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Ecological conditions predict the intensity of Hendra virus excretion over space and time from bat reservoir hosts

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Abstract

The ecological conditions experienced by wildlife reservoirs affect infection dynamics and thus the distribution of pathogen excreted into the environment. This spatial and temporal distribution of shed pathogen has been hypothesised to shape risks of zoonotic spillover. However, few systems have data on both long-term ecological conditions and pathogen excretion to advance mechanistic understanding and test environmental drivers of spillover risk. We here analyse 3 years of Hendra virus data from nine Australian flying fox roosts with covariates derived from long-term studies of bat ecology. We show that the magnitude of winter pulses of viral excretion, previously considered idiosyncratic, are most pronounced after recent food shortages and in bat populations displaced to novel habitats. We further show that cumulative pathogen excretion over time is shaped by bat ecology and positively predicts spillover frequency. Our work emphasises the role of reservoir host ecology in shaping pathogen excretion and provides a new approach to estimate spillover risk.

KEYWORDS
bats, generalised additive models, nutritional stress, pathogen spillover

INTRODUCTION

Cross-species transmission can be conceptualised as a series of hierarchical barriers that must be overcome for a pathogen to move from a reservoir host to a recipient host such as a human (Plowright et al., 2017). At the beginning of this cascade, the ecological conditions experienced by reservoir hosts shape susceptibility to infection and the likelihood of pathogen shedding (i.e. excretion) (Becker et al., 2020; Plowright et al., 2021). For example, droughts have been associated with increased nematode shedding in ungulates (Ezenwa, 2004), and *Salmonella* shedding by ibis is more likely in urban than rural habitats (Hernandez, 2016). These factors in turn determine pathogen pressure, the distribution or amount of pathogen available to recipient hosts over space and time, which theoretically affects the force of infection

and probability of spillover (Washburne et al., 2019). Identifying the ecological conditions that drive pathogen pressure could accordingly improve reservoir host surveillance efforts, help predict where and when spillover events are most likely, and guide the design of ecological interventions to reduce cross-species transmission (Becker, Washburne, et al., 2019; Sokolow et al., 2019).

Multiple challenges restrict our ability to identify the ecological drivers of pathogen pressure. On the one hand, many host populations must be repeatedly sampled to capture spatial and temporal variation in shedding (Plowright et al., 2019). This presents logistical difficulties and often requires optimised allocation of sampling effort (Becker, Crowley, et al., 2019; Nusser et al., 2008). On the other hand, the complex ecological dynamics used as predictors are best characterised with long-term research (Giron-Nava et al., 2017;

Lindenmayer et al., 2012). For example, more than two decades of annual monitoring were necessary to identify the ecological conditions that predict the density of infected nymphal ticks and human Lyme disease risk (Ostfeld et al., 2006). As such, the general sparsity of long-term, ecological data in many wildlife pathogen systems limits inference about the possible factors that shape the distribution of pathogen shed from reservoir hosts into the environment. Systems with data on both pathogen shedding and long-term ecological conditions are critical for advancing our mechanistic understanding of spillover and to identify likely drivers in less studied systems. Furthermore, theory predicts that the distribution of pathogen shedding into the environment is a key determinant of spillover risk (Plowright et al., 2017; Washburne et al., 2019), but empirical tests are limited owing to the general rarity of observed spillover events. Systems with a relatively high frequency of cross-species transmission are thus critical for testing the causal links between ecological conditions, the spatial and temporal distribution of shedding, and spillover risk.

Hendra virus (HeV) shedding from Australian flying foxes (*Pteropus* spp.) provides an ideal system to identify the ecological conditions that predict pathogen pressure and how these ultimately relate to spillover risk (Plowright et al., 2015). Hendra virus emerged in 1994, causing an outbreak of a lethal disease in horses and then in humans, which has been followed by more than 60 spillovers through 2019 across eastern Australia (Eby et al., 2020; Murray et al., 1995). Flying foxes are the natural reservoirs of HeV (Halpin et al., 2011), and transmission to horses likely occurs via contact with pasture, feed or water contaminated with urine, saliva or faeces (Field et al., 2001). Horses amplify and then transmit HeV to humans via close contact; no cases of bat–human transmission have yet been observed (Plowright et al., 2015). Spatiotemporal sampling of bat roosts has revealed shedding pulses during the Austral winter in subtropical regions, yet their occurrence varies both spatially and interannually (Field et al., 2015). This suggests that seasonal processes such as births and climate shifts cannot solely drive pathogen pressure (Páez et al., 2017). The mechanisms underlying shedding pulses remain poorly understood but have been hypothesised to stem from synchronous ecological conditions that affect bat behaviour and within-host processes (Kessler et al., 2018; Peel et al., 2019; Plowright et al., 2011, 2016).

