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The dynamics and evolutionary underpinnings of zoonotic transmission and emergence

Author
Guth, Sarah

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The dynamics and evolutionary underpinnings of zoonotic transmission and emergence

By
Sarah Guth

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Committee in charge:
Dr. Mike Boots, Chair
Dr. Britt Koskella
Dr. Peter Sudmant
Dr. John Marshall

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Abstract

The dynamics and evolutionary underpinnings of zoonotic transmission and emergence

By

Sarah Guth

Doctor of Philosophy in Integrative Biology

University of California, Berkeley

Dr. Mike Boots, Chair

Emerging infectious diseases (EIDs) pose a significant threat to public health, with the frequency of emergence events increasing in recent decades. The vast majority of EIDs are zoonotic, meaning they transmit between animal and human hosts. We applied methods in genomics, epidemiology, and ecology in field, laboratory, and computational settings to study the dynamics and evolutionary underpinnings of zoonotic transmission and emergence in wildlife and human populations. Specifically, we used statistical modeling to analyze zoonotic risk, developed a DNA methylation assay for estimating chronological age in bats to support age-seroprevalence modeling of bat viruses, and mathematically analyzed pathogen-host coevolution in an adaptive dynamics framework.

Prior to this doctoral dissertation research, my master's thesis highlighted the growing risk that humans introduce zoonoses to new geographic areas where the imported pathogens can then 'spill back' to infect local wildlife. We reviewed ecological mechanisms underlying the emergence of novel enzootic cycles and made the case that modeling approaches can provide critical insights in the face of empirical limitations. This paper set the stage for my doctoral dissertation focus on the interdisciplinary study of zoonotic pathogens.

The first dissertation chapter statistically analyzed zoonotic risk across mammalian, directly transmitted zoonotic viruses, delineating host and viral traits predictive of the severity of disease they engender in the human population ('virulence') and in their capacity for sustained human-to-human transmission post-emergence. We found that animal hosts most distantly related to humans—in particular, order Chiroptera (bats)—harbor the most virulent zoonoses with a lower capacity for endemic establishment in human populations.

The second chapter built off this analysis, increasing our sample size to include avian and vector-borne viruses and additionally analyzing variation in the total number of human deaths caused by each virus ('death burden'). We found that bats still harbored the most virulent viruses, but death burden did not correlate with any reservoir group and instead, was a function of viral traits. Nevertheless, these statistical analyses suggest that bats offer a study system that is both highly relevant to public health and valuable for understanding the evolutionary and transmission dynamics of zoonotic pathogens.

The third chapter presented a hybridization capture-based target enrichment strategy for estimating the chronological age of bats from DNA methylation (DNAm) profiles. Many mechanistic epidemiological and population models in wildlife rely on age data, traditionally estimated via mark-recapture surveys, body measurements, lethal sampling of bone density, or tooth analyses—methods that are often prohibitively resource-intensive, imprecise, or

impractical. Our DNAm assay offers a cutting-edge alternative, requiring just a single, non-invasive DNA sample. This chapter is an important first step in increasing epidemiological and population modeling capacity in bat populations.

The fourth and final chapter contributed a theoretical perspective to our understanding of the evolutionary dynamics of zoonotic transmission and emergence through an adaptive dynamics framework. Specifically, we analyzed how the interplay between parasite-host coevolution, population dynamics, and epidemiology influence the optimal parasite growth strategy and host investment in constitutive (always present and costly) as opposed to induced (activated and costly only upon infection) defense. Critically, we provide the first theoretical framework that considers both coevolutionary and population-level dynamics, examining trends across host competition and natural mortality rates when the parasite does not directly affect host fertility, as well as when the parasite is a castrator. We show that incorporating host-parasite coevolution into our model captures feedbacks between the host immune and parasite growth strategies that are missed when only the host is allowed to evolve. Furthermore, we find that whether the parasite affects host reproduction significantly impacts host-parasite coevolution; when the parasite is a castrator, selection on the host is often largely geared towards minimizing reproductive costs—either by investing in immunity to avoid infection or recover when parasite prevalence is high, or by reducing investment in reproductively costly constitutive defense when the parasite prevalence is low.

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CURRICULUM VITAE

Sarah Guth
Department of Integrative Biology, University of California, Berkeley
phone: (917) 364-1877; **email:** sarah_guth@berkeley.edu

Education

2017-2022	**PhD in Integrative Biology, University of California-Berkeley** *The dynamics and evolutionary underpinnings of zoonotic transmission and emergence*
2017-2022	**Certificate of Teaching and Learning in Higher Education, University of California-Berkeley**
2017-2021	**Master of Arts in Integrative Biology, University of California-Berkeley** *A framework for assessing the risk of enzootic establishment—arboviruses in the neotropics as a case study*
2011-2015	**Bachelor of Arts, Joint Biology and Environmental Science, Middlebury College** *Conservation Biology, 3.76 GPA, All-American Academic*

Teaching Experience

Fall 2022	**Adjunct Instructor** for General Biology 10, Laney College *Lead instructor, non-majors biology. Developed and customized curriculum for Hack the Hood scholars enrolled in the CIS program at Laney College.*
Fall 2022	**Graduate Student Instructor** for Biology 1B, UC Berkeley *Taught lab sections, general introduction to Ecology and Evolution for Biology majors.*
May 2022	**Instructor** for Cal Genomics Summer Research Experience *Hands-on field and lab experience for undergraduate students in developing research questions and applying cutting-edge molecular biology techniques—including sample collection, nucleotide isolation and amplification, real-time DNA sequencing, and bioinformatics.*
2021-2022	**Graduate Student Instructor** for the Firebaugh Scholars Program, University of California-Berkeley *Selected from a highly competitive pool of applicants from across all graduate programs at UC Berkeley. Co-lead weekly seminars and provide mentoring to 15 undocumented, AB540, systems-impacted, and formerly*

incarcerated undergraduate students who are conducting independent research projects.

2020-2022 **Guest Lecturer** for Biology 134, Emerging World Diseases, Lake Forest College
Invited every semester (3) to give a full lecture on various subjects, including epidemiological modeling, field research, and formulating a research question.

Summer 2021 **Instructor** for Summer Math and Science Honors (SMASH) Academy, University of California-Berkeley
Residential summer program for low-income high school students, focusing on "How scientists are responding to COVID-19".

Summer 2021 "Bring out the Best in your Undergraduate Researchers: Teaching & Mentoring in Scientific Research Groups (BURET)" workshop series
Participated in NSF summer workshop series for Berkeley graduate students that provides training for teaching and mentoring undergraduate students.

Spring 2021 **Guest Lecturer** for IB114: Infectious Disease Dynamics
Invited to give a full lecture on modeling bat-borne zoonoses, spillover events, and debunking xenophobic discussions of zoonotic risk.

Spring 2021 **Graduate Student Instructor** for IB114: Infectious Disease Dynamics, University of California-Berkeley
Taught discussion sections, developing lectures and worksheets that reinforced course concepts. Wrote all assessments for the course, including weekly quizzes, two midterm exams, a final exam.

Fall 2020 **Graduate Student Instructor** for IB164: Human Genetics and Genomics, University of California-Berkeley
Taught discussion sections, developing lectures and worksheets that reinforced course concepts.

October 2020 **Guest Lecturer** with honorarium for Introduction to Environmental Analysis, Pomona College
Invited to give a full lecture on the ecology of zoonotic pathogens and spillover events to humans.

May 2020 **Reader** for IB114: Infectious Disease Dynamics, University of California-Berkeley
Developed and graded a remote final exam for the course that, in a series of short essay questions, asked undergraduate students to relate key concepts from the class to a novel epidemic of their choice (including SARS-CoV-2).

January 2019, 2020	**Instructor** for Ecological and Epidemiological Modeling in Madagascar (E2M2) *Introductory programming (R) and modeling workshop for Malagasy students in ecology, medicine, and public health.*
April 2019	**Guest Lecturer** for Sustainable Development Goals: Challenges and Opportunities, University of St. Andrews *Invited to give a full lecture on the connections between environmental change and infectious disease, as well as participate on a panel.*
Fall 2017	Teaching Colloquium, Department of Integrative Biology, University of California-Berkeley *Semester-long pedagogy course for Graduate Student Instructors that provided discussions and hands-on experiences with undergraduate-level course design and delivery.*
2015-2017	**Program and Undergraduate Coordinator** for the Planetary Health Alliance (PHA), Harvard University *Supported the Planetary Health Education fellow in the development of their planetary health education platform and led efforts to engage Harvard undergraduates in several PHA projects.*

Student Advising

2021-2022	**Priscilla Zhang** **Independent project**: *Key drivers of virulence in intracellular vs. extracellular pathogens*
2020-present	**Katia Renault**, *UC Berkeley undergraduate student*

Awards and Fellowships

2019	Fulbright DDRA program, alternate candidate for Madagascar
2019-2021	Andrew and Mary Thompson Rocca Scholar
2019	Thomas C. Alber Science & Engineering for Global Health Fellow
2018	Infectious Disease Evolution Across Scales Research Exchange Program, Brazil
2017-2022	National Science Foundation, Graduate Research Fellowship
2015	All-American Miler and All-American Academic, NCAA Indoor Track and Field

Peer-Reviewed Publications

***Undergraduate co-author**

2022

Brook CE, Rozins C, **Guth S**, and Boots M. Reservoir host immunology and life history shape virulence evolution in zoonotic viruses. PLoS Biology. *In review.*

Zhang P*, Renault K*, Boots M, and **Guth S**. 2022. Analysis of key drivers of virulence in human-infecting viral and bacterial pathogens. Berkeley Scientific Journal. *In review*.

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Visher E, Evensen C, **Guth S**, Lai Edith*, Norfolk M*, Rozins C, Sokolov NA, Sui M*, and Boots M. 2021. The three Ts of virulence evolution during zoonotic emergence. *Proceedings of the Royal Society B* 288: 20210900. doi.org/10.1098/rspb.2021.0900

Andrianiaina A, Andry S, Gentles A, **Guth S**, Heraud JM, Ranaivoson HC, Ravelomanantsoa NAF, Treuer T, and Brook CE. 2021. Reproduction, seasonal morphology, and juvenile growth in three Malagasy fruit bats. *Journal of Mammalogy* XX(X): 1-15. doi.org/10.1093/jmammal/gyac072

2020

Ravelomanantsoa NAF, **Guth S**, Andrianiaina A, Andry A, Gentles A, Ranaivoson HC, and Brook CE. 2020. The zoonotic potential of bat-borne coronaviruses. *Emerging Topics in Life Sciences* 4(4): 365-381. doi.org/10.1042/ETLS20200097.

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Selected Oral Presentation

Invited talk. *Host phylogenetic distance drives trends in virulence and transmissibility across the animal-human interface*. Disease Seminar, University of Glasgow, Scotland. March 2019.

Talk. *Risk of Arbovirus Spillback in Brazil*. Princeton and Japan Joint Meeting at the Marine Mammal Center, Sausalito, CA. September 2018.

Research Grants

2021	**IB Summer Grant.** *PI.* $2000
2020-2021	**Rocca Dissertation Grant.** *PI.* $6000
2019-2020	**Rocca Dissertation Grant.** *PI.* $6000
2019-2020	**Thomas C. Alber Science & Engineering for Global Health Fellowship Grant.** *PI.* $4000
2020	**IB Summer Grant.** *PI.* $2000
2019	**IB Summer Grant.** *PI.* $2000

Skills

Languages:	Spanish (minimum professional proficiency), Malagasy (limited working proficiency)
Programming:	R, Python, Bash, ArcGIS, Microsoft Office (PowerPoint, Word, Excel)

CHAPTER 1

Host phylogenetic distance drives trends in virus virulence and transmissibility across the animal-human interface

Abstract

Historically, efforts to assess 'zoonotic risk' have focused mainly on quantifying the potential for cross-species emergence of viruses from animal hosts. However, viruses clearly differ in relative burden, both in terms of morbidity and mortality (virulence) incurred and the capacity for sustained human-to-human transmission. Extending previously published databases, we delineated host and viral traits predictive of human mortality associated with viral spillover, viral capacity to transmit between humans following spillover and the probability of a given virus being zoonotic. We demonstrate that increasing host phylogenetic distance from humans positively correlates with human mortality but negatively correlates with human transmissibility, suggesting that the virulence induced by viruses emerging from hosts at high phylogenetic distance may limit capacity for human transmission. Our key result is that hosts most closely related to humans harbour zoonoses of lower impact in terms of morbidity and mortality, while the most distantly related hosts—in particular, order Chiroptera (bats)—harbour highly virulent zoonoses with a lower capacity for endemic establishment in human hosts. As a whole, our results emphasize the importance of understanding how zoonoses manifest in the human population and also highlight potential risks associated with multi-host transmission chains in spillover.

Introduction

Emerging infectious diseases (EIDs) pose a significant threat to public health with the frequency of emergence events increasing in recent decades (1). The vast majority of EIDs are zoonotic, meaning they transmit from animal to human hosts (2). Several studies have conducted meta-analyses to characterize trait profiles associated with zoonotic hosts and viruses (2–6). The majority of this work has focused on quantifying "zoonotic potential"—the probability that a pathogen, or a pathogen originating from an animal species or region of interest, could emerge into the human population. Such a binary categorization effectively treats all zoonoses as risks of equal magnitude, but it is clear that not all zoonoses are created equal. Emerging zoonoses vary both in the severity of disease they engender in the human population ('virulence') and in their capacity for sustained human-to-human transmission post-emergence. A more nuanced understanding of how zoonoses establish in their human hosts will be critical to any public health effort to combat EIDs—as diseases with different severities and transmissibilities will require uniquely targeted intervention and control strategies.

Recent meta-analyses have begun to explore the impact of zoonoses post-spillover, though, to date, only two studies have assessed variation in zoonotic severity. Geoghegan et al. (7) and Brierley et al. (8) demonstrated that zoonoses engendering higher case fatality rates (CFRs) are associated with limited ability for human-to-human transmission. Brierley et al. (8) identified viral traits predictive of virulence but categorized zoonotic severity only in binary terms—"severe" or "nonsevere". A slightly larger body of work has identified viral traits predictive of a zoonotic pathogen's capacity for between-human transmission post-spillover (7–10). However, the majority of these studies use a binary classification scheme (i.e. can transmit between humans or cannot) in their analyses. In nature, there is considerable variation in a

pathogen's capacity for human-to-human transmission, ranging from none (humans are dead end hosts) to stuttering chains to sustained transmission (11). Woolhouse et al. (12) and Lloyd-Smith et al. (13) classify this variation according to a zoonotic pathogen's basic reproduction number (R_0) among human hosts, delineating the number of cases engendered by a single primary case in the human population. In this classification scheme, both dead-end and stuttering chain zoonoses are described by subcritical (<1) R_0 values, while zoonoses that sustain human-to-human transmission have R_0 values >1. For example, human-to-human transmission has been reported for Andes virus, but transmission chains are rare and typically short-lived ($R_0 < 1$) (14), while other viruses, such as Ebola, have the capacity for effective between-human transmission, resulting in long transmission chains and large epidemics ($R_0 > 1$) (15). Additionally, some zoonotic pathogens, such as Hepatitis E, can establish endemic transmission in human populations (16). To our knowledge, Brierley et al. (8) is the only meta-analysis to date that begins to capture this nuance, using a 3-point ranking system to distinguish between zoonotic pathogens without human transmission, and those with limited and sustained transmission.

All previous meta-analyses investigating zoonotic severity and transmissibility have focused almost exclusively on identifying viral predictors of zoonotic impact post-spillover. Currently, no comparative studies of zoonotic virulence and transmissibility consider the role of the animal host, though both virulence and transmission are shaped by viruses' interactions with their hosts (17–19). A virus must replicate to overcome host defenses and transmit to other individuals in the host population, but replication can also produce maladaptive virulence that damages the host, thus shortening the infectious period and jeopardizing opportunities for future transmission (20,21). According to this virulence-transmission tradeoff, viruses optimize virulence incurred in their hosts so as to effectively transmit to new hosts. This optimal balance depends on how hosts respond to the virus ('host selective pressure') (22). If increased viral replication is needed to overcome more robust host defenses, host immunity will select for higher virulence (23–25). Host population structure can also facilitate or hinder transmission, further influencing the evolution of virulence (26,27).

In this study, we addressed the need for a more nuanced understanding of how emerging zoonoses manifest in the human population. Focusing on directly transmitted viruses with a recent history of spillover to humans, we extended databases published by Olival et al. (28), detailing the mortality ('virulence') induced by a given virus upon spillover to humans and quantifying each virus's capacity for human-to-human transmission. We used generalized additive models to identify mammalian host and viral traits predictive of: (a) the human CFRs induced by viral zoonosis, (b) the extent of human-to-human transmission resulting from zoonotic spillover, and (c) the probability of a given virus being zoonotic.

Our analysis of zoonotic risk extends beyond the simple probability of emergence and improves on previous analogous studies to investigate both host and viral trait predictors for non-binary metrics of virulence and transmissibility. This is the first meta-analysis of its type to distinguish between viruses' reservoir host species (i.e., primary selective environment), and secondary host species that have been infected, but do not maintain zoonotic transmission. We hypothesized an inverse relationship between CFR and human transmissibility and anticipated that both reservoir host and viral traits would significantly predict zoonotic risk. In particular, we investigated the hypothesis that host species distantly related to humans would host more virulent viruses with lower capacities for between-human transmission.

Methods

Detailed methods and datasets are given in the *SI Methods* and *SI Data and Results*.

Compiling the databases. We compiled a list of 420 associations between 67 directly-transmitted zoonotic viruses and 278 mammalian hosts, drawing primarily from an extensive database of virus-mammal associations published by Olival et al. (28) (*SI Data and Results,* Table S1). For each virus-mammal association, we conducted literature searches to collect two metrics of zoonotic risk: CFR in the human population, following viral spillover (a proxy for virulence), and viral capacity for human-to-human transmission, which we ranked according to a four-point scale, ranging from "1" for viruses never recorded as transmitting between humans to "4" for viruses known to maintain endemic human transmission (8,11,13,29).

In addition to collecting targeted metrics of virulence and transmissibility, we classified each virus-mammal association according to the mammal's role in viral transmission. We used a binary code to distinguish between mammal species that maintain viruses endemically (reservoir hosts) and species that harbor the virus but are not implicated in zoonotic maintenance (secondary hosts, *see Table 1.1 for definitions of all terms in this study*). We assigned a second binary code to define each host's role in zoonotic spillover ("spillover capacity"), distinguishing between mammal species that serve as human infection sources and species with no record of transmission to humans. Combining these two codes, we defined a third "spillover type" code to distinguish between "primary spillover" from reservoir host species and "secondary spillover" from secondary host species, or "bridge hosts".

In addition to the virus-mammal association database, we extracted a dataset of 345 directly-transmitted mammalian viruses (both zoonotic and non-zoonotic) from Olival et al. (28) (*SI Data and Results,* Table S2).

Using previously published databases (7,9,28–33), we next collected a series of host and viral traits that we hypothesized might predict the observed variation in zoonotic virus dynamics in humans. For hosts, we focused on four life history traits, quantifiable across mammal species, that have been linked to host-pathogen coevolution: body mass, litter size, gestation duration, and lifespan. Host body mass has been linked with the rate of disease progression (34), reservoir competence (35), and pathogen replication rate (36,37); host reproductive effort trades off with immune investment and shapes susceptible host population demography (38,39); and protracted host lifespans are associated with heightened population-level transmission (40,41). Replicating Olival et al. (28), we additionally considered host phylogenetic order and distance from humans. Evolutionary distance between novel and previously documented host species has been identified as a predictor of disease-induced mortality post-spillover in domesticated animals (42). For viral traits, we focused on traits that Olival et al. (28) previously linked to zoonotic infectivity, collecting viruses' host phylogenetic breadths as proxies for viral host ranges. We additionally included the position of a virus' host breadth relative to humans by considering the maximum host phylogenetic distance from humans across a virus' host range. All datasets with metadata and references are available in the *SI Data and Results,* Tables S1–4. Table 1.2 describes predictor and response variables used in our analysis.

Statistical analysis. Because nonlinear relationships were anticipated, we used generalized additive models (GAMs) in the mgcv package in R (43) to assess host and viral predictors of zoonotic risk. GAMs are flexible generalized linear models that, rather than manually specifying higher order polynomial functions, use smooth functions to capture nonlinear relationships

between response and predictor variables. We fit two sets of GAMs, assessing host predictors of zoonotic risk in one group of models and viral predictors in the other.

Our global models included all host and viral trait predictors outlined in Table 1.2. We used automated term selection by double penalty smoothing for variable selection. This method constructs an additional penalty for each GAM smooth function, effectively removing terms without predictive power and has been recognized as superior or comparable to alternative approaches (44). We set an effective degree of freedom cutoff of 0.001 to identify which terms had been penalized and effectively removed from the model (28).

Host models

We restricted our analysis of host predictors of zoonotic risk to known reservoir host species with demonstrated evidence of animal-to-human spillover, thus only considering species implicated as both the primary selective environment and source of human infection for a given virus. Because the specific host species responsible for a spillover event is not always identified, we were frequently unable to collect human CFR and transmissibility data that varied depending on the specific spillover host. Thus, to avoid pseudoreplication, we further restricted our analysis to include only unique entries for each host order per virus in a simplified dataset, summarizing information across hosts encapsulated in each unique entry by taking the maximum value for each host trait metric.

Using this simplified dataset, we first asked *what host traits best predict CFRs in human hosts following spillover?* Specifically, we used a GAM to query the predictive capacity of the host-specific traits outlined in Table 1.2 on the response variable of mean CFR in a human host.

We next asked, *what host variables best predict the extent of human-to-human transmission of a given virus following spillover?* In this case, we used a GAM to query the predictive capacity of the same host traits outlined in Table 1.2 on the response variable of human transmissibility.

While our previous GAMs only included reservoir host species, we next investigated both reservoir and secondary hosts with evidence of spillover to humans (bridge hosts). Fitting a separate GAM for each response variable, we explored the relationship between host phylogenetic distance from humans and both CFR and transmissibility as a function of 'spillover type' ("primary spillover" from reservoir hosts vs. "secondary spillover" from bridge hosts). In each case, we queried the response variable against the predictor variable of host phylogenetic distance, modeled as two distinct smoothers separated by spillover type (43).

Virus models

For our analysis of viral predictors of zoonotic risk, we first asked *what viral traits best predict the probability that a virus is zoonotic*? We constructed a binomial GAM, testing the predictive capacity of viral traits outlined in Table 1.2 against the response variable of zoonotic status (0-1, is versus is not).

Our analysis of viral predictors of CFR and human transmissibility largely mirrored that of our host analysis. To avoid pseudoreplication, we again only considered unique entries, grouping trait information by discrete CFR and transmissibility values per virus.

As with host models, we then applied GAMs to this simplified viral dataset to ask, *what viral traits best predict case mortality rates in human hosts following spillover?* and *what viral traits best predict the extent of human-to-human transmission of a given virus following spillover?*

Results

Host predictors of human CFRs and capacity for human-to-human transmission.

The selected model to predict human CFR explained 51.4% of the total deviance and included maximum host body mass, gestation period, lifespan, and phylogenetic distance from humans as significant predictors, as well as a term for host phylogenetic order (*SI Data and Results,* Table S5*a*). We observed a correlation between increasing CFR and increasing mammalian host phylogenetic distance from humans (Figure 1.1a): hosts most distantly related to humans harbor the most virulent viruses. The model additionally indicated that CFR decreases with increasing host body mass and decreasing host gestation period, though host life history traits are inherently correlated with each other and with host phylogenetic distance from humans (*SI Data and Results,* Table S6).

The selected host model for human transmissibility, which explained 26.5% of the deviance, included maximum host lifespan, litter size, and phylogenetic distance from humans as significant predictors, as well as a term for maximum host body mass (*SI Data and Results,* Table S5*b*). In contrast to CFR, capacity for human-to-human transmission decreases as host phylogenetic distance increases (Figure 1.1b).

Effect of phylogenetic distance on CFR and transmissibility as a function of spillover type.

While our first analysis focused on primary spillover events, in which viruses spill over to humans directly from reservoir hosts, we added important nuance to our analysis by running additional GAMs that considered all virus-mammal associations, including cases of secondary spillover. We explored whether zoonotic risk changes with the position of the immediate spillover source in the transmission chain from reservoir host to human.

The selected model to predict human CFR with interaction terms for host phylogenetic distance and spillover type explained 66.8% of the deviance and included host maximum lifespan, gestation period, and phylogenetic order as significant terms (*SI Data and Results,* Table S5*c*). Spillover type affects the relationship between host phylogenetic distance from humans and human CFR (Figure 1.2). For primary spillovers from reservoir hosts, CFR increases with host phylogenetic distance from humans (Figure 1.2a), consistent with results in Figure 1.1a. In contrast, spillovers from secondary (bridge) hosts are associated with elevated CFR across all secondary host species, irrespective of phylogenetic distance from humans. The largest positive effect sizes are recovered from host species both closely and distantly related to humans (Figure 1.2b). Heightened CFR in secondary hosts at high phylogenetic distances is consistent with the trends observed for reservoir hosts—host species most distantly related to humans harbor the most virulent viruses. However, elevated virulence in secondary hosts at low phylogenetic distances conflicts with our prior observation for reservoir hosts that species most closely related to humans harbor zoonoses of lower impact in terms of morbidity and mortality.

The selected model for human transmissibility with interaction terms for host phylogenetic distance and spillover type explained 34.5% of the deviance and included host order and maximum body mass as significant terms (*SI Data and Results,* Table S5*d*). With primary spillover, transmissibility decreases with increasing host phylogenetic distance from humans, consistent with the virulence-transmission relationship outlined in Figure 1.1. However, variable selection effectively omitted the secondary spillover predictor from this model, suggesting that gaps in data—particularly our limited ability to trace viral outcomes in humans to specific spillover hosts—or a more muddled evolutionary tradeoff among viruses that have a less

extensive shared evolutionary history with their secondary host species may limit our inference capacity.

Examining bats as 'special' viral reservoirs. Broadly, we observed that viruses within a given viral family preferentially infect a discrete subset of host orders (i.e. some viral families are predominantly primate or rodent or bat viruses, etc.) and that distinct mammalian host orders cluster at distinct phylogenetic distances from humans (Figure 1.3). Of the five major animal source orders for zoonotic viruses (Figure 1.3a: Primates, Cetartiodactyla, Carnivora, Rodentia, and Chiroptera), order Chiroptera ('bats') is, on average, the most phylogenetically distant from humans (Figure 1.3b). As such, bats could underpin the heightened virulence of viruses harbored by hosts at high phylogenetic distance (Figure 1.1a). Here, we examine bats as a possible 'special' host order in our database. Bat-derived zoonoses cluster in the upper the right-hand corner of the plane of phylogenetic distance and human CFR (Figure 1.4); our GAM for host traits predictive of virulence demonstrated that order Chiroptera has the strongest positive effect on CFR (*SI Data and Results,* Table S7).

Viral predictors of virus zoonotic potential, human CFRs, and capacity for human-to-human transmission. Because particular viral families associate non-randomly with particular host orders, variation in zoonoses derived from these orders could be equally attributed both to traits of the hosts themselves or traits of the viruses that infect those hosts. Incorporation of viral predictors into our GAM framework demonstrated significant predictive power for several viral traits on three metrics of zoonotic risk. In our probability analysis of a virus's zoonotic potential, the selected model, which explained 32.5% of variation in the data, included significant predictors for cytoplasmic replication capacity, viral genome composition, maximum host phylogenetic distance from humans, and maximum host phylogenetic breadth (*SI Data and Results,* Table S5*e*). Our results mirror previous findings which indicate that viruses with cytoplasmic replication and broader host phylogenetic breadths are more likely to be zoonotic (Figure 1.5b) (6,28). We further observed that viruses derived from hosts at the closest phylogenetic distances to humans had the highest zoonotic potential (Figure 1.5a), consistent with Olival et al.'s (28) finding that hosts closely related to humans harbored the most zoonoses. Both host breadth and the phylogenetic position of that breadth relative to humans significantly predict whether a virus is zoonotic.

