i.e. the same salinity of the ocean), but were able to survive 3-4 days if incubated with soil overnight. These data, together with considerations of rapid dilution in circulating ocean water, suggest that the window of opportunity for environmental transmission would be quite short for dolphins. However, under certain optimal conditions (large aggregations of animals, or behaviors involving particularly close contact with urine) environmental transmission might occur in marine settings. This could have implications for understanding *Leptospira* ecology in pinnipeds as well, where transmission has broadly been assumed to occur while animals are hauled out on land.

Ultimately, to better understand the extent to which *Leptospira* infections occur in the marine ecosystem and the epidemiological linkages between and among marine and terrestrial host species, surveillance and sampling must be expanded across these ecosystems. Sequencing of *Leptospira* genomes from different host species will yield crucial information about possible transmission links, either through sequencing of isolates obtained via culture of prospective samples, or by application of DNA enrichment techniques to both banked and prospectively collected samples that test positive by PCR. The advent of these new techniques ushers in a new era for understanding and untangling the complex ecology of this important zoonotic pathogen.

7.6. References

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8. Leveraging a natural fadeout event to estimate long-term antibody decay of an endemically circulating pathogen in a wildlife system

8.1. Introduction

The measurement of antibodies in blood is a critical tool in the field of wildlife disease ecology. Antibodies persist longer than most other biomarkers of infection and can usually be detected with relatively limited resources when compared to molecular techniques. Because of that, serological data has been widely relied upon to infer many aspects of the temporal or spatial dynamics of wildlife disease systems.

In the past, serological tests were preoccupied primarily with detection and diagnosis, which is mostly satisfied with a binary test result indicating presence or absence of a specific antibody in the sample. More recently, a growing field of quantitative serology has recognized that antibody concentration, rather than binary results, can be used to parameterize more detailed models of titer kinetics connecting individual and population infectious disease biology. These models can provide valuable insight and generate improved estimates of important variables that are not directly observable, such as individual time since infection (Borremans et al., 2016), force of infection (the rate at which susceptible individuals become infected) (Reiner et al., 2014), and total incidence (Teunis et al., 2012).

Immunological studies have found that in the absence of repeated antigenic stimulation, individual titer kinetics generally transitions between an initial phase of abundant production of antibodies by short-lived cells to a regime of slower decay where a lower titer is maintained by long-lived plasma cells (Amanna & Slifka, 2010). The long-term antibody decay rate is particularly relevant to ecological applications of titer kinetics models, but challenging to estimate from the data we are typically able to collect. This rate determines how individual and population-scale serologic indicators will vary through time in the timescale that is often most relevant to the ecology of the system. For ethical and practical reasons, captive animal studies rarely last long enough to collect longitudinal data on long-term titer kinetics, and the complications and uncertainties of field data are only made worse when we consider a span of multiple years.

Another important aspect of dealing with serologic data is the conditions under which the host animal was sampled. In natural conditions, we rarely have the opportunity to obtain samples from the same animal post-exposure, and describing individual titer kinetics from cross-sectional data presents many challenges. Furthermore, these animals are susceptible to unknown amounts of repeated exposure which can significantly alter the antibody response in ways that are highly dependent on both host and pathogen. Longitudinal studies of captive animals provide some of the best data we can hope for to build titer kinetics models, so naturally many parameters estimated from longitudinal data are employed in models of disease dynamics in the wild. However, captive animals experience very different conditions compared to their free-ranging kin who are exposed to environmental conditions that can directly impact immune function, specially through changes in nutrition (Acevedo-Whitehouse & Duffus, 2009; Derango et al., 2019).

If we wish to describe titer kinetics in accordance with the data we are able to collect, it is important we do not overlook technical aspects of how titers are measured. Although widely accepted criteria for testing and diagnosis have long recognized the problem of measurement error in serologic assays (Cauchemez et al., 2012; Teunis et al., 2012), a frontier in modeling serological data is to deal with this process by incorporating an explicit observation model that describes the serologic assay and the errors it may introduce. Specific serological assays can differ significantly for example in which antibodies can be detected, how precise the test is to the target pathogen, what the limit of detection is, and even what the magnitude really represents (Gilbert et al., 2013), and models must be built with understanding and consideration of the data generating process.

In this study we have leveraged a uniquely valuable opportunity to estimate long-term antibody kinetics of disease in a wildlife system. We use data from a period when endemically circulating pathogenic bacteria *Leptospira interrogans* disappeared from California sea lions (*Zalophus californianus*) after multiple decades of yearly seasonal outbreaks of varied intensity (Figure 1) (Gulland et al., 1996; Lloyd-Smith et al., 2007). Intensified sampling starting in 2010 has found strong evidence that the disease naturally stopped circulating in the system starting in early 2013. The disease re-emerged in 2017, ultimately causing one of the largest outbreaks on record in 2018. We refer to this period from 2013 to 2017 as the fadeout period.

This wildlife system has received considerable attention due to a very rare 24-year time series of age-structured serology, paired with decades of data on demography and incidence of leptospirosis and other mortality inducing conditions (DeLong et al., 2017). Studies of this system have relied on serological data as the main source of information on the circulation and impact of leptospirosis, and quantitative modeling approaches have the potential to tackle some of the harder inherent challenges presented by this type of data.

A robust data-driven estimate of how antibody kinetics proceeds within a host is a key component of quantitative serology analysis. Here, we provide a better estimate of the long-term antibody decay rate in this system, which can be used to derive even more value from an established and expanding dataset. Studies aiming to investigate the disease dynamics of leptospirosis in CSL have dealt with uncertainty about re-exposure and long-term antibody decay by restricting the dataset to younger animals, or using estimates based on longitudinal data. Here we provide a quantitative estimate of long-term antibody decay rates that will enable future studies to use serology data over longer time spans more accurately.

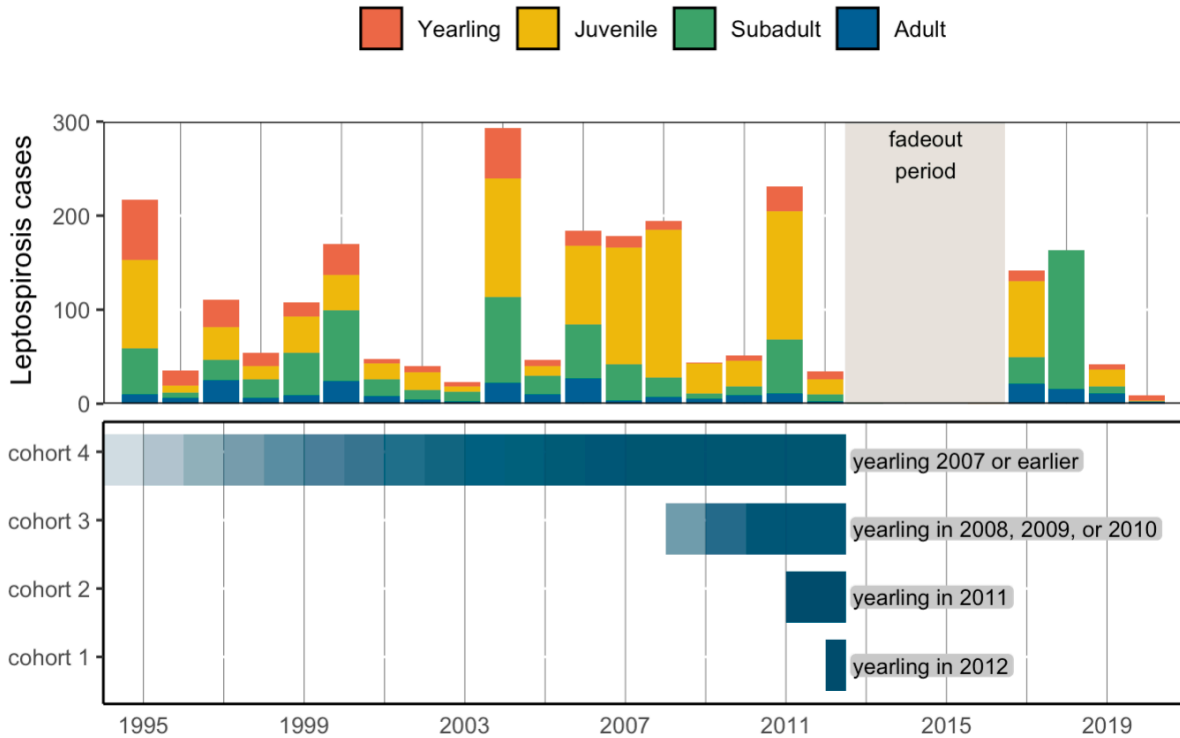


Figure 8.1. Age-structured incidence of leptospirosis in CSL, and definition of cohorts. Total observed incidence of acute leptospirosis in CSL in central California showing the “fadeout” period from 2013 to 2017 where no cases were diagnosed in the system despite increased sampling effort. Horizontal bars on the bottom show the pre-fadeout period during which animals of each age cohort could have been exposed to leptospirosis based on their age class at time of sampling. Animals in cohort 1 could only have been infected during the 2012 outbreak because that was the only pre-fadeout year where they were alive and mixing with the general population (assuming no disease circulation occurs in pre-weaned pups). Cohort 2 includes animals old enough to have been exposed in 2011 and 2012. Cohort 3 includes animals that could have been exposed starting in 2008, and cohort 4 includes all adult animals, for which we do not have an upper boundary on age (other than expected natural lifespan). For Cohorts 3 and 4, where a range of birth years is possible for each animal, transparency indicates that not every animal in that cohort was alive during the year in the x axis.

8.2. Data

We consider serological data collected from California sea lions during a period when the endemic circulation of *Leptospira interrogans* serovar Pomona was interrupted in this population. While the precise start and end dates of this interruption are unknown, in this study we consider the period from 2013-01-01 to 2017-01-01 and henceforth call it the 'fadeout period'. We analyze all available data from the fadeout period, with the following restrictions: (i) we exclude samples for which endpoint titers are not available; (ii) we exclude three samples from stranded sea lions flagged as potential leptospirosis cases, which can be attributed either to misdiagnosis or a failed re-introduction of the disease to the system; (iii) we exclude samples from animals born in 2012 or later, which were too young to have been exposed to *Leptospira* in

the last outbreak before the fadeout period, in fall 2012. Prior studies have found evidence of low to no exposure to *Leptospira* in the rookeries where sea lion pups spend their first year, and no sign of leptospirosis in young pups (Greig et al., 2005), so it is accepted that widespread exposure to *Leptospira* is limited to yearlings, (i.e. 1-year-olds) and older age classes. Seronegative samples from animals old enough to have been exposed prior to the fadeout are included, since those could have decayed below the limit of detection and therefore provide some information on long-term waning.

Serum microscopic agglutination tests (MAT) were performed at the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, using a live-cultured reference strain of *Leptospira interrogans* serovar Pomona, and titrated in two-fold dilutions from 1:100 to endpoint. Endpoint titer for the MAT assay is defined as the highest two-fold dilution of the serum sample at which the sample exhibits at least 50% agglutination with the reference strain. Titer results are sometimes $\log_2$ transformed for ease of interpretation using the following formula: $\log_2(\text{titer}/100) + 1$, thus a titer of 1:100 = 1, 1:200 = 2, 1:400 = 3, etc. Serovar Pomona is the only strain to have ever been isolated from this population in decades of active sampling (Zuerner & Alt, 2009), and historically has almost always been the highest MAT titer in samples for which a panel of multiple serovars was performed (Prager et al., 2013).

Our study uses 1,263 endpoint MAT titers from individual California sea lions of which 610 were wild-caught and 653 stranded along the central coast of California. Stranded animals were rescued, diagnosed, treated, and sampled by staff at The Marine Mammal Center (TMMC) in Sausalito, California. Animals stranded during the fadeout period for a variety of reasons including malnutrition, trauma, and pneumonia, but not acute leptospirosis. Wild-caught animals are seemingly healthy individuals caught across the coast of California and in two locations in Oregon and Washington and sampled under the authorization of Marine Mammal Protection Act Permit No. 932-1905-00/MA-009526.

8.3. Methods

8.3.1. Modeling Framework

We developed a mathematical model to predict the antibody concentration in each serum sample, based on sample date and animal age, using prior knowledge of disease dynamics and antibody decay in this system. A second mathematical model converts the antibody concentration into a predicted MAT titer value. These predicted titers are compared to measured titers (the data) to fit the model to infer model parameters including the long-term antibody decay rate.

A fundamental quantity in our modeling framework is AC_{50} , the concentration of antibody at which 50% agglutination is observed, given standardized procedures for the MAT assay and perfect observation. Any concentration of antibodies can be expressed in AC_{50} units, such that concentrations above 1 AC_{50} unit will cause >50% agglutination, and concentrations below 1 AC_{50} unit will cause <50% agglutination. We will also use $\log_2 AC_{50}$ units, where a value of 0 corresponds to 50% agglutination.

8.3.2 Model to predict MAT titer from antibody concentration

We relate predicted antibody concentrations to the observed MAT titer values using a model of the MAT observation process, as represented in Figure 8.3. All our MAT titer data were generated using a standard MAT protocol starting with a 1:100 serum dilution and proceeding with two-fold dilutions until less than 50% of the live *Leptospira interrogans* serovar Pomona cells added agglutinate. The amount of agglutination is estimated visually using dark-field microscopy. If the observed dilution agglutinates above the 50% threshold, then another two-fold dilution is performed and inspected, otherwise the test is concluded, and a result is recorded. The reported value is the last dilution that read above 50% agglutination (Figure 3). Equivalently, if the MAT titer is expressed using the standard $\log_2$ transformation, the titer corresponds to the number of dilutions that read above the AC_{50} (Figure 3). Therefore, for an MAT of k to be observed, k dilutions must have been observed to agglutinate over 50% of the bacteria and dilution $k+1$ must have agglutinated below 50%. Further dilutions may be performed beyond $k+1$, for example in the case where an entire plate is used with the number of wells exceeding $k+1$, but typically that information is not recorded by the tester.

Agglutination assays are not deterministic. Sources of random variation include the visual perception of the tester, small variations in concentration due to sample manipulation, laboratory conditions, or any stochasticity inherent to the agglutination process. To construct a model of this measurement noise, we first calculate the true underlying antibody concentration in the diluted sample, a/d_i , where a is the antibody concentration in the undiluted serum sample (in AC_{50} units) and d_i is the dilution factor (e.g. $d_0 = 100$ at 1:100 dilution, $d_1 = 200$ at 1:200 dilution, etc). To model MAT measurement noise, we treat the amount of agglutination observed for a given sample at a given dilution level as probabilistically related to the true antibody concentration. That is, we assign each dilution i a realized “effective antibody concentration” c_i in $\log_2 AC_{50}$ units, which is normally distributed about the true concentration in the dilute sample a/d_i with standard deviation σ_m . More than 50% agglutination is observed whenever this effective concentration $c_i > 0$ in $\log_2 AC_{50}$ units (i.e. the effective concentration is above the 50% agglutination threshold, since 0 in $\log_2 AC_{50}$ units equals 1 AC_{50}), and less than 50% agglutination will be observed whenever $c_i < 0$ in $\log_2 AC_{50}$ units.

Formally, c_i is a normally distributed random variable with units of $\log_2 AC_{50}$

$$c_i \sim \text{Normal}\left(\frac{a}{d_i}, \sigma_m\right) \quad (1)$$

and we observe 50% agglutination if and only if $c_i > 0$.

Recall that the MAT titer result is the last dilution that read above 50% agglutination. The probability of observing an MAT titer of k (in $\log_2$ units) is therefore:

$$\begin{aligned} P(\text{MAT} = k | \text{true titer} = a) &= P(c_0 > 0)P(c_1 > 0) \dots P(c_{k-1} > 0)P(c_k < 0) \\ &= \left(\prod_{i=0}^{k-1} P(c_i > 0) \right) P(c_k < 0) \end{aligned} \quad (2)$$

The total log of the probability in equation (2) given that the c_i are normally distributed as in equation (1) is:

$$\log(P(k|a, \sigma_m)) = \sum_{i=0}^{k-1} \text{Normal_lccdf}(0|a - \log_2 100 - i, \sigma_m) + \text{Normal_lcdf}(0|a - \log_2 100 - k, \sigma_m) \quad (3)$$

where $\text{normal_lcdf}(x | \mu, \sigma)$ represents the log of the normal cumulative distribution function at x with mean μ and standard deviation σ and normal_lccdf represents the log of the normal complementary cumulative distribution function with equivalent parametrization. We subtract $\log_2 100$ to account for the first dilution tested (1:100). We place a half-normal prior on the standard deviation parameter, $\sigma_m \sim \text{Half-Normal}(1, 0.25)$, to reflect the noise in the agglutination assay, with hyperparameters chosen such that there is a reasonable chance that the MAT result is off by 1 unit but a small chance that it is off by 2 units or more, in alignment with empirical observations of replicate MAT measurements.