Traditionally, flying foxes move nomadically to find ephemeral sources of nectar in native forests (Eby, 1991; Eby et al., 1999). Their roosts are thus inhabited continuously, seasonally, or irregularly, depending on available resources (Lunn et al., 2021). However, few dietary plants reliably flower in the Austral winter (Eby & Law, 2008; Eby et al., 1999). These plants occur in forests that have been mostly cleared for agriculture and urbanisation, making native winter feeding habitat scarcer and more dispersed (Wilson et al., 2002). Eucalypt flowering

is especially vulnerable to disruption from temperature and rainfall variability (Eby & Law, 2008; Giles et al., 2016), and occasionally winter or spring nectar fails, leading to acute food shortages and periods of nutritional stress (Eby et al., 1999; Giles et al., 2018; Plowright et al., 2008). Bats have responded to these changes by splitting into smaller roosts that are closer to reliable, often introduced, food in agricultural areas and urban gardens (Eby et al., 1999; Páez et al., 2018). Bats in these newly established and continually occupied roosts supplement their diet with non-native plants (Markus & Hall, 2004; Mcdonald-Madden et al., 2005), which are likely of poorer nutritional quality than native nectar (Kessler et al., 2018; Pulscher et al., 2021). This change in host behaviour has expanded the winter distribution of flying foxes in subtropical Australia into areas that do not provide native winter food resources (Lunn et al., 2021; Tait et al., 2014).

Reliance on agricultural and urban food resources when native food resources are unavailable could combine with other energetic stressors, such as seasonal reproduction and extreme temperatures (McMichael et al., 2017; Van Der Ree et al., 2006; Williams et al., 2006). These stressors could alter the within-host dynamics of infection (e.g. via immunosuppression) in ways that increase susceptibility to HeV or facilitate reactivation from persistent infections (Peel et al., 2019; Plowright et al., 2011, 2016; Wang et al., 2013). Periods of nutritional stress have been associated with poor condition and elevated HeV seroprevalence in little red flying foxes (*Pteropus scapulatus*) (Plowright et al., 2008); moreover, poor condition predicted HeV infection in black flying foxes (*Pteropus alecto*) but was negatively correlated with HeV seroprevalence in *P. alecto* and grey-headed flying foxes (*Pteropus poliocephalus*) (Boardman et al., 2020; Edson et al., 2019). The relationships between food shortages and HeV shedding thus remain poorly understood, especially in the context of changing ecological conditions experienced by bat hosts. Recent work suggests such ecological changes have increased the HeV spillover frequency (Eby et al., in review) (Eby et al., 2020; Peel et al., 2017), but the mechanisms linking food shortages and shifts in bat behaviour with virus shedding remain unresolved.

Here, we analyse data from a spatially replicated, multi-year study of HeV in flying foxes in light of long-term data on bat ecology to test whether food shortages and recent shifts in flying fox behaviour amplify pathogen shedding. We first ask if these ecological conditions can explain seemingly idiosyncratic seasonal shedding pulses while also accounting for underlying spatiotemporal variation in infection dynamics and roost composition, including species with high competence (e.g. *P. alecto*) (Edson et al., 2015; Goldspink et al., 2015; Smith et al., 2014). We next quantify the area under each annual shedding curve as an approximation of pathogen pressure and assess how bat ecology relates to cumulative

seasonal shedding intensity across the landscape. Finally, we test the prediction that roosts and years with greater cumulative shedding have a greater frequency of realised HeV spillover events. Our work thus provides a mechanistic approach to understanding bat-borne virus spillover risk as well as a new method for estimating these risks from multiple ecological predictors. Bats throughout the eastern hemisphere are experiencing similar stressors owing to land conversion (Kessler et al., 2018; McKee et al., 2021) and climatic extremes (Pruvot et al., 2019; Sherwin et al., 2013), underscoring the general need to better understand the likely consequences for pathogen shedding and spillover risk.

MATERIALS AND METHODS

Spatiotemporal data on HeV shedding

We analysed a previously published longitudinal data set of HeV shedding from flying foxes spanning July 2011 to November 2014 across New South Wales and Queensland (Field et al., 2015). Sampling consisted of urine collection from quadrants of 3.6 m × 2.6 m plastic sheets placed under roosts at mostly monthly intervals (Edson et al., 2015; Field et al., 2011). From each quadrant, 1.25 ml urine was pooled and screened by a high-sensitivity quantitative RT-PCR targeting the HeV M gene to determine the presence or absence of viral shedding in a roost, for which samples yielding a Ct value less than 40 were positive (Field et al., 2015; Smith et al., 2001). Typically, ten plastic sheets were deployed per sampling event, with each sheet collecting urine from an estimated 5–20 individuals depending on roost density. In the subset of roosts and years included in our analyses (see below), a median 10 sheets were used per sampling event (±0.14; 68% used 10 sheets), each of which had pooled urine collected from a median of three quadrants (±0.04). We aggregated data to weeks and determined the proportion of HeV-positive urine pools (i.e. urine pool prevalence). We caution that urine pool prevalence indicates the frequency of HeV shedding into the environment in any given roost but not viral load. Similarly, urine pool prevalence does not necessarily directly correspond to infection prevalence in bats, given that multiple individuals could contribute to any one quadrat urine pool. Simulations have suggested this pooled quadrant design could overestimate infection prevalence by 3.2 times, although this bias is higher for designs that pool urine from entire sheets (Giles et al., 2021).