Using the zoonotic subset of our virus database, we next considered the effect of viral traits on the response variables of human CFR and capacity for sustained human-to-human transmission. Our selected viral trait model for human CFR explained 53.8% of observed variation in the data and included terms for genome composition, disease-related citations, the presence/absence of a viral envelope, and the maximum phylogenetic distance from humans of all hosts observed for a given virus (*SI Data and Results,* Table S5*f*). The absence of viral envelope was associated with lower virulence upon spillover to humans. Consistent with trends reported in Figure 1.1a, host phylogenetic distance was positively correlated with higher human CFRs post-spillover (Figure 1.5c; though note that viral host phylogenetic distance was calculated differently than the host distances used in Figure 1.1 analyses, see *SI Methods*). Notably, max host phylogenetic breadth per virus was not a significant predictor in our final CFR model (Figure 1.5d).

Finally, the selected viral model for human transmissibility described 55.1% of variation in the data and included terms for genome and DNA/RNA composition, the presence/absence of

a viral envelope, the maximum host phylogenetic distance from humans, and the maximum host phylogenetic breadth observed for each viral species (*SI Data and Results,* Table S5*g*) (Figure 1.5e, 1.5f). Consistent with Geoghegan et al. (7), the absence of viral envelope was positively associated with increased capacity for human-to-human transmission. Consistent with our host model in Figure 1.1b, we observed that capacity for human-to-human transmission decreased with increasing host phylogenetic distance from humans. The relationship between host phylogenetic breadth and human transmissibility was more complicated: human-to-human transmissibility increased with heightened host phylogenetic breadth up to a point (.85), then declined sharply at extremely high breadths. This trend is likely driven by only a few viruses – most notably, rabies, for which zoonotic infection and transmission dynamics are complex (45). Human-to-human transmission may be limited for rabies due to effective control strategies and a bite-dependent transmission route rather than lack of innate viral capacity; rabies virus is known to be present in human tissues relevant for transmission, and, indeed, between-human transmission has been suspected but not confirmed for a few infections (46,47).

Discussion

Our work introduces a novel multidimensional framework for assessing and predicting zoonotic risk. Building off previous analyses (*SI Data and Results,* Table S8), we extended databases published by Olival et al. (28) to delineate host and viral traits predictive of (*a*) the mortality burden associated with viral spillover into the human population, (*b*) the extent of sustained human-to-human transmission following zoonosis, and (*c*) the probability that a virus is zoonotic. For each metric of zoonotic risk, we identified a unique set of host and viral predictor variables, which highlight the critical role of host phylogenetic distance and virus-host evolutionary history in driving zoonotic outcomes.

Our work uncovers a positive correlation between host phylogenetic distance and the mortality incurred by viruses derived from these hosts upon spillover to human populations; zoonoses emerging from more distantly related hosts are more virulent. This result is consistent with empirical work that has demonstrated an association between host phylogeny and disease-induced mortality in a novel host following a pathogen host shift (48,49). Nonetheless, while distantly related hosts may harbor more virulent viruses, our results suggest that these viruses are less likely to engender zoonoses capable of establishing sustained human-to-human transmission. Virulence appears to constrain transmission, limiting zoonotic spread within the human population. Host phylogenetic distance from humans appears to modulate this relationship, predicting virus-induced mortality in a human host and the transmission "cost" associated with that virulence.

The correlation between increasing phylogenetic distance, elevated virulence, and diminishing capacity for transmission is additionally modulated based on the pathway of zoonotic emergence. This study represents the first meta-analysis of its type to distinguish between primary spillover from reservoir hosts—the primary selective environment of viruses—and secondary spillover from bridge host species that have been infected, but do not maintain zoonotic transmission. Primary spillover from reservoir hosts to humans follows the general observed trends, with the highest transmission cost incurred at high phylogenetic distance and high virulence. Intriguingly, secondary spillover hosts, including those closely related to humans, produce elevated CFRs across phylogenetic distances. Because our analysis did not identify secondary spillover as a significant predictor of human transmissibility—potentially due to a limited sample size or more muddled evolutionary relationships between viruses and their

secondary hosts—we cannot infer whether spillover from secondary hosts is constrained by the same virulence-transmission tradeoff observed in reservoir hosts. However, increased CFRs in secondary host species closely related to humans suggest that these hosts could function as "gateways" for virulent viruses originally derived from more distant reservoirs. The virulence-inducing viral traits driving CFR upon spillover from secondary hosts may largely reflect virus's long-term evolutionary history in more phylogenetically distant reservoirs. By contrast, short-term history within more closely related secondary hosts could facilitate pre-adaptation to human transmission. Thus, secondary spillover—by which virulent viruses from distant phylogenetic backgrounds spill over to humans through more closely-related, secondary hosts—may pose the greatest zoonotic threat.

Across mammalian host orders, host species most closely related to humans harbor zoonoses of lower impact in terms of morbidity and mortality but with an elevated propensity for human-to-human transmission. More distantly related hosts—in particular, order Chiroptera—harbor highly virulent zoonoses that nonetheless have a lower capacity for between-human transmissibility. Chiroptera, previously posited to represent a 'special' order of mammalian hosts for zoonotic viruses (50), does appear to uniquely shift zoonoses into otherwise uncharted territory on the virulence axis. Though occupying the same general region of the host phylogenetic distance axis as order Rodentia, bat zoonoses yield CFRs more than double those resulting from rodents.

Results from the viral trait models support previous findings that multi-host pathogens are more likely to emerge and transmit in human populations (2,10,28,51,52). Consistent with findings from Olival et al. (28), we identify maximum phylogenetic host breadth, a marker of viral generalism, as a powerful predictor of zoonotic behavior for the directly-transmitted mammalian viruses queried in our dataset. However, our results indicate that, in addition to the breadth of a virus's host range, the positioning of that range, with respect to phylogenetic distance from humans, has important predictive capacity. Thus, predictions of zoonotic risk will differ for a generalist virus that evolved in a reservoir environment that is phylogenetically close to humans (i.e. primates) versus one that is distant (i.e. bats). This distinction is important, since our work further emphasizes that distinct viral families tend to preferentially infect distinct host orders. Furthermore, in keeping with host model results, we observed that viral traits predicting zoonotic behaviour following emergence demonstrate opposite impacts on virulence and transmissibility in human hosts—indicating trade-offs between these two traits. Host phylogenetic distance, corresponding to the evolutionary host environment in which a virus evolved, is positively correlated with mortality incurred by emerging virus in human hosts, but negatively correlated with viral capacity for human-to-human transmission.

The multidimensional definition of zoonotic risk considered in this analysis highlights limitations in our understanding of emerging zoonoses to date. Historically, most work in this field has been restricted to descriptions and predictions of viral, host, and geographic 'hotspots' for zoonosis, with little consideration of variation in the impact of a given spillover event post-emergence. Our work emphasizes the power of a more nuanced approach to assessing zoonotic risk, but also uncovers gross gaps in data and reporting which will present challenges for future research extensions. To facilitate tracking of directionality in transmission chains, we restricted our database to mammal host-virus associations previously confirmed via PCR or viral isolation, excluding unconfirmed or serologically identified mammalian hosts. For example, compelling serological evidence suggests that bats likely function as the natural reservoir host for Middle Eastern respiratory syndrome (MERS-CoV), a coronavirus similar to bat-derived SARs-CoV

(53), but evidence is insufficient to support inclusion of this association in our study. Indeed, many animal hosts for emerging zoonoses remain unidentified or unconfirmed, and for the majority of known hosts, we lack data regarding transmission and infection dynamics. These gaps in reporting limit our capacity for inference.

The extent to which human mortality and transmissibility may differ depending on the reservoir or spillover host in question has yet to be documented for the vast majority of zoonoses—indeed, we identified distinct measures of CFR and transmissibility that could be traced to specific spillover hosts only for two viruses: Nipah and Marburg. As both of these examples support our finding that changes in a spillover transmission chain are associated with shifts in virulence and transmissibility, collection of data from additional systems demonstrating both secondary and primary zoonotic sources is thus a major research priority. Ebola virus outbreaks, for example, have varied extensively in both virulence and transmissibility across the past four decades of emergence (15,54). The extent to which these variations may be attributable to phylogenetic differences in the source host or transmission chain remains, to our knowledge, unexplored. A more nuanced framework for understanding variation in severity and transmissibility across all potential zoonotic hosts will nonetheless be central to any future effort to develop targeted and effective public health strategies for intervention and control.

CHAPTER 2

Bats host the most virulent—but not the most dangerous—zoonotic viruses

Abstract

Identifying virus characteristics associated with the largest public health impacts on human populations is critical to informing zoonotic risk assessments and surveillance strategies. Efforts to assess "zoonotic risk" often use trait-based analyses to identify which viral and reservoir host groups are most likely to source zoonoses but have not fully addressed how and why the impacts of zoonotic viruses vary in terms of disease severity ('virulence'), capacity to spread within human populations ('transmissibility'), or total human mortality ('death burden'). We analyzed trends in human case fatality rates, transmission capacities, and total death burdens across a comprehensive dataset of mammalian and avian zoonotic viruses. Bats harbor the most virulent zoonotic viruses even when compared to birds, which alongside bats, have been hypothesized to be "special" zoonotic reservoirs due to molecular adaptations that support the physiology of flight. Reservoir host groups more closely related to humans—in particular, Primates—harbor less virulent, but more highly transmissible viruses. Importantly, disproportionately high human death burden, arguably the most important metric of zoonotic risk, is not associated with any animal reservoir, including bats. Our data demonstrate that mechanisms driving death burdens are diverse and often contradict trait-based predictions. Ultimately, total human mortality is dependent on context-specific epidemiological dynamics, which are shaped by a combination of viral traits and conditions in the animal host population and across and beyond the human-animal interface. Understanding the conditions that predict high zoonotic burden in humans will require longitudinal studies of epidemiological dynamics in wildlife and human populations.

Significance statement:

The clear need to mitigate zoonotic risk has fueled increased viral discovery in specific reservoir host taxa. We show that a combination of viral and reservoir traits can predict zoonotic virus

virulence and transmissibility in humans, supporting the hypothesis that bats harbor exceptionally virulent zoonoses. However, pandemic prevention requires thinking beyond zoonotic capacity, virulence, and transmissibility to consider collective 'burden' on human health. For this, viral discovery targeting specific reservoirs may be inefficient as death burden correlates with viral, not reservoir, traits, and depends on context-specific epidemiological dynamics across and beyond the human-animal interface. These findings suggest that longitudinal studies of viral dynamics in reservoir and spillover host populations may offer the most effective strategy for mitigating zoonotic risk.

Introduction

The vast majority of human pathogens are derived from animal populations (2). In response to increasingly frequent zoonotic spillovers and their substantial public health risks (1), there has been a movement to identify the ecological systems and taxonomic groups of animals and pathogens that are most likely to source the next emerging zoonosis in the human population (10,28,55–59). However, most of this work has centered on a binary definition of zoonotic risk—whether particular pathogens are capable of infecting humans—without considering how pathogens vary with respect to their impacts on humans after spillover. The ongoing SARS-CoV-2 pandemic has re-emphasized the reality that not all zoonoses pose risks of equal magnitude—some are exceptionally more dangerous than others due to the severity of disease they cause ('virulence') or their capacity to spread within human populations ('transmissibility'), which combined, influence the total number of human deaths ('death burden') (60). Given the extraordinary diversity of both animal hosts and the viruses they harbor, understanding which animal and virus groups are more likely to source dangerous zoonoses is an important public health aim. Many high-profile zoonotic viruses—including Nipah and Hendra henipaviruses; Ebola filovirus; SARS, MERS, and SARS-CoV-2 coronaviruses; pandemic avian influenzas; West Nile virus; and Eastern Equine encephalitis virus—have emerged from Chiropteran (bat) or avian reservoirs (61). The high number of zoonotic viruses found in bats and birds has been attributed to their large gregarious populations, mobility, ability to colonize anthropogenic environments, and sheer species diversity (57,61). Nonetheless, the question remains: are bat- and/or bird-borne viruses disproportionately dangerous?

A recent analysis (60) found that mammalian reservoir hosts most closely related to humans harbor zoonoses of lower impact in terms of mortality relative to more phylogenetically distant hosts. These results were consistent with phylogenetic trends in virulence that have been reported in cross-species pathogen emergences in other systems (48,62), and likely reflect mismatches in host biology, physiology, and ecology. Notably, order Chiroptera (bats)—one of the more distantly related host orders—had the highest positive effect size on case fatality rate in humans. Nevertheless, this analysis considered only directly transmitted viruses and viruses derived from mammalian hosts, despite the existence of several high-profile vector-borne and avian zoonoses (61). In particular, birds occupy a separate taxonomic class from humans—a phylogenetic distance that might correlate with heightened virulence in humans.

In vitro work has suggested that molecular adaptations that support the physiology of flight, a trait unique to bats among mammals, may allow bats to tolerate rapidly-replicating viruses that express heightened virulence upon emergence in less tolerant hosts such as humans (63)—thus offering a possible explanation for bat virus virulence. Bats and birds share a suite of convergent flight adaptations—both taxa are remarkably long-lived for their body size and appear to circumvent metabolic constraints on longevity through cellular pathways evolved to

mitigate oxidative stress induced by flight (61). These metabolic adaptations are hypothesized to be linked to the evolution of virulent viruses in bats, but only typically discussed with respect to their effect on lifespan in birds (64). A few papers have reviewed birds' role as special zoonotic reservoirs (61,65), but the virulence of avian zoonoses remains largely unexplored. Nonetheless, though the most virulent zoonotic viruses may garner the most publicity, these pathogens are not necessarily the most 'dangerous' to human health. Rather, human health is most impacted by viruses that cause large volumes of cases and deaths ('burden'). While some viruses such as Ebola and rabies are associated with both high case fatality rates and burden in the human population, pandemic viruses are often characterized by relatively low case fatality rates but high human transmissibility. The 2009 H1N1 influenza pandemic was estimated to have caused 60.8 million cases and more than 12,000 deaths in the United States alone with a case fatality rate of less than 1% (66), and as of July 9th, 2021, SARS-CoV-2 has caused over 185 million cases and 4 million deaths worldwide with a case fatality rate of just 2.2% (67). To prevent the next zoonotic pandemic, it is important to think beyond the individual measures of zoonotic capacity, virulence, and transmissibility to consider collective 'burden' on public health.

We applied generalized additive models (GAMs) to a dataset of mammalian and avian zoonotic viruses to identify reservoir host and viral traits predictive of the (a) case fatality rate (CFR), (b) capacity for forward transmission, and (c) death burden induced by infections in the human population—with the goal of characterizing sources of zoonotic viruses that pose the greatest danger to global health. Our work builds on a small body of analyses that have begun to explore variation in the virulence and between-human transmissibility of zoonotic viruses (7,9,10,68). We provide the first analysis of burden and the largest sample size—with trends examined across the majority of known zoonotic viruses. We hypothesized that birds—given their capacity for flight and phylogenetic distance from humans—might rival bats for the association with the most virulent zoonotic viruses. However, we did not expect bats or birds to be responsible for the greatest burden on global health, instead anticipating high burden to be largely a function of viral traits and associated with reservoir orders that harbor less virulent, more transmissible viruses.

Materials and Methods

Constructing the database (Figure S1 in *SI Appendix*). We curated a comprehensive dataset of mammalian and avian zoonotic viruses—and the taxonomic orders of the reservoir hosts from which they were derived—from published databases (57,60,69). Reservoirs were defined as the primary host species that is responsible for maintaining zoonotic transmission. Using the information provided in these databases and supplementing with literature searches, we extracted viruses that met a strict definition of zoonotic, requiring at least one published human infection in which the virus species was confirmed by PCR, sequencing, or isolation as well as evidence of animal-to-human directionality in transmission (*SI Appendix*, *Exclusion criteria*). With this strict inclusion criteria, we compiled 89 unique virus species (Table S1 in *SI Data and Results*).
For each virus-reservoir association, we collected both human case fatality rate (CFR) as a proxy for virulence, and the cumulative global death count as a proxy for burden on the human population. For CFR, we collected two estimates. First, we recorded existing estimates of global CFRs from the literature, calculating averages when ranges were reported. Second, for each virus species, we calculated country-specific CFRs from death and case counts in countries that have reported the largest outbreaks of that virus—to assess and account for potentially confounding effects of regional differences in health care and overall infrastructure (*SI Appendix*,

Supplementary analyses). For our death burden response variable, we collected the total number of deaths recorded across the world since 1950. In many cases, our death count began after 1950, either because a zoonosis first emerged in humans after 1950 or reliable death records were only available for a subset of the timeline. To standardize, we added a variable for the number of years over which death counts were recorded to use as an offset in our models. Death and case counts were derived, when available, from the Global Infectious Diseases and Epidemiology Network (GIDEON) (70)—which contains outbreak data from case reports, government agencies, and published literature records—and supplemented with literature searches. We additionally ranked each zoonosis' capacity for transmission within human populations—a correlate of R_0—on a four-point scale (60). All variable descriptions are provided in Table S4 in *SI Data and Results*.

Drawing from previously published databases (57,60), we collected seven variables (*SI Data and Results*, Table S7) that we hypothesized might predict observed variation in human CFR, capacity for transmission within human populations, and death burden. Given published correlations between phylogenetic distance and virulence in cross-species spillovers (48,49,60,62,71), we included the reservoir host group cophenetic distance from Primates. We considered both reservoir host and virus taxonomy, recording host order and virus family. However, only ten avian zoonoses were distributed across several avian reservoir host orders. To test our hypotheses regarding avian zoonoses, we addressed this small sample size by aggregating avian reservoir orders into a single "Aves" group, while maintaining separate host orders for the mammalian reservoirs. Given that the number of zoonoses harbored by a reservoir group appears to correlate with species diversity within that group (57), we hypothesized that species diversity might influence reservoir effect size on CFR in humans; thus, we included reservoir species richness, which we derived from the Catalogue of Life using version 0.9.6 of the taxize library in R (57,72), taking the sum of values across bird orders for the Aves reservoir group. We defined a "spillover type" variable to account for the zoonotic transmission chain of each virus, distinguishing between zoonoses that jump into humans directly from the reservoir population and those that spillover to humans from bridge hosts (60). While the majority of zoonoses were linked to single zoonotic transmission chains, there were a few exceptions with both "direct" and "bridged" spillover. For example, zoonotic Influenza A virus and Nipah virus (73,74) have spilled over into the human population directly from their avian and bat reservoirs, respectively, as well as from domestic pig bridge host populations. In such cases, each spillover type (i.e., transmission chain) was entered separately in the database. We included an additional binary variable that identified whether viruses were vector-borne, as both theory (20) and previous analyses (7,68) have suggested a relationship between vector-borne transmission and virulence. Finally, as has been done in other similar analyses, we included virus species publication count to account for any potential publication bias (28,60,71).

To pair with our country-specific CFR data, we collected an eighth predictor variable—gross domestic product (GDP) per capita—as a proxy for geographical differences in the quality of health care and epidemiological control measures.

We additionally collected, for each virus species, the transmission route that contributes the majority of human infections, extending data published by Brierley et al. (68). We then assessed trends in death burden across transmission types, hypothesizing that density-dependent transmission, as characteristic of transmission via respiratory droplets, would be associated with the highest death burdens in human populations.

Statistical analysis. Given the non-normal distribution of our data, expected nonlinear relationships, and nested data structures within our predictor variables (75), we applied generalized additive models (GAMs) in the mgcv package in R (76) to assess predictors of CFR, transmissibility, and death burden in human populations. Rather than manually specifying higher order polynomial functions, GAMs permit the use of smooth functions to capture nonlinear relationships between response and predictor variables (75,76). We fit continuous variables (i.e., reservoir group species richness and phylogenetic distance from Primates, and virus species publication count) as smoothed effects, and all binary (i.e., vector-borne status and spillover type) and categorical (i.e., reservoir order and virus family) variables as random effects, as has been done in previous analyses (10,28,57,60). For variable selection, we ran all possible model combinations, ranked by AIC, and selected the models with the lowest AIC values. We confirmed our results by rerunning variable selection with automated term selection by double penalty smoothing. This method bypasses AIC-maximization procedures by constructing an additional penalty for each GAM smooth function, effectively removing terms without predictive power, and has been recognized as superior or comparable to alternative approaches (44). We set an effective degree of freedom cutoff of 0.001 to identify which terms had been penalized and effectively removed from the model (28). The validity of all models was checked using standard methods implemented in the mgcv library (76).

We first asked, *which reservoir host and virus types are associated with elevated CFRs in human populations following spillover?* We constructed GAMs in the beta regression family to query the predictive capacity of our predictor variables (*SI Data and Results*, Table S7) on CFR in humans. We compressed our CFR range to the beta distribution interval (0,1) by applying the recommended data transformation $y" = [y'(N-1) + 1/2]N$, where N is the sample size (77,78). For all CFR analyses, we modeled unique zoonotic transmission chains—which we defined as unique reservoir orders and spillover type combinations per virus. As a result, zoonoses with a single reservoir host order and spillover type were modeled as a single CFR entry, while those with multiple reservoir orders and/or spillover types (e.g., Influenza A and Nipah viruses) were modeled as multiple CFR entries. We excluded five viruses for which only one human case has been recorded (Table S1 in *SI Data and Results*), deciding that we could not accurately represent a single observation as a CFR. Our final GAM analysis of global CFR estimates included 82 unique virus species with a total of 86 unique zoonotic transmission chains (Table S5a in *SI Data and Results*).

We next asked, *which reservoir host and virus types are associated with elevated capacity for transmission within human populations?* We constructed a GAM in the 'ocat' ('ordered categorical data') family to query the predictive capacity of our predictor variables on transmissibility, defining the vector of categorical cut points, θ, to match our four-point ranking scale (θ = 1,2,3,4). We again excluded the five viruses for which only one human case has been recorded (Table S1 in *SI Data and Results*), deciding that we could not accurately determine between-human transmissibility based on a single observation. Thus, like our CFR analysis, our transmissibility analysis included 82 unique virus species with a total of 86 unique zoonotic transmission chains (Table S5b in *SI Data and Results*).

Lastly, we asked, *which reservoir host and virus types are associated with high death burdens in human populations?* The death count data demonstrated strong overdispersion (Figure S11 in *SI Appendix*). Thus, we constructed a negative binomial GAM with the scaled observation period (i.e., number of years over which the death count was recorded) as an offset. We considered simpler Poisson GAMs, as well as zero-inflated models, but enhanced residual quantile-quantile

(QQ) plots (79) suggested that these distributions fit poorly. Unlike our CFR analysis, we did not exclude viruses for which only one human case has been recorded. However, we did exclude a single virus species—Rotavirus A—for which we were unable to distinguish between deaths caused by zoonotic strains versus deaths caused by endemic human strains. Thus, our death burden models included 86 zoonotic viruses with a total of 90 transmission chains (Table S5c and S6f in *SI Data and Results*).

Results

Drawing from existing databases (57,60,69), we compiled a dataset of all mammalian and avian zoonotic virus species that met a strict definition of zoonotic—requiring a post-1950 record of natural human infection confirmed by PCR or sequencing and animal-to-human directionality in transmission (*Materials and Methods, Constructing the database*). Virus species linked to multiple independent reservoir groups (e.g., canine and bat rabies) or those which spillover to humans both directly from their reservoir and through bridge hosts (e.g., Nipah virus) were subdivided into separate entries for each unique transmission chain ending in spillover, creating a final dataset of 89 viruses with a total of 93 transmission chains (*SI Data and Results,* Table S1). We then applied generalized additive models (GAMs) to assess predictors (*SI Data and Results*, Table S7) of three metrics of zoonotic risk: global estimates of case fatality rates (CFRs) in humans (proxy for virulence), capacity for forward transmission within the human population ranked on a four-point scale (human transmissibility), and post-1950 cumulative death counts (death burden) (Figure S1 in *SI Appendix*). We used both AIC-maximization model selection (results reported in the main manuscript) and automated term selection by double penalty smoothing (*SI Data and Results*, Table S8), and found that all key results were consistent across the two variable selection techniques.

Predictors of human CFRs. In our virulence analysis, we observed a left-skewed distribution of CFRs, with 33.7% of virus species linked to no fatalities (0% CFR) and more than half (57.8%) linked to a CFR of less than 10% (Figure S2 in *SI Appendix*). Bat reservoirs contributed more than two thirds (68.8%) of the identified viruses with CFRs higher than 50%. The top selected GAM to predict global estimates of CFR in humans—across the 87 unique zoonotic transmission chains for which at least two human cases have been recorded—explained 75.3% of the deviance and included reservoir host group, virus family, bridged spillover, and vector-borne transmission (Figure 2.1, Table S5a in *SI Data and Results*). Consistent with previous work (60) and the hypothesis that bats are "special" zoonotic reservoirs, order Chiroptera had the largest positive effect size on CFR in humans (Figure 2.1b). The top selected model predicted a CFR of 65.6% for zoonotic viruses derived from order Chiroptera, representing a more than 50% increase from the next highest predicted CFR (Figure S3). Nevertheless, overlapping confidence intervals for both CFR predictions (Figure S3) and effect sizes (Figure 2.1b) indicated that without larger sample sizes, we cannot eliminate all uncertainty regarding the virulence of bat viruses relative to viruses from reservoir groups with very few known zoonotic viruses. Contrary to our flight hypothesis, avian reservoirs were not similarly associated with disproportionately virulent zoonoses; order Aves had a neutral effect size on human CFR that was not significant. Order Cetartiodactyla—which in our dataset, included on domesticated animal species (i.e., cattle, pigs, and camels)—had the largest negative effect size on CFR.

Past analyses have observed that particular viral families associate non-randomly with particular host groups (60,80), suggesting that virus taxonomy may underlie trends in virulence across reservoir orders. For example, the high number of virulent bat-borne zoonoses (Figure S2

in *SI Appendix*) may be entirely a result of the virus groups that preferentially infect bats, rather than the bats themselves. However, here, reservoir host group and virus family significantly predicted CFR within the same models (Figure 2.1a), indicating that both reservoir and virus taxa contributed to the observed variation in virulence. Chiroptera had the highest positive effect size on CFR despite being associated with virus families that ranged from the most (Rhabdoviridae) to least (Coronaviridae) virulent (Figure 2.1c). Removing the 100% fatal lyssaviruses (n=5) from the dataset resulted in large reductions in the CFR predicted for bat-borne zoonoses (Figure S5), though order Chiroptera still had the highest and most significant positive effect size on CFR—indicating that observed patterns were not driven by rabies alone (Figure S4 in *SI Appendix*, Table S6a in *SI Data and Results*).

Previous work has demonstrated a positive correlation between reservoir host phylogenetic distance from humans and the case fatality rates of zoonoses derived from those reservoirs (60); in our analysis, however, reservoir host group phylogenetic distance from Primates was not correlated with CFR, dropping entirely from the top ranked model and not ranking significantly in any of the top 15 selected models (Figure 2.1a). The combined effect of reservoir host group and virus family as predictor variables in the same model likely overwhelmed any correlation between host phylogeny and CFR, particularly given the lack of granularity in our phylogenetic distance variable, based on a time-scaled phylogeny, which produced only six unique distance values across nine host groups, with Chiroptera and four of the other mammalian orders clustering at a single distance level (*Materials and Methods*). Nevertheless, trends in effect size on CFR (Figure 2.1b) and predicted CFR (Figure S3) across reservoir host groups suggest that, in general, virulence increases with phylogenetic distance, but this positive correlation may collapse at "extreme" distances.