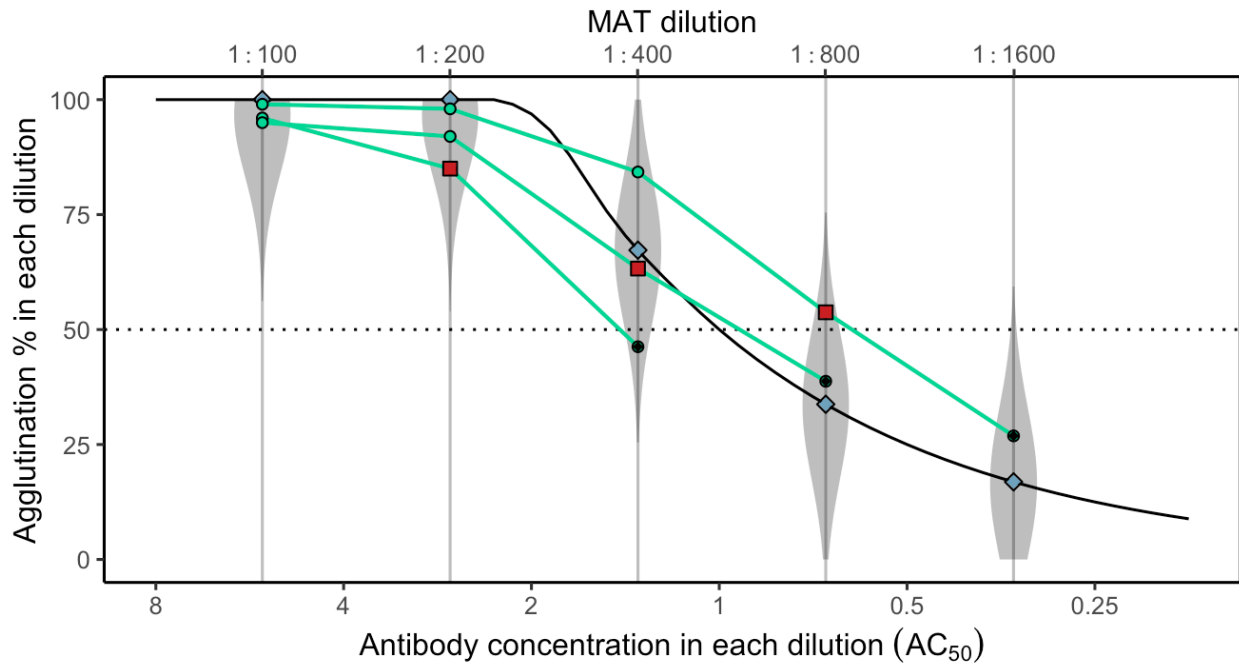


Figure 8.2. Probabilistic model for MAT serology assay. Schematic representation of the MAT observation process for one hypothetical sample tested three separate times, each time yielding a different MAT titer due to small variations in the amount of agglutination read for each dilution. Each successive point in a connected green line shows on the y-axis a decreasing amount of agglutination observed as the sample is increasingly diluted to the ratios shown in the top x-axis. The bottom x-axis shows the antibody concentration in each diluted sample in AC₅₀ units. Blue diamonds represent the (unobserved) ground truth agglutination per dilution for this hypothetical sample, which if measured without noise via MAT should result in a read of 1:400. Circles and squares represent observations of the amount of agglutination in 3 realizations of the MAT process for the same sample. The gray violin plots represent possible random variability in the observed amount of agglutination in the assay for every dilution performed. Black circles represent the last dilution performed for each test, which is the first dilution to register less than 50% agglutination. Red squares represent the measured test results of 1:800, 1:400, and 1:200.

8.3.3 Model to predict antibody concentration from sample date and animal age

Each sample (corresponding to a unique animal) is classified into a birth-year cohort based on the age of the animal at time of sampling (Figure 1). This classification is based on the age class attributed to the animals and previous studies showing that most California sea lions are born in June of each year (Melin et al., 2012). For older age classes, which span several years of age and hence several possible birth years, we consider the earliest possible birth-year for that age class (up to a maximum of 20 years before the sample date, for adults).

Because of the lack of resolution for older animals, and the inability to distinguish primary versus boosted antibody responses in MAT data, we pooled the older cohorts for further analysis (Figure 1A). We define four cohorts: cohort 1 includes animals born in 2011, so they

were yearlings in 2012, and hence had exposure to only one leptospirosis outbreak before the fadeout began; cohort 2 includes animals born in 2010, so they were yearlings in 2011, and had exposure to outbreaks in 2011 and 2012; cohort 3 includes animals that were yearlings in 2008, 2009 or 2010 and had exposure to several outbreaks before 2011 and 2012; and cohort 4 includes all older animals, who had potential exposure for many years before the fadeout.

We then take each datapoint and its cohort classification and generate predicted antibody concentrations based on four scenarios representing the key exposure histories for any animal sampled during the fadeout: last exposed in 2012 (a_{12}), last exposed in 2011 (a_{11}), last exposed prior to 2011 (a_{waned}), or naive (a_0). Predicted antibody concentrations are generated by the model in $\log_2 AC_{50}$ units. Because the model generates continuous antibody concentration values, negative values are possible, representing sample concentrations below 1 AC_{50} , which are therefore below the limit of detection of MAT.

For samples from truly naive animals we impute the value $a_0 = -10$ (in $\log_2 AC_{50}$ units) as a baseline negligible amount of agglutination. A strictly positive value is more numerically stable for computation and is plausible since the value has a very high probability of reading an MAT of 0 under our MAT model, and residual non-specific agglutination is even in naïve animals.

For the two most recent exposure histories (a_{12} and a_{11}), we represent antibody kinetics using a biphasic decay model based on results from a few individuals that were sampled longitudinally (Prager et al., 2013, 2020). This study found that anti-*Leptospira* antibody titers in sea lions decay in a pattern of two log-linear phases, with an initial fast decay where titers decay by 4 to 6 MAT $\log_2$ units in a period of less than 90 days, followed by a long period of slow decay during which low positive MAT titers can be detected years after seroconversion [Prager20]. So for a given sample collected from individual j at date t_s , we calculate the predicted antibody concentrations a_{11}^j and a_{12}^j

$$\begin{aligned} a_{12}^j &= \max \left(m_{12}^j - d_f \Delta t_{f,12}^j - d_s \Delta t_{s,12}^j, a_0 \right) \\ a_{11}^j &= \max \left(m_{11}^j - d_f \Delta t_{f,11}^j - d_s \Delta t_{s,11}^j, a_0 \right) \end{aligned} \quad (4)$$

where m is the peak titer, d_f and d_s are the decay rates corresponding to fast decay and long-term slow decay, Δt_f^j is the amount of time spent by j in the fast decay regime, and Δt_s^j is the amount of time spent in the slow decay regime (by the time of sampling). Titers are not allowed to decay below the naive baseline value a_0 . Peak titers m_{11} and m_{12} are sampled from a normal distribution with different mean and standard deviation hyper-parameters for 2012 and 2011.

For older infections where the exposure time is at least 2 years prior to the start of the fadeout, we assume that the slow decay phase began before the fadeout, so we do not model the fast decay phase at all. Instead, for these samples, we impute an antibody concentration at the start of the fadeout and assume that the slow decay regime applies until date of sampling.

$$a_{\text{waned}}^j = \max \left(w^j - d_s \Delta t_{s,\text{waned}}^j, a_0 \right) \quad (5)$$

where $\Delta t_{s, \text{waned}}^j$ is the time interval between the start of fadeout and the day the sample was collected.

Antibody concentration values a_{11} , a_{12} , a_{waned} and a_0 are weighed by quantities f_{11}^i , f_{12}^i , f_{waned}^i , and f_0^i , each representing the estimated fraction of the animals in our dataset infected in 2011, 2012, 2010 or earlier, and never (respectively). These values are specific for each cohort i , matching the age of the animal when the sample was obtained as explained previously. Each f quantity is a beta-distributed parameter in our model, which serves primarily to penalize classifications that are implausible due to animal age.

$$\begin{aligned} \log(P(k|a, f, \sigma_m)) = & \sum_{j=0}^N \log \left(f_{12}^{i(j)} P(k_j | a_{12}^j, \sigma_m) \right) + \log \left(f_{11}^{i(j)} P(k_j | a_{11}^j, \sigma_m) \right) + \\ & \log \left(f_{\text{waned}}^{i(j)} P(k_j | a_{\text{waned}}^j, \sigma_m) \right) + \log \left(f_0^{i(j)} P(k_j | a_0^j, \sigma_m) \right) \end{aligned} \quad (6)$$

In Figure 8.3 we show a visualization of the model output and the inference process. An example data point shown in yellow represents a sample collected in the Fall of 2013 that resulted in an MAT of 5 $\log_2$ units. In panel 3A, we show a sample of model-generated longitudinal trajectories leading to the predicted titers for an animal sampled at that same data under the 4 different classifications explained above. A wide range of possible MAT results is represented, excluding only the highest values which cannot be generated without the possibility of more recent exposures (i.e. active circulation during the fadeout period). Panel 3B exemplifies the process of weighing the possibilities shown in 3A by the age-informed fractions f_j^i , as shown in equation (6). This process all but excludes one of the classifications (waned) which is incompatible with animal age at time of sampling. A very small probability of error in age-class assignment is considered to create a healthier parameter space for the MCMC. Then, using our MAT titer model for the observed sample MAT, we generate the density function shown in 3C, which can then be compared to the distribution of predicted titers in 3D (resulting directly from the outcomes shown in 3B).

8.3.4 Markov Chain Monte Carlo

Parameter estimates come from posterior samples drawn using RStan (Stan Development Team, 2023), which implements a No-U-Turn Sampler Markov Chain Monte Carlo. We assessed convergence by examining trace plots, $\hat{R}$ values, and effective sample sizes. We confirmed appropriateness of prior distributions with prior predictive checks and goodness-of-fit through posterior predictive checks. All code used to perform the analysis is available in our github repository https://github.com/acrgomez/fadeout_titer_model

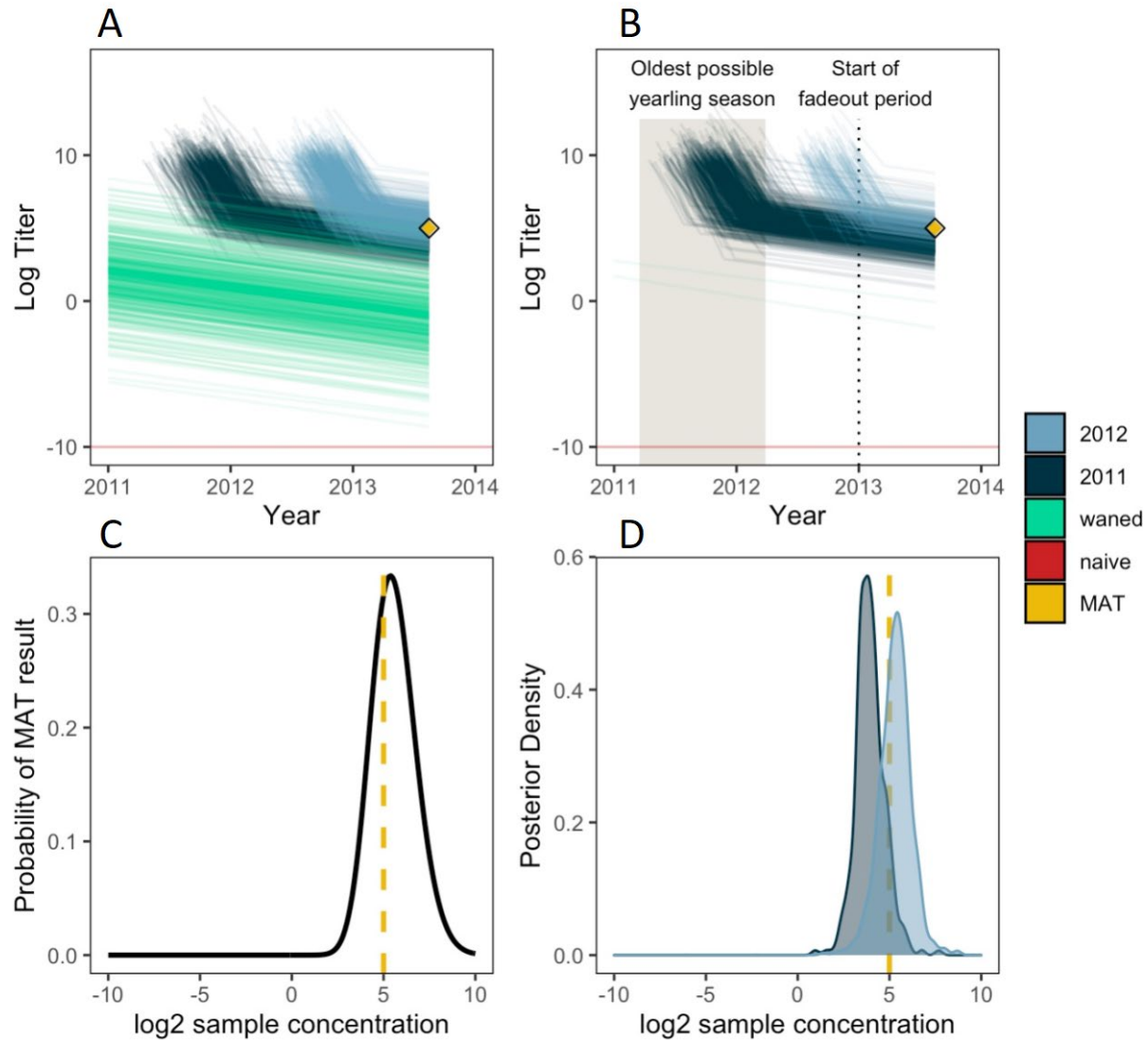


Figure 8.3. Representation of mixture model of antibody titers based on age classified samples.

Panel A. Model proposed antibody decay trajectories for one datapoint. Yellow diamond represents an observed MAT value sampled during the fadeout period. Lines represent 300 randomly selected trajectories for the antibody decay process leading to the day the sample was collected. Colors represent different hypothesis on when the animal was last exposed: 2012, 2011, 2010 or before (waned), or never (naïve). Panel B. Trajectories remaining from panel A when the animal's age is considered. Panel C. Densities of predicted titers at date of sampling for the two most likely infection histories for this animal. Dashed yellow line represents the measured MAT titer in $\log_2$ units. Panel D. Probability of measuring an MAT of 5 in a sample with the antibody concentration shown in the x-axis.

8.4. Results

Of a total of 1326 MAT results from individual California sea lions sampled during the fadeout period from 2013 to 2017, 164 (12.4%) were positive, ranging from 1:100 to 1:12800 (Figure 8.4). Sampled animals were classified as yearlings if between 1 and 2 years of age, juvenile if between 2 and 4, subadult if between 4 and 8, or adult if over 8 years of age when sampled. All age classes are represented but not evenly distributed throughout the fadeout period, since animals too young to have been alive during the last season of regular leptospirosis circulation (Fall 2012) were filtered out of the inference dataset. For that reason, yearlings can only appear in the data in 2013 and juveniles up until 2015, while subadults and adults are present during the entire timeframe.

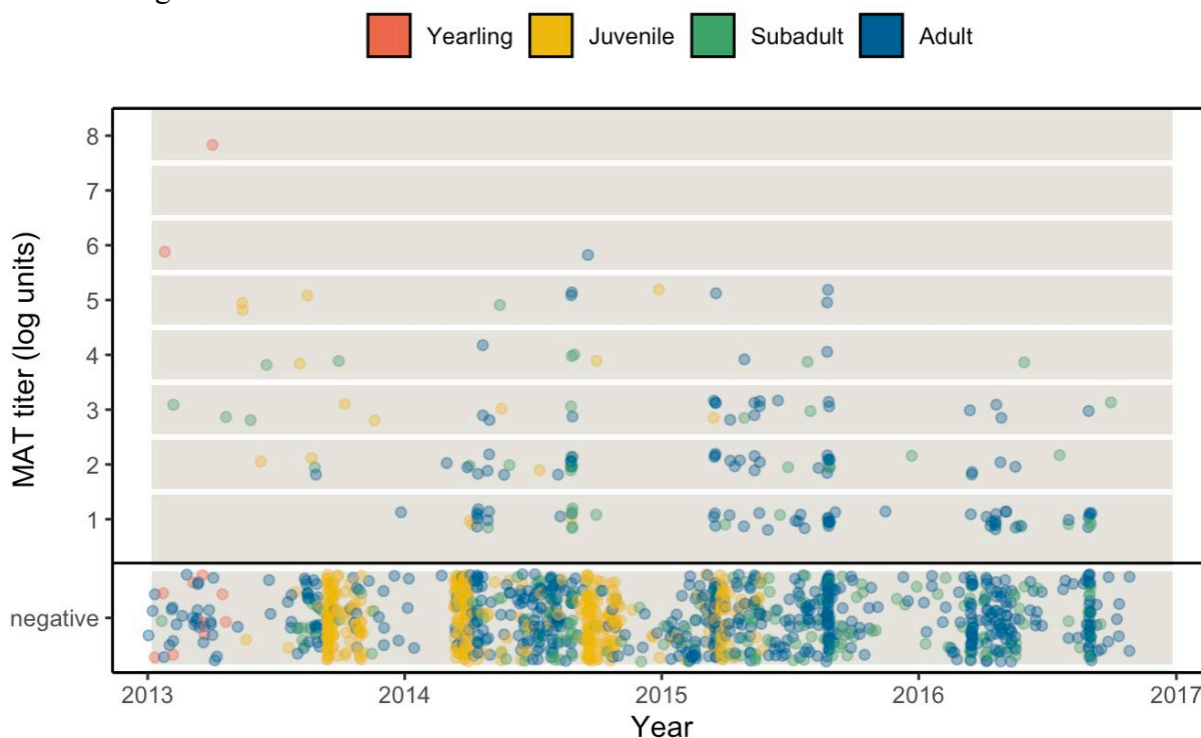


Figure 8.4. Antibody titers against *Leptospira interrogans* serovar Pomona for serum samples obtained from California Sea Lions during the fadeout period. All titers shown are measured by microscopic agglutination test (MAT) using *L. interrogans* serovar Pomona and plotted on a log scale. Significant jitter was added to reduce overplotting and white horizontal lines delimit the jittered values to their corresponding discrete titer on the y axis. “Negative” represents all samples that fail to agglutinate above the usual threshold for MAT at the lowest dilution of 1:100. Colors represent animal age class at time of sampling, defined as yearling (1 to 2 years old), juvenile (2 to 4 years old), subadult (4 to 8 years), or adult (>8 years).

The cross-sectional serology data in Figure 8.4 suggests an overall pattern of decaying antibody concentrations in the absence of reexposure, which is best observed in the lowering upper bound of observed values going from 8 in 2013 to 4 in 2016. Importantly, this pattern is not at visible when only seropositivity is considered: 8.6% of the samples shown are positive in 2013, where 12.1% are positive in 2014, and 18% in 2015 and 2016 respectively.

In the top panel of Figure 8.5 we show the univariate posterior distribution for our main inference target: the long-term antibody half-life. The median estimated value was 319.6 days, with a 95% credible interval of 268.2 to 381.5 days. This represents the number of days that it takes for an individual to decay by one MAT unit once the initial faster decay period has passed. The estimated value agrees both with previous results (Prager et al., 2020) and the general pattern seen in Figure 8.4, where many MAT results of 1 or more are observed several years into the fadeout period.