In addition to HeV urine pool prevalence, we also calculated the median number of all flying foxes and for each Australian *Pteropus* species per roost per time interval. We restricted these data to an area of subtropical eastern Australia (i.e. mid-to-northeast New South Wales, southeast Queensland) that represents our study region for long-term data on bat ecology (Figure S1;

see the following section) and includes the locations of almost all subtropical HeV spillovers (Plowright et al., 2015). We further restricted analyses to 2012 through 2014, as 2011 sampling only occurred at a single roost (Field et al., 2015). To properly identify seasonal patterns within roosts and to allow seasonal replication per roost across years, we also limited our analyses to roosts sampled in at least three time points per year for least two of the three years. We, thus, analysed urine pool prevalence data from nine roosts sampled in 2012 through 2014 (*n* = 196 total weeks across all roosts; Figure 1).

Roost-level ecological conditions

We paired HeV urine pool prevalence data within our study region with long-term data on flying fox ecology. As described in full in Eby et al. (in review), we used data held by Australian state governments, flying fox surveys conducted between 1998–2005 and 2012–2019, records held by local land managers and experienced observers, and direct observation by the authors to characterise the ecological conditions experienced by bat populations over time (Eby & Law, 2008; Eby et al., 1999; Lunn et al., 2021). Roosts were classified as belonging to their historic winter range (and, thus, where bats rely on native winter dietary plants) or to newly established overwintering regions (and, thus, where native food is less available). We also used these data to identify regional food shortages, acute periods associated with reduced flowering of native dietary plants or no flowering (Table 1).

Analyses of HeV shedding

To test if roost type (i.e. historic or new overwintering regions) and regional food shortages (see Table 1) affect seasonal HeV shedding, we fit generalised additive mixed models (GAMMs) with a binomial response to urine data (i.e. counts of positive and negative pools) with the *mgcv* package in R (R Core Team, 2013; Wood, 2006). GAMMs can flexibly approximate the temporal dynamics of infection, even when underlying transmission mechanisms are unknown (Epstein et al., 2020; Lisovski et al., 2017). Our fixed effects included a cyclic cubic spline for week, ordered factors of roost type and regional food shortage, and all two-way interactions. We used regional food shortages from the prior year (i.e. October 2012 and October–November 2013; Figure 1) owing to prior associations between spring and summer food shortages and subsequent winter spillovers of HeV in 2010–2011 and in 2016–2017 (Peel et al., 2017).

We also adjusted for presence of *P. alecto* using two smooth covariates: (i) relative abundance and (ii) total abundance (square-root transformed) per roost from weekly median flying fox counts (Figures S2 and S3). The seasonal occupancy patterns of this species varied
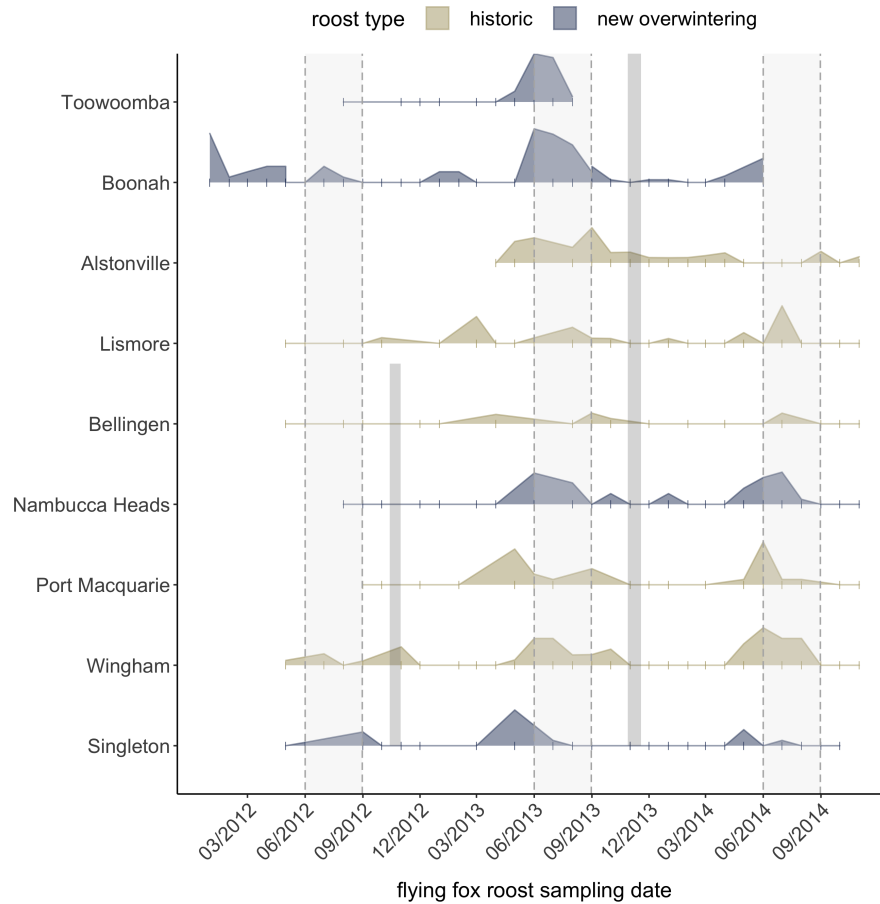
FIGURE 1 Spatiotemporal variation in HeV shedding for the nine Australian flying fox roosts sampled from 2012 through 2014. Curve height indicates the weekly proportion of HeV-positive urine pools, with roosts shown in order of latitude and coloured by roost type. Ticks show sampling time points. Dark grey shading indicates regional acute food shortage events, and dashed lines with light grey shading indicate the Austral winter (i.e. June through August)