To test whether these results held across a larger sample size, we ran a CFR analysis that included viruses that met a more lenient definition of zoonotic—specifically, viruses with only serological evidence of infection in humans, viruses that have only caused human infections in laboratory settings, and viruses for which only one human case has been recorded—increasing our dataset to 121 virus species with a total of 126 unique zoonotic transmission chains (Figure S6 in *SI Appendix*, Table S6b in *SI Data and Results*). This supplementary analysis echoed the results from our first analysis of global CFR estimates (Figure S6a in *SI Appendix*)—both reservoir and virus taxonomy contributed to the observed variation in CFR; and Chiroptera had the highest positive effect size on CFR, whereas Aves had a neutral nonsignificant effect (Figure S6b in *SI Appendix*).

To assess whether CFR trends might be biased by viruses' geographic ranges (e.g., differences in health care infrastructure and case ascertainment), we tested whether Gross Domestic Product per capita (GDP per-capita) significantly predicted country-specific CFR estimates—calculated from death and case counts in countries that have reported the largest outbreaks of each given virus species, with up to three country estimates for each species for a total of 119 estimates across the 87 unique zoonotic transmission chains. First, we modeled all 119 country-specific CFR estimates separately to test whether GDP per-capita predicts country-level variation in CFR (Figure S7 in *SI Appendix*, Table S6c in *SI Data and Results*). We then modeled GDP per-capita and country CFR estimates aggregated at the level of the 87 unique zoonotic transmission chains (Figure S8 in *SI Appendix*, Table S6d in *SI Data and Results*). In both analyses, GDP per-capita was not significant in any of the top models, often dropping entirely during model selection (Figure S7a and S8a in *SI Appendix*), suggesting that viruses' geographic ranges most likely do not bias Figure 2.1 trends. Nevertheless, as with the

supplementary analyses presented in Figure S4 and Figure S6, both analyses of the country CFR estimates echoed all key results presented in Figure 2.1.

Predictors of transmissibility within human populations. We found that most zoonotic viruses (71.3%) have not been reported to transmit within the human population following spillover (i.e., transmissibility rank = 1, or $R_0 = 0$) (Figure S9). Only 14.9% of virus species had demonstrated capacity for endemic transmission among humans, of which the majority (61.5%) were sourced from Primates. The top selected GAM to predict the ordinal rank of transmissibility within human populations—across the 87 unique zoonotic transmission chains for which at least two human cases have been recorded—explained 56.6% of the deviance and included virus family, the phylogenetic distance between each viruses' reservoir host group and Primates, vector-borne transmission, and the virus species publication count (Figure 2.2, Table S5b in *SI Data and Results*). Transmissibility declined with phylogenetic distance from Primates, but the estimated trend was highly uncertain (Figure 2.2c). We therefore reran the analysis with reservoir host group as the only host taxonomic predictor (excluding the phylogenetic distance variable). This model explained 55.9% of the deviance and identified Primates as the only host order significantly associated with heightened transmissibility in humans, suggesting that this group is the primary driver of the phylogenetic trend observed in the top selected model (Figure S10a in *SI Appendix*, Table S6e in *SI Data and Results*).

Predictors of post-1950 death burden in the human population. For our death burden analysis, we modeled the total number of deaths resulting from a given zoonosis recorded worldwide since 1950 (and up until March 7th, 2021). In cases where our death count could only begin after 1950, either because a zoonosis first emerged in humans after 1950 or because reliable death records were only available for a subset of the timeline, we standardized analyses by including an offset for the number of years over which the death counts were recorded. The raw death count distribution was highly left-skewed, with 39.8% of virus species linked to zero deaths and more than half (62.5%) linked to fewer than 50 deaths (Figure S12 in *SI Appendix*). We observed significant overdispersion in death counts, even when standardized by the number of years over which the deaths were recorded, with deaths per year ranging from zero to almost 2 million for SARS-CoV-2. Just two viral predictors—virus family and species publication count—explained most of the variation in death burden among the 93 zoonotic transmission chains across all the top GAMs (Figure S13a in *SI Appendix*). Host predictors explained a very low percentage of the variation in death burden across all the top selected models, often dropping entirely during term selection. Virus species publication count tempered virus family effects (Figure S13c in *SI Appendix*) because virus species with high death burdens were also associated with high publication counts, likely because high death burdens motivate increased research efforts. In contrast, there was little evidence that only poorly studied viruses were limited to unusually low death burdens, implying that a lack of diagnostic effort is not a major driver of low death burdens in our data (Figure S13c in *SI Appendix*). After excluding the virus species publication predictor, we found that Coronaviridae, Orthomyxoviridae and Rhabdoviridae had the highest positive effect sizes on death burden, driven by, respectively, the SARS-CoVs, the Influenza A transmission chains, and Rabies virus (Figure 2.3b*,* Table S5c in *SI Data and Results*). With virus publication count removed, the top four models included two reservoir traits—phylogenetic distance from Primates and species richness—as significant predictors. Reservoir groups most closely related to Primates were associated with heightened death burdens relative to more distantly related reservoirs, consistent with results from our transmissibility analyses that indicated that reservoirs most closely related to Primates harbored more

transmissible viruses (Figure 2.3c). Reservoir species richness positively correlated with death burden, as we would expect given that species richness has been found to correlate with the number of viruses associated with a given reservoir order (Figure 2.3d) (57). However, both reservoir predictors explained a small fraction of the variation in death burden relative to virus family, confirming that death burden is largely a function of viral traits (Figure 2.3a).

While some reservoir groups—bats, primates, rodents, and birds—have sourced more high burden viruses than others (Figure 2.4a), both our model results and raw data suggested that high burden viruses appeared to be function of viral traits, not the reservoirs themselves. No single reservoir stood out as a consistent source of high burden viruses, with every reservoir that harbors high burden viruses also harboring substantially more viruses that cluster at the lowest death burdens (Figure 2.4a). This was not the case for virus family (Figure 2.4b) or primary transmission route (Figure 2.4c); Coronaviridae and Orthomyxoviridae and a respiratory transmission route were associated only with high burden zoonotic viruses. In general, the viruses linked to the lowest death burdens were associated with the lowest transmission capacity. As a deviation from this trend, Primates—which our models indicate harbor the most transmissible, but generally less virulent zoonotic viruses—harbored several highly transmissible viruses with low death burdens (Figure 2.4a).

The highest death burdens were overall associated with zoonotic viruses that are less virulent but highly transmissible in human populations (Figure 2.4d). Respiratory pathogens with capacity for human-to-human transmission have often incurred massive burdens over short timeframes as a result of rare, but catastrophic spillover events that spark widespread transmission in humans. Critically, while our dataset included only six viruses with respiratory droplets as a primary transmission route—SARS-CoV-1, SARS-CoV-2, MERS CoV, Influenza A, Nipah, and Monkeypox—these viruses accounted for more than 85.9% of the deaths recorded for the 86 viruses in our death burden analysis, highlighting respiratory transmission as a high-risk zoonotic trait. However, these data were derived from a notably small sample size, as three of the six respiratory viruses have caused only a single major epidemic. There was also substantial variation among these respiratory viruses, with the death burdens associated with SARS-CoV-1 and SARS-CoV-2 differing by more than 2.5 million people.

Discussion

A key insight from our work is that bats harbor the most virulent zoonotic viruses relative to other mammalian and avian reservoirs (Figure S2 in *SI Appendix*). Given that birds represent the only other flying vertebrates and that flight adaptations are hypothesized to influence the evolution of viruses virulent to humans in bat reservoirs (61), we expected avian viruses to similarly be associated with heightened CFRs in humans. However, we found that only order Chiroptera had an exceptionally high positive effect size on CFR in humans, while Aves had a neutral nonsignificant effect. It is of course possible that we observed this association between Chiroptera and high CFRs in part because low virulence zoonotic viruses have gone undetected in bat reservoirs; however, other poorly studied reservoirs are not comparably associated with heightened virulence, suggesting that detection bias cannot explain our results. Like CFR, transmissibility in humans was also correlated with reservoir traits, but in this case, Primates—the reservoir group most closely related to humans—sourced the zoonotic viruses with the highest capacities for forward transmission in human populations. While a combination of both virus and reservoir taxonomy predicted virulence and transmissibility, death burden did not correlate with any reservoir group and instead, was a function of viral traits. Nevertheless, our

data indicated that mechanisms driving high death burdens are diverse and often contradict trait-based predictions. Several high-profile zoonotic viruses linked to significantly higher death burdens than we would expect based on their capacity for forward transmission in the human population (Figure 2.4d), suggesting that death burden is highly dependent on both the contact rate at the human-animal interface and epidemiological dynamics within the human population—factors which are not fully captured by the broad explanatory variables considered in trait-based analyses.

Evolution of virulence theory typically assumes a tradeoff between virulence (death rate due to infection) and transmission rate on the basis that while high within-host growth rates increase infectiousness, they also increase damage to the host, increasing virulence and thus shortening the infectious period and reducing opportunities for future transmission (20,21). Critically, CFR is not equivalent to virulence, but instead, a proxy that can be reliably quantified. As defined by Day 2002 (81), CFR is a function of both pathogen virulence (α) and clearance rate (σ), in which $CFR = \alpha/(\alpha + \sigma)$. Thus, virulent pathogens (high α) with high clearance rates (high σ)—e.g., acute, short-lived infections such as Chikungunya virus (82)—could produce low CFRs. In contrast, less virulent pathogens (low α) with low clearance rates (low σ)—e.g., persistent infections such as HIV (83)—could produce high CFRs. Nevertheless, in our data, we observed a relationship between CFR and transmissibility in humans that roughly supports the fundamental theoretical tradeoff between virulence and transmission rate (Figure S11 in *SI Appendix*). Viruses causing the highest CFRs in humans (>75% CFR) clustered in the lower right corner with the lowest capacity for forward transmission in the human population, implying maladaptive virulence. Conversely, the least virulent viruses (0% CFR) clustered at either the lowest transmission capacity—likely indicative of poor compatibility with humans—or the highest transmission capacity—suggesting transmission uninhibited by virulence.

The surprisingly low virulence of avian zoonotic viruses in contrast to bat-borne viruses may reflect the extreme phylogenetic distance that separates birds from Primates. In our previous analysis, we found that mammalian reservoir hosts most closely related to humans harbor less virulent zoonotic viruses relative to more distantly related mammalian hosts such as bats (60). This positive correlation between reservoir phylogenetic distance from humans and viral virulence is consistent with trends that have been reported in cross-species pathogen emergences in other systems (48,60,62), and likely reflects maladaptive virulence resulting from mismatches in host biology, physiology, and ecology. Clearly, while bats are distantly related to humans, they are still mammals, whereas birds occupy a separate taxonomic class. It is possible that the positive correlation between phylogenetic distance and virulence collapses at distances beyond mammals, because viruses are expected to have a limited capacity to replicate in host environments that are very different from that of their reservoir, leading to ‘non-host resistance’ (84,85). Phylogenetic distance dropped from all CFR models likely due to a lack of granularity in our phylogenetic distance data, which described reservoir host cophenetic distance from Primates on a time-scaled phylogeny (57), producing only six unique distance values across all of the reservoir groups in our database. Trends across reservoir host groups overall support the hypothesis that the positive correlation between phylogenetic distance and virulence collapses at “extreme” distances. Nevertheless, more studies are needed to parse the effect of phylogenetic distance on virulence trends in animal-to-human spillovers. The time-scaled phylogeny represents the only available phylogeny that includes both mammals and birds. Future studies would benefit from developing additional phylogenies of mammalian and avian reservoirs,

which prioritize immunological or physiological traits that may more accurately proxy virologically relevant differences in host environments.

Chiroptera represented an outlier among distantly related reservoirs, with an undeniably positive effect size on CFR more than triple that recovered for any other mammalian order. Consistent with the hypothesis that bats represent a 'special' viral reservoir (50), the order Chiroptera does appear to harbor zoonotic viruses that are uniquely virulent upon spillover to humans, even when considering virulence effects that might be attributed to their phylogenetic distance from Primates. In bats, flight adaptations have been linked to viral tolerance, which previous work suggests may select for high growth rate viruses that could manifest as virulent upon emergence in less tolerant hosts such as humans (63). Notably, bats experience limited morbidity or mortality from intracellular infections with only a few known exceptions (50,86–88). Conversely, while birds harbor several zoonotic viruses that are virulent in humans such as Highly Pathogenic Avian Influenza (HPAI), West Nile, and Equine Encephalitis viruses, only some avian species are tolerant of these infections—many avian species experience morbidity and mortality (89). Bats and birds are expected to experience similar selective pressures from flight—they have been found to incur comparable energetic costs while flying, despite different forms and physiologies (64,90). However, the two taxonomic groups, within disparate vertebrate classes, may have responded differently to these selective pressures. Specifically, there is a possibility that bats evolved cellular pathways that protect against both aging and immunopathology, whereas birds evolved pathways that only protect against aging. For example, bats have been found to host a suite of cellular-level anti-inflammatory adaptations—including enhanced cellular autophagy and downregulated signaling pathways linked to the induction of inflammatory antiviral defenses—which may both mitigate cellular damage induced by bat metabolism and inhibit immunopathology incurred upon viral infection (50,91–95). On the other hand, birds may rely primarily on systemic antioxidant responses (96), which mitigate oxidative stress, but do not interact so tightly with cellular-level processes that impact viral pathology. Critically, birds appear to be missing anti-inflammatory protein tristetraprolin (TTP) (97), and immunopathology is often the cause of death in birds that die from viral infections such as HPAI and West Nile virus (89). Differences between mammalian and avian immune systems may additionally play a role in their differing infection outcomes. The immune system is broadly conserved in amniotes, but some avian immunological features diverge from those of bats and other mammals: notably, birds lack lymph nodes and instead develop B cells in a specialized lymphoid organ, the bursa of Fabricius; have heterophil in their white blood cells as opposed to neutrophil; and produce only three classes of immunoglobulin in contrast to the five produced by mammals (61). Nevertheless, the differing effects of Chiropteran and avian metabolic adaptations on viral tolerance and viral evolution remain largely uncharacterized and more basic research in this field is needed (98).

Order Cetartiodactyla had the largest negative effect size on CFR, but notably, Cetartiodactyl hosts in our dataset included only domesticated animal species—cattle, pigs, and camels. The long coexistence of domestic animals and humans likely facilitated increased research effort for this clade, which have may have led to greater detection of low virulence zoonoses in domestic animal species. A long history of domestic animal-human coexistence may also have supported the development of preexisting human immunity to some livestock diseases, resulting in lower virulence infections.

We found that both reservoir host and virus taxonomy predict the virulence and transmissibility of a virus in the secondary human host, consistent with the expectation that a

virus evolves virulence to maximize reproduction in its reservoir population (99). The optimal balance between virulence and transmission depends on how the reservoir host population responds to the virus (the 'host selective pressure'), which is determined by the ecological, physiological, and biological traits of the reservoir. While we identified "special" reservoirs of virulent and transmissible zoonotic viruses, we found that the human death burden incurred by viral zoonoses does not correlate with any one reservoir host order, including bats, and instead, is a function of viral traits. Our data demonstrate that mechanisms driving high death burdens are diverse and often contradict trait-based predictions. High death burdens have resulted from rare spillover events of highly transmissible viruses that spread widely in the human population; small, but frequent spillovers of the least transmissible viruses; and historically low-burden pathogens that take off given the right ecological and evolutionary conditions. This suggests that ultimately, death burden depends on epidemiological circumstances, which should be shaped, not by reservoir host traits, but by a combination of viral traits and conditions in the animal host population and across and beyond the human-animal interface. Notably, the pandemic spread of SARS-CoV-2 can be attributed to its highly effective respiratory transmission between humans, a trait linked to its identity within Coronaviridae, rather than its bat origins (indeed, CoVs demonstrate gastrointestinal tropism in bat reservoirs) (100).

However, several outliers demonstrated that capacity for forward transmission in human populations does not always predict death burden; it is critical to also consider epidemiological dynamics across and beyond the human-animal interface. Less transmissible viruses can accumulate large death burdens over many small, but frequent spillovers, particularly in systems in which humans regularly interact with animal reservoirs. Rabies, Hantaan (HTNV), and Japanese Encephalitis viruses have been associated with some of the highest death burdens induced by viral zoonosis despite lacking forward transmission in human populations (Figure 2.4d). This is likely because these viruses spill over to humans from animal host populations that live amongst human communities—Rabies burden is largely driven by spillover from endemic circulation in domestic dogs (101), HTNV spills over from striped field mouse (*Apodemus agrarius*) populations that inhabit agricultural fields (102), and Japanese encephalitis is amplified via domesticated pigs (103). Outbreaks in these spillover host populations source human infections that are dead ends for further transmission but add up to large numbers. Emphasizing the importance of understanding system-specific dynamics, HTNV had a death burden more than 18 times greater than the combined death burden of all ten other rodent-borne hantaviruses in our dataset, most likely because other rodent reservoirs of hantaviruses tend to overlap less with human populations (102). Furthermore, zoonotic viruses that have historically been low burden pathogens can "unexpectedly" cause high death burdens in the case of virus evolution or unique epidemiological circumstances (104). For example, Ebola virus first emerged in humans in 1976, causing deadly, but local outbreaks up until late 2013, when suddenly, emergence in a region with dense and interconnected human populations, coupled with virus adaptation (15), allowed an Ebola spillover event to spark a transnational epidemic that in just 2 years, caused more than 6.5 times the total number of deaths recorded from 1976-2013 (104,105). These outliers suggest that understanding epidemiological dynamics—within wildlife populations and across and beyond the human-animal interface—in specific systems is a critical component of predicting death burden and consequently, danger to human health.

Over the course of the last decade, a significant amount of funding and research effort has been dedicated to identifying correlates of zoonotic risk, often with a long-term aspiration of identifying ways to anticipate and prevent emerging zoonoses in the future (106–108). This

research increasingly prioritizes viral discovery over longitudinal studies of epidemiological dynamics and targets animal populations such as bats that have been identified as key zoonotic reservoirs. While our analysis corroborates the hypothesis that bats are a 'special' reservoir for virulent zoonotic viruses, we also demonstrate that viral traits—not bat reservoirs—pose the greatest danger to human health. We argue that burden, which does not correlate with any animal reservoir and instead appears to be a function of transmission conditions to and within the human population, more correctly approximates "danger" to human health than does virus virulence. While reservoir and viral traits can predict zoonotic capacity, virulence, and transmissibility, death burden is dependent on system-specific epidemiological dynamics, which are shaped by a combination of viral traits and conditions in the animal host population and across and beyond the human-animal interface. Thus, understanding and controlling the mechanisms that drive high death burdens in humans—high rates of human-animal contact and/or epidemiological dynamics in the human population that allow discrete spillover events to trigger human epidemics—requires longitudinal surveillance of specific zoonotic or potentially zoonotic viruses in both animal and human populations. There is a pressing need for more longitudinal studies of transmission dynamics in human and wildlife populations to better understand and prevent the epidemiological conditions that cultivate the most dangerous cases of zoonotic viral emergence.

CHAPTER 3

Hybridization capture for DNAm age estimation in bats: a cost-efficient, high-throughput design to support epidemiological and population modeling analyses across bat species.

Abstract
Mathematical models are powerful tools for understanding both viral dynamics and population trends in wildlife populations. Many mechanistic epidemiological and conservation models depend on age data, traditionally estimated via mark-recapture surveys, body measurements, lethal sampling of bone density, or tooth analyses—methods that are often prohibitively resource-intensive, imprecise, or impractical. DNA methylation (DNAm) assays offer a cutting-edge alternative, requiring just a single, non-invasive DNA sample. The activity of DNA methyltransferases (DNMTs) changes over the course of an animal's lifespan, producing age-correlated trends in the percentage of DNA methylation (DNAm) at particular cytosine guanine dinucleotides (CpGs)—clock-type DNAm positions—that can be leveraged to construct an "epigenetic clock" for age estimation. A scalable age estimation method that could support epidemiological and conservation modeling would be particularly powerful in bat populations, given their zoonotic and conservation importance. Based on the Wilkinson et al. (2020) microarray, we developed a cost-efficient, high throughput in solution hybridization capture for assaying clock-type DNAm profiles in bats. Our method can be applied across species and the large sample sizes needed for robust modeling analyses.

Introduction
Age data offer key insights into wildlife ecology. Epidemiological modeling can infer the transmission dynamics of wildlife disease from age-structured seroprevalence data (109–111). Population viability analyses quantify wildlife population trajectories by using age-frequency data to estimate adult annual fecundity and survival rates (112,113). While age data can inform both zoonotic risk and conservation assessments, it is notoriously difficult to obtain from most wild animal species. Historically, chronological age has been estimated using methods that are prohibitively resource-intensive, imprecise, and/or unfeasible across sample sizes needed for modeling analyses—such as long-term mark-recapture surveys, body measurements, lethal sampling of bone density, and histological analysis of *cementum annuli* layering in tooth samples (114,115).

Molecular age markers offer a cutting-edge alternative, often requiring just a single, non-invasive DNA sample (116). In particular, the activity of DNA methyltransferases (DNMTs) changes over the course of an animal's lifespan, producing age-correlated trends in the percentage of DNA methylation (DNAm) at particular cytosine guanine dinucleotides (CpGs)—clock-type DNAm positions—that can be leveraged to construct an "epigenetic clock" for age estimation (114). DNAm-based age estimation has been reported to outperform other molecular methods in humans (117) and more recently, applied in wildlife populations (114,118–120).

Wilkinson et al. (121) developed a microarray-based epigenetic clock for bats, identifying a set of 1,994 CpG sites that are conserved and correlated with chronological age across bat genomes. They identified these bat age-correlated sites from a custom mammalian microarray based on 35,541 50bp CpG-terminal sequences that are conserved across 62

mammals (including five bat species) and 1,951 sequences that have been identified in prior human biomarker studies (122).

Such an epigenetic clock could support epidemiological and conservation modeling in bat populations, which is highly important for both global health and conservation (123,124). Bats are asymptomatic reservoir hosts for a number of zoonotic viruses that are virulent in humans, including the Ebola filoviruses, Nipah and Hendra henipaviruses, and SARS coronaviruses (50,123). Bats are also a highly ecologically and taxonomically diverse group, in many systems, providing key ecosystem services as seed dispersers, pollinators, insect-eaters, and nutrient recyclers; however, 80% of bats assessed by IUCN are considered threatened or data deficient (125).

At $150 per sample, the Wilkinson et al. (126) microarray is prohibitively expensive across sample sizes needed for bat conservation and epidemiological modeling analyses. We adapted the Wilkinson et al. (126) microarray probe set to a cost-efficient, high throughput in solution hybridization capture. Our method can be applied across a diversity of bat species and the large sample sizes needed for robust epidemiological and population modeling analyses.

Methods

Designing the probe set. For target enrichment, we designed a set of 80bp myBaits biotinylated probes (Custom DNA-Seq, Arbor Biosciences) from the Wilkinson et al. (126) 50bp microarray probe sequences. Using the Burrows-Wheeler Aligner (BWA-MEM) (127), we mapped the 1,994 Wilkinson et al. (126) microarray probe sequences containing the top 2,000 age-correlated sites to 17 bat species genomes: *Antrozous pallidus, Desmodus rotundus, Eptesicus fuscus, Hipposideros galeritus, Lasiurus cinereus, Molossus molossus, Myotis lucifugus, Myotis myotis, Phyllostomus discolor, Pipistrellus kuhlii, Pteropus vampyrus, Rhinolophus ferrumequinum, Rousettus aegyptiacus, Tadarida brasiliensis, Rousettus madagascariensis, Eidolon dupreanum*, and *Pteropus rufus*. We extended all perfect 50bp alignments by 30bp up- and downstream, and using the 'msa' package in R (128), ran a multiple sequence alignment of the extended sequences across all 17 species. We defined our 80bp probe sequences by identifying the most conserved 80bp frame within the 110bp multiple sequence alignment (i.e., the frame that had the highest number of bp matches and lowest number of gaps summed across species) and recording the most common nucleotide across species at each base pair within that frame. To design our probe set for post-bisulfite-conversion capture, we made 9 variants for each 1,994 probe sequence—4 bisulfite converted sequences for both strands and 1 unconverted sequence for a bisulfite conversion control—for a total of 17,946 probes.

DNA extractions. Wing tissue samples were digested in 1.5mL tubes containing 120uL Biofluid & Solid Tissue Buffer (Zymo Research), 40uL Proteinase K (20 mg/mL), and 2uL DTT (X mM), and incubated overnight with rotation at 55°C. Digested samples were centrifuged at 10,000 rpm for 10 minutes to pellet remaining undigested material and lysates were transferred to a 96-well deep well plates. The remainder of the extraction protocol was conducted on the BenchSmart 96 semi-automated pipetting system. To allow for RNA degradation, 20uL of RNAse A (from a pre-prepared RNAse A 96-well plate) was added to the lysates. Note that RNAse A was added post-digestion and at a high volume because DTT—added to facilitate digestion of the wing tissue hair—interferes with RNAse A activity. A 1:1 ratio of SPRI beads (180uL) were added to each sample and incubated with rotation for 20 minutes at room temperature. Plates were placed on magnetic racks to pellet the SPRI beads and washed twice with 80% ethanol—first with 380uL, and then with 180uL. For elution, plates were removed from the magnetic rack, and 50 uL of

nuclease-free water was added to the sample and bead solutions. After a 1-hour incubation with rotation at 55C, plates were placed on magnetic racks to pellet the beads, and the DNA samples were transferred to 96-well elution plates.

Library preparation. Pre-capture libraries were constructed using the Kapa HyperPlus Library Preparation Kit (Kapa Biosystems), optimized to quarter volumes of all reagents unless otherwise specified. Briefly, DNA fragmentation reactions were incubated at 37°C for 35 minutes. End repair, A-tailing, adapter ligation, and post-ligation cleanup were performed according to the Kapa protocol. Ligated adapters were xGen Stubby Adapters (Integrated DNA Technologies), which are pre-methylated and thus unaltered by bisulfite conversion. Indexing PCR reactions were assembled with 15uL Kapa 2x Ready Mix, 10uL of adapter-ligated product, and 5uL of the corresponding indexing oligo. After amplification, a double-sided Rx/Lx ratio of 0.90x/0.75x was applied to select for a fragment size distribution mean of roughly 200bp. Following size selection, bisulfite conversion was performed according to the protocol outlined in the Zymo EZ-96 DNA Methylation-Lightning Kit (Zymo Research). Lastly, the targeted probe capture was performed according to the Arbor myBaits Hybridization Capture for Targeted Methylation Sequencing protocol. Sequencing coverage requirements for accurate DNAm percentages were determined by following Ziller et al. (129).

Probe set validation. We assessed the efficacy of our probe set in silico by mapping all unique 1,994 probe sequences back to the 17 bat genomes and analyzing coverage. In the lab, we prepared pre-capture sequencing libraries from four bat tissue samples—representing four individuals across three bat species (*Rousettus aegyptiacus,* 1; *Eidolon helvum*, 1; *Pteropus mariannus yapensis*, 2)—provided by the Museum of Vertebrate Zoology at University of California-Berkeley. We split each pre-capture library, continuing onto the probe capture with only one of the aliquots before sequencing. This approach was designed to allow us to validate DNAm data from our custom in-solution hybridization capture against standard whole genome Methyl-Seq data (130).

Results

The majority of our probes included multiple CpG sites, but in most cases, only one of the CpG sites were identified as age-correlated by Wilkinson et al. (126), and no probes had more than two age-correlated sites.

Arbor Biosciences myBaits can hybridize to target regions with small indels and up to a 5% mismatch in sequence. We predicted a probe would successfully capture a target if the target sequence—aligned against the probe sequence—was 5% or less divergent and contained less than three gap openings and no more than one gap extension. Using these criteria, we estimated that our probe set should capture more than 1,700 of the 1,994 target sequences in all 17 bat species genomes, and more than 95% of target regions in 12 of the genomes (Figure 3.1). These results indicate that our probe set should provide more than sufficient coverage for age analysis. We also found that 67% of probes mapped to all 17 genomes and 99.5% of probes mapped to more than half of the genomes, suggesting that our probe set is highly cost efficient (Figure 3.2).