The bottom panel of Figure 8.5 shows bivariate posterior distributions for the antibody half-life and eight other parameters estimated in our model. We observe some correlation with the mean peak titer for the 2011 outbreak, where our data might be insufficient to select a slightly higher peak with faster decay over a lower peak with slower decay, although we emphasize that the range of values is relatively small when compared to the range of quantitative titers seen in this system.

In Figure 8.6 we show a subsample of values for all f^i_j parameters, as described in equation (6), as well as the median posterior values for the whole MCMC output. The model was able to correctly infer, from serology data only, that the 2011 outbreak had a larger impact on the animals than the 2012 outbreak. It is also noticeable that the naïve classification represents a high proportion of each cohort. That is expected for cohort 1, which only experienced a small outbreak, but more puzzling for the oldest age cohort, which has experienced several outbreaks, with at least two large ones. Since our model does not exclude seronegative samples and does not have historical exposure rate data, we expected the hardest task would be to differentiate between very old infections and unexposed animals. That is confirmed by a preponderance of naïve animals in cohort 4 (Figure 8.6), which is the age cohort most likely to include animals that have lived long enough to have seroconverted and decayed back to an MAT of 0 before we sampled them. The unrealistically high value for $f^4_{\text{naïve}}$ should not impact our estimates. Animals that were very likely to have been seronegative at the start of the fadeout period are non-informative to this model and cannot effectively be distinguished from animals that are truly naïve with the data we used.

The f^i_j rates received noninformative priors that heavily penalized samples where the exposure would have occurred before the animal was born. We allowed low probabilities of a mistake in the animal age classification (as shown by the small amounts of green in cohorts 1 and 2 in Figure 8.6), which is realistic and generates a more well-behaved posterior for the MCMC.

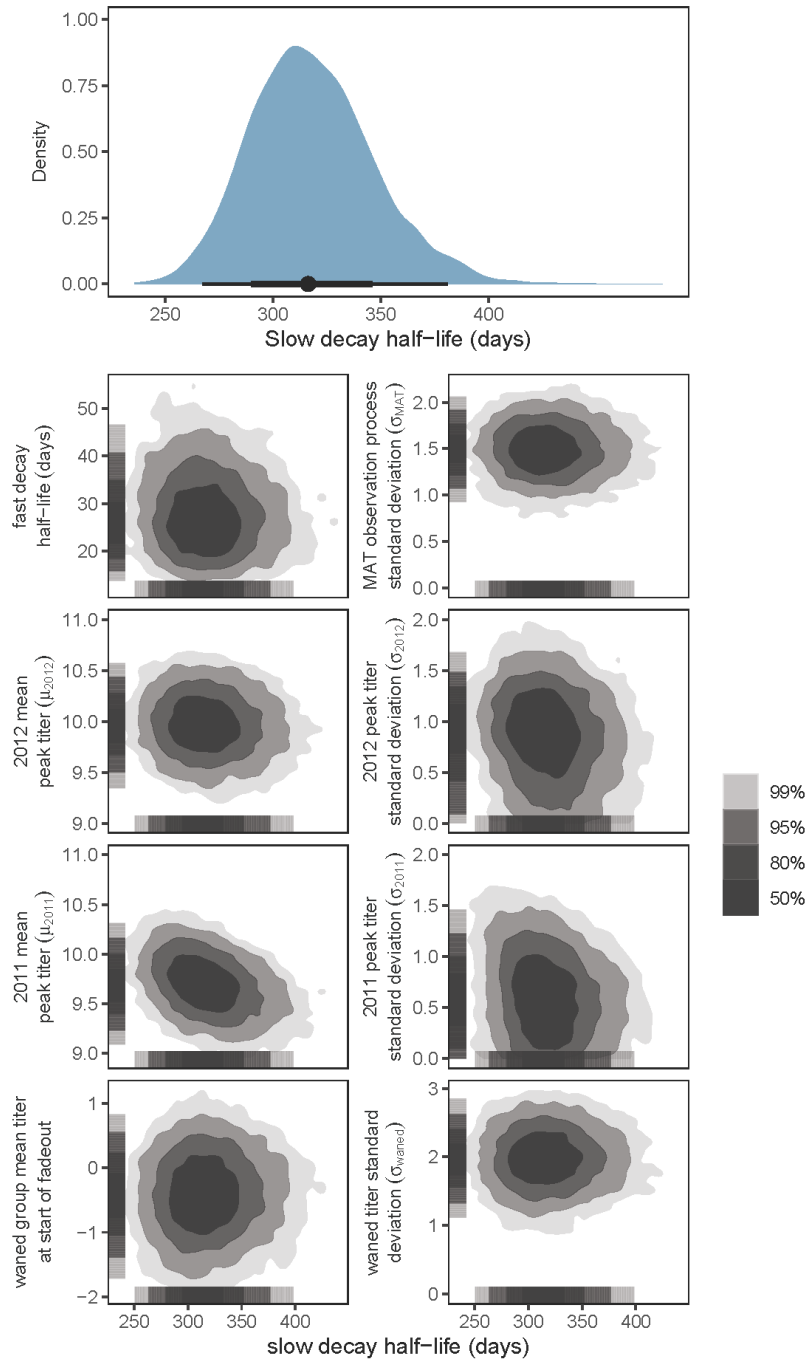


Figure 8.5. Posterior distribution of long-term half-life of antibodies against *Leptospira* in CSL. On the top panel, the univariate posterior, with median estimate shown in a black circle with the 68% and 95% credible intervals. On the bottom panels, we show the highest density regions for the bivariate posterior distributions of long-term half-life (x-axis) with other key model parameters on the y-axis.

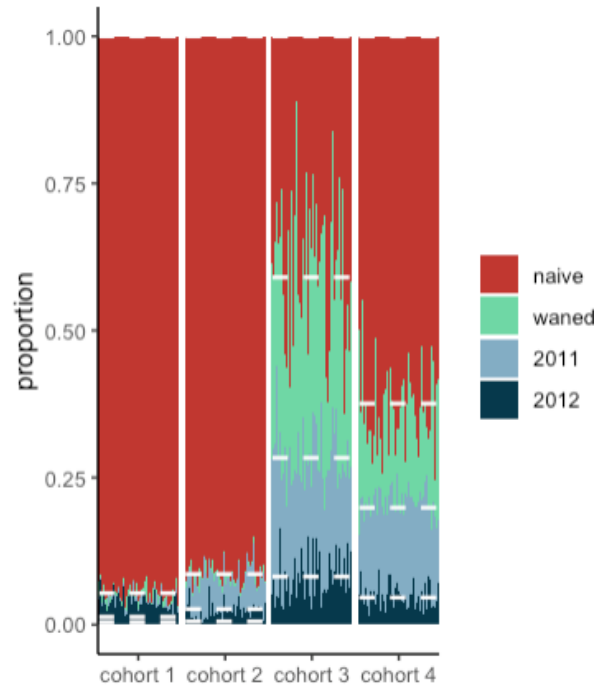


Figure 8.6. Model-estimated proportions of different exposure histories for serum samples from each CSL cohort. Columns show breakdown by cohort based on age at the time of sampling from 1 (youngest) to 4 (oldest). Colors show the which proportion values come from animals infected in 2012, 2011, or prior to 2010 (waned) and the estimated proportion of samples from animals that were never infected naïve). Each column shows 100 collated random draws from the posterior distribution of the proportions as vertical lines. The dashed lines show the median of the cumulative sums of each posterior draw ordered from lowest to highest value.

8.5. Discussion

In this study, we capitalize on the four-year fadeout of *L. interrogans* from a coastal mammal wildlife species to estimate the long-term decay rate of antibodies against the pathogen. Because our team has extensively documented the absence of *L. interrogans* during this period, we can interpret serological patterns during the fadeout as purely driven by decay, without complexities arising from possible re-exposure. To do so, we developed a model that combines the biphasic antibody decay kinetics reported previously with prior knowledge of likely periods of exposure for different cohorts during the pre-fadeout era (Figure 8.3).

Alongside this model of the ecological system and antibody kinetics, we developed a new model for the serological assay used worldwide to quantify anti-*Leptospira* antibodies: the microscopic agglutination test (MAT) (Figure 8.2). By taking into consideration particular aspects of how the test is performed and how measurement error may apply to this multi-step serologic assay, our approach our work opens the door to wider application of quantitative serology techniques using MAT data.

By fitting the combined model to quantitative serological data collected during the fadeout period, we estimated the long-term antibody decay rate (or half-life) under field conditions, for the first time (Figure 8.5). This analysis supplies a crucial ingredient for future quantitative

serology studies in this system – of particular value due to the 24-year time series of age-structured serology data, paired with decades of data on demography and incidence of leptospirosis and other conditions, that our team has accrued.

Studies aiming to investigate the disease dynamics of leptospirosis in CSL have dealt with uncertainty about re-exposure and long-term antibody decay by restricting the dataset to younger animals, or using estimates based on longitudinal data. Here we provide a quantitative estimate of long-term antibody decay rates that will enable future studies to use serology data over longer time spans more accurately, extracting even more value from a rare dataset. And while we rely on a well-documented but unique natural fadeout phenomenon, we propose a novel approach to estimating long-term decay rates from cross-sectional serology data which can potentially be applied to other host-pathogen systems with intermittent ‘hot’ and ‘cold’ periods of incidence spread across multi-year periods.

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9. Inferring time of infection from field data using dynamic models of antibody decay

9.1. Abstract

1. Studies of infectious disease ecology would benefit greatly from knowing when individuals were infected, but estimating this time of infection can be challenging, especially in wildlife. Time of infection can be estimated from various types of data, with antibody level data being one of the most promising sources of information. The use of antibody levels to back-calculate infection time requires the development of a host-pathogen system-specific model of antibody dynamics, and a leading challenge in such quantitative serology approaches is how to model antibody dynamics in the absence of experimental infection data.
2. We present a way to model antibody dynamics in a Bayesian framework that facilitates the incorporation of all available information about potential infection times, and apply the model to estimate infection times of Channel Island foxes infected with *Leptospira interrogans*.
3. Using simulated data, we show that the approach works well across a broad range of parameter settings and can lead to major improvements of infection time estimates that depend on system characteristics such as antibody decay rate and variation in peak antibody levels after exposure. When applied to field data we saw reductions up to 83% in the window of possible infection times.
4. The method substantially simplifies the challenge of modeling antibody dynamics in the absence of individuals with known infection times, opens up new opportunities in wildlife disease ecology, and can even be applied to cross-sectional data once the model is trained.

9.2. Introduction

Knowing when individuals got infected with a pathogen can dramatically boost insights into infectious disease biology, at both population and within-host scales (Handel & Rohani, 2015; Pepin et al., 2017). This knowledge allows estimation of incidence and force of infection, fundamental quantities for understanding and modeling transmission dynamics (Heisey, Joly, & Messier, 2006; Leonard Held, Hens, O'Neill, & Wallinga, 2019; Hens et al., 2010) and developing mitigation strategies (Caley & Hone, 2004; Weitz et al., 2020).

Individual infection times are relevant to a broad range of pathogen-related factors, including interpreting timelines of clinical disease (Hawley, Grodio, Frasca, Kirkpatrick, & Ley, 2011), vaccine efficacy (Antia et al., 2018), infection risk factors (Borremans et al., 2011; Pepin et al., 2019), pathogen spillover (Smith, Skelly, Kung, Roberts, & Field, 2014), effects of disease on wildlife health and survival (Tersago, Crespin, Verhagen, & Leirs, 2012), host immunity (Epstein et al., 2013) and tracing infection sources (Craft, 2015). However, even though a variety of data can theoretically be used to estimate infection time (e.g. clinical disease signs, antibody concentration, outbreak seasonality, contact tracing), there are significant challenges that limit the widespread adoption of time-of-infection approaches, particularly in wildlife.

Key challenges include incorporating individual variation in response to infection (Simonsen et al., 2009; Teunis et al., 2002), integrating different data sources (Borremans, Hens, Beutels, Leirs, & Reijnders, 2016), dealing with interval-censored data (Wilber et al., 2020), modeling the anamnestic response to reinfection (Pothin, Ferguson, Drakeley, & Ghani, 2016) and dealing

with antibody cross-reactivity. A currently unresolved major challenge is how to model the dynamics of biomarkers such as antibodies or pathogen DNA/RNA when there are no individuals with a known infection time, such as a group of animals infected experimentally and tracked longitudinally; this challenge is particularly common in wildlife studies.

Models of biomarker dynamics constitute the foundation of most time-of-infection estimation methods (Brookmeyer & Gail, 1988; Gilbert et al., 2013; Teunis, van Eijkeren, de Graaf, Marinović, & Kretzschmar, 2016), and are central to the rapidly expanding field of quantitative serology (Boni, Molbak, & Krogfelt, 2019; Hay et al., 2020; Pepin et al., 2017; Teunis et al., 2012). The presence and concentration of biomarkers can contain information about whether and when an individual has been infected (Borremans et al., 2016), the degree of immunity (Röltgen et al., 2021), infection severity (Vaughn et al., 2000), and whether and for how long they are infectious (Hardestam et al., 2008; Prager et al., 2020). Crucially, a biomarker can be used for such purposes only after its relevant properties have been quantified and when a model exists of how its presence or concentration correlates with the information of interest (e.g. time since infection).

Antibody dynamics can be a particularly rich source of information about time of infection. Following infection, antibody levels decline after reaching a peak level some time after infection, and this decay tends to follow a particular functional form, with a characteristic decay rate, specific to a host-pathogen system and antibody type. When this functional form and rate are known, antibody levels measured at some later point can potentially be used to determine how long ago an individual was infected (Boni et al., 2019; Hay et al., 2020; Teunis et al., 2012). This in turn opens up the possibility to improve estimates of incidence in the population (Hay et al., 2020; Pepin et al., 2017; Wilber et al., 2020) and perhaps to estimate whether and for how much longer an individual is immune to reinfection (Borremans et al., 2015). Notably, R package *serosolver* (Hay et al., 2020) provides a range of functions to infer infection histories based on antibody dynamics, and offers a framework that is able to account for cross-reactivity and antibody boosting following re-exposure. The models in this package are powerful, but their usefulness depends on how well the underlying antibody dynamic model can be estimated. Antibody dynamic models typically follow a general pattern: an increase phase (often ignored because it is short), a peak level, and a decay phase (Teunis et al., 2016). Quantifying this pattern and its associated parameters requires specific data. The optimal situation for parameter estimation is to have experimental data with known infection times for multiple individuals that are sampled longitudinally until antibodies are no longer detectable. For example, experimental infection of the African rodent *Mastomys natalensis* with an arenavirus, followed by frequent sampling for the entire lifetime, enabled the development of an antibody dynamic model that could then be used to estimate infection times of wild rodents based on a limited number of samples (Borremans et al., 2016, 2015). Similarly, experimental data on influenza A in snow geese and mallards have been used to model the antibody response following infection, and subsequently estimate infection times and force of infection (Pepin et al., 2017).

Unfortunately, in most wildlife disease studies such experimental data are not feasible to collect, and infection times in the field are unknown. This is particularly problematic when periods between sampling are long and sample sizes are small (Gilbert et al., 2013). This has been a major reason that quantitative serology methods have not yet been widely adopted in wildlife disease ecology (Gilbert et al., 2013). Instead, a standard approach to determining infection time has been to take the midpoint between the interval bounded by the last antibody-negative and the first positive samples (Begon et al., 2009; van den Bergh, Venter, Swanepoel, &

Thompson, 2019), or to consider this interval as a uniform probability distribution for infection (i.e., any time is equally possible). Intervals of multiple months or even years are common in wildlife systems, leading to potentially large errors surrounding time of infection estimates, especially when a uniform distribution between sampling times is assumed. This offers opportunities for improvement, as quantitative serology models that improve on these interval-censored uniform distributions have the potential to provide better estimates of infection time, which could dramatically improve related estimates of epidemiological quantities such as incidence.

Here, we present a general approach for modeling antibody dynamics when sampling is sparse and infection times are unknown (Fig. 9.1). The approach uses hierarchical Bayesian MCMC inference to integrate different sources of information about model parameters, with full consideration and propagation of uncertainty, and accounting for population heterogeneity in antibody decay characteristics. Additionally, we show how the simultaneous integration of the dynamics of additional biomarkers can improve parameter fitting and infection time estimation. We apply this approach to island foxes (*Urocyon littoralis*) infected with *Leptospira interrogans* serovar Pomona, using standard non-experimental field data only. The framework provides a way to estimate infection times by modeling biomarker dynamics even in the absence of experimental data, which we hope will stimulate more widespread use of quantitative serology in disease ecology.