among roosts and among years. Because flying foxes roost in aggregations of up to hundreds of thousands of individuals, and because the survey methods used in the initial data collection were coarse, the counts and species proportions are crude estimates. We did not account for occupancy patterns of co-roosting *P. poliocephalus* and *P. scapulatus*, as *P. alecto* is the dominant species associated with HeV shedding across our study region (Goldspink et al., 2015; Smith et al., 2014). Our model also included a random factor smooth of week per roost per year, which functions akin to a nonlinear random slope (Pedersen et al., 2019). We set this random effect such that each annual time series can differ in its weekly HeV shedding dynamics but is penalised if it deviates too strongly from the global trend.

Given the complexity of our GAMM relative to our sample size, we used an information theoretic approach to select among a small candidate set of competing and nested fixed effects. We considered our full model alongside a simplified structure without the two-way interaction between roost type and prior year food shortage. For these two structures, we considered separate models that adjusted for either the relative or total abundance

of *P. alecto*. We fit these four GAMMs with maximum likelihood (ML) and compared among models with corrected Akaike information criterion (AICc) and Akaike weights (w_i). We considered models within two ΔAICc to be competitive (Burnham & Anderson, 2002). We then refit models with restricted ML (REML) to reduce overfitting (setting $\gamma = 1.4$ to further minimise such risks), derive R^2, and estimate parameters (Kim & Gu, 2004; Wood, 2006).

To quantify cumulative annual shedding intensity as an approximation of pathogen pressure, we fit univariate GAMs with binomial response and cyclic cubic splines for week to our HeV urine pool prevalence data per year for each roost ($n = 25$; Figure S4). In rare cases with few (e.g. $n = 3$) sampling events and no virus detection (i.e. in two annual time series; Figure S4), we used thin plate splines to improve convergence. As with our GAMMs, we used REML and $\gamma = 1.4$. We then calculated the area under each annual shedding curve (AUC) by integrating fitted values and confidence intervals using the *Bolstad2* package (Bolstad, 2010; Lisovski et al., 2017). AUC summarises the magnitude and duration of shedding in a single metric and thus

TABLE 1 Spatiotemporal ecological conditions of flying fox roosts included in the analysis

Roost	Longitude	Latitude	Year	Food shortage in prior year	Roost type
Toowoomba	151.954	−27.564	2012	0	New overwintering
			2013	0	
Boonah	152.681	−27.992	2012	0	New overwintering
			2013	0	
			2014	1	
Alstonville	153.439	−28.481	2013	0	Historic
			2014	1	
Lismore	153.277	−28.807	2012	0	Historic
			2013	0	
			2014	1	
Bellingen	152.897	−30.452	2012	0	Historic
			2013	1	
			2014	1	
Nambucca Heads	153.003	−30.642	2012	0	New overwintering
			2013	1	
			2014	1	
Port Macquarie	152.908	−31.431	2012	0	Historic
			2013	1	
			2014	1	
Wingham	152.376	−31.871	2012	0	Historic
			2013	1	
			2014	1	
Singleton	151.175	−32.562	2012	0	New overwintering
			2013	1	
			2014	1	

suggests more cumulative positive pools per roost per year. We used the *metafor* package to assess heterogeneity in AUC through a random-effects model weighted by sampling variance, which we derived from the number of sampling events per time series and the estimated standard deviation (i.e. using sample size and the AUC confidence interval width) (Viechtbauer, 2010). We then used REML to quantify I^2, which measures the contribution of true heterogeneity rather than noise to the total variance in AUC (Senior et al., 2016).

We then assessed the ecological drivers of annual AUC with another set of GAMs using a Tweedie-distributed response and weighting by sampling variance. We included roost type and prior food shortage as predictors. As in our GAMMs, we used AICc to compare separate models adjusting for the annual relative or total abundance of *P. alecto* per roost with thin plate splines, considering the mean, median and winter maximum for both abundance metrics. Because four annual time series had short sampling durations that did not include the Austral winter (Figure S4), we refit our top GAMs after excluding these annual AUCs derived from 20 or fewer weeks.