We were unable to complete the lab portion of our probe set validation—sequencing data was not available in time to compare DNAm data between the targeted and whole genome Methyl-Seq libraries.

Discussion

The hybridization capture-based target enrichment strategy presented in this chapter is a key first step in increasing epidemiological and population modeling capacity in bat populations—important for both public health and conservation. At the time of writing, our protocol, from DNA extraction through sequencing, was predicted to cost roughly $33 per sample, which is substantially cheaper than the Wilkinson et al. (126) microarray, priced at $150 per sample. Cost-efficient and high throughput, our method makes it possible to estimate bats' chronological age across large sample sizes—critical for accurately modeling bat population viability and potentially zoonotic virus dynamics. By building off the Wilkinson et al. (126) microarray probes that target conserved CpG sites, we have maintained the flexibility of their assay and thus, our protocol can flexibly be applied to obtain age data for any bat species of interest.

The targeted sequencing library preparation protocol proposed in this chapter requires a minimum of five days. A multi-day protocol is unavoidable—a double capture, which requires a minimum of three days, is recommended bisulfite-converted libraries because for the conversion process depletes input material (131,132). Nevertheless, future work may consider exploring alternate strategies to reduce protocol length. For example, recently, another group published a tagmentation-based methylation sequencing method for DNAm age estimation (133). Tagmentation does significantly reduce the library preparation protocol length; however, Tn5 can be expensive, requires more troubleshooting, and may not be accessible to model-guided field projects in remote countries.

Our in-silico analysis demonstrated that our capture design is both highly flexible and efficient, predicting that our probe set should capture a high percentage of target sequences across bat genera and species. However, before our hybridization capture can be leveraged to obtain age data for modeling analyses, future work will need to complete the lab portion of our probe set validation. Specifically, DNAm percentages across target CpG sites must be compared between our targeted and whole genome Methyl-Seq libraries to validate whether our probe set accurately captures DNAm data and identify any data biases. Our capture-based target enrichment protocol should also be applied to known-age samples to demonstrate that our method—combined with the Wilkinson et al. (126) clock—can be used to accurately determine chronological age. For this age validation step, it is important that the known ages are precise; for example, derived from captive individuals or age estimation methods such as mark-recapture (as opposed to less precise methods such as histological analysis of *cementum annuli* layering in tooth samples) (114,115,134). However, if ages from captive individuals are used it is important to consider that previous work has found that environment influences biological aging patterns (135) and thus DNAm data may differ between captive and wild animal populations.

CHAPTER 4

The coevolution of parasite virulence, and host investment in constitutive and induced defense

Introduction

Parasites are ubiquitous in nature, affecting organismal evolution and ecology at all phylogenetic levels (136). Thus, a good understanding of how parasites and their hosts coevolve is critical for human and animal health, as well as our understanding of how infectious disease shapes natural systems (18,137). In particular, parasite and hosts impact each other at both the individual and population level, which has repercussions for both epidemiology and animal population dynamics (18,138).

Parasites influence host life history traits (e.g., mortality rates), population characteristics (e.g., carrying capacities), and investment in immunity (138,139). In particular, hosts have evolved a range of diverse immune defenses against parasites including both tolerance and resistance (140–143). Resistance mechanisms which act to reduce the fitness of the parasite while increasing that of the host can be usefully divided into two types: constitutive mechanisms, which are persistently active and typically act to prevent infection in the first place such that hosts do not become infectious (avoidance), and induced mechanisms, which are only activated during an infection and typically drive the recovery process (144,145). In this definition, constitutive defenses include innate mechanical barriers, complement and antimicrobial proteins, and phagocytic, granulocyte, and natural killer (NK) white blood cells, as well as the natural antibodies which bridge innate and adaptive immunity—whereas induced defenses include innate inflammatory responses, as well as adaptive cytokines and antibody responses (146). This distinction between constitutive and induced defense is important to host evolution at both the individual and population level. At the individual level, maintaining constitutive defenses that are always ready to act is costly even in the absence of pathogens, but avoids damage by preventing infection altogether; in contrast, activating induced defenses during an infection may be more energetically efficient since they are only used in the presence of the pathogen, but risks incurring damage from both the infection itself and very typically from the immune response (immunopathology) (147,148). At the population level, constitutive defense reduces the infection rate, while induced defense only shortens the infectious period and therefore there is the potential for different epidemiological feedbacks. These population level effects are important because effectively the host immune investment influences parasite epidemiological traits such as the prevalence and force of infection, which feedbacks into selection for immune defense in the first place (144,149).

Host characteristics, in turn, influence parasite evolution—in particular, modulating the transmission costs and benefits of virulence (38,150). Classic evolution of infectious disease theory assumes a tradeoff between virulence and transmission rate on the basis that while high within-host growth rates increase infectiousness, they also increase damage to the host, thus shortening the infectious period and reducing opportunities for future transmission through increasing mortality (virulence) (20,21). Host mortality rates and carrying capacities impact the density of susceptible individuals available to the parasite, regulating opportunities for transmission and thus the transmission cost of virulence (38). In our context with respect to the host immune strategy, constitutive defense reduces infectiousness—heightening the transmission benefit of virulence—whereas induced defense introduces host damage from immunopathology—heightening the transmission cost of virulence. Thus, understanding evolution in parasite-host

systems requires considering the costs and benefits of different parasite and host strategies, as well as parasite-host coevolution—at both the individual level and in the broader epidemiological and population dynamic context. Empirical work has described a complex web of interactions between parasite-host coevolution and ecological feedbacks (18,137,138). Eco-evolutionary theory (151) allows us to parse how these interactions actually shape the diversity of parasite and host strategies that we observe in nature (140,144).

Here, we develop eco-evolutionary theory that makes general predictions regarding how the interplay between parasite-host coevolution, population dynamics, and epidemiology impact host investment in constitutive and induced defense, and parasite growth. There has been theory on how parasites influence host investment in constitutive and induced immune defense (152,153), and how these two arms of defense influence the evolution of other host characteristics (154) and parasite growth (147). However, few theoretical studies have incorporated parasite-host coevolution in their models (145). Furthermore, to our knowledge, only one evolutionary model of host constitutive and induced defense has accounted for epidemiology and population dynamics; however, this model did not consider parasite-host coevolution (144). We therefore lack a general theory on the evolution of host constitutive and induced defense and parasite growth that accounts for both coevolution and population and epidemiological dynamics. Our goal is to address this gap and provide a framework for understanding host immune defense and parasite growth strategies in natural systems.

Methods

We explore epidemiological and coevolutionary feedbacks to the evolution of host constitutive and induced immune defense, and parasite growth using a classic compartmental epidemiological model (155–157):

$$\frac{dS}{dt} = \big(a[c] - q(S+I)\big)(S+fI) - bS - \beta[c,p]SI + \gamma[h]I$$

$$\frac{dI}{dt} = \beta[c,p]SI - (b + \alpha[h,p] + \gamma[h])I$$

All hosts reproduce at rate a, which is reduced due to competition by a density-dependent factor, q. Infected hosts can potentially suffer an additional reduction in birth rate by a sterilizing factor, f (when f = 0, the parasite is a castrator). Specifically, when the parasite castrates the host ($f = 0$), infected hosts lose their reproductive capacity until they recover back to the susceptible class. All hosts die at a natural mortality rate, b. Transmission is a density-dependent mass-action process with a coefficient, β. Infected hosts suffer increased mortality, or virulence, at rate α, and can recover back to susceptibility at rate γ.

We allow both host and parasite parameters to evolve. Specifically, three key traits are subject to selection: 1) host constitutive defense (c), defined as reduced susceptibility to infection (resistance); 2) host induced defense (h), defined as an increased ability to clear disease (an increased recovery rate); and 3) the parasite growth rate (p). Each of the three evolving traits carries a cost. We assume that constitutive defense—persistently active and thus energetically costly to maintain—reduces the birth rate (140,144,149,154,158). In contrast, we assume that induced defense—activated only after infection—incurs an immunopathology cost from immune activation, increasing mortality in infected hosts (144,146). Thus, only infected hosts pay the cost of induced defense, whereas all hosts pay the cost of constitutive. Induced defense may incur some

costs in the absence of disease (154), but we deliberately maintain simplistic assumptions to develop a baseline model from which future work that includes more complex assumptions about costs can be developed. Lastly, we assume that parasite growth leads to higher transmission (β), but also increases virulence (α) (144,152). All three evolving traits are also tied to the population-level epidemiology—constitutive defense reduces transmission (β), induced defense shortens the infectious period (by increasing the host recovery rate, γ), and parasite growth increases transmission (β) (144,149).

We define the host recovery rate as a simple function of induced defense, such that,

$$y[h] = h + \gamma_0$$

Transmission and virulence are functions of both host and parasite parameters. Specifically, we assume the transmission coefficient, β, is a multiplicative, 'universal' function of constitutive defense and parasite growth, such that,

$$\beta[c,p] = (\beta_0 - c)B[p] + k$$

which has been commonly used in previous studies (140,149). Similarly, we define virulence as a multiplicative function of immunopathology (the cost of induced defense) and parasite growth, such that,

$$\alpha[h,p] = \Gamma[h]p + \alpha_0$$

Thus, constitutive defense trades off with host reproduction, induced defense trades off with increased mortality of infected hosts, and parasite growth trades off with transmission. These three trade-offs are given by exponential functions, such that:

$$\mathrm{a}[c] = \mathrm{a}_0 - \frac{(\mathrm{a}_1)^2}{\mathrm{a}_2}\left(1 - \exp\left[\frac{\mathrm{a}_2}{\mathrm{a}_1}(c - c_0)\right]\right),$$
$$\Gamma[h] = \Gamma_0 - \frac{(\Gamma_1)^2}{\Gamma_2}\left(1 - \exp\left[\frac{\Gamma_2}{\Gamma_1}(h - h_0)\right]\right),$$
and,
$$\mathrm{B}[p] = \mathrm{B}_0 - \frac{(\mathrm{B}_1)^2}{\mathrm{B}_2}\left(1 - \exp\left[\frac{\mathrm{B}_2}{\mathrm{B}_1}(p - p_0)\right]\right),$$

where $\mathrm{a}_1 = {da}/{dc}$, $\mathrm{a}_2 = {d^2a}/{dc^2}$ and similarly for Γ_1, Γ_2, B_1 and B_2 The advantage of this form is that for a chosen singular point at (h_0, Γ_0) we can fix the gradient as Γ_1 and the curvature as Γ_2, allowing us to easily manipulate the trade-off (159). Importantly, constitutive and induced defense do not trade off with each other and instead, evolve independently.

We model evolution using the adaptive dynamics framework (160,161). As such, we assume that rare mutants with a small phenotypic difference attempt to invade a (monomorphic) resident at endemic equilibrium. The success of the mutant depends on its invasion fitness, defined as the growth rate in the environment set by the resident. For the parasite, this is simply the growth of mutant-infected individuals. For the two host arms, we instead use the fitness proxy of the negative

determinant from the mutant's part of the Jacobian, which has been shown to be sign equivalent to the true fitness (159). In isolation, each of the traits will evolve in the direction of its local selection gradient; for example, for the parasite, $[\partial r/\partial p_m]_{p_m=p}$ where p_m is the mutant trait. The three mutant fitness gradients together then form a dynamical system of ordinary differential equations (for simplicity we assume equal mutation rates):

$$\left.\frac{\partial s_{ind}}{\partial h_m}\right|_{h_m=h} = (a[c] - q(S^* + I^*) - b - \beta[c,p]I^*)\left(\frac{d\gamma[h_m]}{dh_m} + \frac{\partial \alpha[h_m,p]}{\partial h_m}\right) + \frac{d\gamma[h_m]}{dh_m}\beta[c,p]I^*$$

$$\left.\frac{\partial s_{con}}{\partial c_m}\right|_{c_m=c} = \left(\frac{da[c_m]}{dc_m} + \frac{\partial \beta[c_m,p]}{\partial c_m}\right)(b + \alpha[h,p] + \gamma[h]) - \frac{\partial \beta[c_m,p]}{\partial c_m}\left(y[h] + f(a[c] - q(S^* + I^*)\right) + \frac{da[c_m]}{dc_m} f\beta[c,p]I^*$$

$$\left.\frac{\partial r}{\partial p_m}\right|_{p_m=p} = \frac{\partial \beta[c,p_m]}{\partial p_m}S^* - \frac{\partial \alpha[h,p_m]}{\partial p_m}$$

When all three equations are 0 simultaneously (i.e., none of the traits are experiencing directional selection), there will be an 'equilibrium' of the evolutionary dynamics, termed a singular strategy in adaptive dynamics. The behavior at this point depends on second-order fitness terms (160,161). In particular, if the strategy for each trait cannot be invaded by any nearby mutants, then it is termed evolutionarily stable. If the singular strategy is locally attracting from nearby initial conditions, then it is termed convergence stable. Here, we check for these stability conditions numerically. Code to produce the plots in Python is available on Github.

Results

a) Coevolution of parasite growth and host investment in constitutive and induced defense when the parasite has no impact on host fertility ($f = 1$)

i. Varying mortality (b) when $f = 1$

We first consider coevolutionary dynamics when the parasite has no impact on host fertility ($f = 1$). In response to increased background mortality in the host population, there can be selection for investment in both higher constitutive and induced defense (Figure 4.1a-b) which means that shorter rather than longer lived hosts invest in more defence. Notably, there is a faster increase in constitutive defense, such that shorter-lived hosts invest relatively more in constitutive than induced defense; and longer-lived hosts invest relatively more in induced than constitutive defense. Although it is often thought that longer lived organisms are more at risk of infection, these results reflect how immune defenses incur fewer total costs over shorter lifespans, particularly with respect to the constant reproductive cost of constitutive defense. Furthermore, heightened background mortality rates reduce the host population density (Figure 4.1d), reducing transmission risk in a density dependent parasitic and furthermore higher background mortality also reduces parasite prevalence (Figure 4.1e) again reducing risk. The key

to these effects is that with long lived hosts, prevalence is very high in these models, and therefore the risk of infection is so high even with strong immunity, that the costs are defense make outweigh the benefits. Our other key result is that counter to classic theory, the parasite is not strongly selected to increase exploitation as host mortality increases. This is likely driven by the increased investment in defense on the part of the host. Furthermore, once host investment in induced defense reaches a threshold (Figure 4.1a), immunopathology costs select for reduced parasite growth (Figure 4.1c).

Our result that decreasing lifespan selects for higher overall immune investment—with a steeper increase in constitutive defense—is consistent with results from our previous model in which only the host evolved (144). However, in this prior modeling analysis, hosts invested relatively more in constitutive than induced defenses across all natural mortality rates. In contrast, here, with parasite-host coevolution, we find that as the background mortality rate decreases, the relative investment between induced and constitutive defense flips such that longer-lived hosts invest relatively more in induced than constitutive defenses (Figure 4.1a-b). This result is a product of coevolutionary dynamics. Lower background mortality increases host population density (Figure 4.1d), which supports a higher parasite prevalence (Figure 1e)—as a result, the parasite reduces its growth rate (Figure 4.1c), decreasing virulence, which simultaneously reduces the advantage of constitutive avoidance (Figure 4.1b) and the immunopathology cost of induced defense (Figure 4.1a). Furthermore, longer lifespans accumulate higher costs from investing in immune defense, particularly constitutive.

ii. Varying competition (q), f = 1

Increasing the host birth rate sensitivity to crowding (i.e., increasing competition, or decreasing the carrying capacity) reduces the host population density (Figure 4.2d), leading to a pattern where investment in immunity is somewhat constant until we reach very high densities (low q) and the hosts reduce investment in defence (Figure 4.2a-b). A key driver of this is that extreme host population densities (Figure 4.2d) and parasite prevalence (Figure 4.2e) begin to make infection inevitable, selecting for low investment in immune defense as hosts "give up" to reduce costs (Figure 4.2a-b). Parasite growth (Figure 4.2c), is selected to be high at very high densities, then falls off before increasing again with very strong competition. Notably, we previously found that when only the host evolves, there are monotonic increases in both arms of defense (144). In contrast, adding parasite-host coevolution produces non-monotonic changes such that investment in both induced (Figure 4.2a) and constitutive (Figure 4.2b) defense begins to decrease slightly when competition exceeds a threshold.

The parasite strategy is independent of competition itself and is thus purely driven by the host evolutionary response. Specifically, in response to the immunopathology costs from increasing host induced defense, there is selection for reduced parasite replication (Figure 4.2c), which reduces prevalence (Figure 4.2d). However, as the host immune investment levels off, selection on the parasite reverses such that the replication rate increases (Figure 4.2c), which subsequently increases immunopathology costs, selecting for a reduction in host immune investment (Figure 4.2a).

b) Coevolution of parasite growth and host investment in constitutive and induced defense when the parasite is a castrator ($f = 0$)

i. Varying mortality (b), $f = 0$

Overall, the castrating parasite generally selects for higher host defense across mortality levels (Figure 4.3a-b) relative which is in clear contrast to when the parasite is non-castrating (Figure 4.1a-b). This reflects the strong selective pressure for hosts to protect their reproduction. However, because immune defense is costly, hosts moderate their investment in immunity in response to the infection risk—reducing constitutive defense (Figure 4.3b) to avoid unnecessary reproductive costs as parasite prevalence declines (Figure 4.3e)—and the parasite virulence—reducing induced defense (Figure 4.3a) to avoid immunopathology as parasite growth rate increases (Figure 4.3c). In contrast to the case where there is no castration, although prevalence still increases with reduced mortality, it does not reach such high levels that the host begins to 'give up' on immune defense until host are very long lived, when there is some evidence of induced defenses declining (Figure 4.3a). The key difference between the case when infecteds reproduce and when they don't is the much stronger selection for higher parasite growth rates when hosts suffer higher background mortality in castrators. A key cause of this is the difference in the selection for immune defence in the host in the two cases.

Parasites that castrate the host flip trends across host natural mortality rates. Notably, when only the host is allowed to evolve, castrators select for increased investment in induced defense, but decreased investment in constitutive defense (144). This occurs because increasing host background mortality and infection-induced castration makes the additional reproductive cost of constitutive defense unsustainable; but then, given that only susceptible hosts can reproduce, induced defense is critical for allowing infected individuals to recover and reproduce. However, here, when the parasite is allowed to coevolve, there is selection for higher parasite growth (Figure 4.3c). This increasing parasite growth in turn selects for declining investment in induced defense after immunopathology costs exceed a threshold (Figure 4.3a). Once investment in induced defense begins to decline (Figure 4.3a), preventing infection-induced castration becomes the key mechanism for maintaining host reproduction and thus, there is selection for higher constitutive defense (Figure 4.3b). However, investment in constitutive defense peaks at intermediate host lifespans—because host castration increases with parasite growth and reaches a threshold at which the reproductive cost of constitutive immunity outweighs its infection avoidance benefit (Figure 4.3b-c), especially given the decline in parasite prevalence (Figure 4.3e).

ii. Varying competition (q), $f = 0$

Parasites that castrate the host also flip trends across levels of host birth rate susceptibility to crowding. At low competition levels, extreme host population densities (Figure 4.2d and Figure 4.4d) and parasite prevalence (Figure 4.2e and Figure 4.4e) make infection inevitable. When the parasite does not affect host reproduction, this heightened infection risk selects for low investment in immune defense as hosts "give up" to reduce costs (Figure 4.2a-b). However, when the parasite is a castrator, hosts cannot afford to "give up"—instead, this heightened infection risk selects for high investment in immunity (Figure 4.4a-b) to protect reproduction, at whatever cost.

However, again, because immune defense is costly, hosts moderate investment in induced and constitutive immunity in response to the parasite infection risk and virulence. Increasing competition decreases the host population density (Figure 4.4d) and consequently, the risk of infection (Figure 4.4e), which allows hosts to reduce investment in defense (Figure 4.4a-b). Initially, at mid to low levels of competition, there is selection for increased investment in

constitutive defense, as only preventing infection altogether directly protects the host from castration (Figure 4.4b). However, as competition increases and further reduces the birth rate, constitutive defense becomes too reproductively costly and is selected against (Figure 4.4b). It is energetically impossible to achieve complete constitutive immunity, or absolute infection avoidance—thus, infection-induced reduction of the birth rate is unavoidable, making the additional reproductive cost of constitutive defense unsustainable, especially as the infection risk declines (Figure 4.4e).

Induced defense acts on the recovery rate, after the host is already infected; thus, while hosts can regain their reproductive ability through recovering, induced defense itself does not directly protect against castration. Notably, when only the host is allowed to evolve, there is selection for decreased investment in constitutive defense, but induced defense remains unaffected (144). However, when the parasite is allowed to coevolve, the decreasing host birth rate selects for higher parasite growth (Figure 4.4c), increasing immunopathology costs and selecting for decreasing investment in induced defense (Figure 4.4a).

Discussion

We have analyzed how the interplay between parasite-host coevolution, population dynamics, and epidemiology influence the optimal parasite growth strategy and host investment in constitutive (always present and costly) as opposed to induced (activated and costly only upon infection) defense. Critically, we provide the first theoretical framework that considers both coevolutionary and population-level dynamics. We examine trends across host competition and natural mortality rates when the parasite does not directly affect host fertility, as well as when the parasite is a castrator. We show that incorporating host-parasite coevolution into our model captures feedbacks between the host immune and parasite growth strategies that are missed when only the host is allowed to evolve. Furthermore, we find that whether the parasite affects host reproduction significantly impacts host-parasite coevolution; when the parasite is a castrator, selection on the host is often largely geared towards minimizing reproductive costs—either by investing in immunity to avoid infection or recover when parasite prevalence is high, or by reducing investment in reproductively costly constitutive defense when the parasite prevalence is low.

When hosts coevolve with a non-castrating pathogen, increasing host background mortality selects for overall higher investment in immunity, with a faster increase in constitutive defense. These results, as well as the results from our prior host evolution model, are consistent with the Lee (146) pace-of-life prediction, which posits that fast-living species should invest relatively more in constitutive than induced defense because short lifespans neither accumulate the energetic costs of non-specific constitutive defense nor benefit from more specific induced defenses. However, only the coevolutionary model recaptures the pace-of-life prediction that slow-living species should invest relatively more in induced than constitutive defense because constitutive immunity is particularly costly over long lifespans. Empirical support for these Lee (146) predictions has been found in mammals (162), birds (163), and invertebrates (164). Additionally, short-lived stickleback populations demonstrated higher overall immune activity relative to their long-lived counterparts (165), and crucian carp shifted immune investment to the cheapest constitutive defense in response to increasing mortality rates (166). Nevertheless, the empirical literature is not conclusive—some studies have supported a contrasting theory that shorter lifespans may instead constrain immune investment overall, prioritizing resources to meet development and reproductive demands (167–170). In natural systems, the relationship between lifespan and immune strategies is likely confounded by environmental factors, pathogen diversity,

and other host life history strategies such as reproductive strategies and body size (146,165). Our work also emphasizes that there are different predictions when the pathogen impacts host reproduction, and this is not always taken into account in these studies.

With respect to the parasite evolution, increasing natural mortality in the host population initially selects for increased parasite growth; until, at intermediate host lifespans, immunopathology costs select for reduced parasite growth. This result that short host lifespans select for reduced parasite growth contradicts previous theory that the cellular rate of pathogen growth is instead slower in larger-bodied, slower-living species (36,171). However, this previous theory is based on a body of work that compares parasite replication rates with host metabolism and body mass—metrics that are generally correlated with lifespan, but ultimately, reflect physiology. The physiological conditions within fast-living hosts may indeed select for increased parasite replication rates. However, our model indicates that the ecological and coevolutionary processes associated with fast-living hosts—higher background mortality rates and immune investment—select for parasites with reduced growth rates to avoid depleting the susceptible host population. Nevertheless, the relationship between host mortality and pathogen replication rates remains, to our knowledge, unexplored in empirical systems and thus, future research is needed to test our eco-evolutionary model predictions.

Increasing host competition (i.e., host birth rate sensitivity to crowding) selects for overall increasing host investment in defense; although when competition is intense, investment falls again. Notably, this non-monotonic trend in defense is only recovered by our model when allowing parasite-host coevolution—and is likely a more realistic representation of real-world trends. There is a lack of empirical literature on how parasite-host coevolution is influenced by birth rate sensitivity to crowding; however, there is literature on the effects of crowding. Specifically, the density-dependent prophylaxis (DDP) theory posits that high host density increases the risk of infection, selecting for higher immune investment (172). Empirical support for the DDP theory has been derived primarily from insect systems (173), but has also been found in some animal populations such as elk (174). At extreme host densities, our model finds the opposite of the DDP theory—"give up" on immune defense to reduce costs. It is possible that the extreme densities in our model are not observed in real world systems—at intermediate densities, our model trends in host defense are more consistent with the DDP theory, suggesting that these intermediate density levels may reflect more realistic conditions. However, potentially consistent with the non-monotonic trends observed in our model, empirical work suggests that the stress and limited resource availability of high density host populations can also reduce immune function (175–177). Critically, the empirical literature reports that the relationship between host competition and investment in immune defense is driven by density-dependent changes in parasite risk and resources available to support the energetic demands of immune function, whereas our model only accounts for density-dependent changes in the birth rate. Thus, our results highlight that density-dependent decreases in birth rate may also contribute to the observed correlation between high host density and increased immune investment. Notably, the empirical literature has identified a possible tradeoff between reproduction and immune function, where, in line with our model results, lower reproductive output may increase energetic resources for immune investment (169,178). Additionally, our model may explain why empirical pace-of-life predictions regarding immune function are inconclusive. Host pace-of-life is determined by a combination of natural mortality and birth rate, and our model suggests that these two factors have opposing effects on immune investment—we found that decreasing host pace-of-life by decreasing natural mortality reduces overall immune investment, whereas decreasing pace-of-life through density-dependent

decreases in birth rate (i.e., increasing birth rate sensitivity to crowding) increases overall immune investment.

Critically, these trends flip when the parasite castrates the host—there is a clear distinction between parasites that castrate their hosts and those that do not. Specifically, we found that castrators select for overall lower host investment in immune defense. When the parasite is a castrator, the reproductive cost of constitutive immunity often outweighs its infection avoidance benefit. When the castrator itself is allowed to coevolve, selection for higher parasite growth heightens immunopathology costs, selecting for decreased investment in induced defense. To our knowledge, immune defense strategy in empirical host systems affected by castrating parasites remains unexplored. Nevertheless, snail populations exposed to castrating nematodes have been found to invest more in reproduction (179), mature and reproduce at smaller sizes (180,181), and increase reproductive output (182)—suggesting that if reproduction trades off with constitutive immunity (as in our model), hosts exposed to castrating parasites would be expected to decrease investment in constitutive defense as observed in our analyses of both mortality rates and competition. Nevertheless, the direct relationship between infection-induced host castration and parasite-host coevolution remains, to our knowledge, unexplored in empirical systems and thus, future research is needed to test our eco-evolutionary model predictions.

Importantly, our model does not capture how exposure to pathogens changes with lifespan—hypothesized to be a key mechanism underlying observed variation in immune defense strategies; while short-lived hosts can rely on non-specific constitutive defense, long-lived hosts are likely to live to encounter pathogens more than once and thus benefit from specific adaptive induced defense (146). For future analyses, incorporating model structure that allows the level and specificity of pathogen exposure to vary with lifespan and other host life history characteristics may help parse contrasting results in the empirical literature. Furthermore, our model assumes that constitutive and induced defense do not directly trade off with each other and instead, evolve independently. However, in some systems, there is evidence of a constitutive-induced trade-off, which has been hypothesized to generate and maintain observed diversity in host defense both within and between species (144,183,184). When only the host evolves, we found that assuming a direct trade-off between constitutive and induced defense does not generate evolutionary branching and coexistence between genotypes (144). Future modeling analyses should assess whether incorporating parasite-host coevolution allows a direct constitutive-induced tradeoff to generate evolutionary branching in host defense and parasite growth strategies.