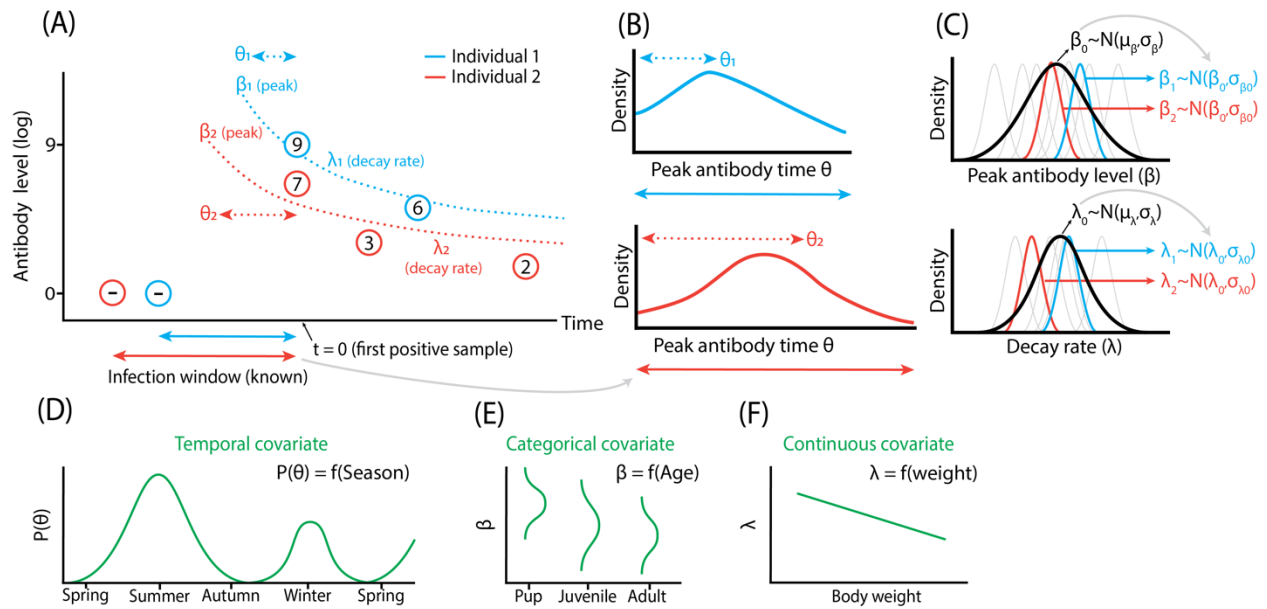


Figure 9.1. Hierarchical Bayesian modeling of biomarker data to estimate infection times. Bayesian inference offers a framework to use multiple sources of information to construct biomarker models and estimate individual time of infection. Panel (A) illustrates observed $\log_2$ antibody level data (circled numbers) for two individuals that are used to estimate model parameters β (peak antibody level) and λ (decay rate), with the ultimate goal of estimating time of infection for each individual i (which is determined by the peak antibody time θ_i , measured relative to the time of the first positive sample). Dotted lines show possible unobserved antibody trajectories. Intervals between the last negative and first positive samples can be used as prior information to bound possible peak antibody times θ_i (Panel B: posterior probabilities indicating the most likely peak antibody times). Model parameters can be estimated at the individual level (β_i and λ_i), while simultaneously estimating the mean and variation of these

parameters at the population level ($\beta_0, \sigma_{\beta_0}, \lambda_0, \sigma_{\lambda_0}$) in a hierarchical way (C). When available (not in our study), other types of information can be used to improve estimates of the different model parameters, e.g. seasonal fluctuations in infection risk provide information about θ_i (D), while age-dependent infection risk (E) or a continuous covariate such as body weight (F) can provide information about β_i or λ_i .

9.3. Materials and methods

9.3.1. Data collection

Serum samples from island foxes were collected on Santa Rosa Island (California, USA) as part of a reintroduction and monitoring program (Ti. J. Coonan, Guglielmino, & Shea, 2015). Sampling was done annually between July and February, from 2004 through 2019. Foxes were trapped using Tomahawk Live Traps (Tomahawk, WI, USA). Passive integrated transponder (PIT) tags were used for identification. Foxes were weighed, aged and assessed for body condition. Samples were collected during capture, taking up to 10ml of blood. Blood was kept cold until centrifuging 3-5 hours later, after which serum samples were frozen. Samples were tested for antibodies against *Leptospira* using microscopic agglutination testing (MAT). Samples prior to 2016 were tested at the Centers for Disease Control (CDC; Atlanta, Georgia, USA), later samples were tested at the Animal Health Diagnostic Center (Ithaca, New York, USA). Samples were titrated to endpoint titer against serovar Pomona. Samples tested at CDC before 2013 were also titrated to endpoint titer for serovar Autumnalis. Antibody levels were log-transformed so that each unit change corresponds with a two-fold dilution step ($\log_2 \left(\frac{\text{dilution}}{100} \right) + 1$). All trapping and sampling were done by the National Park Service under USFWS permit TE-08267-2. More details can be found in (T. J. Coonan, 2010).

We used antibody levels against *Leptospira interrogans* serovars Pomona and Autumnalis for antibody decay modelling and peak antibody time estimation. While the study population is known to be infected with serovar Pomona (Mummah, 2021), antibodies show a strong MAT signal for both serovar Pomona and serovar Autumnalis due to cross-reactivity (Paul N Levett, 2003). We leveraged both data sources to improve model parameter fitting and infection time estimation.

9.3.2. Candidate models

Prior to model fitting, candidate models of antibody decay had to be chosen. Aspects to keep in mind when selecting candidate models are the possible shapes a function can have and the number of unknown function parameters. A model with more parameters has increased flexibility, but this can increase the risk of overfitting and reduce the model's ability to predict new data (Bolker, 2008). Several functions have been used to model antibody decay, with the single (constant decay rate) and double (gradually decreasing decay rate) exponential functions being the most common (Boni et al., 2019; Teunis et al., 2016). When initial decay is significantly faster than later decay, alternatives such as a power function can be used (Teunis et al., 2016). When empirical antibody kinetics do not resemble existing functions, a flexible function such as a smoothed spline can be used (Borremans et al., 2016). Note that it is useful to consider and compare multiple models, as this will reduce the chances of relying on a misspecified model that results in false confidence in parameter estimates. Even though Bayesian

models offer the advantage of providing a full posterior distribution that allows proper quantification of estimation uncertainty, it is nevertheless important to choose the underlying models wisely.

Based on initial data exploration we selected three candidate functions. Single exponential: $\mu_{i,t} = \beta_i e^{-\lambda_i(t+\theta_i)}$; double exponential: $\log(\mu_{i,t}) = \log(\beta_i) e^{-\lambda_i(t+\theta_i)}$; power: $\log(\mu_{i,t}) = \frac{1}{1-r_i} \log(\beta_i^{(1-r_i)} - (1-r_i)v_i(t+\theta_i))$; where $\mu_{i,t}$ is the observed antibody level of individual i sampled at time t . Here, t is defined as the time since an individual's first positive sample and θ_i is the time of peak antibody level relative to the first observed positive sample of individual i (i.e., the number of days between an individual's estimated time of peak antibody level and its first observed positive sample). β_i and λ_i are the peak antibody level and antibody decay rate of individual i . r_i and v_i are the shape and scale parameters of the power function. Functions were fitted separately to Pomona and Autumnalis, not considering mixed functions (e.g., a single exponential for Pomona and a double exponential for Autumnalis) based on the assumption that serovars have the same underlying decay process.

Note that we did not attempt to model the initial period during which antibody levels increase, which is a limitation imposed by the low temporal resolution of our data relative to the duration of the increase period, which is not well known but is likely somewhere between 1 and 4 weeks (Langston & Heuter, 2003; P N Levett, 2001). When data do allow quantification of the increase period, the increase and decrease phases are typically modeled as two different functions connected at the peak antibody level time (de Graaf, Kretzschmar, Teunis, & Diekmann, 2014; Teunis et al., 2016).

9.3.3. Model fitting

Model fitting was done using Bayesian MCMC as implemented in rJAGS (Plummer, 2019). A log-normal error distribution was assumed for antibody levels. Six parallel chains were run for 60,000 iterations (10,000 burn-in), assessing chain convergence visually and with the Gelman-Rubin convergence diagnostic (Brooks & Gelman, 1998).

A major advantage of a Bayesian approach is that it allows the explicit incorporation of additional information about parameters through prior distributions. We used informative priors for time of infection (i.e., peak antibody level time θ_i) and peak antibody level β_i , as described below. We further aimed to capture the biological variation in model parameters across the population, to derive both individual and population-level estimates. This was possible by making the model hierarchical, where the population-level parameters (hyperparameters) were estimated explicitly, and the individual-level parameters were drawn from these population-level distributions (Gelman & Hill, 2007). Individual-level parameters were modeled using prior distributions $\log(\beta_i) \sim \mathcal{N}(\beta_0, \sigma_{\beta_0})$, $\lambda_i \sim \mathcal{N}(\lambda_0, \sigma_{\lambda_0})$, $\log(r_i) \sim \mathcal{N}(r_0, \sigma_{r_0})$ and $\log(v_i) \sim \mathcal{N}(v_0, \sigma_{v_0})$, where β_0 , σ_{β_0} , λ_0 , σ_{λ_0} , r_0 and σ_{r_0} are the hyperparameters of the model: population-level means and standard deviations (sd) of peak antibody level (mean β_0 , sd σ_{β_0}), exponential decay rate (mean λ_0 , sd σ_{λ_0}) and power function shape (mean r_0 , sd σ_{r_0}) and scale (mean v_0 , sd σ_{v_0}). Each of these hyperparameters had its own prior distribution (Table S1). Note that when parameters need to be constrained (e.g. $\beta_0 \geq 1$), this must either be modeled using an appropriate prior distribution, or iterations with impossible samples must be removed from the posterior distributions.

Parameter estimation and model performance can be improved by combining data from multiple biomarkers or other covariates such as age, incidence seasonality or other infection risk factors (Borremans et al., 2016). As an example, we implemented an additional biomarker: antibody levels against serovar *Autumnalis*. This was possible using a joint-likelihood approach (Isaac et al., 2020). This is a simple extension of single-biomarker fitting, where two separate models (for *Pomona* and *Autumnalis*) are fitted simultaneously, with distinct values allowed for all parameters except the peak antibody time θ_i , which becomes a shared parameter. This approach increases the likelihood of accepting parameter values that are supported by the different biomarker datasets and can result in more precise posterior estimates for θ_i . Last, to account for the correlated *Pomona* and *Autumnalis* antibody levels a multivariate normal distribution for both peak antibody parameters was used.

Because samples were processed at two different labs an additive lab effect parameter was added to the model. This allows for antibody levels of the labs to differ systematically. The JAGS code used for model fitting has been provided online. Posterior 95% credible intervals (CrI) were calculated as highest density intervals using the function ‘dens’ of R package HDInterval (Meredith & Kruschke, 2018).

9.3.4. Prior distribution of peak antibody time

Peak antibody time θ_i was bounded by the interval between the most recent negative sample (negative test or birth date) and the first positive sample (the infection window). This information was incorporated as a uniform prior distribution for θ_i with minimum θ_{min} and maximum 0. Alternative data to inform θ_i could be average lifespan, known seasonality in infection risk, onset of clinical signs of disease, or any other variable that provides information about the possible infection time. The probability distribution translating this information to a prior distribution can assume any shape and is not restricted to a uniform distribution as used here for the bounded infection window. We emphasize again that peak antibody time θ_i is used as a proxy for time of infection, since the time resolution of the dataset makes it highly unlikely that sufficient samples were taken in the period between infection and peak antibody time.

9.3.5. Prior distribution of peak antibody level

Another source of information that was used to improve model fitting is the distribution of peak antibody levels of recently infected foxes, which informs the population-level mean β_0 and standard deviation σ_{β_0} . This prior distribution was chosen by selecting a subset of foxes with relatively short infection windows, balancing the trade-off between sample size, which must be sufficiently large to provide a useful distribution, and recent infection time. We chose a maximum time of 250 days between the first positive and last negative sample as “recently infected”. Although this was still a large window, this was a limitation resulting from the study’s sampling frequency, which provides an opportunity to illustrate the strength of the approach under realistic field conditions. This resulted in 54 foxes that could be used to inform the prior distribution. Normal distributions were fitted to the frequency distribution of the antibody levels of the first positive samples. Fitted means were increased slightly to account for antibody decay within the 250-day window, as were standard deviations to allow for additional uncertainty, resulting in prior distributions with mean 7 and sd 2.5 $\log_2$ dilutions for serovar *Pomona* and mean 7.5 and sd 3 $\log_2$ dilutions for serovar *Autumnalis*. Normal distributions were fitted using

the `fitdistr` function in R package MASS (Venables & Ripley, 2002). More details are provided in Supplementary Information (Section 9.6.1). Note that the fact that this subset of individuals is used for informing a prior as well as being included in the model technically means that these data are being used twice to estimate peak antibody level. For this reason, the standard deviation of the prior distribution was deliberately chosen to be larger, which minimizes this effect. This is a way to allow this subset of individuals, which is known to provide better information about peak antibody level than the rest of the dataset, to weigh slightly more heavily on the estimation of peak antibody level. As shown in Supplementary Information (Section 9.6.2), the shape of the prior has very limited influence on parameter estimates. In addition, we tested whether this approach affected the model estimates by using exactly the same procedure for everything, except the prior distribution of peak antibody level was selected using only the 10 animals that had the shortest time between last negative and first positive, and then those 10 animals were withheld from all model fitting. This resulted in nearly identical parameter estimates (results not included).

9.3.6. Data for model fitting

Model fitting was done using 1,381 data points from 305 foxes that had at least 2 positive samples preceded by a negative one (or a known birth date) that determines the infection window. Because the antibody model does not accommodate secondary increases in antibody level, samples that exhibited signs of possible antibody boosting (possibly due to re-exposure to the pathogen, not necessarily leading to infection), defined here as an antibody level increase $\geq 2 \log_2$ units between samples, were removed from the dataset together with all samples from that individual that were taken afterwards. This occurred in 2 out of the 305 foxes, resulting in the removal of 4 data points.

9.3.7. Model comparison

Model fits were compared using the leave-one-out cross-validation information criterion LOOIC (Aki Vehtari, Gelman, & Gabry, 2017) using R package `loo` (A Vehtari et al., 2020), where lower values indicate a better fit. Additionally, we used three measures of the degree to which a model improves estimation of peak antibody time θ_i relative to the uniform prior infection window. The first is “% infection window reduction” which is the percentage by which the size of the infection window was reduced in the posterior distribution compared to the original uniform window size, for credible intervals of 95% (a commonly used interval) and 50% (a better representation of the most concentrated area of the posterior). For example, if the original window size is 250 days, and the model generates a posterior distribution of θ_i for which the 95% CrI ranges from 200 to 20 days prior, the % reduction would be $100 - \left(\frac{200-20}{250*0.95} \right) * 100 = 24\%$. If the 50% CrI of the posterior ranges from 110 to 75 days prior, the % reduction would be $100 - \left(\frac{140-65}{250*0.5} \right) * 100 = 40\%$. While these measures are useful because they are easy to interpret, they do not consider that probabilities within the credible intervals are not equal, and some θ_i will have a higher probability than others. To capture this, we used relative entropy (or Kullback-Leibler divergence) (Kullback & Leibler, 1951) as a third measure. Relative entropy (units = “bits”) quantifies the difference in information content between two distributions, which in this case are the uniform prior distribution (infection window) and the posterior distribution of

θ_i . Relative entropy $D_{KL}(P||Q) = \sum_x P(x) \log_2 \frac{P(x)}{Q(x)}$, where $P(x)$ and $Q(x)$ are the posterior and prior distributions defined over the same range of values x (Burnham & Anderson, 2002). The values of x are individual-specific and adopt every possible value of θ_i as determined by the uniform prior infection window. A higher value corresponds with more information in the posterior relative to the uniform prior. For reference, given a uniform prior (original infection window) of 250 days, a posterior normally distributed around a mean of 100 days with a standard deviation of 20 would result in a relative entropy value of 1.6 bits, compared to 0.8 bits if the standard deviation is 35; i.e., a higher relative entropy value indicates a greater reduction in uncertainty about estimated infection time.

9.3.8. Simulation study

To assess model behavior to different assumptions and conditions, we simulated data mimicking the real dataset. Antibody levels were simulated for 75 individuals, randomly sampling log peak antibody level and decay rate from normal distributions and drawing 2 to 5 samples (up to 2,000 days after peak antibody level). Random noise was added to antibody levels to simulate real variation. The peak antibody sample was excluded from the simulated dataset, again to mimic reality. Parameter estimation was done for multiple simulated datasets that were generated using a range of standard deviations for peak antibody level and decay rate. We then tested how different combinations of peak antibody level variation and decay rate affect model performance, as faster decay and/or smaller variation in peak antibody level may constrain the possible peak antibody time window, which in turn would affect the precision of the peak antibody time θ_i estimates. Simulation details and results are provided in Supplementary Information (Section 9.6.2).

9.3.9. Additional supplementary analyses

Some analyses were performed to provide additional information and context and are only shown in Supplementary Information. Section 9.6.5 shows tested correlations between peak antibody level and a number of covariates, Section 9.6.6 shows the correlation between serovars Pomona and Autumnalis, and Section 9.6.7 shows peak antibody level estimates for individuals without a prior negative sample and when using only the first positive sample for each individual.

9.3.10. Software

All data preparation, analysis and plotting was done in R (R Core Team, 2019) using packages ggplot2 (Hadley Wickham, 2016), rjags (Plummer, 2019), ggrridges (Wilke, 2020), dplyr (H Wickham, François, Henry, & Müller, 2019), patchwork (Pedersen, 2019), loo (A Vehtari et al., 2020), R2OpenBUGS (Sturtz, Ligges, & Gelman, 2005) and HDInterval (Meredith & Kruschke, 2018).

9.4. Results

9.4.1. Model fits to antibody decay data

Of the three candidate models (single exponential, double exponential, power) the best fits were observed for the two that allow faster initial decay that slows with time since peak antibody level (double exponential and power), for both serovar Pomona and Autumnalis (Fig. 9.2A). The double exponential model had the lowest LOOIC value (single exponential = 5933, double exponential = 4705, power = 5757). All models were fit to the dataset including both serovar Pomona and Autumnalis, resulting in one overall LOOIC value but separate parameter estimates for each serovar. Results are shown for the double exponential model only. The fitted double exponential functions for Pomona and Autumnalis are shown in Fig. 9.2B with the observed data after adjusting time (x-axis) for each individual based on the estimated peak antibody time θ_i . The mode was used as the posterior estimate for θ_i to accommodate the skewed posterior distribution.