HeV spillover analyses

To lastly test whether annual AUC (as an approximation of pathogen pressure) positively predicts spillover frequency, we collated data on the location and date of all known HeV cases in horses in our study region between 2012 and 2014 (Plowright et al., 2015). As described in full in Eby et al. (in review), these data were obtained from government notices (i.e. New South Wales Department of Primary Industries, Queensland Department of Agriculture and Fisheries; Business Queensland), ProMED, local media reports and personal communications. We assigned all HeV case locations to the nearest town or regional centre for confidentiality.

We derived spatial buffers using the *rgeos* package to collate the total number of HeV spillover events near each of our nine roosts per sampled year. Australian *Pteropus* are highly mobile (Giles et al., 2018; Roberts et al., 2012), and prior analyses have shown weak spatial synchrony in HeV shedding among flying fox roosts up to approximately 500 km (Páez et al., 2017). We therefore used spatial buffers of 50, 100, 200, 300, 400 and 500 km. We modelled counts of spillovers within each

Term	β	z	EDF	χ^2	p
Intercept	−3.73	−29.21			<0.001
New overwintering	−0.14	−0.79			0.43
Prior food shortage	−0.29	−1.66			0.10
s(week)			0.62	1.01	0.01
s(week): new overwintering			3.95	26.94	<0.001
s(week): prior food shortage			2.94	19.01	<0.001
s(relative abundance of *P. alecto*)			2.32	18.14	<0.001

TABLE 2 Results of the top GAMM for predicting seasonal HeV urine pool prevalence from Australian flying foxes ($n = 196$), fit using REML. Fixed effects are presented as ordered factors with coefficients (categorical) or the estimated degrees of freedom (EDF) and test statistics
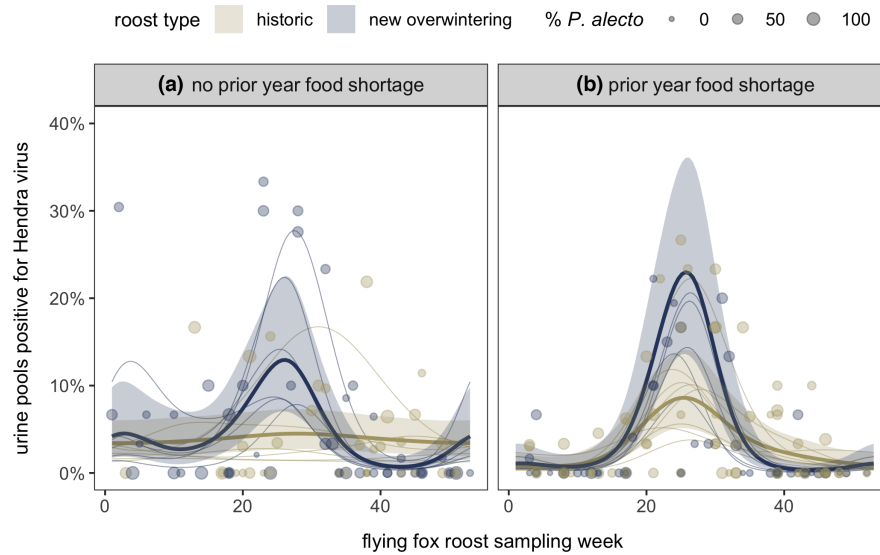
FIGURE 2 Fitted HeV urine pool prevalence and 95% confidence intervals from the most parsimonious GAMM with week, seasonal interactions with roost type and previous food shortages, and an adjustment for relative abundance of *Pteropus alecto*. Weekly data are overlaid, coloured by roost type, and sized by corresponding *P. alecto* relative abundance. Thin lines show the fitted curves from the random factor smooth including each roost per year

buffer through a set of GAMs that used Poisson distributions and penalised splines for AUC, again using REML and $\gamma = 1.4$ to limit overfitting (Kilinc & Asfha, 2019). To more specifically estimate the shape of the association between AUC and spillover, we also applied nonlinear least squares to fit linear, saturating, and quadratic relationships using the NLS package for each spatial scale and compared models with AICc (Seber & Wild, 2005). For both the GAMs and nonlinear least squares, we weighted by our sampling variance of AUC.

RESULTS

Ecological predictors of seasonal HeV shedding pulses

Our GAMMs revealed that previously observed seasonality in HeV shedding (e.g. Field et al., 2015) is markedly explained by recent changes in bat behaviour and by food shortages. The most parsimonious model explained 66%