We have applied eco-evolutionary theory to make a series of predictions regarding the coevolution of parasite growth and host defense strategies. Our analysis demonstrates the importance of considering coevolution and population-level dynamics and provides a framework for future research. In particular, our work would benefit from modeling analyses that examine whether our trends change when adding additional dynamics such as spatial structure (185) and multiple infections (186). There is also a need to experimentally test our theoretical predictions, as well as collect comparative data in natural systems. Overall, we have provided the theoretical groundwork for building a mechanistic understanding of how parasites and hosts coevolve at both the individual and population level—contributing to the study of human and animal health, as well as how infectious disease shapes natural systems.

FIGURES

CHAPTER 1 FIGURES

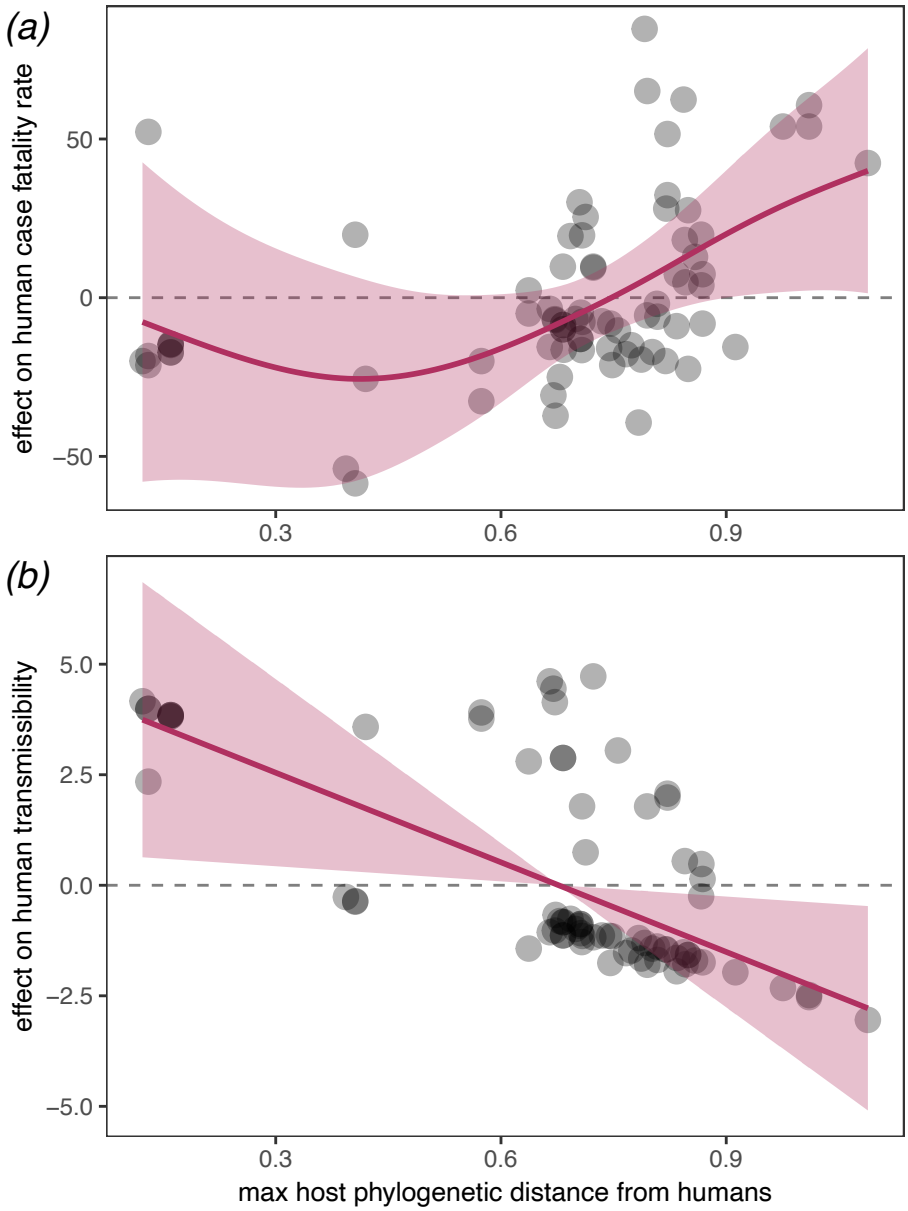

Figure 1.1. Host phylogenetic distance from humans predicts zoonotic risk. Partial effect plots from our selected GAMs show the relative effect of host phylogenetic distance from humans on *(a)* human CFR (virulence); and *(b)* capacity for human-to-human transmission (human transmissibility). Data points represent partial residuals, and shaded regions represent 95% confidence intervals around mean partial effects. Full model descriptions are provided in *SI Data and Results,* Table S5*a-b* .

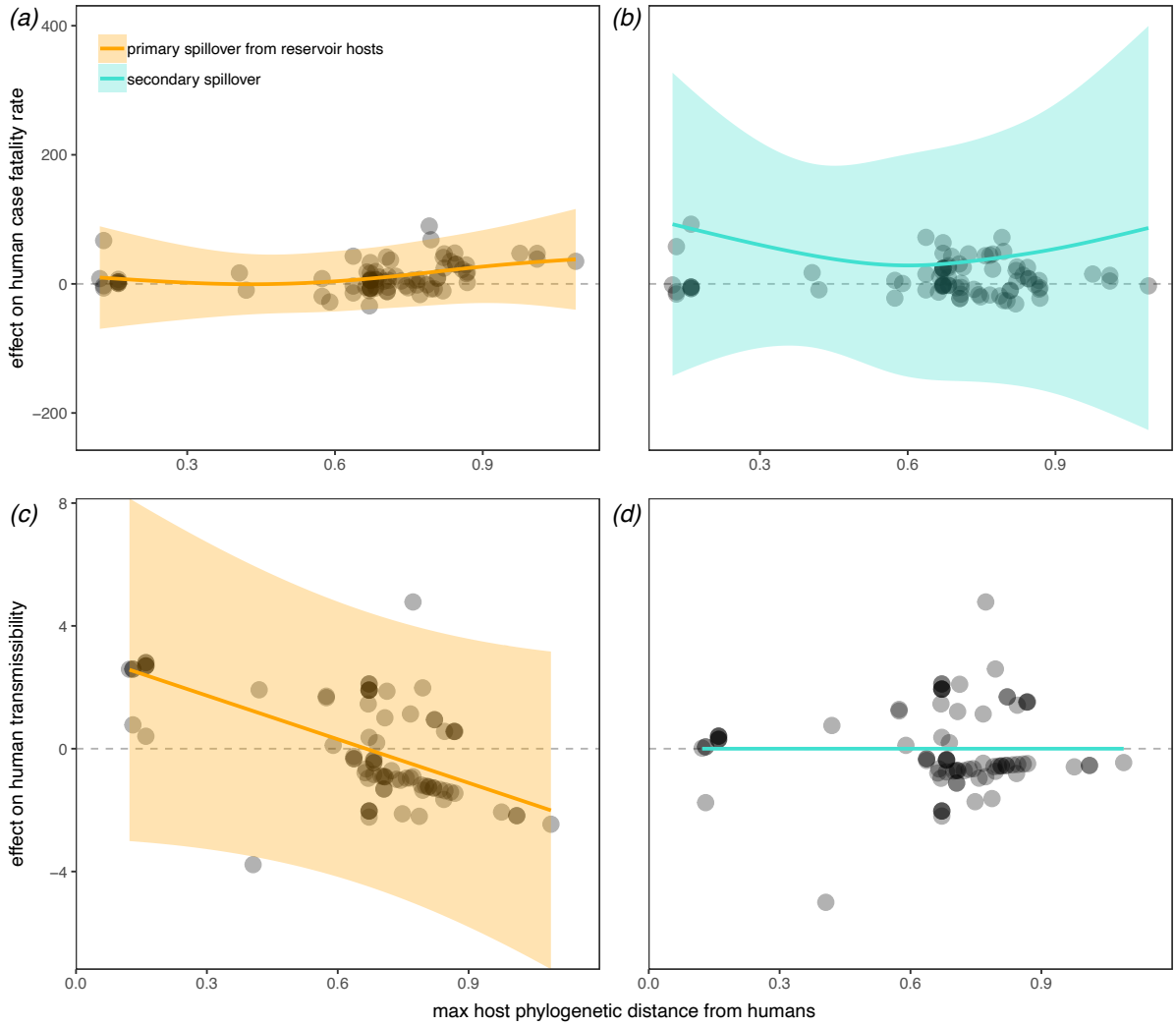

Figure 1.2. Spillover type modulates host predictors of zoonotic risk. Partial effect plots from our selected GAMs show how direct (orange) versus intermediate (blue) spillover changes the relative effect of host phylogenetic distance from humans on *(a, b)* human CFR (virulence); and *(c, d)* the capacity for human-to-human transmission (human transmissibility). Data points represent partial residuals, and shaded regions represent 95% confidence intervals by standard error around mean partial effects. Full model descriptions are provided in *SI Data and Results,* Table S5*c-d*.

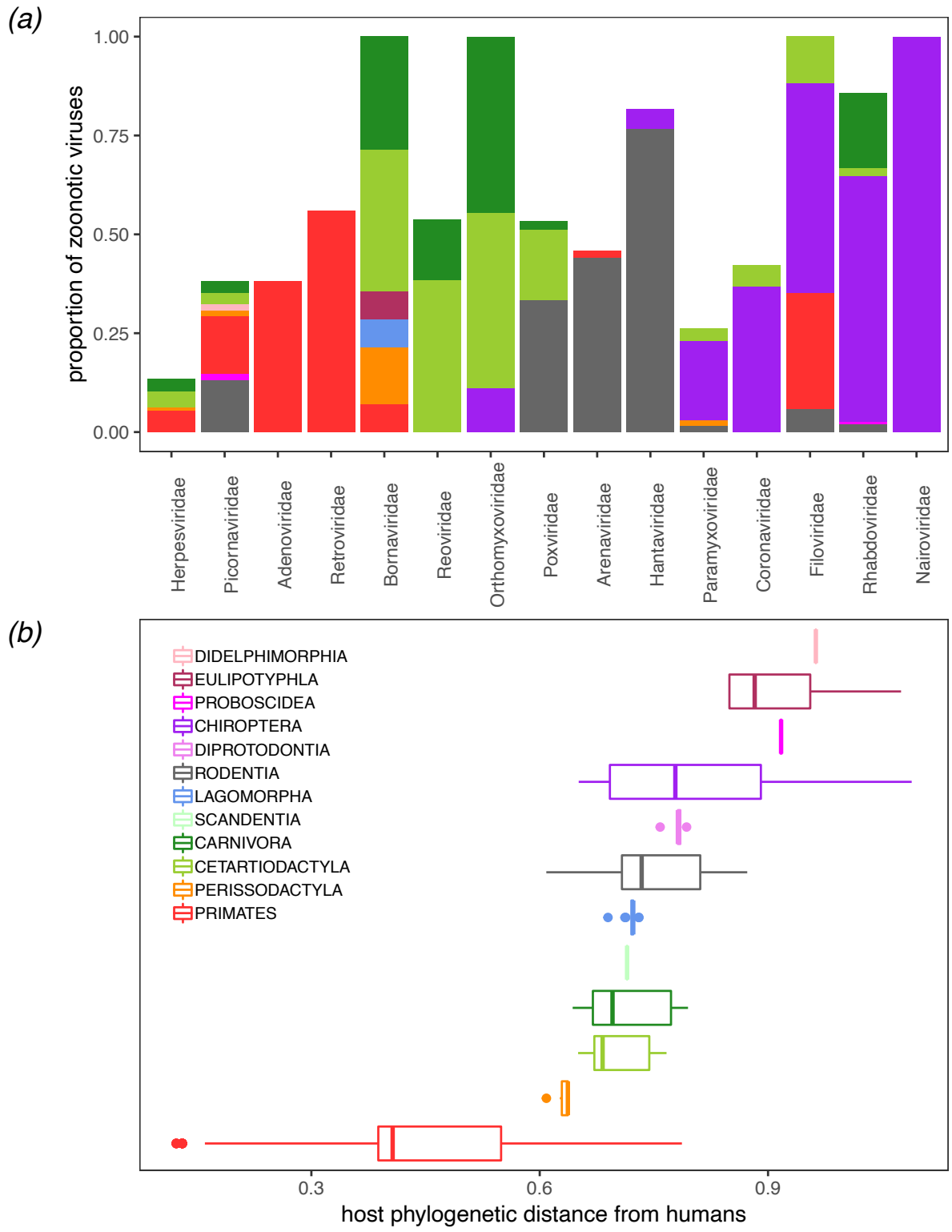

Figure 1.3. Correlation between virus and host types. Across host phylogenetic distance from humans, the distributions of *(a)* zoonotic virus species, grouped by family; and *(b)* mammalian host species, grouped by host order. Colors correspond to host order.

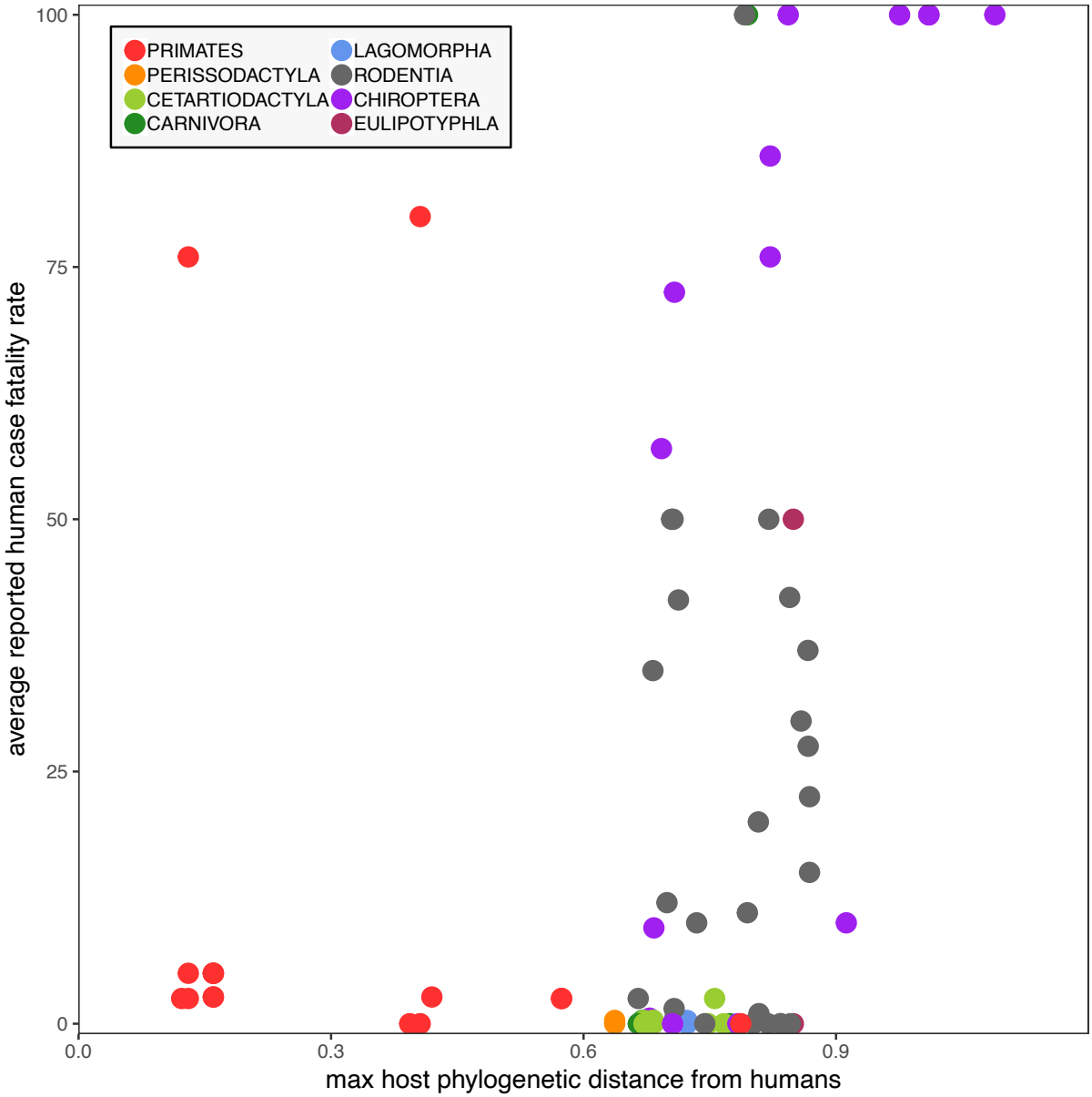

Figure 1.4. Bat-borne viruses are the most virulent of all mammalian zoonoses. Phylogenetic distance and CFR data for all mammalian zoonoses with colors indicating host order. Each point represents a unique CFR per host order for each virus.

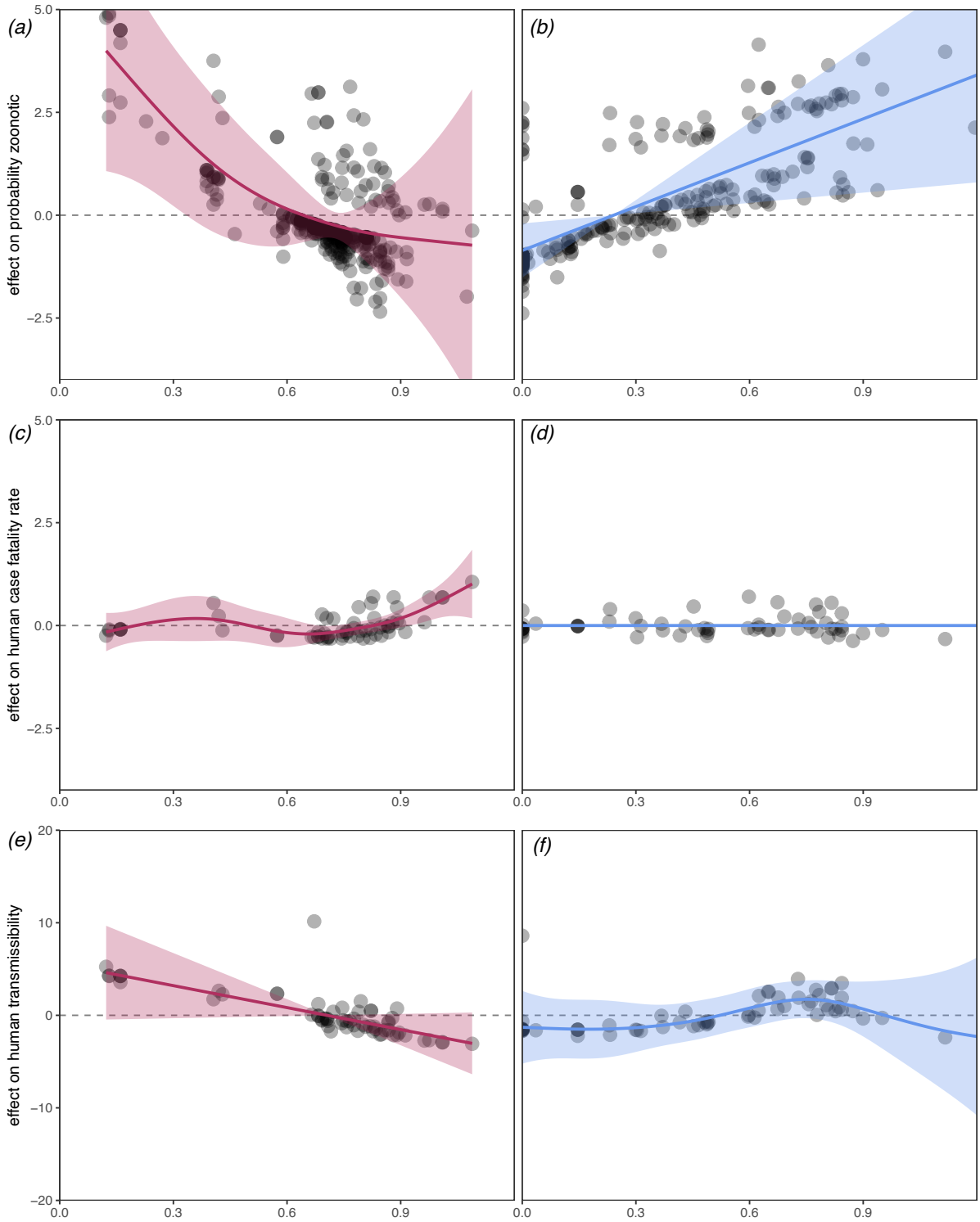

Figure 1.5. Viral predictors of zoonotic risk. Partial effect plots from our selected GAMs show the relative effect of maximum host phylogenetic distance from humans (red) and host phylogenetic breadth (blue) on *(a, b)* the probability that a virus is zoonotic; *(c, d)* human CFR (virulence); and *(e, f)* capacity for human-to-human transmission (human transmissibility). Data points represent partial residuals, and shaded regions represent 95% confidence intervals by

standard error around mean partial effects. Full model descriptions are provided in *SI Data and Results,* Table S5*e-g.*

CHAPTER 2 FIGURES

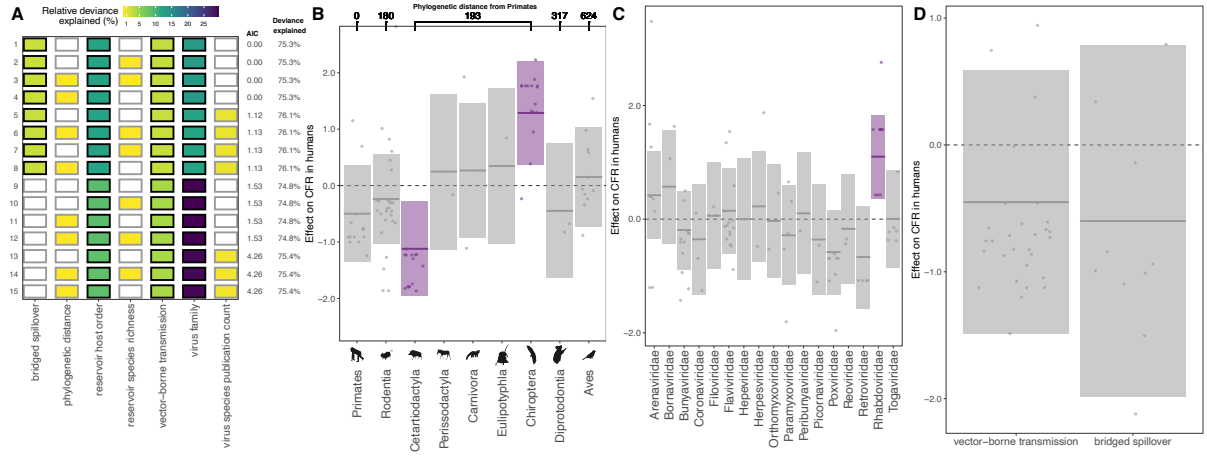

Figure 2.1. Predictors of global CFR estimates. (A) Top 15 models ranked by AIC. Rows represent individual models and columns represent predictor variables. Cells are shaded according to the proportion of deviance explained by each predictor. Cells representing predictor variables with a p-value significance level of <0.1 are outlined in black. (B-D) Effects present in the top model: reservoir host group, virus family, vector-borne transmission, and bridged spillover. Lines represent the predicted effect of the x-axis variable when all other variables are held at their median value (if numeric) or their mode (if categorical). Shaded regions indicate 95% CIs by standard error and points represent partial residuals. An effect is shaded in gray if the 95% CI crosses zero across the entire range of the predictor variable; in contrast, an effect is shaded in purple and considered "significant" if the 95% CI does not cross zero. Full model results are outlined in Table S5a in *SI Data and Results.* (B) Reservoir host groups are ordered by increasing cophenetic phylogenetic distance from Primates (in millions of years), as indicated on the top axis.