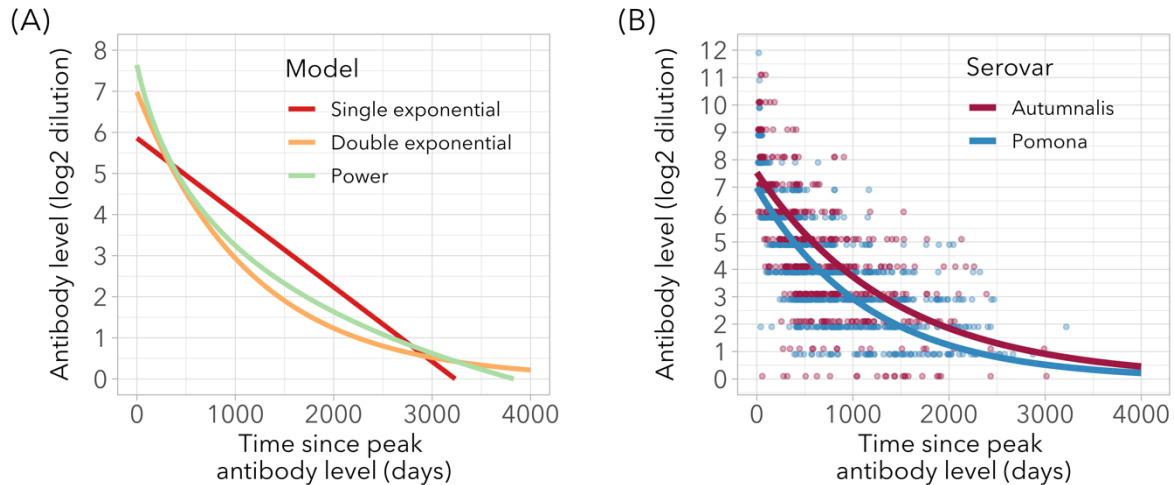


Figure 9.2. Candidate functions fitted to observed data. (A) Fitted functions for the three candidate models, using the posterior means for serovar Pomona peak antibody level and decay rate. (B) Fitted double exponential functions for serovars Pomona and Autumnalis, overlaid on observed data for 307 individuals after changing time since first positive sample to estimated time since peak antibody level.

9.4.2. Posterior estimates of decay model parameters

The population-level posterior means of the peak antibody level distribution parameters for serovar Pomona were $6.89 \log_2$ units (95% CrI 6.61 to 7.15) for the mean and $2.00 \log_2$ units (95% CrI 1.79 to 2.24) for the standard deviation. The estimated peak antibody level distribution for serovar Autumnalis was slightly higher, with a mean of $7.45 \log_2$ units (95% CrI 7.11 to 7.88) and standard deviation of $2.08 \log_2$ units (95% CrI 1.81 to 2.35) (Fig. 9.3A). The population-level distribution of the decay rate parameter for serovar Pomona had a mean of $0.00086 \log_2$ units/day (95% CrI 0.00079 to 0.00093) and standard deviation of $0.00035 \log_2$ units/day (95% CrI 0.00028 to 0.00042). For serovar Autumnalis the mean was $0.00069 \log_2$

units/day (95% CrI 0.00061 to 0.00078) and the standard deviation was 0.00028 $\log_2$ units/day (95% CrI 0.00021 to 0.00037) (Fig. 9.3B). There was no statistically meaningful correlation between individual peak antibody level and decay rate (posterior mean of the fitted regression coefficient φ in $\theta_i = \varphi \lambda_i$: 90, 95% CrI -456 to 646; Fig. 9.3C).

The mean percentage by which the individual prior infection windows were reduced after model fitting (using 50% credible intervals of the posterior distribution) was 22.1% (± 1.1 SE, range 2.5 to 83.2%) for the model that includes both serovars (with nearly identical results for the model using serovar Pomona only). When using 95% credible intervals, mean window size reduction for the model including both serovars was 6.1% (± 0.5 SE, range 0.5 to 64.8%) for the model that includes both serovars (with nearly identical results for the model using serovar Pomona only). Mean relative entropy values were 0.100 bits (± 0.011 SE, range 0.006 to 1.185) and 0.107 bits (± 0.014 SE, range 0.006 to 1.691).

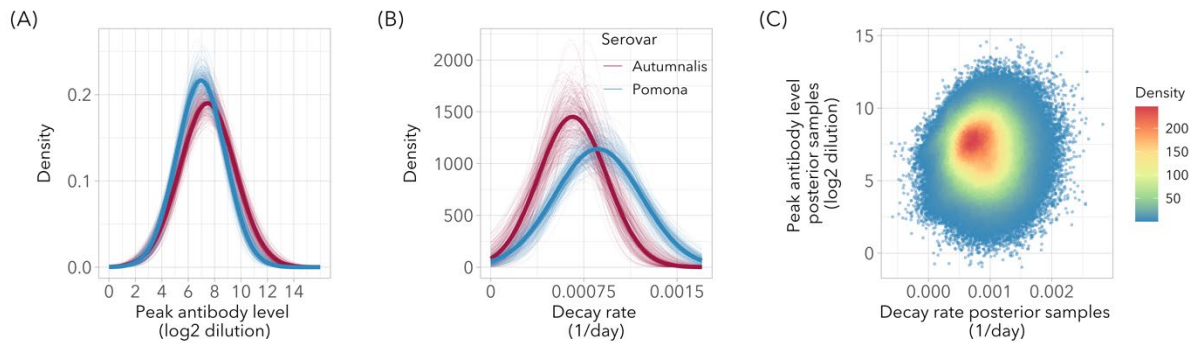


Figure 9.3. Estimated population-level distributions of peak antibody level (A) and decay rate (B), for serovars Pomona and Autumnalis. Bold lines are the distributions based on the posterior means of the mean and the standard deviation of each parameter. Thin lines are drawn from a random selection of 200 MCMC iterations, to show the magnitude of the variation around the posterior mean values. Panel (C) shows posterior samples for peak antibody level and decay rate, with colors depicting the density of points.

9.4.3. Correlates of model precision

We observed strong individual variation in how much information the model was able to provide about peak antibody time θ_i , with reductions in infection window size ranging from 2.5 to 83.2% (or 0.5 to 64.8% using a 95% credible interval), and relative entropy values from 0.006 to 1.185 bits. To explore the factors underlying this variation, we tested the correlation between window size reduction (using the 95% CrI, which is highly correlated with the 50% CrI) and a number of variables: prior infection window size, number of samples, time range covered by the samples, estimated peak antibody level and estimated decay rate for serovar Pomona.

Correlations were tested using linear models with a log-transformed infection window reduction outcome variable. Greater improvements were observed when the prior infection window was larger (effect size (rel. change) = 1.28, 95% CrI 1.17-1.40), when the estimated decay rate was higher (effect size (rel. change) = 1.47, 95% CrI 1.36-1.59) and when the estimated peak antibody level was higher (effect size (rel. change) = 1.13, 95% CrI 1.04-1.24). Model comparison using LOOIC showed the best fit for a regression model including a combination of decay rate, peak antibody level and infection window size, with decay rate occurring in all top models (Supplementary Information Section 9.6.3). In short, more information is gained when

the early antibody levels are high (leading to high estimates of peak titer, and rapid decay) and when the possible infection window is long (meaning there is more room for improvement). This is illustrated in Fig. 9.4, which shows the detailed output for two different individuals, while Fig. 9.5 shows the posterior distributions of θ_i for each individual.

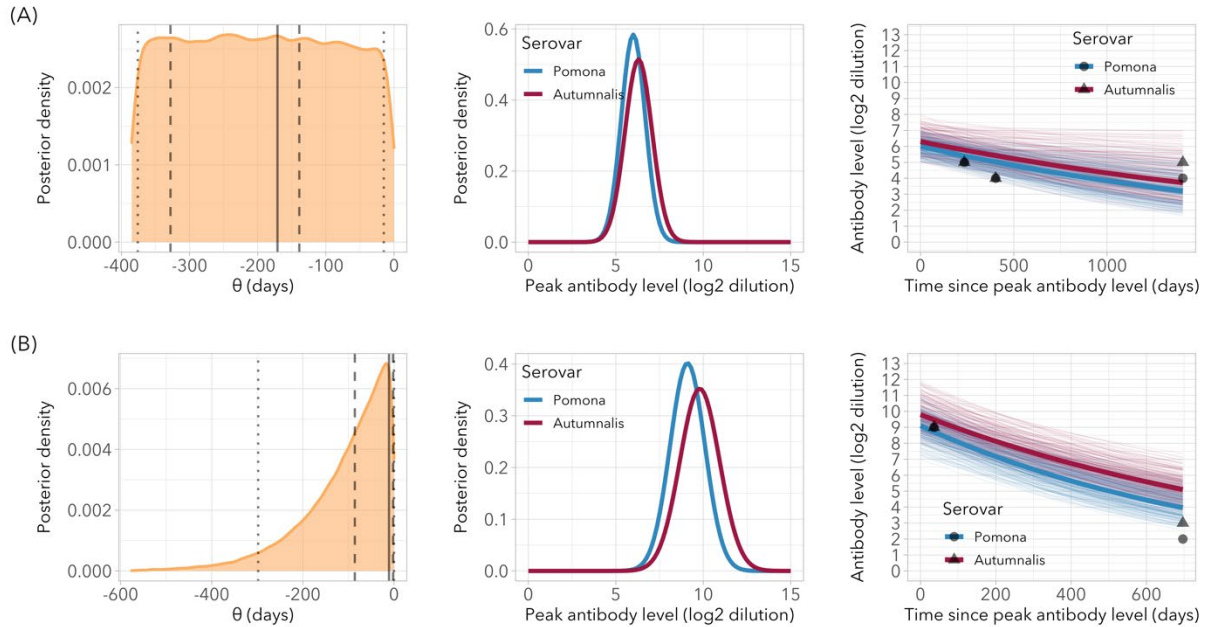


Figure 9.4. Posterior results for two individual foxes with low (A) and high (B) gain in information about peak antibody time θ . Left: posterior distribution of θ , the time between the first positive sample and the estimated peak antibody time, with maximum density (bold line) and credible intervals (95% = dotted line, 50% = dashed line). Center: posterior density for peak antibody level. Right: fitted functions using posterior estimates (bold lines) overlaid on 200 randomly selected iterations to show the distribution; black points are the observed samples.

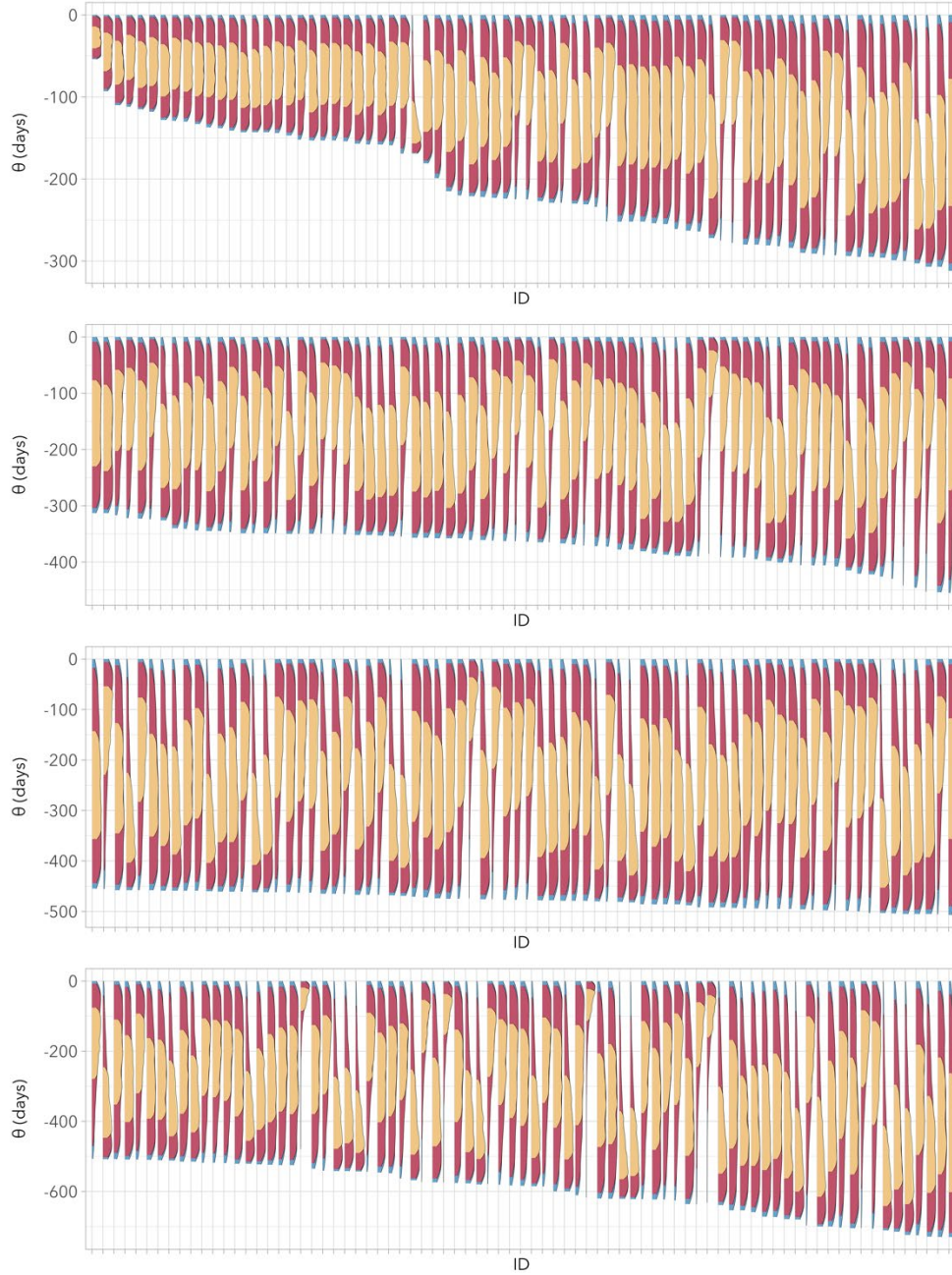


Figure 9.5. Posterior densities of peak antibody time θ for all foxes. Densities were plotted along the y-axis to enable showing all individuals, ordered along the x-axis by prior infection window size (full posterior density = blue, density within 95% CrI = red, density within 50% CrI = yellow). Note that the y-axis ranges increase from the top to bottom plots.

9.4.4. Simulation study

The models fitted to simulated data were able to accurately estimate the population-level parameters (β_0 , σ_{β_0} , λ_0 and σ_{λ_0} ; Fig. 9.7) across a broad range of assumptions about peak antibody level mean and standard deviation, showing that the modeling approach works well.

The estimation of parameter values at the individual level was good overall, but performance declined with more extreme values of the “true” individual peak antibody level (i.e., when the real simulated level was much lower or higher than the population mean; Fig. 9.8 to 9.11). Of broader relevance to biomarker dynamics in general, we found that model performance was strongly dependent on both decay rate and variation in peak antibody level (σ_{β_0}). Infection window reduction increased strongly with decay rate and correlated negatively with peak antibody level variation (Table 9.1). Fig. 9.13 illustrates how estimates of peak antibody time θ_i will be more accurate with faster decay and/or smaller variation in peak antibody values in the population.

Peak Ab SD (log ₂ units)	Infection window reduction (%)			
	Decay rate (log ₂ units/day)			
	0.005	0.001	0.0005	0.0001
0.5	46	49	36	6
1.0	44	37	23	3
2.0	37	26	12	2
3.0	34	17	9	1

Table 9.1. Effect of variation in peak antibody level and decay rate on infection window estimation. Gained information on peak antibody time relative to the prior knowledge, a uniform interval bound by the last negative and first positive samples. Values show the percentage by which 95% of the infection window size was reduced, where the 95% CrI of the posterior distribution was taken as the new window. Results were similar when using relative entropy (Table 9.6). Fig. 9.12 illustrates the different decay rates.

9.5. Discussion

One of the outstanding challenges in quantitative serology is how to estimate time of infection from antibody levels in the absence of experimental data (Borremans et al., 2016; Pepin et al., 2017). By integrating data from different sources using a Bayesian approach, we were able to estimate time of infection and model antibody decay despite highly imprecise knowledge about when individuals were infected. Two key sources of prior information were used in the model. The first is information about possible peak antibody levels provided by a subset of foxes known to have been infected relatively recently. The second is the infection window, an interval that bounds each individual’s possible times at which antibody levels must have peaked, defined by a negative sample preceding the first positive one. Additionally, the Bayesian approach enabled leveraging data from multiple biomarkers, in this case antibody level data on *Leptospira interrogans* serovar Autumnalis in addition to serovar Pomona.

Model fit statistics showed a clear preference for the two models in which antibody decay decelerates with time, and of those the more parsimonious double exponential model received the highest level of support. Using the posterior means for the double exponential model, the decelerating decay when starting at a Pomona antibody dilution 1:6400 would translate to 198 days to decay to 1:3200, 431 days to 1:1600, and 2495 days to 1:100. Initially rapid antibody decay followed by a slow phase seems to be relatively common, and has for example been observed for leptospirosis in California sea lions (Prager et al., 2020), *Bordetella pertussis* in

humans (Teunis et al., 2016) and arenavirus in multimammate mice (Borremans et al., 2015). This phenomenon may be a consequence of the heterogeneous dynamics of antibodies produced at different sites and different cell populations in the body, at different rates and driven by different mechanisms (Andraud et al., 2012; Teunis et al., 2016; Traggiai, Puzone, & Lanzavecchia, 2003).

There was a strong correlation between antibodies against serovars Pomona and Autumnalis, which was particularly strong for peak antibody levels. This meant that the information gained by the addition of data on serovar Autumnalis was limited, as that information was for the most part already provided by serovar Pomona. We indeed found that the reduction in infection window size achieved using our model was not much better when integrating data on both serovars versus only using serovar Pomona. This is a useful general insight into what to expect when considering incorporating multiple biomarkers for quantitative serology, and suggests that a useful strategy would be to prioritize biomarkers that do not correlate strongly. For instance, combining antibody data with data on the presence of a pathogen (or its genetic material) is likely to be much more informative than data on an antibody exhibiting the same dynamics, as shown for arenavirus infection in the rodent *Mastomys natalensis* (Borremans et al., 2016), hantavirus in *Peromyscus sp.* mice (Abbott, Ksiazek, & Mills, 1999) and influenza A in swine (Strelis et al., 2013). Alternatively, biomarkers that reflect the state of disease (e.g. renal health) can add useful information (Prager et al., 2020).