of the deviance and only included interactions between week and both roost type and recent food shortages as well as adjustment for *P. alecto* relative abundance rather than total abundance ($w_i = 0.83$; Table S1 and Table 2). Ordered factor smooths indicated that roosts in newly established overwintering regions had distinct seasonal shedding patterns compared to roosts in the historic wintering range ($\chi^2 = 26.94$, $p < 0.001$); roosts that experienced a recent food shortage also displayed distinct shedding seasonality compared to roosts without recent food shortages ($\chi^2 = 19.01$, $p < 0.001$). In the absence of recent food scarcity, historic roosts had negligible seasonality in shedding, while new overwintering roosts exhibited shedding pulses in winter (Figure 2a). Yet when food shortages occurred in the spring prior to sampling (Figure 2b), both historic and new overwintering roosts displayed stronger shedding seasonality and amplitude the following winter. Furthermore, the winter shedding amplitude for new roosts was greater than historic roosts. Such patterns held after accounting for relative abundance of *P. alecto*, which was a significant, although not monotonic, predictor of HeV prevalence ($\chi^2 = 18.14$,
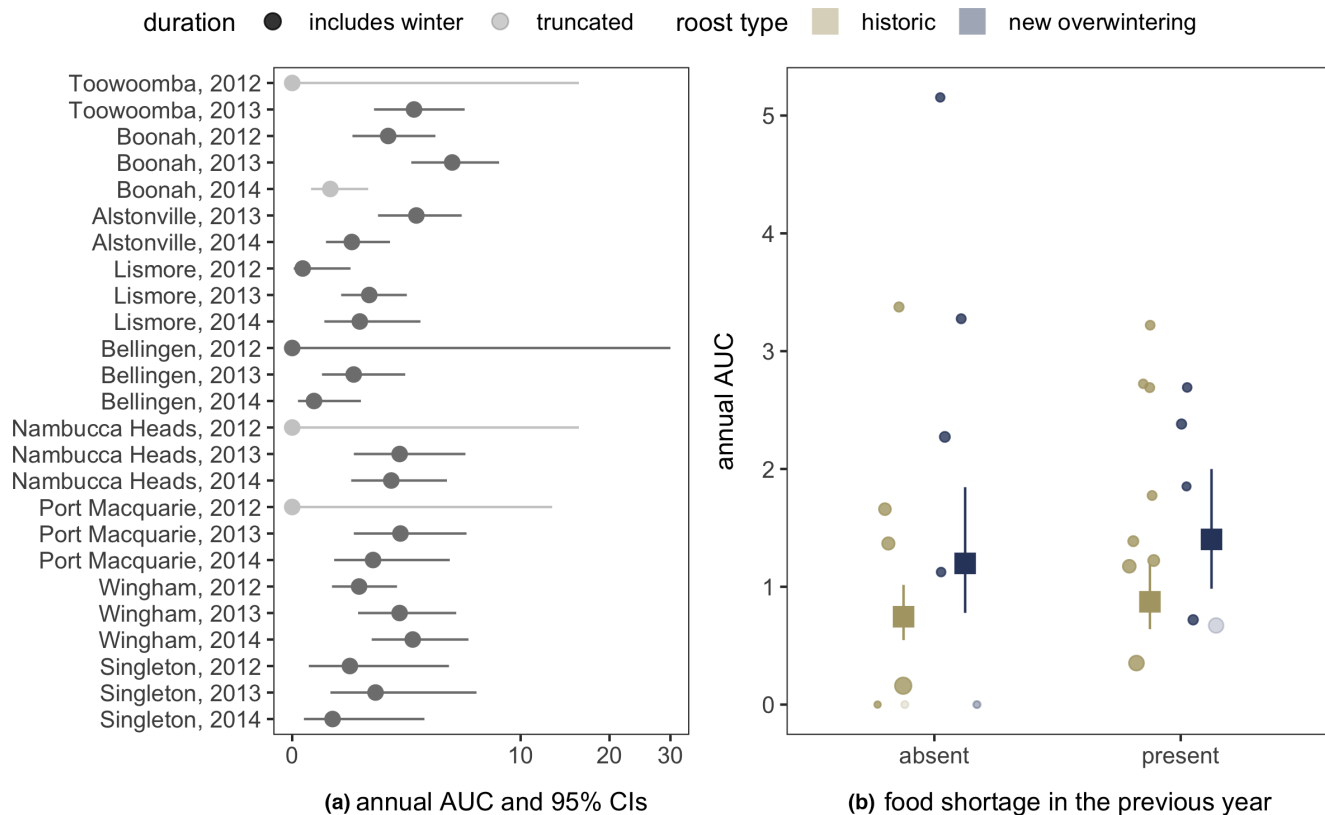
FIGURE 3 Variation in HeV AUC from flying foxes. (A) The forest plot displays annual estimates and 95% confidence intervals ordered by latitude and year; points are scaled by the inverse sampling variance. The horizontal axis uses a modulus transformation to accommodate wide upper bounds of some confidence intervals. (B) Fitted values and 95% confidence intervals for the top GAM, with raw data (scaled by inverse sampling variance) and modelled means coloured by roost type. Transparency denotes AUC derived from truncated time series (≤20 weeks)

$p < 0.001$; Figure S5a). In contrast, an equivalent GAMM that instead included total abundance of *P. alecto* received poor support ($w_i = 0.01$; Table S1), and this measure of bat species presence was a weak albeit positive predictor of shedding ($\chi^2 = 1.01$, $p = 0.07$; Figure S5B).