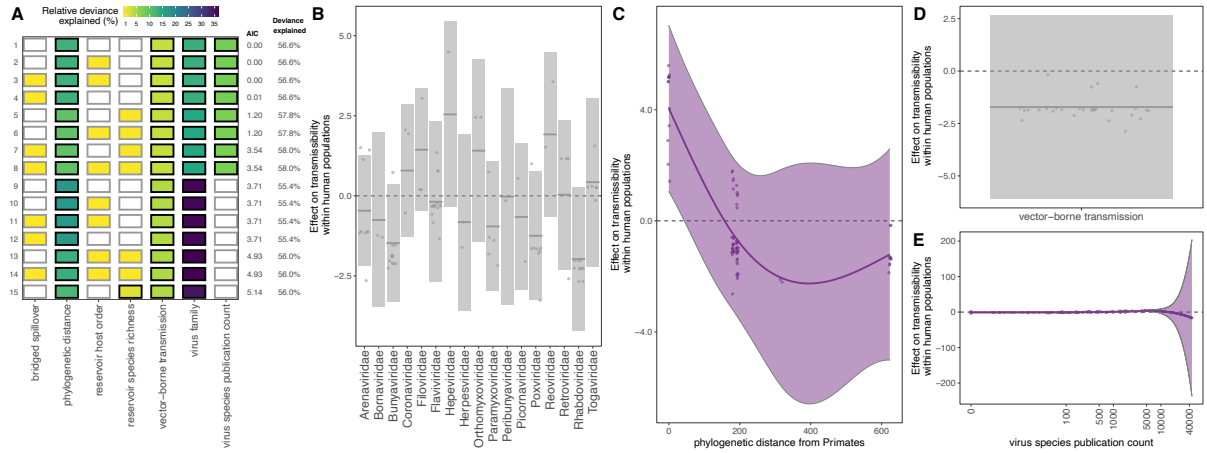

Figure 2.2. Predictors of capacity for forward transmission within the human population following zoonotic spillover. (A) Top 15 models ranked by AIC. Rows represent individual models and columns represent predictor variables. Cells are shaded according to the proportion of deviance explained by each predictor. Cells representing predictor variables with a p-value significance level of <0.1 are outlined in black and otherwise outlined in gray. (B-E) Effects present in the top model: virus family, reservoir group phylogenetic distance from Primates, vector-borne transmission, and log-transformed virus species publication count. Lines represent the predicted effect of the x-axis variable when all other variables are held at their median value (if numeric) or their mode (if categorical). Shaded regions indicate 95% CIs by standard error and points represent partial residuals. An effect is shaded in gray if the 95% CI crosses zero across the entire range of the predictor variable; in contrast, an effect is shaded in purple and considered "significant" if the 95% CI does not cross zero. Full model results are outlined in Table S5b in *SI Data and Results.*

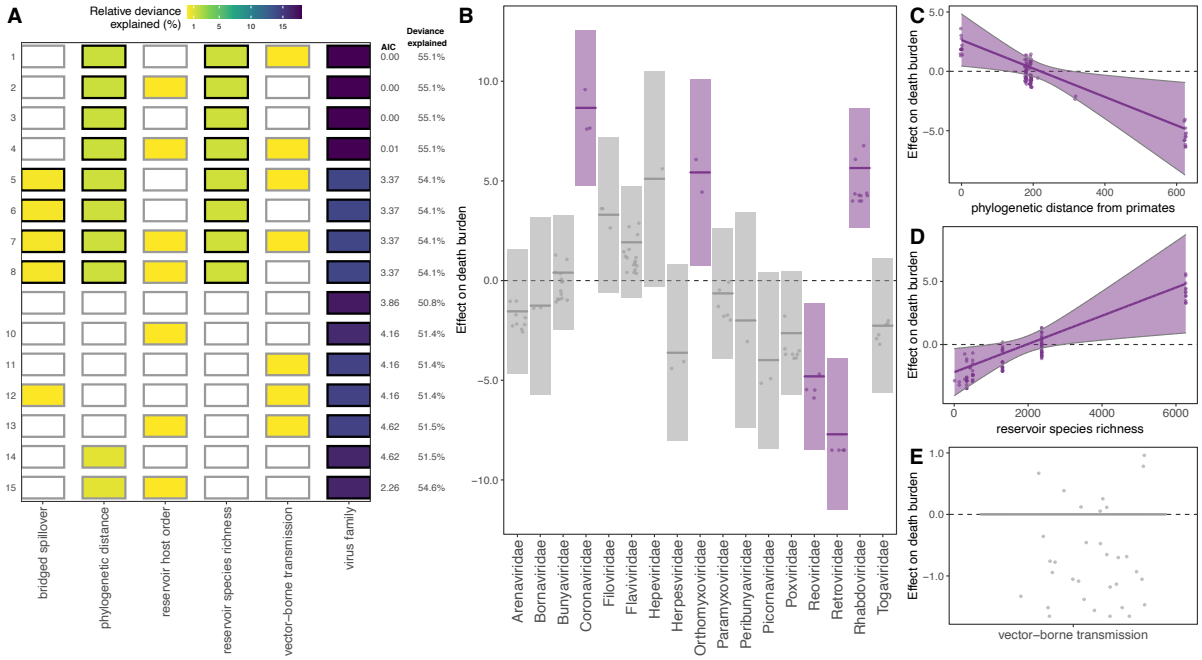

Figure 2.3. Predictors of post-1950 death burden, excluding the virus species publication count predictor. See Figure S12 in *SI Appendix* for inclusion. (A) Top 15 models ranked by AIC. Rows represent individual models and columns represent predictor variables. Cells are shaded according to the proportion of deviance explained by each predictor. Cells representing predictor variables with a p-value significance level of <0.1 are outlined in black and otherwise outlined in gray. (B-E) Effects present in the top model: virus family, reservoir group phylogenetic distance from Primates, reservoir group species richness, and vector-borne transmission. Lines represent the predicted effect of the x-axis variable when all other variables are held at their median value (if numeric) or their mode (if categorical). Shaded regions indicate 95% Cis by standard error and points represent partial residuals. An effect is shaded in gray if the 95% CI crosses zero across the entire range of the predictor variable; in contrast, an effect is shaded in purple and considered "significant" if the 95% CI does not cross zero. Full model results are outlined in Table S5c in *SI Data and Results.*

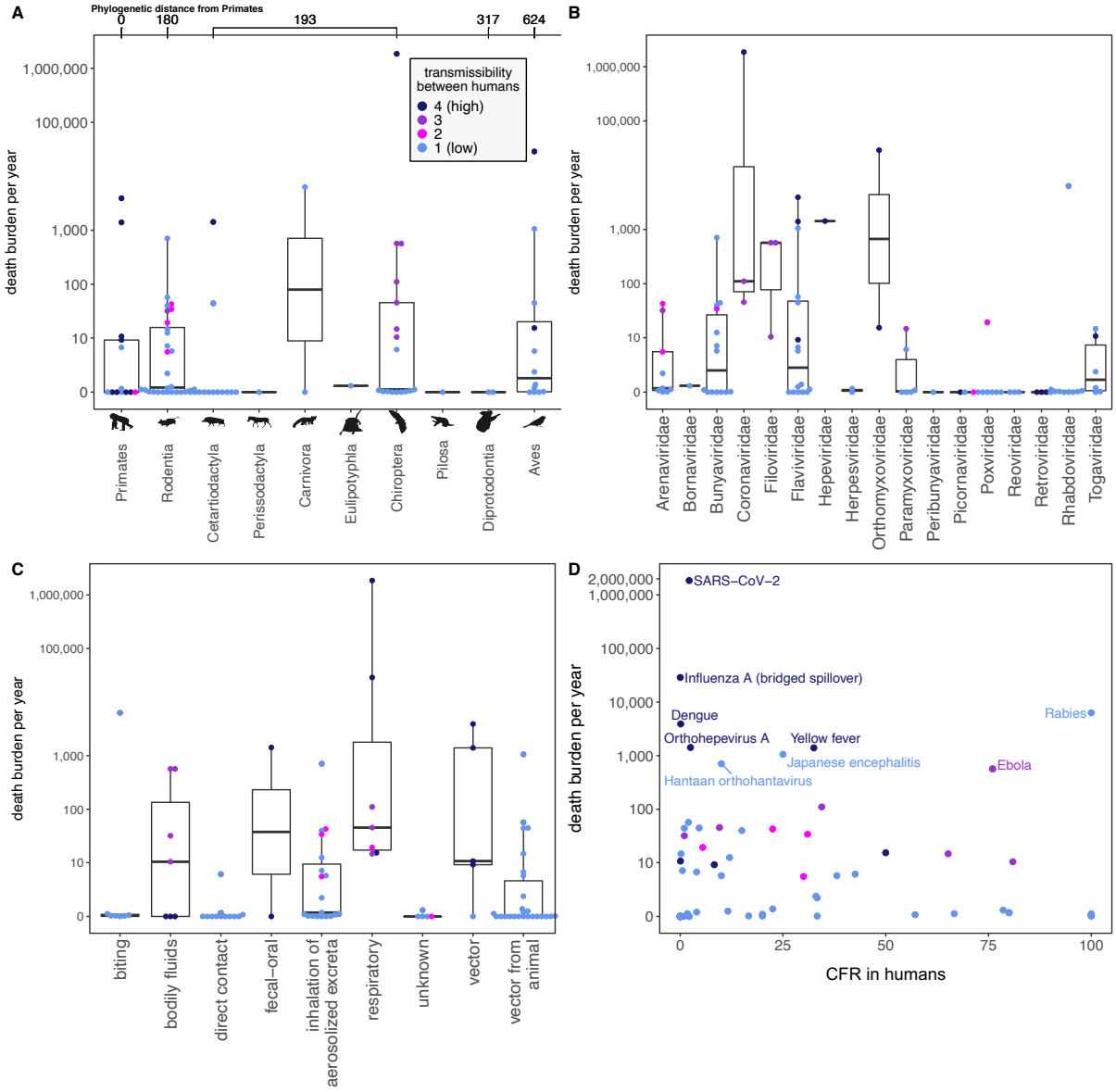

Figure 2.4. Death burden per year (cumulative post-1950 death counts divided by the length of reporting time), grouped by (A) reservoir host group, (B) virus family, (C) primary transmission route, and (D) CFR in humans. Colors indicate transmissibility between humans, with "1" indicating the lowest level of transmission (i.e., no recorded forward transmission in human population post-spillover) and "4" indicating the highest level of transmission (i.e., record of endemic transmission in human populations post-spillover). (A) Reservoir host groups are ordered by increasing cophenetic phylogenetic distance from Primates (in millions of years), as indicated on the top axis.

CHAPTER 3 FIGURES

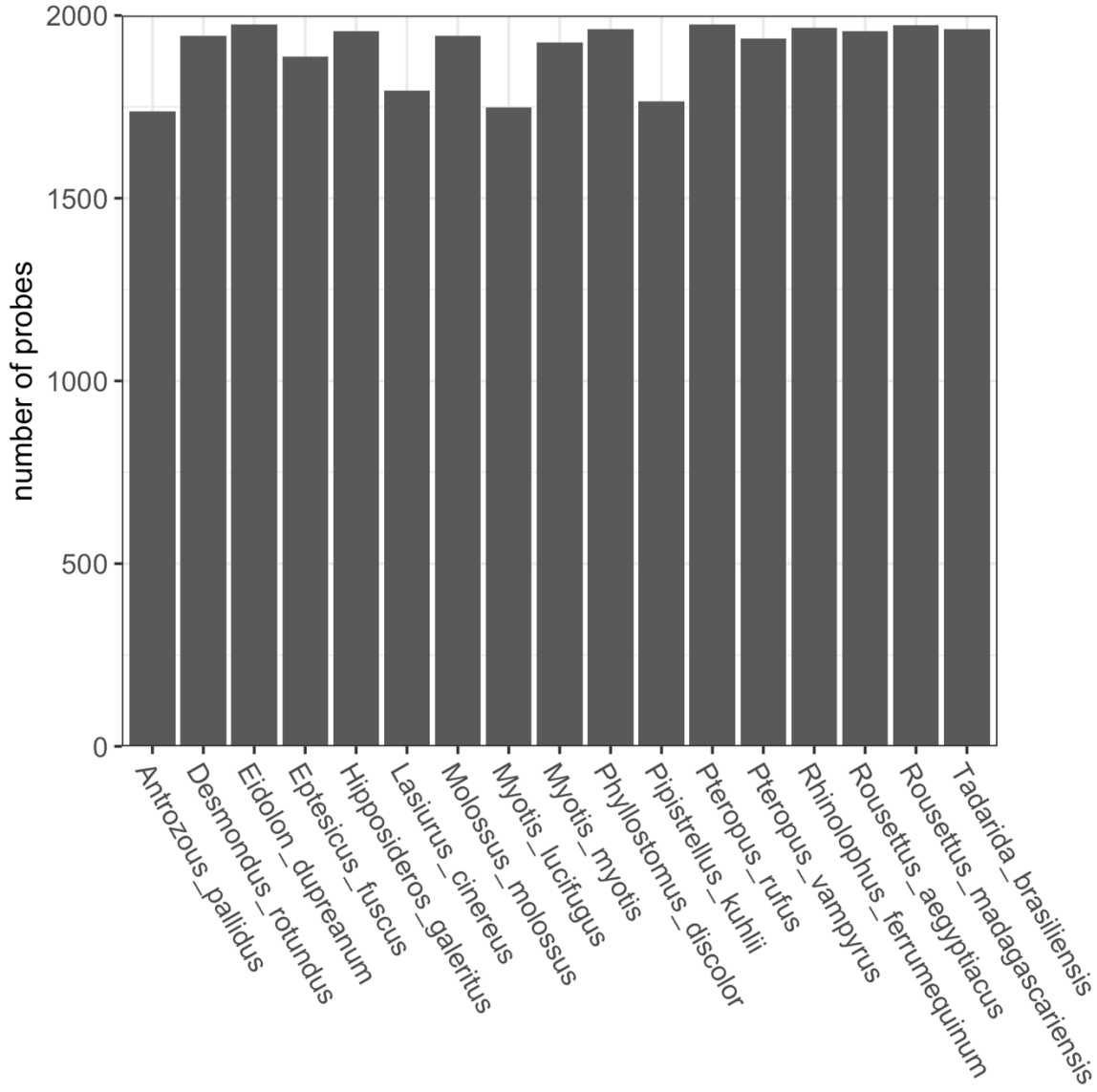

Figure 3.1. Number of probes (out of 1,994) that map to bat genomes across species.

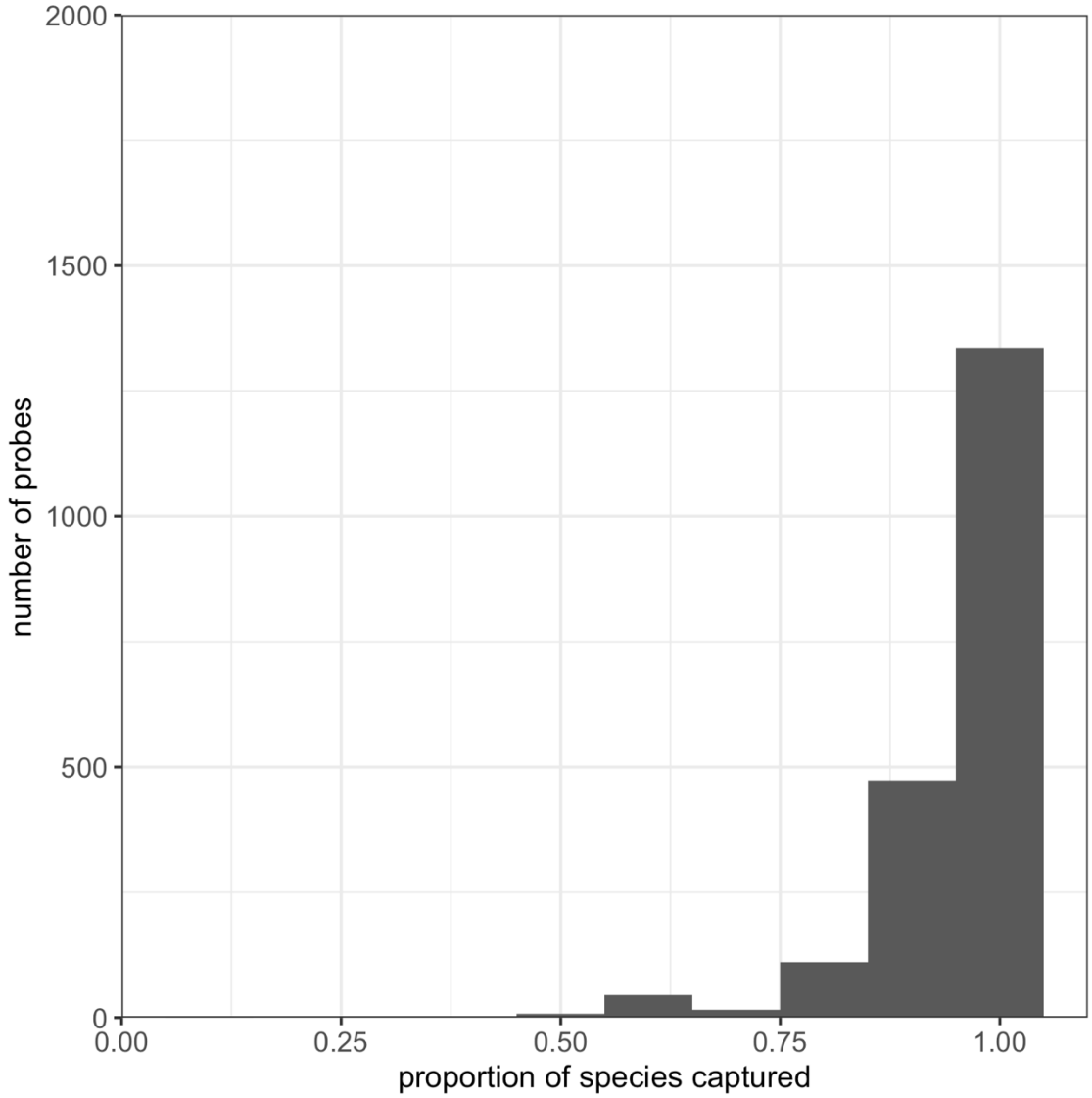

Figure 3.2. Proportion of bat species (out of 17) captured by probes.

CHAPTER 4 FIGURES

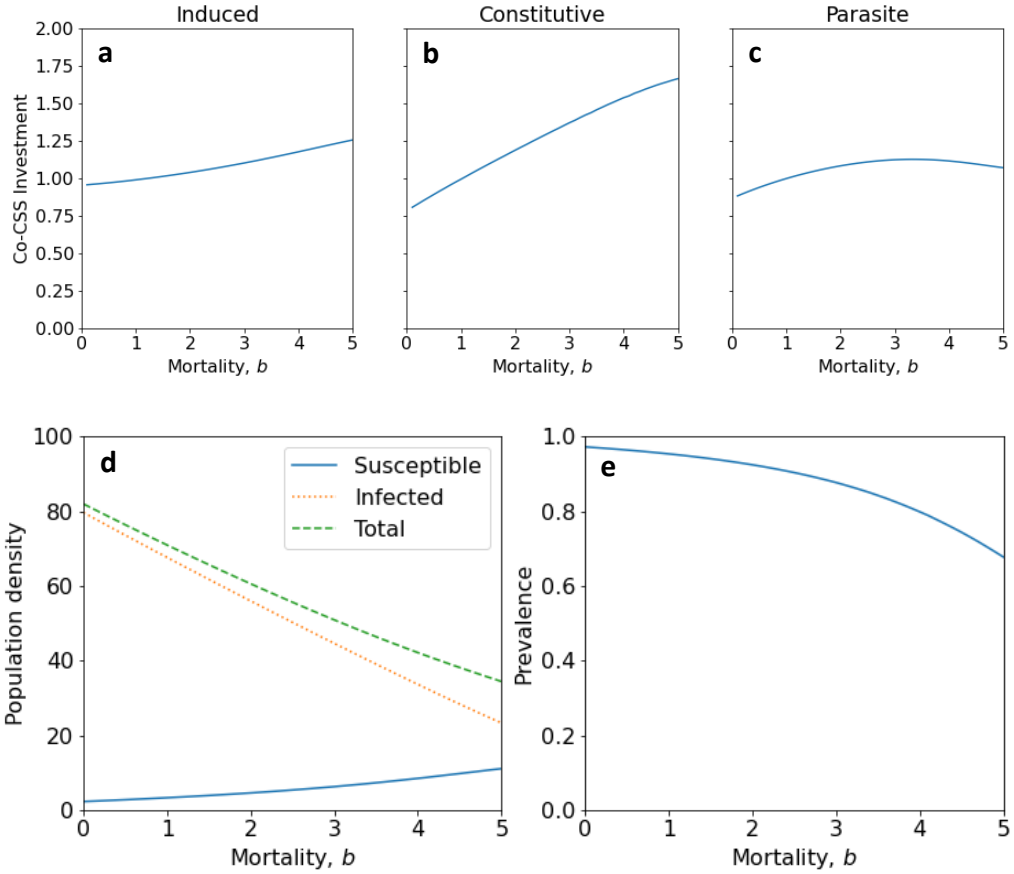

Figure 4.1. Plots of the optimal (continuously stable) strategy in (a) induced defense, (b) constitutive defense, and (c) the parasite growth rate against the natural host mortality rate, b, when the parasite has no impact on host fertility ($f = 1$); and the equilibrium host population densities (d) and parasite prevalence (e). Parameter values: $q = 0.1, \alpha = 1, \gamma = 1, \beta = 2, f = 1$. Constitutive trade-off: $a_0 = 10, a_1 = -0.05, a_2 = -0.1, c_0 = 1$. Induced trade-off: $\gamma_0 = 1, \gamma_1 = 0.02, \gamma_2 = 0.1, h_0 = 1$. Parasite trade-off: $B_0 = 1, B_1 = 0.3, B_2 = -0.4, p_0 = 1$.

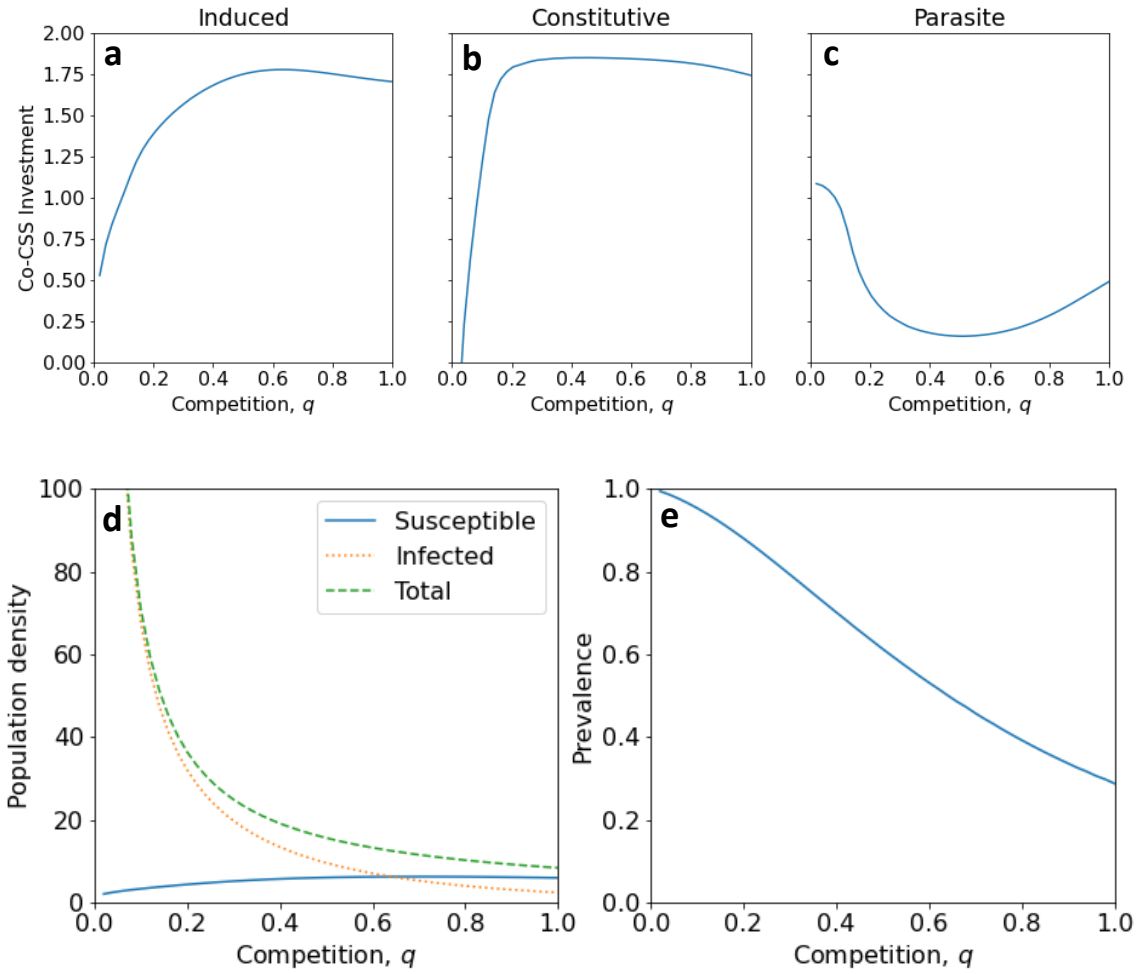

Figure 4.2. Plots of the optimal (continuously stable) strategy in investment in (a) constitutive defense, (b) induced defense, and (c) the parasite growth rate against the host birth rate susceptibility to crowding (competition) when the parasite has no impact on host fertility ($f = 1$) and c_2 = -0.1; and the equilibrium host population densities (d) and parasite prevalence (e). Parameter values: $b = 1, \alpha = 1, \gamma = 1, \beta = 2, f = 1$. Constitutive trade-off: $a_0 = 10, a_1 = -0.05, a_2 = -0.1, c_0 = 1$. Induced trade-off: $\gamma_0 = 1, \gamma_1 = 0.02, \gamma_2 = 0.1, h_0 = 1$. Parasite trade-off: $B_0 = 1, B_1 = 0.3, B_2 = -0.4, p_0 = 1$.

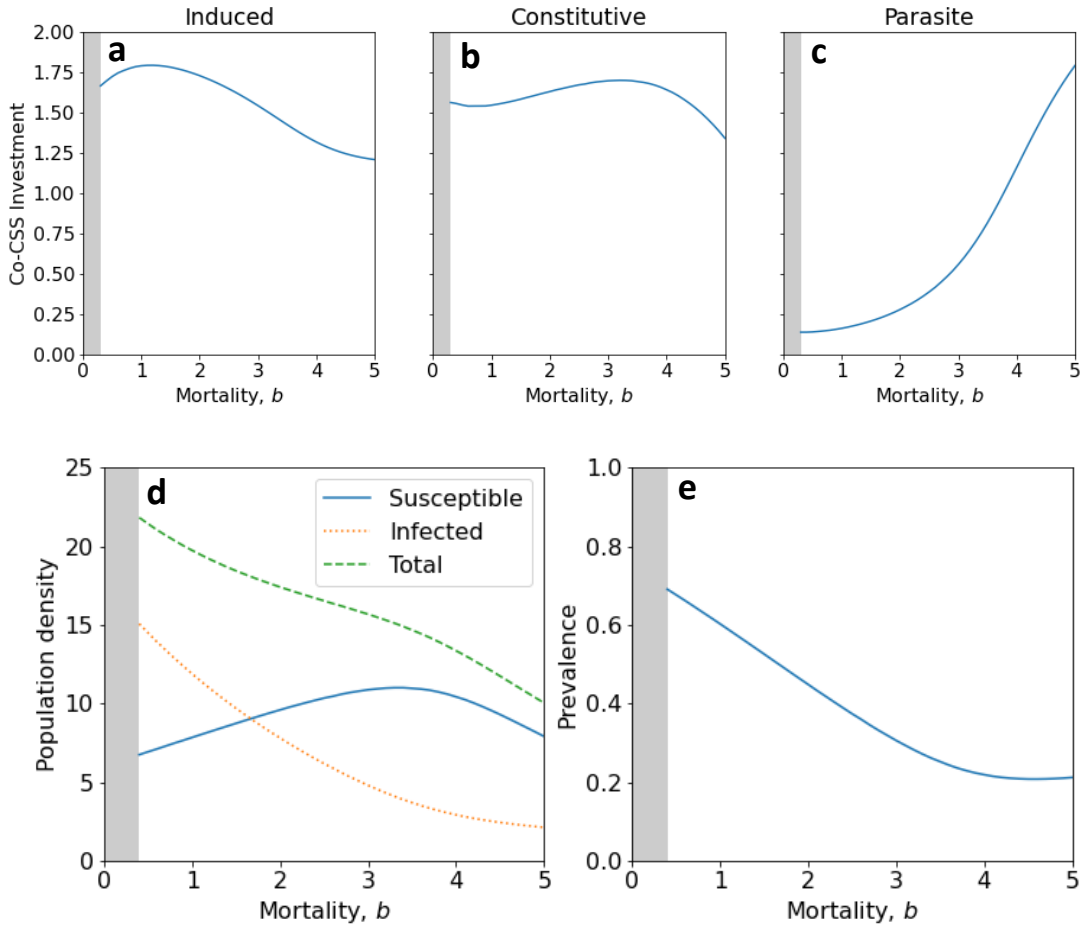

Figure 4.3: Plots of the optimal (continuously stable) strategy in (a) induced defense, (b) constitutive defense, and (c) the parasite growth rate against the natural host mortality rate when the parasite is a castrator ($f = 0$). Parameter values: $q = 0.2, \alpha = 1, \gamma = 1, \beta = 2, f = 0$. Constitutive trade-off: $a_0 = 10, a_1 = -2, a_2 = -0.5, c_0 = 1$. Induced trade-off: $\gamma_0 = 1, \gamma_1 = 1.5, \gamma_2 = 2.5, h_0 = 1$. Parasite trade-off: $B_0 = 1, B_1 = 0.5, B_2 = -0.4, p_0 = 1$.