The degree to which the model was able to improve individual infection windows correlated strongly with a number of individual variables. The model yielded greater reductions in infection window with higher decay rate, higher peak antibody level and greater size of the prior infection window size. The latter can be explained by the fact that when the prior infection window is longer, larger improvements are possible. Higher decay rates on the other hand mean that the information conveyed by antibody levels will be more localized in time, thus resulting in better estimates of peak antibody time (Table 9.1 and Fig. 9.13). The effect of a higher level of peak antibody results from the fact that decay is faster at higher levels. Last, both the data and the simulations showed that a broader distribution of possible peak antibody levels directly translates into a broader distribution of possible peak antibody times, which means that lower individual variation will result in better estimates of time of infection (Table 9.1). It is important to note that while these correlations exist in theory, they will only provide an advantage in practice if sufficient data are available, at sufficiently short sampling intervals relative to the decay rate, to accurately capture the antibody decay parameters. This means that in situations where a high decay rate is expected, the frequency of sampling should ideally be high enough to capture the rapid change in antibody levels. If this is not the case, the decay rate parameter will be estimated with a larger degree of uncertainty, leading in turn to more uncertain estimates of the infection window. For determining an optimal sampling strategy prior to a study, a simulation study is recommended (Blaizot et al., 2019).

These characteristics related to model performance are intrinsic to a pathogen-host system, and will determine the upper level of performance of any model. For our system, the model yielded reductions in infection window size ranging from small (2.5% using 50% credible intervals) to impressive (83%); these reductions were obtained despite the relatively slow decay of antibodies against *L. interrogans* serovar Pomona in island foxes as well as considerable variation in peak antibody level. Indeed, the estimated decay rate of 0.0009 $\log_2$ units/day results in a window of 320 days between antibody levels 8 and 6 $\log_2$ units (assuming a peak level of 11). For comparison, decay rates of 0.005 and 0.01 $\log_2$ units/day would result in

windows of 58 and 29 days, respectively. This means that for other systems such as *L. interrogans* serovar Pomona infection in California sea lions (*Zalophus californianus*) where antibody decay can be as fast as 0.058 (Prager et al., 2020), a similar model would be expected to yield considerably more precise estimates of peak antibody time.

Correct inference about the antibody model dynamic parameters and estimated time of infection depends on the choice of candidate models of antibody decay. While the fit of different candidate models to the available data can be compared so that the best fitting model can be selected, ultimately if all candidate models are highly misspecified this can lead to false confidence in infection times. As for any mode it is therefore important to choose candidate models carefully, based on existing literature, data and expert knowledge. For example, if antibody decay is much faster in the first weeks after infection than in later periods, but none of the candidate models allow for a varying decay rate, the resulting confidence in infection times might be incorrect.

Quantitative serology is a growing field with major potential (Gilbert et al., 2013; Leonard Held et al., 2019; Pepin et al., 2017; Teunis et al., 2012; Wilber et al., 2020), but its broad adoption has been relatively limited due to a number of outstanding challenges. Here, we provided a complete approach to constructing biomarker models entirely from field data with sparse sampling points and unknown infection times, using a hierarchical Bayesian framework that can leverage multiple sources of information and multiple data types (Fig. 9.1). This addresses a major challenge in quantitative serology and time-of-infection estimation that has been one of the key barriers for application to wildlife systems. Within the context of the broader field of quantitative serology the approach can facilitate the adoption in wildlife pathogen systems of methods developed for human pathogens. For example, combining our approach with the powerful methods provided in the *serosolver* R package (Hay et al., 2020) could result in a new toolkit for estimating individual infection history and attack rate in wildlife systems where antibody kinetics have been challenging to model. The package further includes models to deal with antibody boosting follow re-exposure, and exposure to multiple strains that can cause antibody cross-reactivity, which can both be important in wildlife systems.

In conclusion, this work opens new frontiers for quantitative analysis of infectious disease dynamics, including incidence reconstruction with proper error propagation, anamnestic immune response following re-exposure (boosting), dealing with antibody cross-reactivity, integration of infection time estimation and transmission modeling, and software development for easy integration of data sources and incidence reconstruction. The hierarchical structure of the model further provides a natural way to extend it in order to estimate correlates of infection and biomarker dynamics at the population level as well as the within-host level.

9.6. Supplementary information

9.6.1. Data availability statement

The data and code necessary to reproduce the analyses are available at github.com/bennyborremans/antibody_decay_field_data, and the latest release can be found on Zenodo, doi 10.5281/zenodo.7995965 (Borremans et al., 2023).

9.6.2. Prior distribution of peak antibody level, and hyperpriors

A subset of recently negative individuals was used to provide information about what the possible distribution of peak antibody levels could be. Antibody levels were assumed to have peaked during the interval between the last negative timepoint preceding the first positive one. Here, a negative timepoint could be either an earlier negative sample or the animal's birth date. After data exploration, a subset of individuals was selected for which the maximum time between the last negative and first positive timepoints was 250 days. This resulted in a dataset of 54 individuals. Although a period of 250 days is still a relatively long interval, selecting a smaller interval would have resulted in a much smaller dataset due to the seasonality of sampling in our system. To account for this extended interval we used a prior distribution with a slightly larger mean than the distribution fitted to the observed data, but with a broader standard deviation. This resulted in a prior distribution that is weakly informative, thus allowing a large influence of the data on the posterior likelihood. Figure 9.6 shows the distribution of observed antibody levels and the fitted normal distributions. The distribution was fitted using the function `fitdistr` of R package MASS (Venables and Ripley, 2002). Using simulated data with characteristics similar to those of the real data, we found that the effect of the prior distribution specifications was not large, and that the population mean peak antibody level could be estimated well (see below).

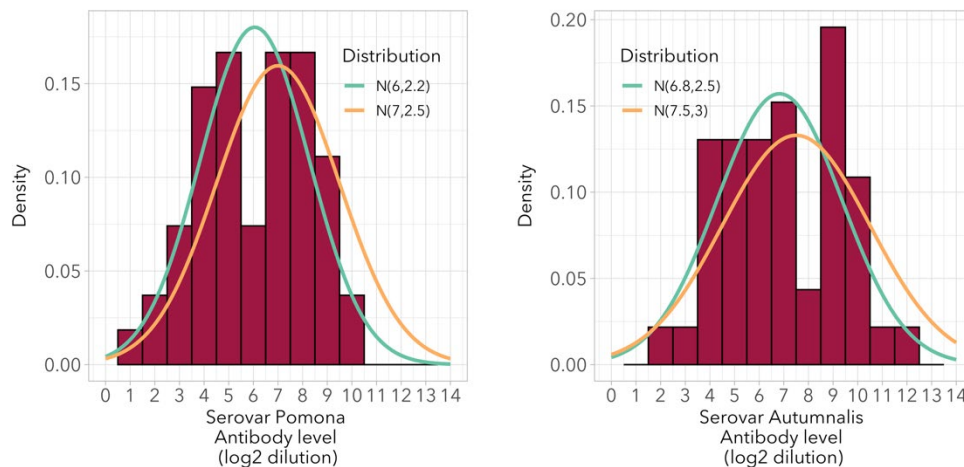


Figure 9.6. Antibody levels for a subset of individual foxes that tested negative within at most 250 days (left: serovar Pomona, right: serovar Autumnalis). Fitted distributions are shown in teal, and distributions used as priors are shown in orange.

Parameter	Prior distribution
$\beta_{0,Pomona}, \beta_{0,Autumnalis}$	$\log(\beta_0) \sim \mathcal{N}(\beta_0^h, \Sigma_{\beta_0}^h)$, multivariate normal distribution with mean vector $\beta_0^h = \begin{bmatrix} 7 \\ 7.5 \end{bmatrix}$ and covariance matrix $\Sigma_{\beta_0}^h = \begin{bmatrix} 2 & 0 \\ 0 & 2 \end{bmatrix}$.
$\sigma_{\beta_{0,Pomona}}, \sigma_{\beta_{0,Autumnalis}}$	$\sigma_{\beta_0} \sim \text{Wishart}(\mathcal{V}, df)$, Wishart distribution with scale matrix $\mathcal{V} = \begin{bmatrix} 2.5 & 0 \\ 0 & 3 \end{bmatrix}$ and degrees of freedom $df = 2$.
λ_0	$\lambda_0 \sim \text{Gamma}(1, 20)$, Gamma distribution with the same parameters for serovars Pomona and Autumnalis.
σ_{λ_0}	$\sigma_{\lambda_0} \sim \text{Gamma}(2, 3)$, Gamma distribution with the same parameters for serovars Pomona and Autumnalis.
r_0	$r_0 \sim \mathcal{N}(\log(0.5), 0.1)$, Normal distribution with the same parameters for serovars Pomona and Autumnalis.
v_0	$v_0 \sim \mathcal{N}(\log(0.0005), 1)$, Normal distribution with the same parameters for serovars Pomona and Autumnalis.

Table 9.2. Hyperpriors for the different parameters.

9.6.3. Simulations and sensitivity analysis

The model fitting approach was tested using simulated data (with characteristics similar to those of the real data) for 75 individuals, using the double exponential function (see main text). For each individual a peak antibody level representing serovar Pomona was randomly generated from a certain normal distribution that depended on the simulated scenario (see below). A second peak antibody level representing serovar Autumnalis was generated from a different distribution of which the mean was $0.84 \log_2$ (with a standard deviation of 0.5 to add realistic variation) units higher. Next, a decay rate was randomly generated from a certain distribution (see below), where the mean of the distribution for serovar Autumnalis was 20% higher than that for serovar Pomona. Using those parameters, between two and five samples (the number was randomly chosen with equal probability) were generated for each individual at random times after the peak antibody time, with a maximum time of 2000 days. A negative sample preceding the first positive sample was also generated to inform the maximum infection window size, where the time was generated from a uniform random distribution with a maximum of 500 days prior to the peak antibody time. Last, noise was randomly added to the data where each sample had a probability of 5% to decrease by one $\log_2$ unit, 90% to remain the same, and 5% to increase by one $\log_2$ unit. This represents plausible variation resulting from the microscopic agglutination assay (see main text) in two-fold dilution units. Each individual's peak time sample was removed from the dataset prior to model fitting, to represent reality.

Simulations were done for two scenarios:

1. **Effect of peak antibody level prior specification.** In order to test the effect of the specifications of the prior distribution of peak antibody level on the posterior estimate, we fitted the same simulated data using different prior specifications of peak antibody. Peak antibody levels (serovar Pomona) were generated from a normal distribution $\mathcal{N}(7,1)$, and decay rates from $\mathcal{N}(0.0008, 0.0002)$. Model parameters were fit for six prior distributions of peak antibody level, to assess the effect of the mean and the standard deviation on the posterior estimates: $\mathcal{N}(6,1)$, $\mathcal{N}(8,1)$, $\mathcal{N}(10,1)$, $\mathcal{N}(8,2)$, $\mathcal{N}(8,3)$, $\mathcal{N}(8,4)$. For all prior distributions the posterior estimates accurately estimated mean peak antibody level at the population level (Figure 9.7). Individual-level estimates of peak antibody time were good overall (Figure 9.8 and 9.9), but when the true (i.e., simulated) peak antibody level was at the extremes of the population-level distribution the estimates became less accurate. Closer inspection showed that this happens when an individual's peak antibody level was much lower or higher than average. Because the model assumes that individual levels are a sample of the population-level distribution, values closer to the mean will have a higher likelihood. This “shrinks” the individual estimates towards the population means, which is generally not problematic unless the real individual peak antibody value is much lower or higher. This is by definition rare when peak antibody levels or decay rates are assumed to be distributed normally; future work could consider hyper-parameter distributions with heavier tails.

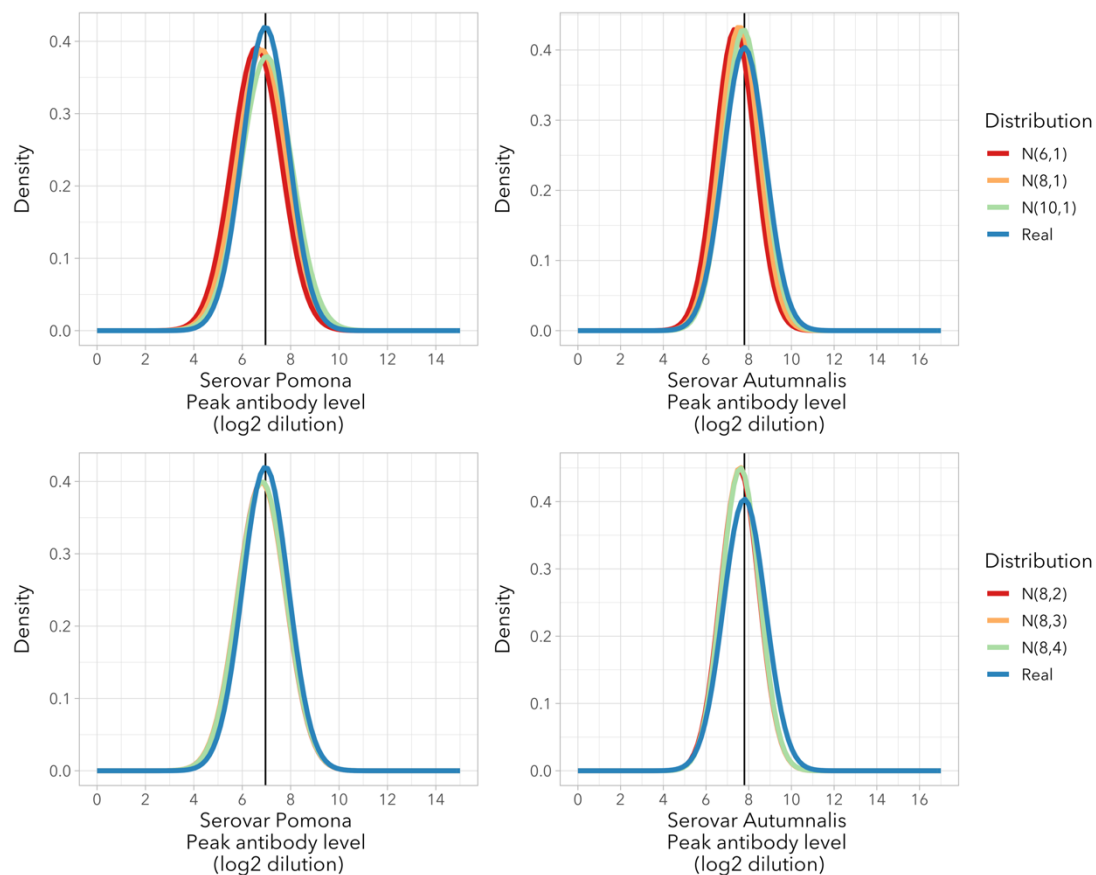


Figure 9.7. Posterior estimates of peak antibody level for different prior distributions. True = generated distributions with means 7 (SD 1) and 7.8 (SD 1) for serovars Pomona and Autumnalis, respectively.

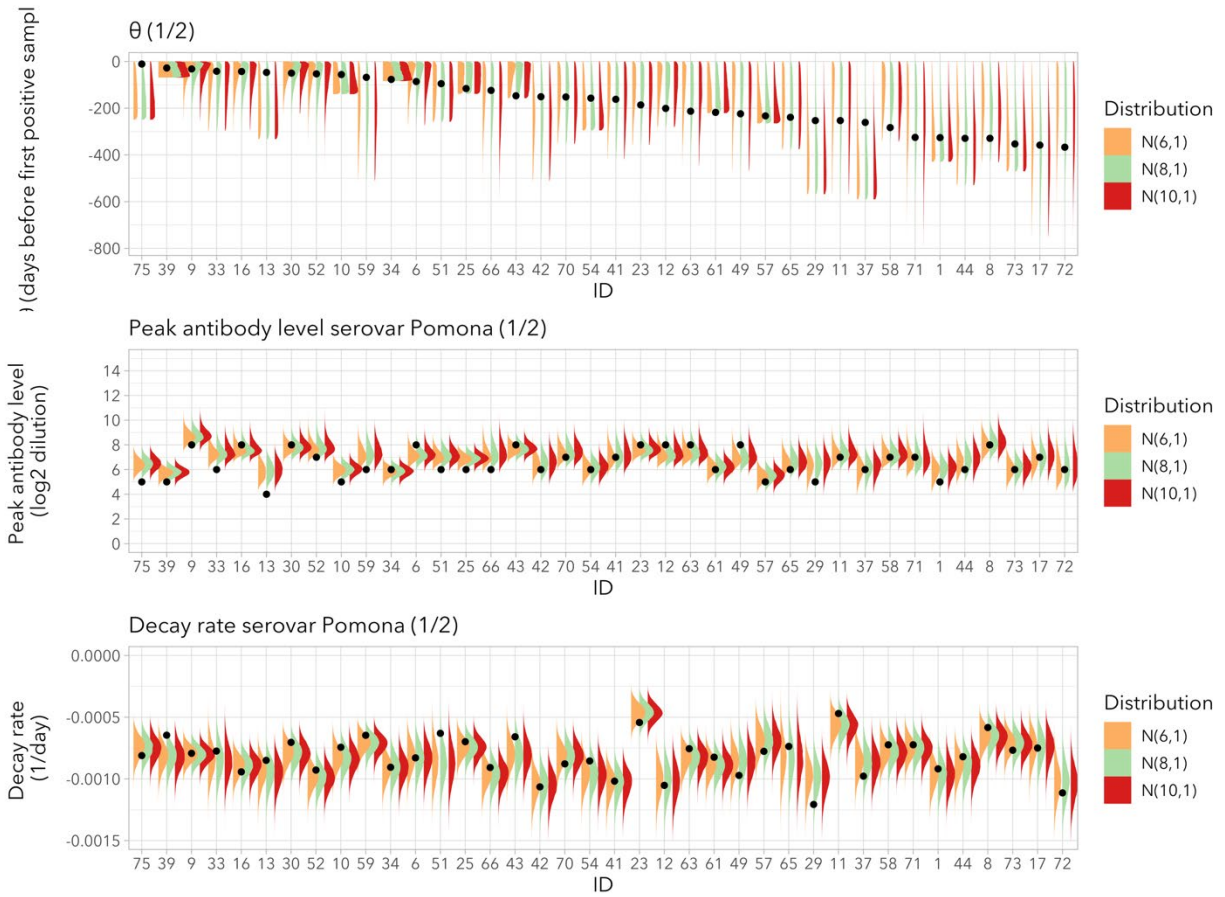


Figure 9.8. Effect of prior peak antibody level means. Posterior densities of peak antibody time, peak antibody level and decay rate for the first half of the 75 simulated individuals. Dots indicate the “real” simulated value, distributions are posterior densities flipped vertically.