Heterogeneity in cumulative shedding

Cumulative HeV shedding (i.e. the area under each annual shedding curve) varied substantially across roosts and years (Figure 3a). Our meta-analysis identified significant but moderate heterogeneity among annual AUC ($I^2 = 0.61$, $Q_{24} = 55.42$, $p < 0.001$). When testing the ecological drivers of this variation, our top GAM adjusted for mean relative abundance of *P. alecto* and explained 41% of the deviance ($w_i = 0.98$); models that adjusted for all other measures of *P. alecto* presence had ΔAICc > 8 and negligible Akaike weight (Table S2). Whereas recent food shortage had weak effects on AUC ($\beta = 0.16$, $t = 0.84$, $p = 0.41$), newly established overwintering roosts had significantly greater cumulative shedding than historic roosts ($\beta = 0.47$, $t = 2.36$, $p = 0.03$; Figure 3b). The mean annual relative abundance of *P. alecto* also significantly predicted AUC ($F_{1.51,3} = 13.13$, $p < 0.001$). AUC increased from low-to-intermediate annual proportions of this

species, but the effect weakened at higher AUC (Figure S6). Excluding the four AUC estimates from truncated time series strengthened the associations observed with both roost type and recent food shortage, although the effect of the former was approximately twice that of the later; the effect of *P. alecto* abundance was also strengthened but displayed a more saturating pattern (Figure S7; Table S3).

HeV AUC and spillover frequency

Within the period of flying fox sampling (2012–2014), 12 spillover events occurred within 500 km of our nine study roosts (Figure 4a; Table S4). When aggregating spillovers within 50 km of each roost, our GAM explained 20% of the deviance, with AUC having an apparent concave relationship with spillover ($\chi^2 = 7.75$, $p = 0.02$; Figure 4b). GAMs also approximated this nonlinear relationship at moderately larger scales (100 km: $\chi^2 = 19.03$, $p < 0.001$, 24% deviance explained; 200 km: $\chi^2 = 19.81$, $p < 0.001$, 28% deviance explained; 300 km: $\chi^2 = 19.77$, $p < 0.001$, 21% deviance explained). All four models estimated small-to-moderate intercepts (Table S5), suggesting relatively minor contributions to spillover from other roosts at these scales. When expanding
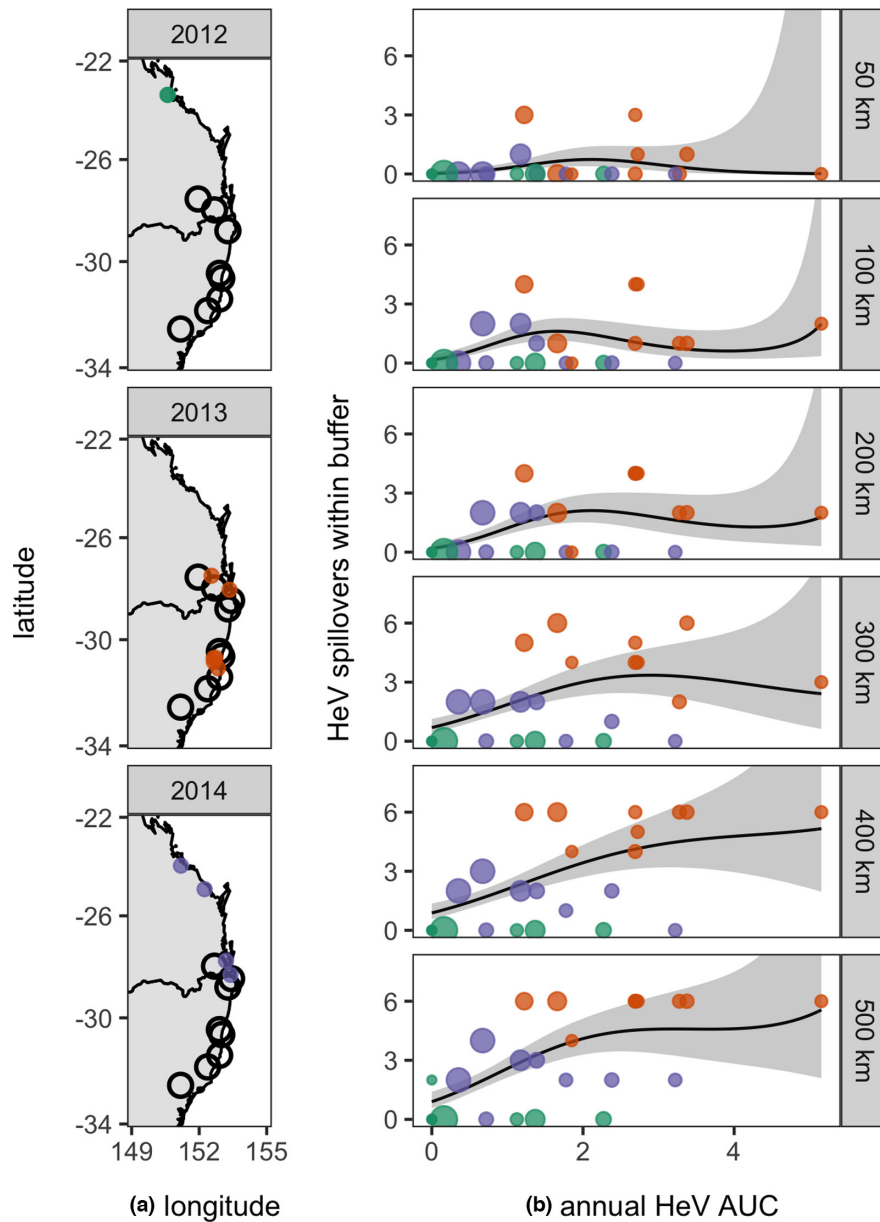
FIGURE 4 Spatiotemporal variation in regional HeV spillover events during the flying fox surveillance period (2012–2014) and its relationship with HeV AUC. (a) Maps display the annual distributions of spillovers (coloured by year) in relation to the nine analysed roosts. (b) Modelled relationships between AUC and spillover counts are shown with fitted values and 95% confidence intervals from GAMs for 50, 100, 200, 300, 400 and 500 km buffers of each roost. Raw data are overlaid and scaled by the inverse of the sampling variance for AUC