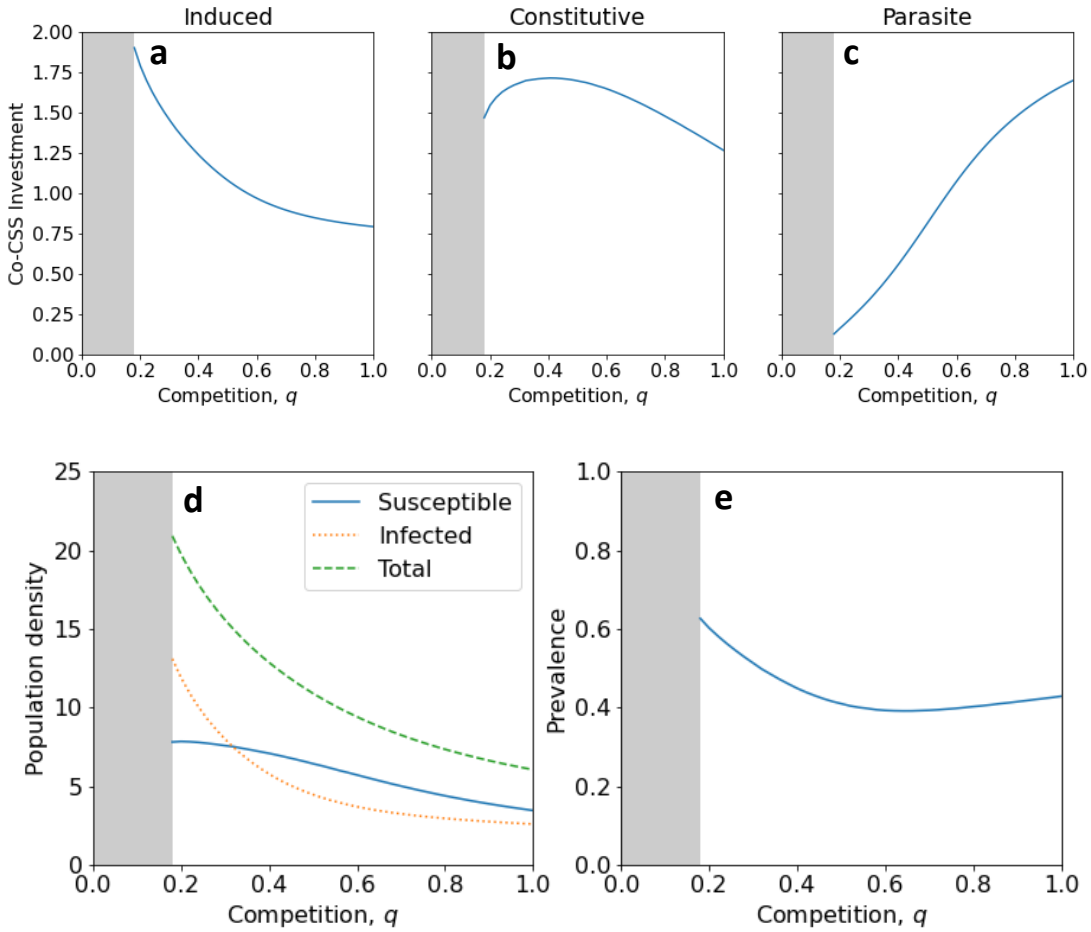

Figure 4.4. Plots of the optimal (continuously stable) strategy in (a) constitutive defense, (b) induced defense, and (c) the parasite growth rate against the host birth rate susceptibility to crowding (competition) when the parasite is a castrator ($f = 0$). Parameter values: $b = 1, \alpha = 1, \gamma = 1, \beta = 2, f = 0$. Constitutive trade-off: $a_0 = 10, a_1 = -2, a_2 = -0.5, c_0 = 1$. Induced trade-off: $\gamma_0 = 1, \gamma_1 = 1.5, \gamma_2 = 2.5, h_0 = 1$. Parasite trade-off: $B_0 = 1, B_1 = 0.5, B_2 = -0.4, p_0 = 1$.

TABLES

CHAPTER 1 TABLES

Table 1.1. Definition of terms used in this study.

term	definition
spillover	transmission of animal viruses to humans
spillback	transmission of human viruses to animals
zoonotic potential	probability that a pathogen—or a pathogen originating from an animal species or region of interest—could emerge into the human population
spillover host	host species that has been recorded to infect humans and thus has served as a source of human infection
reservoir host	primary host species that is responsible for maintaining zoonotic transmission
primary spillover	spillover to humans from a reservoir host species
secondary host	host species that has been infected, but does not maintain zoonotic transmission
bridge host	secondary host species that has been recorded to infect humans
secondary spillover	spillover to humans from a secondary host species
transmission cost	a trade-off that compromises a virus's capacity to transmit between hosts

Table 1.2. Description of predictor and response variables used in the GAM analyses.

Term	Type	Description
Response variables		
Human case fatality rate	numeric	The proportion of human cases for a given virus that are fatal
Human transmissibility	categorical ranking (1-4)	A virus' capacity for human-to-human transmission. See *SI Methods* for a description of the ranking system
Zoonotic potential	binary (0,1)	The probability that a virus has the capacity to infect humans (i.e., is or is not zoonotic)
Host predictors		
Length of gestation period	numeric	Length of pregnancy (in days)
Litter size	numeric	Average number of offspring produced at one time
Maximum lifespan	numeric	Maximum recorded longevity (in months)
Body mass	numeric	Average body mass (in grams)
Phylogenetic distance from humans	numeric	Distance from humans on a cytochrome b phylogenetic tree
Taxonomic order	categorical	Taxonomic classification
Number of disease-related citations	numeric	Number of PubMed citations relevant to zoonotic diseases; to control for any potential publication bias
Viral predictors		
Whether or not the virus is enveloped	categorical	Does the virus have an external envelope?
Whether or not the virus replicates in the cytoplasm	categorical	Does the virus replicate in the cytoplasm?
Average genome length	numeric	Metric for genome size
DNA or RNA	categorical	Is the virus DNA or RNA?
Genome composition	categorical	Viral genome classification
Maximum host phylogenetic distance from humans	numeric	Phylogenetic distance from humans of the most distantly related known host for a given virus
Maximum host phylogenetic breadth	numeric	Maximum phylogenetic distance between the two most distantly related known hosts for a given virus

Number of citations	numeric	Number of relevant PubMed citations; control for research effort bias
Categories for virus-mammal associations		
Reservoir status	binary (1,2)	Host species role in the maintenance of zoonotic transmission. Species that maintain viruses endemically are reservoir hosts (reservoir status = 1). Species that harbor the virus but are not implicated in zoonotic maintenance are secondary hosts (reservoir status = 2).
Spillover capacity	binary (1,0)	Host species role in the spillover of zoonoses to humans. Spillover hosts ("1") defined as species that are a source of human infection. Non-spillover hosts ("0") defined as species that have no record of transmission to humans.
Spillover type	binary (1,2)	Chain of spillover transmission for a given virus-host system. Spillover to humans from a reservoir host is "primary spillover" (reservoir status = 1, spillover capacity = 1, spillover type = 1). Spillover to humans from a secondary host is "secondary spillover" (reservoir status = 2, spillover capacity = 1, spillover type = 2).

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APPENDICES

CHAPTER 1 SUPPORTING INFORMATION

SI Data and Results. Databases with variable descriptions and references; outputs for all selected GAMs; and a review of key findings from past meta-analysis on zoonotic risk. Available at **https://doi.org/10.1098/rstb.2019.0296**.

Supporting Information (SI) Methods. Extended description of the methods used in this study.

A list of viruses and associated mammalian hosts was obtained from an extensive database of virus-mammal associations published by Olival et al. (28). Using the information provided, we extracted associations corresponding to directly transmitted viruses previously PCR-identified or isolated in both mammals and humans, and with evidence of animal-to-human spillover. Viruses classified as zoonotic based on exclusively serological data and viruses with vector-borne transmission or "spillback" from humans to animals were excluded. Viruses such as HIV that have zoonotic origins, but now maintain separate, genetically distinct animal and human transmission cycles (187) were also not included. We supplemented our initial list of virus-mammal associations by cross-referencing other existing virus databases (7,9,29) and conducting literature searches. For each virus, we confirmed the accuracy, detection quality, and completeness of the mammal associations, resolving any inconsistencies between the referenced database and scientific literature. Through our searches, we additionally identified virus and mammal associations that were missing from our list and added those that met the criteria we outlined above. We compiled a list of 420 virus-mammal associations, which included 278 unique host species and 67 unique zoonotic viruses (*SI Data and Results,* Table 1).

For each virus-mammal association in our database, we conducted a series of literature searches to collect two metrics of zoonotic risk: viruses' human case fatality rate and capacity for human-to-human transmission. We collected CFRs as a proxy for virulence, reporting the mean of the maximum and minimum recorded CFR per zoonosis in the literature. When different CFRs for a given virus could be linked to spillover events from different host species, we reported these distinct host-CFR associations as separate entries in our database. This distinction occurred in the case of two viruses only, Nipah virus, which spills over to humans from both pigs (73) and bats (74), and Marburg virus, which spills over to humans from both primates (188,189) and bats (190). We additionally collected information on each zoonosis' capacity for human-to-human transmission according to a four-point scale, adapted from previously defined classification schemes (8,11,13,29). We assigned a human transmissibility level of "1" to viruses for which human-to-human transmission had not been recorded; "2" to viruses for which human-to-human transmission had been recorded, but was described as atypical; "3" to viruses for which human-to-human transmission had occurred regularly, but was restricted to self-limiting outbreaks; and "4" to viruses for which endemic human transmission had been reported. We constructed this ranking system based on literature that has defined epidemic outcomes for different levels of human-to-human transmission (2,11,13). Previous meta-analyses have relied on binary categorization (e.g., pathogens are either capable or incapable of human-to-human transmission) which fails to capture critical nuance. Slight variations in viral capacity for between-human transmission can have a large impact on the outcomes of human epidemics (13). In capturing the full extent of variation in viruses' capacity to transmit between humans, our

classification system provides a better foundation for identifying viruses that pose the greatest threat to human populations.

In addition to collecting our targeted metrics of virulence and transmissibility, we classified each virus-mammal association according to the mammal's role in the transmission of a virus. First, we used a binary code to distinguish between reservoir and secondary hosts ("reservoir status"), assigning "1" to mammal species that maintain viruses endemically (reservoir hosts) and "2" to species that harbor the virus but are not implicated in zoonotic maintenance (secondary hosts). Thus, the "reservoir status" variable identifies the primary selective environment (i.e., reservoir) of viruses in zoonotic transmission cycles. We assigned a second binary code to define each host's role in zoonotic spillover to humans ("spillover capacity"), assigning "1" to mammal species that serve as a source of human infection and "0" to species that have no record of transmission to humans. Thus, the "spillover capacity" variable identifies host species implicated in infecting humans. Combining these two codes, we defined a third "spillover type" code to distinguish between "primary" spillover from a reservoir host species (reservoir status = 1, spillover capacity = 1) and "secondary" spillover from a secondary host species (reservoir status = 2, spillover capacity = 1).

In addition to the virus-mammal association database, we compiled a database of all directly-transmitted mammalian viruses, including both zoonotic and non-zoonotic viruses. We extracted directly-transmitted viruses from the extensive database of mammalian viruses published by Olival et al. (28), again excluding viruses with vector-borne transmission, "spillback" from humans to animals, or exclusively human transmission. We collected 7 additional viruses from Geoghegan et al. (7), compiling a total of 345 unique viruses (supplementary table 2).

Using previously published databases (7,9,28–33), we collected a series of host and viral predictor variables that based on the literature, we hypothesized might explain observed variation in zoonotic virus dynamics in human hosts. The literature has identified a suite of life history, ecological, immunological, and biological host factors that can influence host-pathogen coevolution (17,19,21,191). In this study, we focused on four life history traits that could be quantified across mammal species: body mass, litter size, gestation period length, and lifespan. Host reproductive effort trades off with investment in immunity and shapes the demography of the susceptible host population (38,39). Long-lived hosts are associated with heightened transmission in a population (40,41). The literature has additionally linked host body mass with the rate of disease progression (34), reservoir competence (35), and pathogen replication rate (36,37). Our analysis builds off previous work by Luis et al. (192), which considered host life history traits such as body mass, lifespan, and litters per year in a meta-analysis of zoonotic burden across bats and rodents. In addition to including viral traits such as genome length and whether or not the virus replicates in the cytoplasm, we analyzed viruses' host ranges. Replicating Olival et al. (28), we first considered viruses' host phylogenetic breadth, as a pathogen's degree of generalism has been posited to influence the evolution of virulence (193). We additionally considered the position of a virus' host breadth relative to humans by considering the maximum host phylogenetic distance from humans across a virus' host range.

We obtained these host traits primarily from the PanTHERIA database (31), supplementing missing trait information with the Animal Diversity Web (33), The Encyclopedia of Life (30), AnAge database of animal ageing and longevity (32), and literature searches (supplementary table 3). We proxied unavailable trait data by averaging across other host species in the same genus, or borrowing data from species in the same family that had similar body

masses. Additionally, we collected host taxonomic classification, phylogenetic breadth, and phylogenetic distance from Olival et al. (28); virus recombination rates from Geoghegan et al. (7); and virus taxonomic classification and genome composition from the International Committee on Taxonomy of Viruses (ICTV) database (194). Our full database with references is available in the supplementary information (supplementary tables 1–4). Table 1.2 lists and describes all predictor and response variables used in our analysis.

Olival et al. calculated hosts' phylogenetic distance from humans and viruses' host phylogenetic breadths from a matrix of phylogenetic distances between mammal species. The authors derived the distance values in this matrix from a maximum likelihood phylogenetic tree of mammalian cytochrome *b* sequences using the ape package in R (195,196). Cytochrome *b* is a mitochondrial protein with high sequence variability and availability across mammal species, and as a result, is commonly used to determine phylogenetic relationships between mammals (197). Cytochrome *b* has also been demonstrated to be the most effective mitochondrial genetic marker for reconstructing mammalian phylogenies (198). Many previous studies of parasite sharing between animals and plant species have quantified the degree of phylogenetic difference between host species as the number of years since species diverged in evolutionary history (199–202). Using time since divergence in this context implies that host species share pathogens due to mutual ancestry. However, spillover occurs when a pathogen overcomes genetic barriers to infect a novel host – it does not arise due to shared ancestry between two host species (203). Host genetic factors also determine how the host will respond to infection, which will influence the pathogen's virulence and capacity for transmission in a novel host population (204). Given the lack of a universally identified and available genetic loci for host immune traits, studies of cross-species pathogen emergence have often relied on mitochondrial genetic markers to measure the degree of phylogenetic difference between host species (55,205). Although cytochrome *b* sequences are available for the majority of mammal species, some species in our dataset lacked sufficient sequence data and thus, were excluded from the Olival et al. matrix of phylogenetic distances. We calculated phylogenetic distance values for these missing species by averaging across other host species in the same genus or order, which is noted in supplementary table 3. There were 7 viruses missing phylogenetic breadth values; we used viruses in the same genus as proxies, which is noted in supplementary table 2.

CHAPTER 2 SUPPORTING INFORMATION

SI_Data_and_Results. Databases with variable descriptions and references, and table outputs for all selected models. Available at https://doi.org/10.1073/pnas.2113628119.

Data and materials availability: All data, data references, code, and materials used in the analysis are publicly available in the main text, the supplementary materials, or the following github repository: https://github.com/sguth1993/zoonotic_risk_meta_analysis

SI_Appendix. Supplementary methods and figures (Figure S1-13).
Exclusion criteria. We excluded seven viruses (Table S2 in *SI Data and Results*) that have only caused human infections in laboratory settings. We additionally did not include viruses such as HIV (206) and HCoV-229E (207) that have zoonotic origins, but have maintained separate, genetically distinct human transmission cycles since before 1950 (Table S3 in *SI Data and Results*). We excluded such viruses for several reasons: precise death and case count records are sparse pre-1950; viruses that have circulated within the human population for centuries or decades often have unconfirmed or disputed origins; and over long timescales, viral evolution in the human population is expected to muddle any relationship between zoonotic history and dynamics in the human population (104). With this strict inclusion criteria, we compiled 89 unique virus species (Table S1 in *SI Data and Results*). Each virus species was associated with one reservoir host order, with the exception of Rabies virus and Mammalian 1 orthobornavirus, which are both known to be maintained by two distinct nonhuman animal reservoir orders in independent transmission cycles (57).
Supplementary analyses. For each virus, in addition to collecting global CFR estimates from the literature, we calculated up to three country-specific CFRs from death and case counts in countries that have reported the largest outbreaks of that virus—when available, using data that spanned multiple outbreaks and/or years to maximize sample size and accuracy. We expected that global CFR estimates would be more precise approximations of virulence, while country-specific CFR reports would allow us to assess and account for potentially confounding effects of regional differences in health care and overall infrastructure. To test whether GDP per-capita predicts country-level variation in CFR, we modeled all 119 country-specific CFR estimates separately (Table S6c in *SI Data and Results*). To gage whether variation in GDP per-capita among viruses' geographic ranges might confound the trends in global CFR estimates, we then modeled GDP per-capita and CFR estimates aggregated at the level of the 86 unique zoonotic transmission chains (Table S6d in *SI Data and Results*). For this second model, we calculated a composite GDP per-capita for each aggregated CFR statistic by weighting each country's GDP per-capita by the proportion of cases in the CFR calculation that were recorded in each country and summing the weighted GDPs per-capita.

We assigned a human transmissibility level of "1" to viruses for which forward transmission in human populations post-spillover had not been recorded; "2" to viruses for which forward transmission in humans had been recorded but was described as atypical; "3" to viruses for which transmission within human populations had occurred regularly but was restricted to self-limiting outbreaks; and "4" to viruses for which endemic human transmission had been reported.

Recording death and case data from laboratory-confirmed outbreaks in the literature required maintaining a strict definition of zoonotic, excluding some viruses that have been

included in previous analyses (28,57,68). We compiled excluded viruses that met looser inclusion criteria—specifically, seven viruses that have only caused human infections in laboratory settings and 25 viruses that lacked molecular confirmation of infection of humans, but still had serological evidence of infection in humans—in a supplementary database (Table S2 in *SI Data and Results*). To assess whether our observed trends held across a larger sample of zoonotic viruses, we ran an additional GAM analysis of global CFR estimates with this supplementary database, including 121 unique virus species with a total of 126 unique zoonotic transmission chains (Table S6b in *SI Data and Results*). Viruses included in previous analyses that met neither our loose nor strict inclusion criteria are outlined in Table S3 in *SI Data and Results*.

We calculated reservoir host group cophenetic distance from Primates using a composite time-scaled phylogeny of the mean divergence dates for all reservoir clades, as presented in the TimeTree database (57,208). In our prior analysis (60), phylogenetic distance values were derived from a phylogenetic tree of mammalian cytochrome *b* sequences (28,195,196), which captured significantly more variation between host orders. The time-scaled phylogeny used in this analysis produced only six unique distance values across all reservoir groups in our database but represented the only available phylogeny that included both mammals and birds.

Given that the number of zoonoses harbored by a reservoir group appears to correlate with species diversity within that group (57), we hypothesized that species diversity might influence reservoir effect size on CFR in humans; thus, we included reservoir species richness, which we derived from the Catalogue of Life using version 0.9.6 of the taxize library in R (57,72), taking the sum of values across bird orders for the Aves reservoir group. If increasing a reservoir group's total number of zoonotic viruses also increases their number of virulent zoonoses, reservoir species richness might inflate the mean CFR of zoonotic viruses harbored by species rich reservoir groups—or alternatively, given that most zoonotic viruses have low CFRs in humans, species richness might instead reduce the mean CFR associated with these reservoirs. Nevertheless, we expected that higher numbers of zoonotic virus species would inflate the total death burdens associated with species rich reservoir groups.

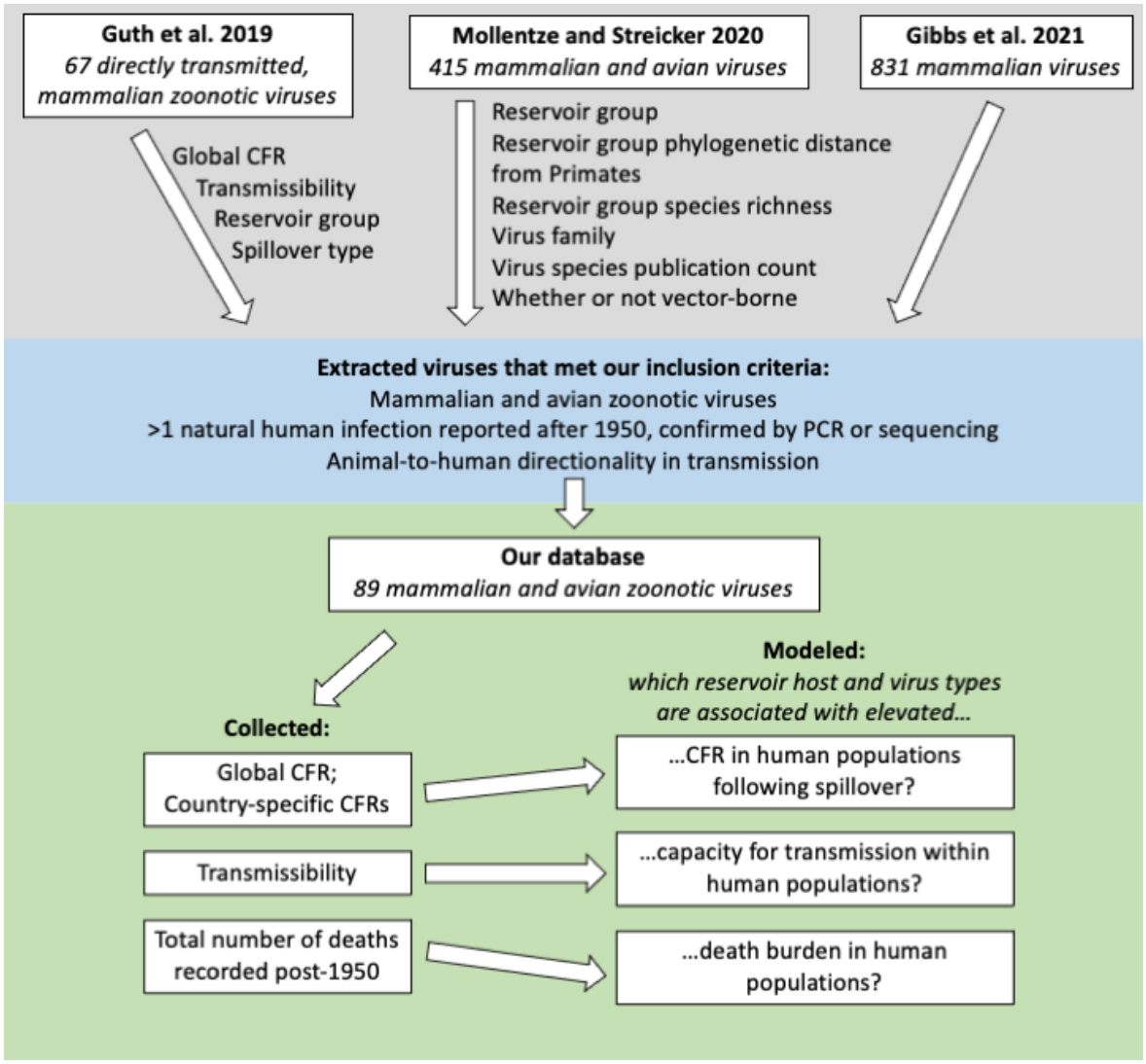

Figure S1. Data provenance and analysis flowchart.

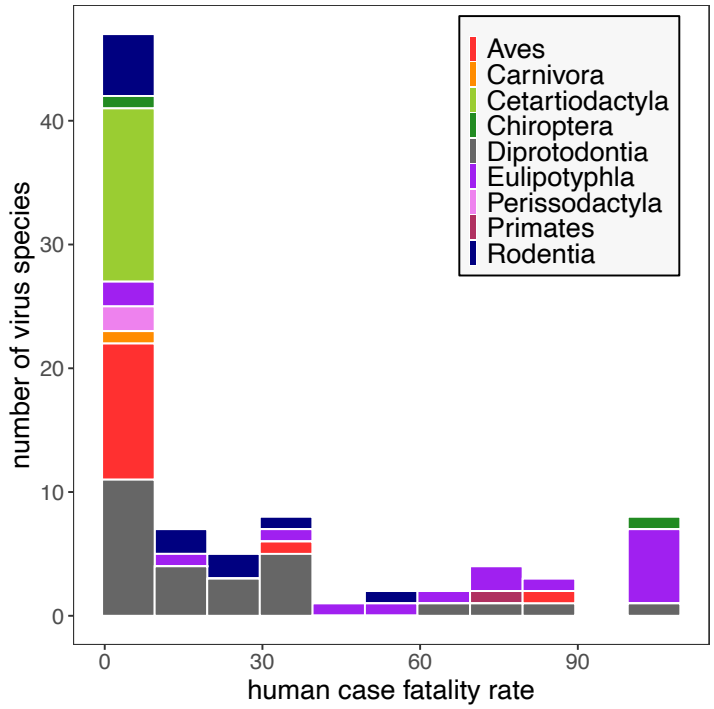

Figure S2. Histogram of human case fatality rates (CFRs) across all zoonotic virus species included in our virulence analysis, grouped by reservoir host type.

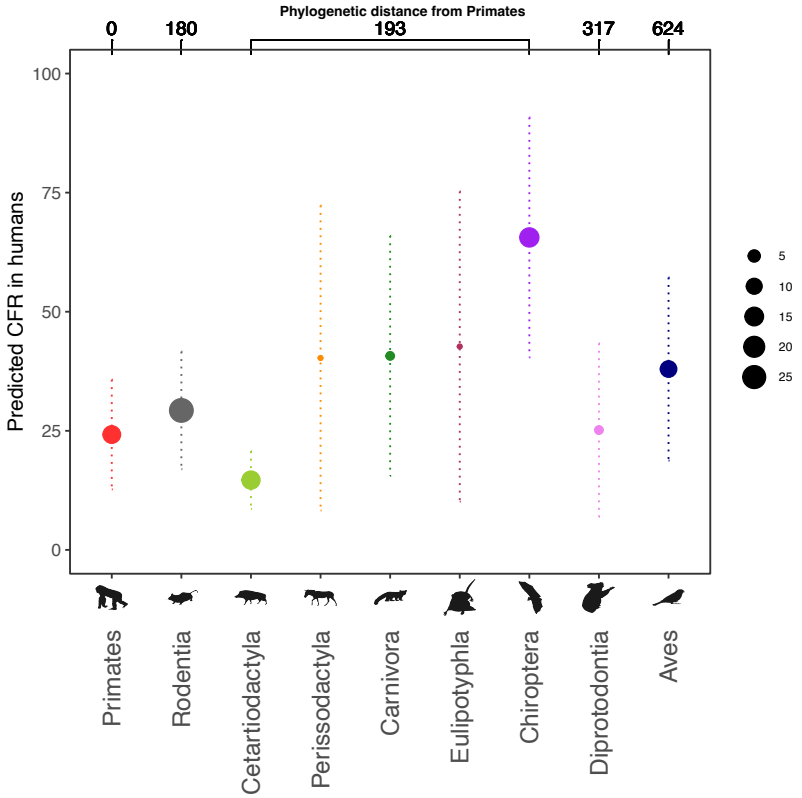

Figure S3. Predicted human CFR of zoonotic viruses sourced from each reservoir host group when using the top selected model of global CFR estimates.

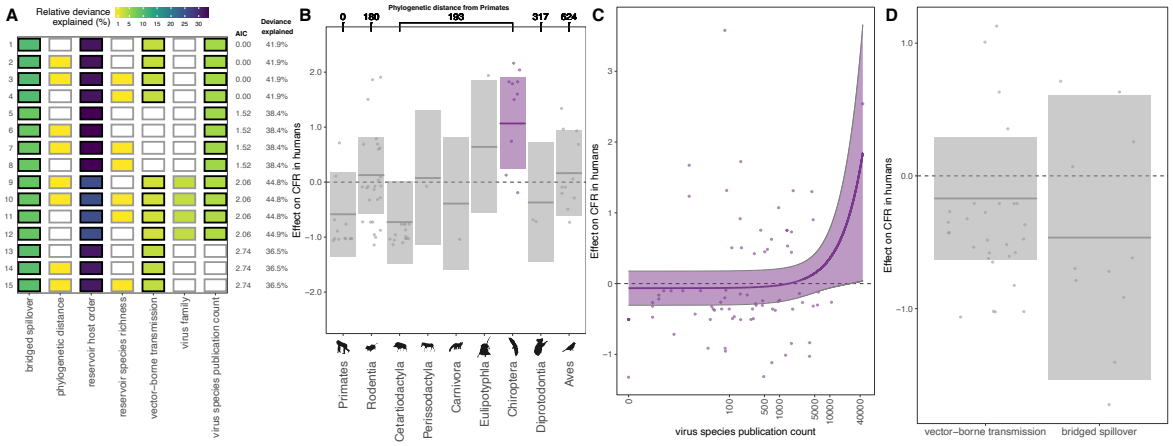

Figure S4. Predictors of global CFR estimates, excluding bat lyssaviruses. (A) Top 15 models ranked by AIC. Rows represent individual models and columns represent predictor variables. Cells are shaded according to the proportion of deviance explained by each predictor. Cells representing predictor variables with a p-value significance level of <0.1 are outlined in black and otherwise outlined in gray. (B-D) Effects present in the top model: reservoir host group, log-transformed virus species publication count, vector-borne transmission, and bridged spillover. Lines represent the predicted effect of the x-axis variable when all other variables are held at their median value (if numeric) or their mode (if categorical). Shaded regions indicate 95% CIs by standard error and points represent partial residuals. An effect is shaded in gray if the 95% CI crosses zero across the entire range of the predictor variable; in contrast, an effect is shaded in purple and considered "significant" if the 95% CI does not cross zero. Full model results are outlined in Table S6a in *SI Data and Results.* (B) Reservoir host groups are ordered by increasing cophenetic phylogenetic distance from Primates (in millions of years), as indicated on the top axis.