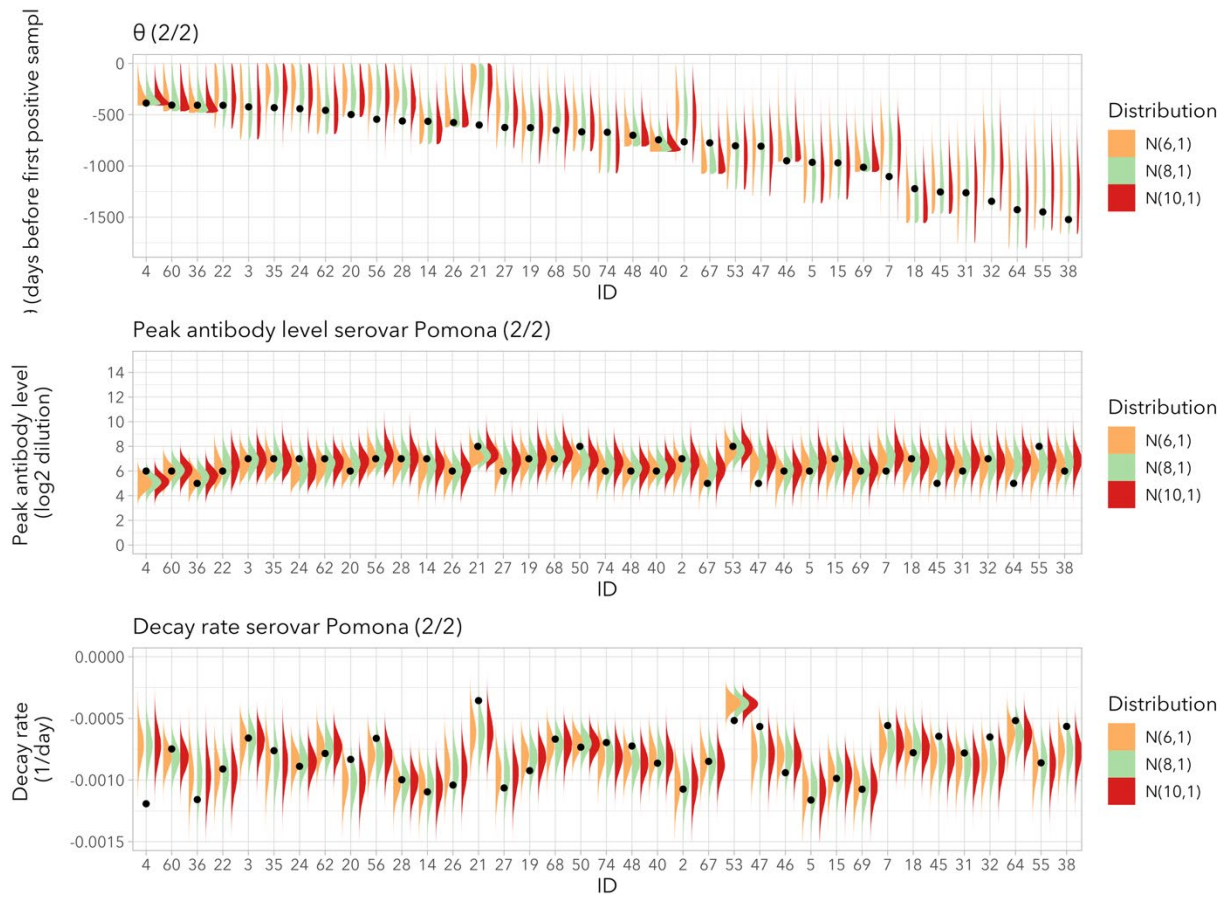


Figure 9.9. Effect of prior peak antibody level means. Posterior densities of peak antibody time, peak antibody level and decay rate for the second half of the 75 simulated individuals. Dots indicate the “real” simulated value, distributions are posterior densities flipped vertically.

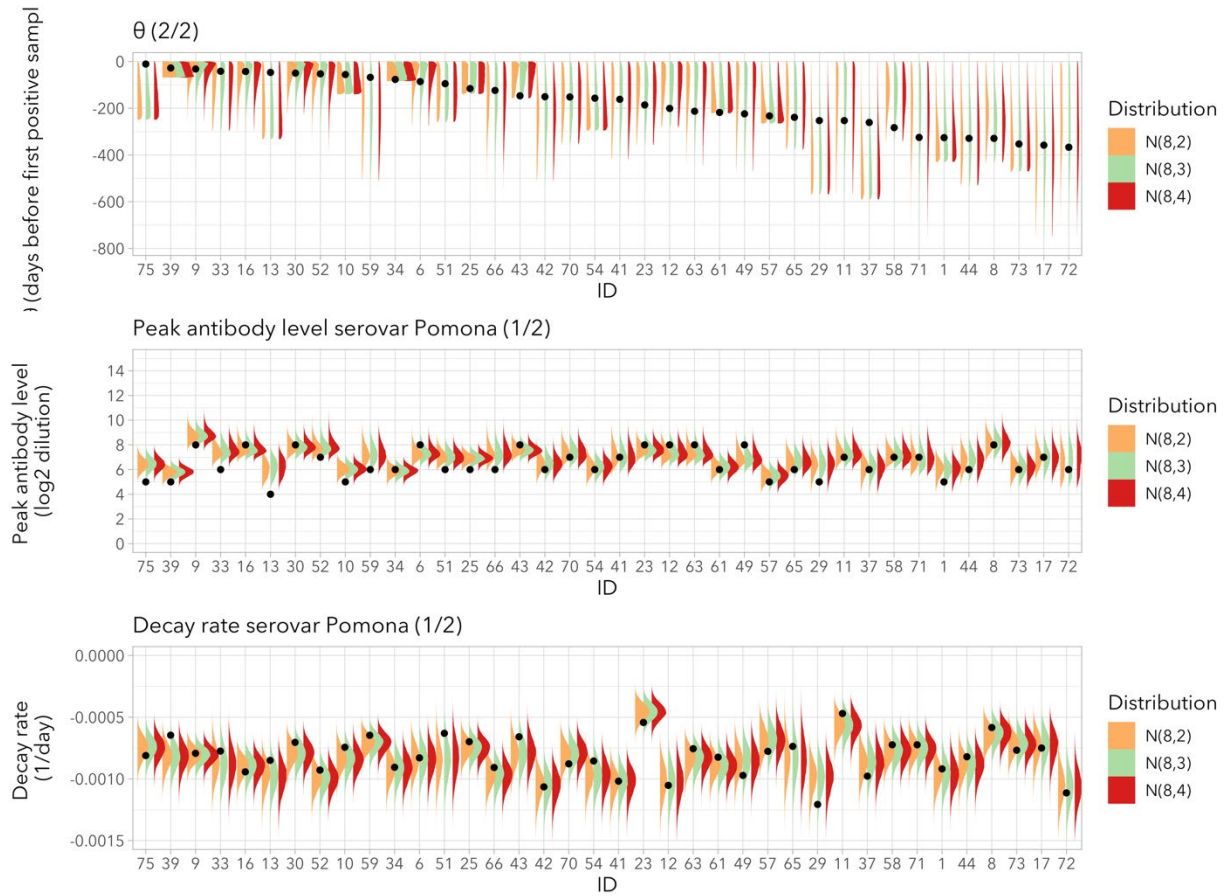


Figure 9.10. Effect of prior peak antibody level standard deviations. Posterior densities of peak antibody time, peak antibody level and decay rate for the first half of the 75 simulated individuals. Dots indicate the “real” simulated value, distributions are posterior densities flipped vertically.

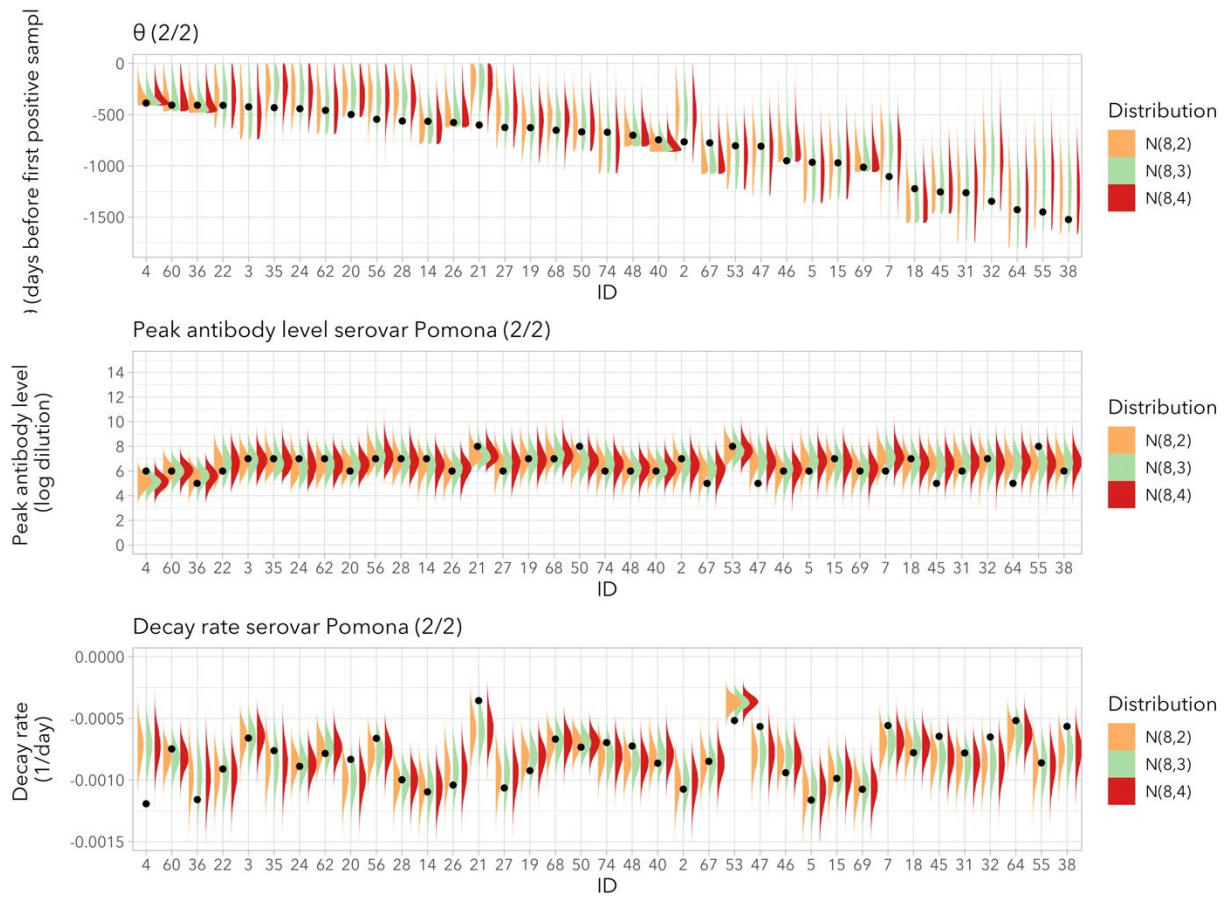


Figure 9.11. Effect of prior peak antibody level standard deviations. Posterior densities of peak antibody time, peak antibody level and decay rate for the second half of the 75 simulated individuals. Dots indicate the “real” simulated value, distributions are posterior densities flipped vertically.

2. **Effect of the magnitude of peak antibody level and decay rate.** Mean peak antibody level as well as decay rate are expected to have a strong effect on how well the models will be able to estimate peak antibody time. In order to quantify this, we fitted models to a series of datasets simulated using a range of peak antibody levels and decay rates. The effect of the different parameter values was then quantified as the mean reduction in the infection window. Peak antibody levels (serovar Pomona) were generated from distributions with mean 7 and standard deviations 0.5, 1, 2 and 3. Decay rates (serovar Pomona) were generated from normal distributions with means 0.005, 0.001, 0.0005, 0.0001 and standard deviation 0.0002. Figure 9.12 shows antibody decay for the different decay rates. Model parameters were fit for each combination of peak antibody level and decay rate distributions. Model performance was clearly dependent on the characteristics of antibody decay, where smaller variation in peak antibody level and/or faster decay resulted in more accurate estimates of peak antibody time, and larger gains of the posterior distribution of peak antibody times relative to the prior uniform interval (Table 9.3).

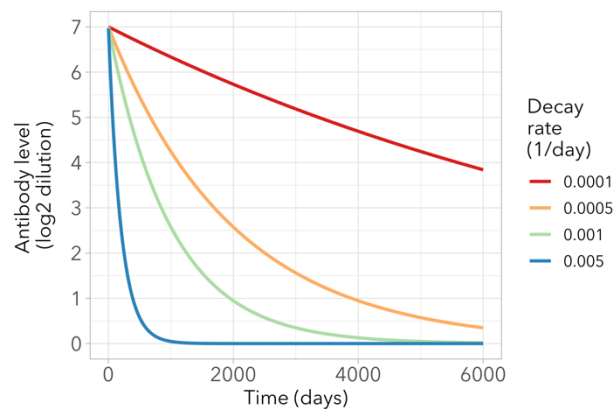


Figure 9.12. Antibody decay curves for different decay rates.

Last, in order to quantify the effect of decay rate on how well the method can be expected to estimate peak antibody time, we calculated the time needed to decay from antibody level 8 to 6 log₂ dilution units for a range of decay rates (Figure 9.13), choosing these two levels because they are close to peak levels but at the mean to lower end which means they are quite common and relevant. A larger time window will result in a less precise estimate of peak antibody time, and Figure 9.13 clearly illustrates that decay rate will be a major determinant of how well a time-of-infection estimation method can be expected to perform.

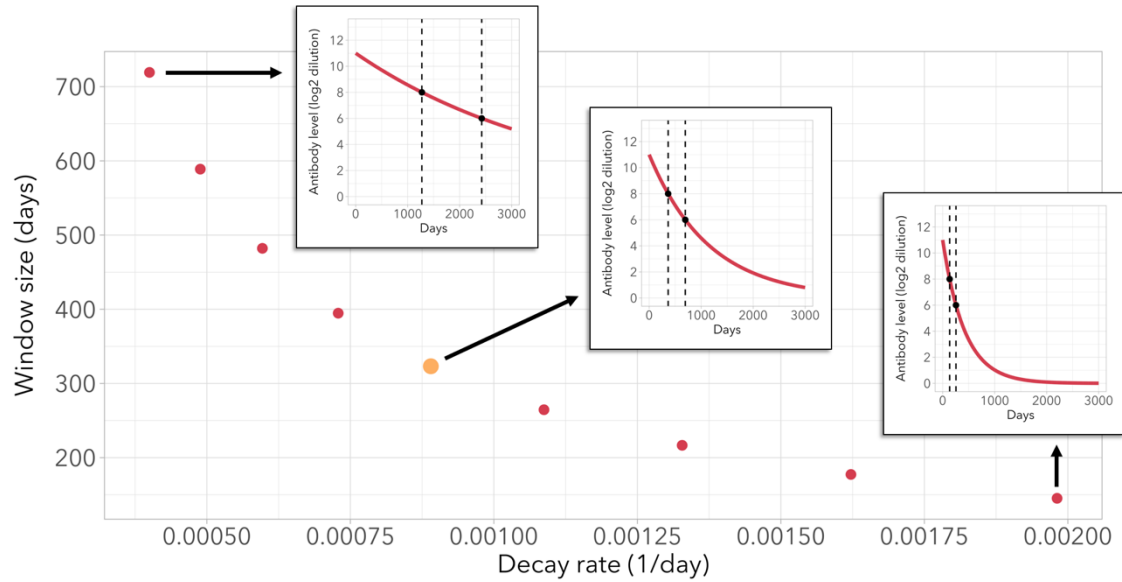


Figure 9.13. The number of days (window size) it takes for antibodies to decay from 8 to 6 $\log_2$ dilution units, for a range of biologically realistic decay rates. The yellow dot marks a decay rate close to the one estimated for serovar Pomona. Inset figures show the decay function for selected decay rates, with the window between antibody levels 8 and 6 indicated with dotted lines.

9.6.4. Predictors of model performance

We tested whether any variables correlated with a reduction in an individual's infection window size. Candidate variables were prior infection window size, number of samples, time range covered by the samples, estimated peak antibody level and estimated decay rate for serovar Pomona. Correlations were tested using linear models with a log-transformed interval reduction outcome variable. Results are shown in Table 9.3, Table 9.4 and Figure 9.14.

Variable	Effect size (exp)	Effect size l- 95%	Effect size u- 95%	LOOIC	LOOIC SE
Prior infection window size	1.28	1.17	1.40	722	37
Number of samples	1.09	0.99	1.20	748	34
Time range of samples	1.09	0.99	1.19	748	34
Peak antibody level posterior mean	1.13	1.04	1.24	744	32
Decay rate posterior mean	1.47	1.36	1.59	674	39
Antibody level of the first positive sample	1.01	0.92	1.11	751	34

Table 9.3. Statistics for the correlations between outcome variable ‘% infection window reduction’ and candidate variables, using a 95% credible interval for the infection window posterior. Effect sizes are exponentiated to get relative change and are shown with 95% credible intervals. LOOIC values are shown with standard errors.

Model	LOOIC	LOOIC SE
Peak level + decay rate + window size	589	45
All variables	592	44
Decay rate + window size	631	46
Peak level + decay rate + number of samples	648	36
Decay rate + peak level	650	36
Decay rate posterior mean	674	39
Decay rate + first positive level	676	38
Peak level + window size	711	34
Prior window size	722	37
Peak level + number of samples	743	32
Peak level posterior mean	744	32
Time range of samples	748	34
Number of samples	748	34
Antibody level of the first positive sample	751	34

Table 9.4. LOOIC values for linear regression models including single and multiple variables fitted to outcome variable ‘% infection window reduction’. Sorted by LOOIC value, which are shown with standard errors.