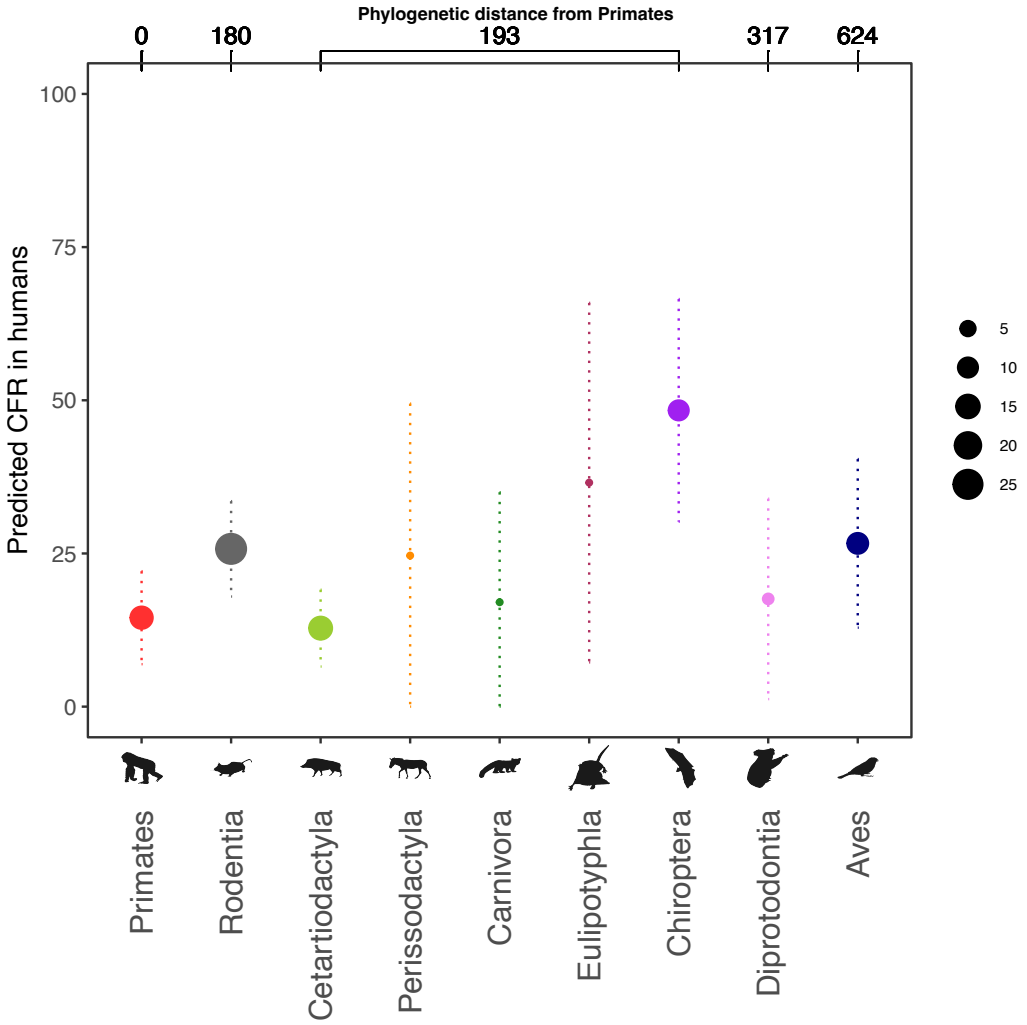

Figure S5. Predicted human CFR of zoonotic viruses sourced from each reservoir host group when using the top selected model of global CFR estimates, excluding bat lyssaviruses.

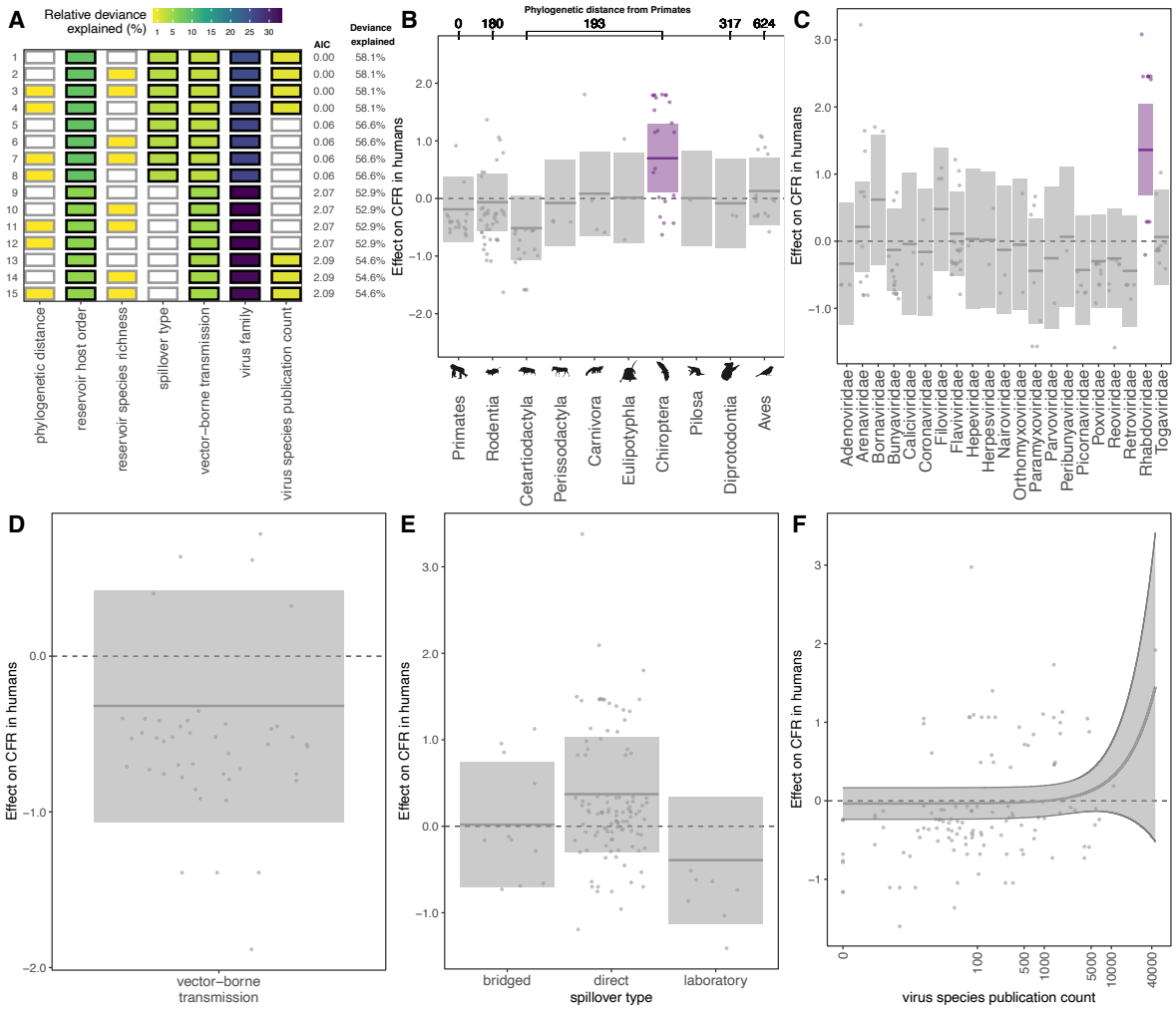

Figure S6. Predictors of global CFR estimates, including virus species that met a lenient definition of zoonotic. (A) Top 15 models ranked by AIC. Rows represent individual models and columns represent predictor variables. Cells are shaded according to the proportion of deviance explained by each predictor. Cells representing predictor variables with a p-value significance level of <0.1 are outlined in black and otherwise outlined in gray. (B-F) Effects present in the top model: reservoir host group, virus family, vector-borne transmission, spillover type, and virus species publication count. Lines represent the predicted effect of the x-axis variable when all other variables are held at their median value (if numeric) or their mode (if categorical). Shaded regions indicate 95% CIs by standard error and points represent partial residuals. An effect is shaded in gray if the 95% CI crosses zero across the entire range of the predictor variable; in contrast, an effect is shaded in purple and considered "significant" if the 95% CI does not cross zero. Full model results are outlined in Table S6b in *SI Data and Results.* (B) Reservoir host groups are ordered by increasing phylogenetic distance from Primates, as indicated on the top axis.

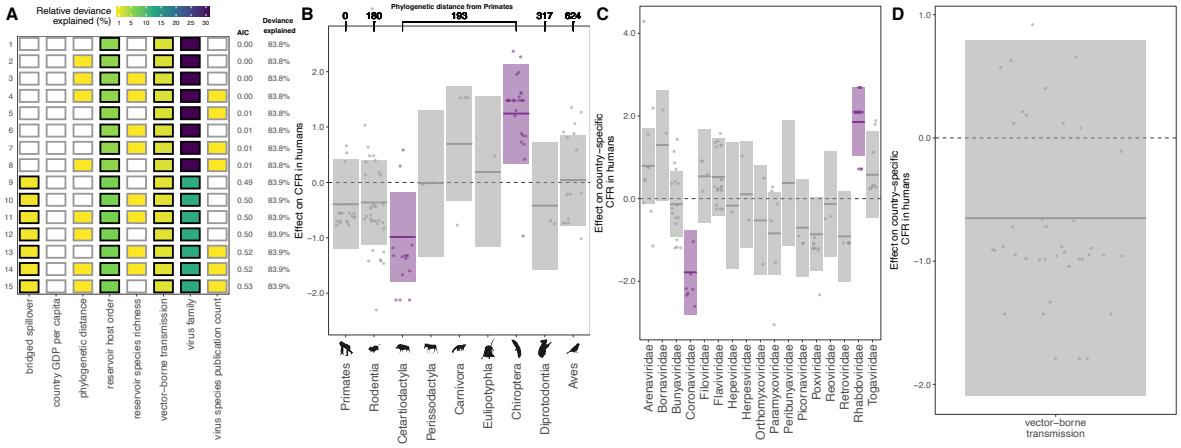

Figure S7. Predictors of variation among the 119 country-specific CFR estimates. (A) Top 15 models ranked by AIC. Rows represent individual models and columns represent predictor variables. Cells are shaded according to the proportion of deviance explained by each predictor. Cells representing predictor variables with a p-value significance level of <0.1 are outlined in black and otherwise outlined in gray. (B-D) Effects present in the top model: reservoir host group, virus family, and vector-borne transmission. Lines represent the predicted effect of the x-axis variable when all other variables are held at their median value (if numeric) or their mode (if categorical). Shaded regions indicate 95% CIs by standard error and points represent partial residuals. An effect is shaded in gray if the 95% CI crosses zero across the entire range of the predictor variable; in contrast, an effect is shaded in purple and considered "significant" if the 95% CI does not cross zero. Full model results are outlined in Table S6c in *SI Data and Results*. (B) Reservoir host groups are ordered by increasing cophenetic phylogenetic distance from Primates (in millions of years), as indicated on the top axis.

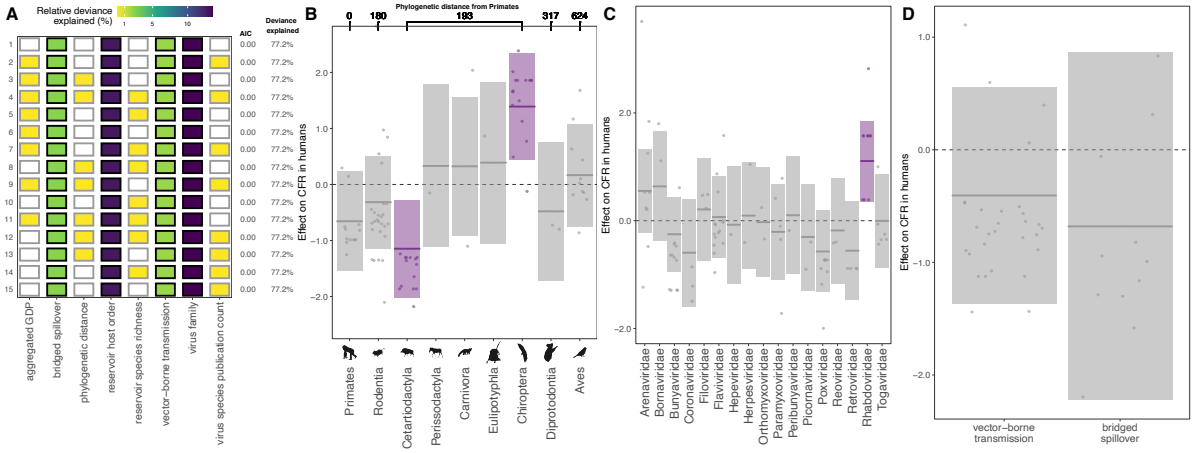

Figure S8. Predictors of CFRs calculated from country-level data aggregated at the level of the 86 unique zoonotic transmission chains. (A) Top 15 models ranked by AIC. Rows represent individual models and columns represent predictor variables. Cells are shaded according to the proportion of deviance explained by each predictor. Cells representing predictor variables with a p-value significance level of <0.1 are outlined in black and otherwise outlined in gray. (B-D) Effects present in the top model: reservoir host group, virus family, vector-borne transmission, and bridged spillover. Lines represent the predicted effect of the x-axis variable when all other variables are held at their median value (if numeric) or their mode (if categorical). Shaded

regions indicate 95% CIs by standard error and points represent partial residuals. An effect is shaded in gray if the 95% CI crosses zero across the entire range of the predictor variable; in contrast, an effect is shaded in purple and considered "significant" if the 95% CI does not cross zero. Full model results are outlined in Table S6d in *SI Data and Results.* (B) Reservoir host groups are ordered by increasing cophenetic phylogenetic distance from Primates (in millions of years), as indicated on the top axis.

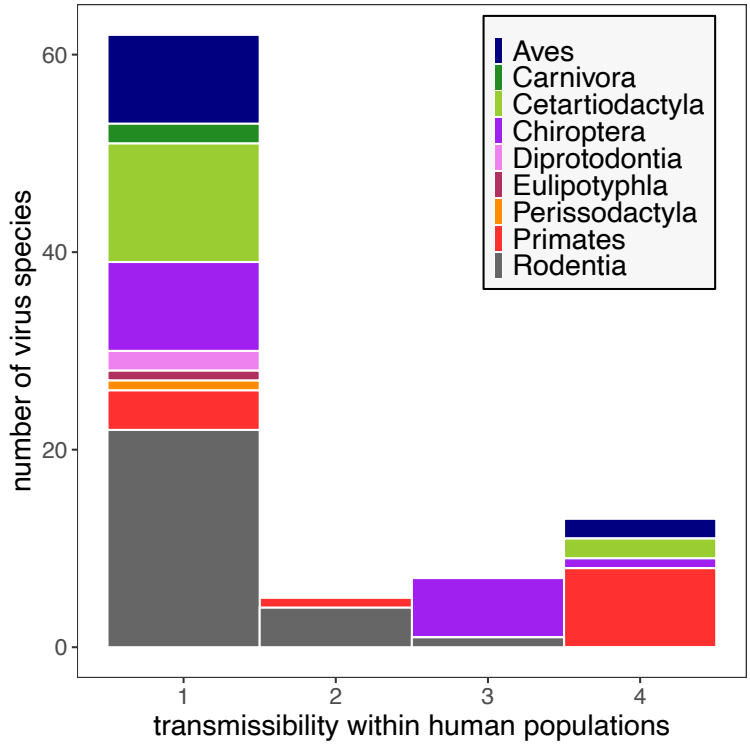

Figure S9. Histogram of human transmissibility rankings across all zoonotic virus species included in our analysis of capacity for forward transmission in humans, grouped by host order.

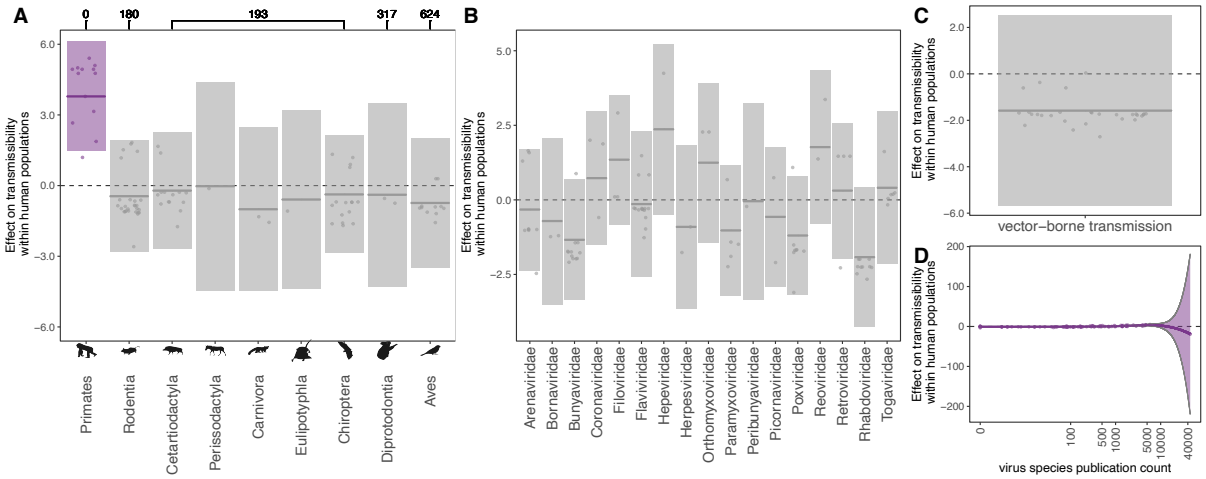

Figure S10. Effects present in the selected model to predict capacity for forward transmission within the human population with reservoir host order as a predictor instead of phylogenetic distance from humans. Effects include reservoir host group, virus family, vector-borne transmission, and virus species publication count. Lines represent the predicted effect of the x-

axis variable when all other variables are held at their median value (if numeric) or their mode (if categorical). Shaded regions indicate 95% CIs by standard error and points represent partial residuals. An effect is shaded in gray if the 95% CI crosses zero across the entire range of the predictor variable; in contrast, an effect is shaded in purple and considered "significant" if the 95% CI does not cross zero. Full model results are outlined in Table S6e in *SI Data and Results.* (A) Reservoir host groups are ordered by increasing cophenetic phylogenetic distance from Primates (in millions of years), as indicated on the top axis.

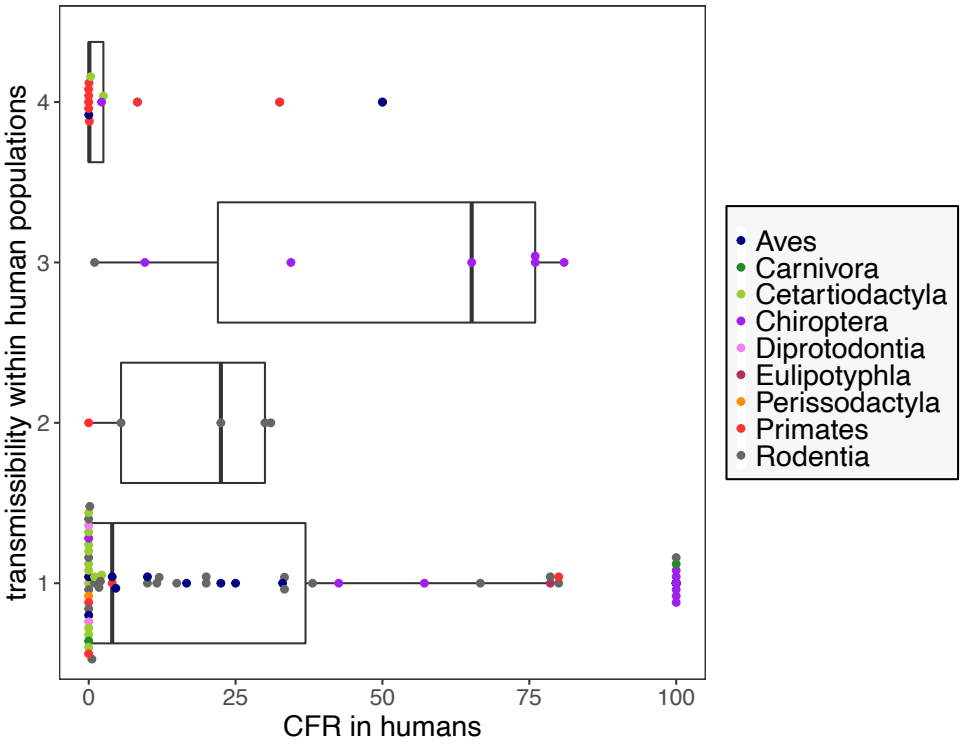

Figure S11. Relationship between CFR and transmissibility in humans.

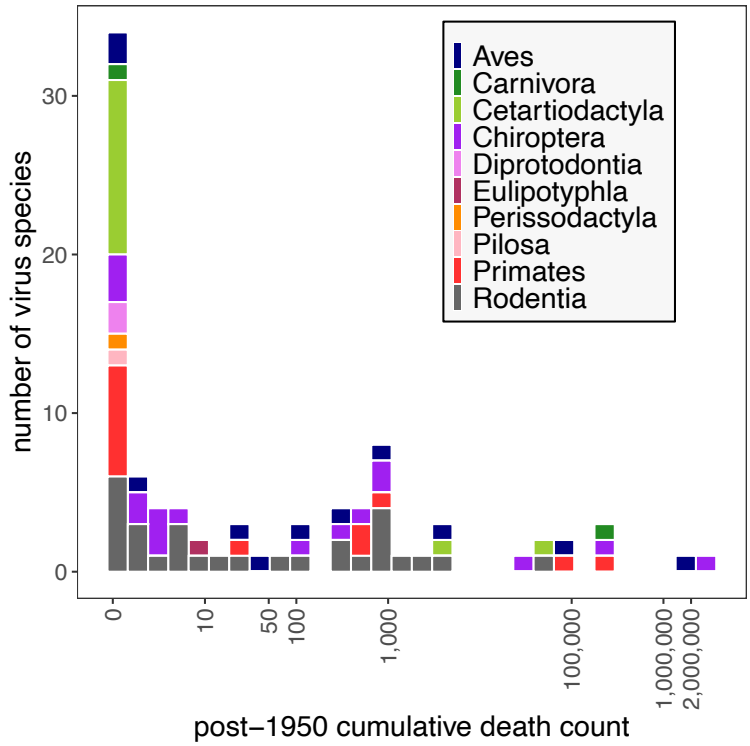

Figure S12. Histogram of post-1950 death counts, grouped by host order. Here, death counts were plotted to display the data distribution and are not adjusted for variation in the length of the reporting timeline. In the death burden model, we normalized counts by including an offset for the exact number of years over which deaths were recorded.

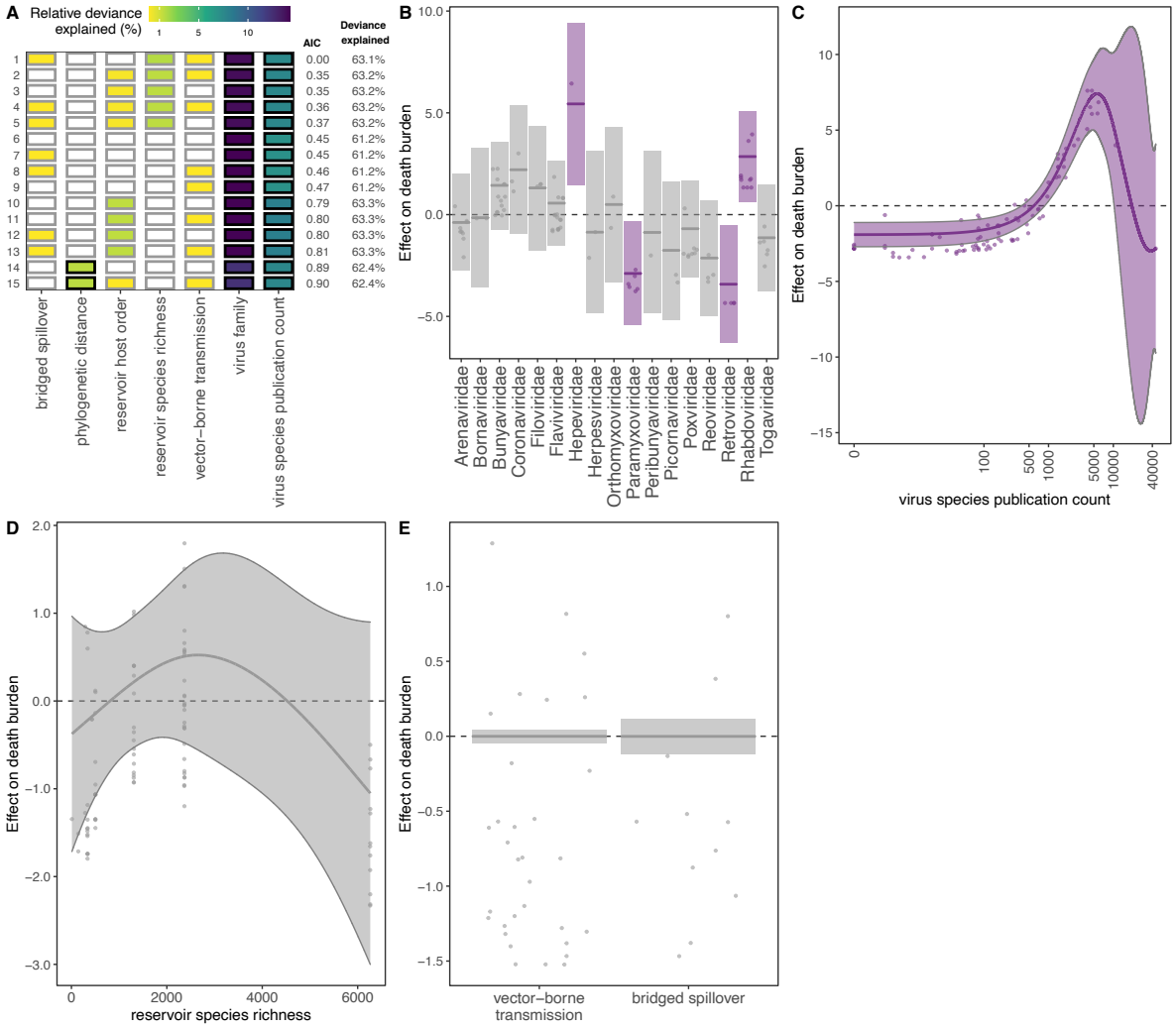

Figure S13. Predictors of post-1950 death burden. (A) Top 15 models ranked by AIC. Rows represent individual models and columns represent predictor variables. Cells are shaded according to the proportion of deviance explained by each predictor. Cells representing predictor variables with a p-value significance level of <0.1 are outlined in black and otherwise outlined in gray. (B-E) Effects present in the top model: virus family, virus species publication count, reservoir group species richness, vector-borne transmission, and bridged spillover. Lines represent the predicted effect of the x-axis variable when all other variables are held at their median value (if numeric) or their mode (if categorical). Shaded regions indicate 95% CIs by standard error and points represent partial residuals. An effect is shaded in gray if the 95% CI crosses zero across the entire range of the predictor variable; in contrast, an effect is shaded in purple and considered "significant" if the 95% CI does not cross zero. Full model results are outlined in Table S6f in *SI Data and Results.* (C) Reservoir host groups are ordered by increasing cophenetic phylogenetic distance from Primates (in millions of years), as indicated on the top axis.