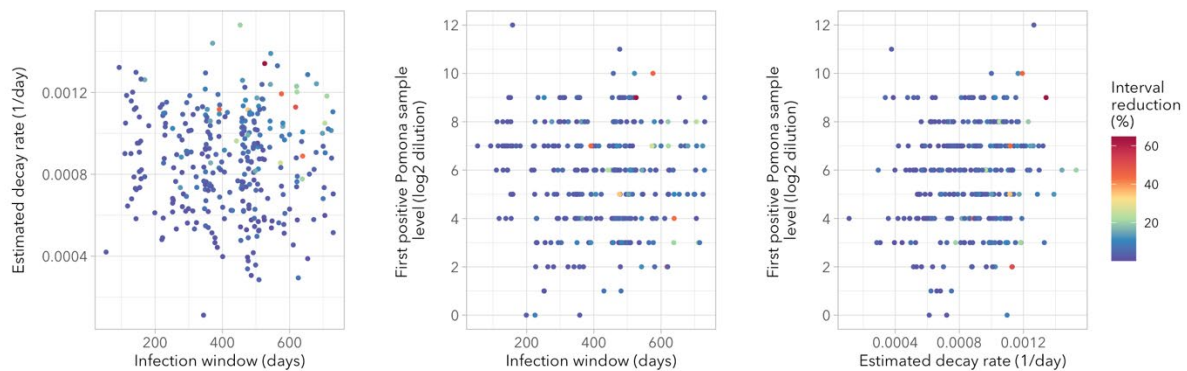


Figure 9.14. Infection window reduction (heatmap colors) for key correlates decay rate, infection window size and level of the first positive sample.

9.6.5. Effect of peak antibody level and decay rate on infection window size

Infection window reduction (%)					Relative entropy (bits)				
Peak Ab SD ($\log_2$ units)	Decay rate ($\log_2$ units/day)				Peak Ab SD ($\log_2$ units)	Decay rate ($\log_2$ units/day)			
	0.005	0.001	0.0005	0.0001		0.005	0.001	0.0005	0.0001
0.5	46	49	36	6	0.5	1.06	1.05	0.71	0.12
1.0	44	37	23	3	1.0	1.01	0.75	0.44	0.07
2.0	37	26	12	2	2.0	0.74	0.50	0.23	0.05
3.0	34	17	9	1	3.0	0.67	0.32	0.17	0.04

Table 9.5. Effect of peak antibody level and decay rate on infection window estimation. Gained information on the size of the infection window relative to the prior knowledge, which is a uniform interval bound by the last negative and first positive samples. Left: the percentage by which 95% of the infection window was reduced, where the 95% CrI of the posterior distribution was taken as the new window. Right: the information gained by the posterior distribution, expressed as relative entropy, where a higher value indicates a larger information gain expressed in ‘bits’ units.

9.6.6. Correlations between peak antibody level and covariates

We tested whether observed peak antibody level correlated with certain variables, as such a correlation could be integrated into a model of peak antibody time and improve the posterior estimate. Peak antibody level was approximated by the level of the first positive sample for the subset of 54 individuals that had been infected recently (described above). Variables tested were sex, body weight, body condition, age class, reproductive status, birth year and sample collection month. Correlations were tested for each variable using a linear model with normal error distribution, using $\log_2$ antibody level as outcome variable. The linear models were fitted using rjags (Plummer, 2019), with uninformative normal prior $\mathcal{N}(0,2)$ for the effect estimates, which were scaled to mean 0 and standard deviation 1 when numeric. There were no variables for which the 95% credible intervals did not include 0.

The same analysis was performed using peak antibody level estimates from the models instead of observed values for recently negative individuals. Similarly, no statistically meaningful effects were observed.

9.6.7. Peak antibody levels of serovars Pomona and Autumnalis

Because a preliminary analysis of the antibody levels for serovars Pomona and Autumnalis suggested a linear correlation, we explicitly incorporated this into the Bayesian model (see below) to test whether this was indeed the case. This was done using a multivariate normal distribution for the peak antibody levels of both serovars. The model indeed found a strong correlation between the two (Figure 9.15). where peak antibody levels for serovar Pomona are on average 0.67 $\log_2$ units lower than those for serovar Autumnalis, with a slope of 0.99 (95% CrI 0.90 to 1.14) and an R^2 value of 0.99 (95% CrI 0.94 to 1.00).

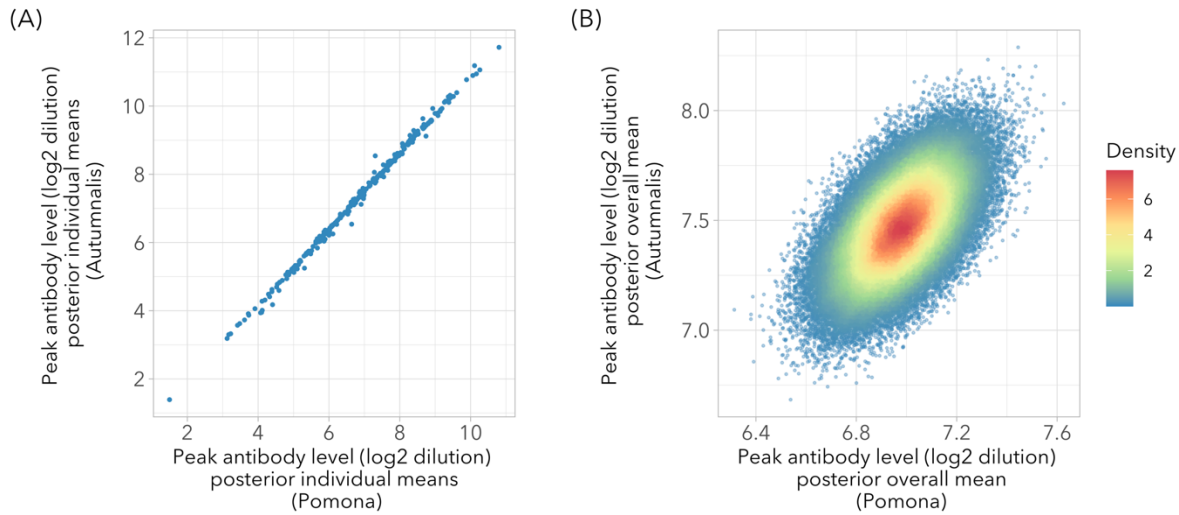


Figure 9.15. Correlation between peak antibody levels for serovars Pomona and Autumnalis. (A): Posterior means of each individual. (B) Posterior MCMC samples of the overall means of peak antibody level for the last 20,000 iterations.

9.6.8. Estimating time of infection for individuals lacking prior negative samples, and from cross-sectional data

We used the model, with parameters estimated from the main data set, to estimate time of infection for individual foxes that lacked the serological data needed for inclusion in the main study. Posteriors for peak antibody time are shown for individuals that did not have a negative sample preceding their first positive (Figure 9.16), and for individuals that had only one positive sample (Figure 9.17). In both cases a bound of 6 years (2190 days) was given for the infection window when a negative sample preceding the first positive was absent. A time of 6 years was chosen because it is longer than any observed infection window, but not so long that it spans an unrealistic time period or a mix of age classes. The situation where only a single positive sample is used resembles that of cross-sectional data, illustrating that the method can be applied to this common situation after training the model using a subset for which multiple samples are available.

Last, we tested whether model performance (measured as window size reduction) correlates with the level of the first positive sample and the original window size, when only the first positive sample is used for estimating time of infection (using only individuals with a negative preceding sample). We found no meaningful correlation, mainly due to the fact that original window size and the level of the first sample were correlated, resulting in an interaction that is not possible to disentangle (Figure 9.18).

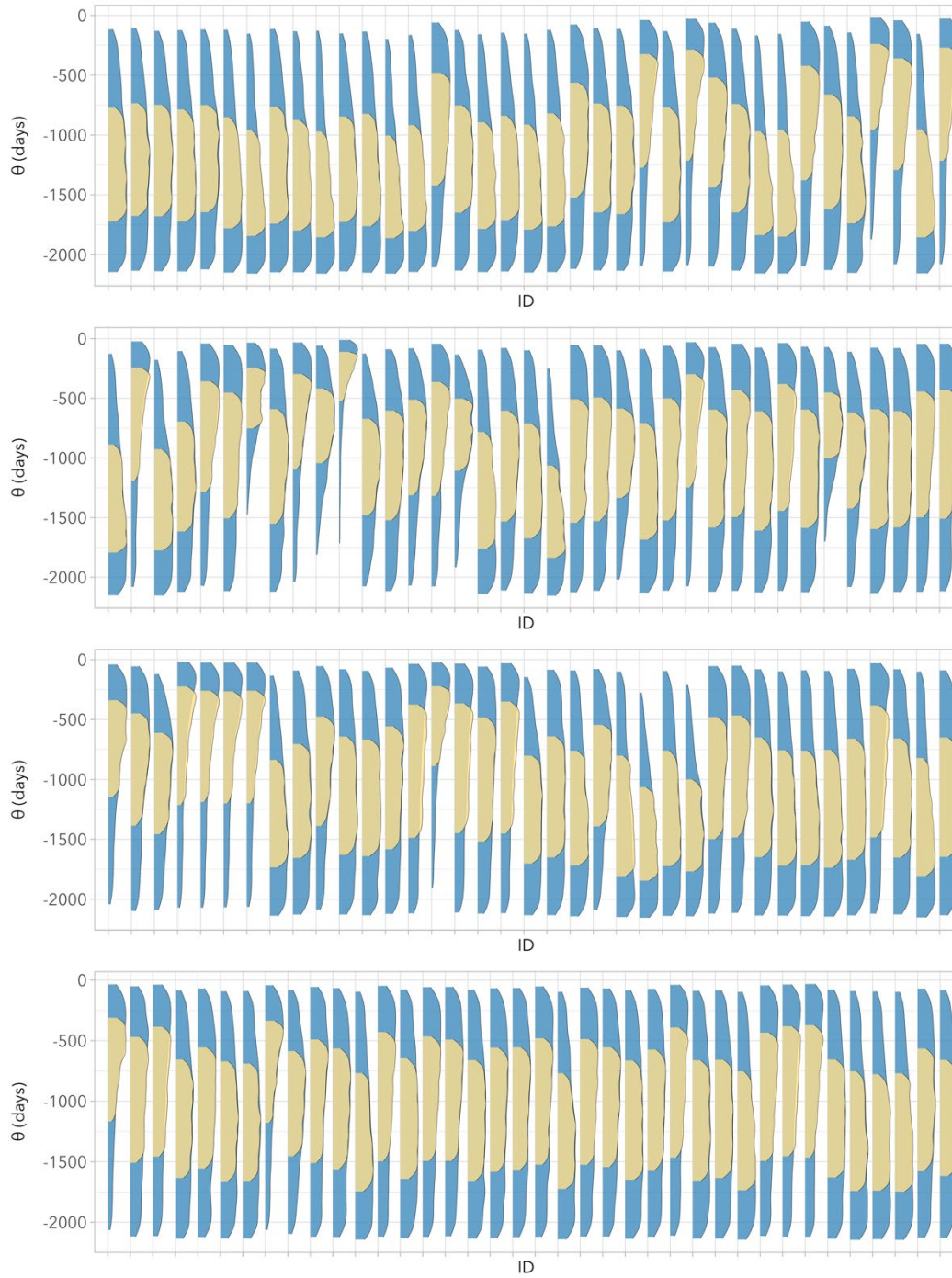


Figure 9.16. Infection window posterior densities for individuals without a negative sample preceding the first positive sample. Colors indicate the size of the credible intervals (blue = 95%, yellow = 50%). Arbitrarily chosen infection windows up to 2190 days (6 years) were possible.

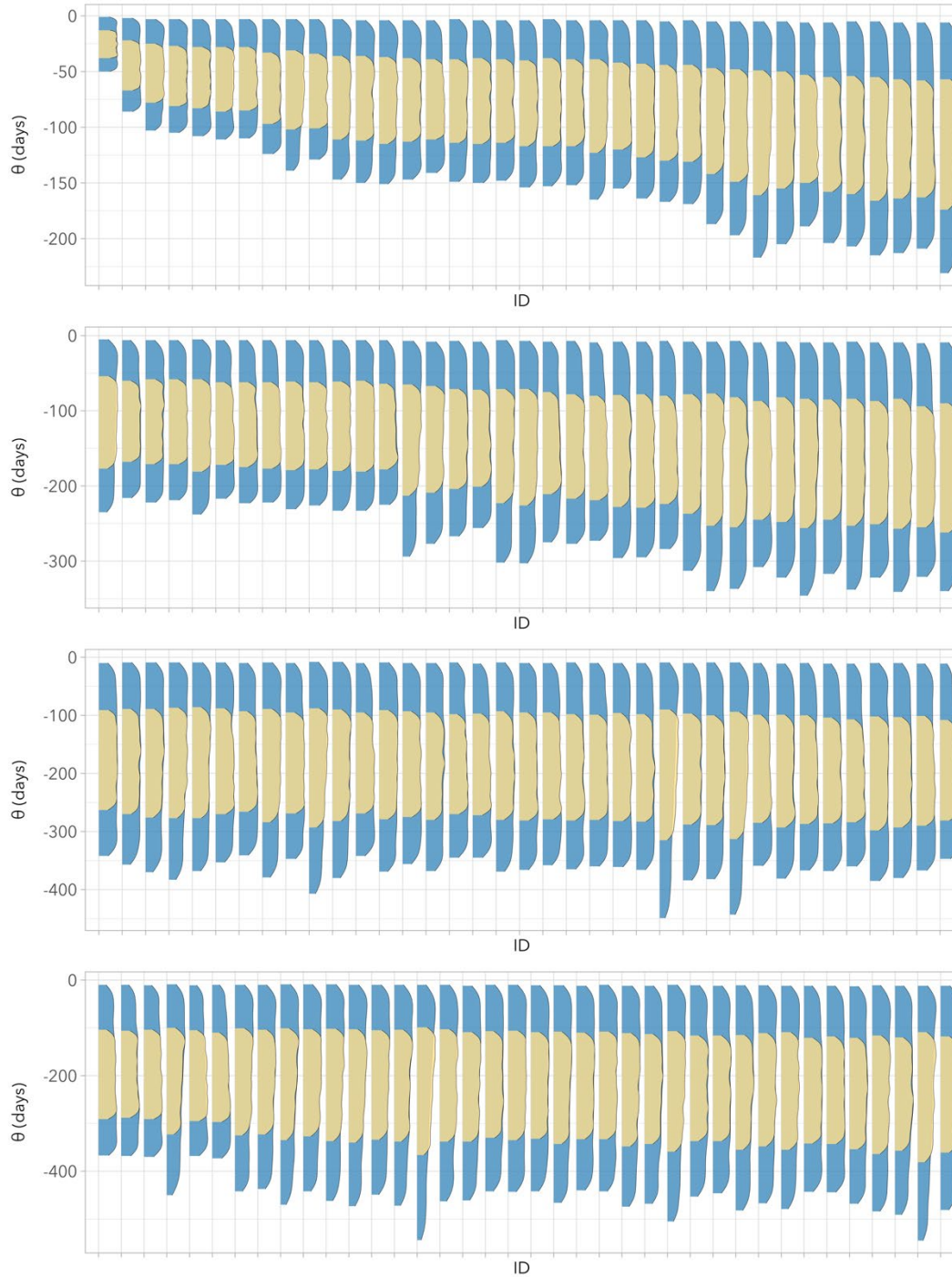


Figure 9.17. Infection window posterior densities for individuals with only a single positive sample. Colors indicate the size of the credible intervals (blue = 95%, yellow = 50%). Arbitrarily chosen infection windows up to 2190 days (6 years) were possible. Only individuals with a preceding negative sample are shown (others are included in Figure S11). Densities are sorted by posterior median.

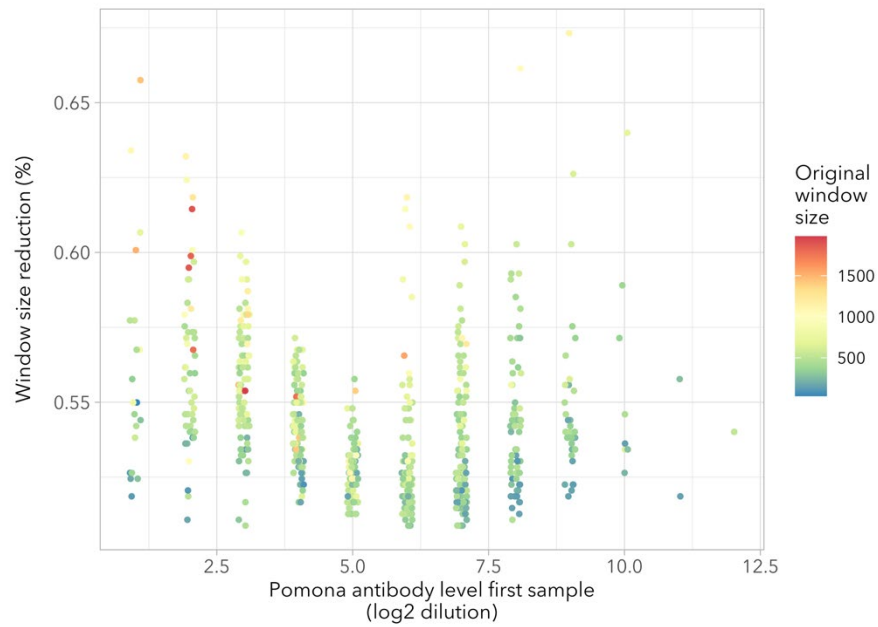


Figure 9.18. Infection window size reduction using only the first positive sample for each individual. Reduction in infection window size plotted against Pomona antibody level ($\log_2$ dilution), with colors indicating the size of the original infection window size (i.e., time between the first positive sample and the last preceding negative).

9.7. References

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