to even larger buffer size, however, GAM intercepts were larger (Table S5). Models at these larger scales also approximated more saturating relationships between AUC and spillover (400 km: $\chi^2 = 33.15$, $p < 0.001$, 23% deviance explained; 500 km: $\chi^2 = 27.57$, $p < 0.001$, 26% deviance explained; Table S5). When complementing the GAMs with a formal comparison of functional relationships, we found that associations between AUC and spillover were primarily best described by a saturating relationships, but the relative support for saturating over linear or quadratic relationships was strongest at the largest considered spatial scales (Table S6; Figure S8).

DISCUSSION

Mechanistic understanding of cross-species transmission risk remains hindered by limited information on the relationships between reservoir host ecology, distribution or amount of pathogen available to recipient hosts over space and time and spillover events (Becker, Washburne, et al., 2019; Plowright et al., 2015, 2017; Washburne et al., 2019). By combining data on long-term ecology with the spatial and temporal distribution of infection, we here demonstrate that the ecological conditions experienced by reservoir hosts shape multiple aspects of pathogen shedding. Prior work has shown that pulses of HeV

excretion from flying foxes often occur in the Austral winter and that drier conditions in prior seasons are associated with peak annual shedding (Field et al., 2015; Páez et al., 2017). However, contextualising these seasonal and climatic patterns with bat ecology here reveals that these periods of intensive virus shedding predominantly occur after food shortages and in new overwintering roosts in urban and agricultural habitats. By quantifying an aggregate proxy of pathogen pressure, we also show that the cumulative intensity of HeV shedding provides distinct insights into these ecological drivers, as AUC was governed more by whether the roost is located in regions with native winter resources than by periods of recent food scarcity. Lastly, annual AUC estimates were positively associated with cases of HeV in horses at a regional scale, which provides support for the spatiotemporal intensity of virus shedding as a mechanistic determinant of spillover risk.

Foremost, our findings emphasise that the ecological conditions experienced by reservoir hosts predict pronounced pulses of virus shedding and overall spatiotemporal distribution of pathogen shed into the environment. Across eastern Australia, habitats for the winter-flowering plants that drive flying fox nomadism have been reduced through land clearance, in turn driving nutritionally stressed bats into novel urban and agricultural environments outside their typical overwintering range (Eby et al., 1999; Giles et al., 2018; Lunn et al., 2021; Tait et al., 2014). Prior work has proposed, but not tested, that HeV shedding from flying foxes is driven by this process (Kessler et al., 2018; Lunn et al., 2021; Peel et al., 2019; Plowright et al., 2016). Acute food shortages, in addition to other energetic and seasonal stressors (e.g. pregnancy, thermoregulation), as well as reliance on poor-quality, non-native food in agricultural and urban habitats, may alter within-host dynamics of viruses in bats (Kessler et al., 2018; Plowright et al., 2011). Prior work has suggested that the physiological demands of winter thermoregulation in parts of the *P. alecto* range could drive observed negative correlations between minimum temperature and urinary cortisol excretion (McMichael et al., 2017). Our results support the idea of cumulative stressors, as HeV urine pool prevalence was greatest not only in winter but also in roosts established in novel overwintering habitats and after a food shortage in the prior spring.

Increased viral shedding associated with suboptimal habitats or food shortages could be driven by immunosuppression, such that cumulative stressors may impair HeV tolerance and facilitate replication or allow latent infections to reactivate (Plowright et al., 2016; Virgin et al., 2009). Reactivated infection of henipaviruses in bats has been proposed to explain shedding pulses that coincide with stressors (Peel et al., 2019; Plowright et al., 2016) and for seroconversions in captivity (Glennon et al., 2019). The mechanism driving the time lag between acute food shortages, which typically occur in spring, and increased HeV shedding in the following winter (i.e. in the subsequent 6–9 months; Figure 1) remain unknown; however, the cumulative impact of multiple stressors (e.g. long-term nutritional stress, cold temperature, pregnancy) offers a potential explanation. Future studies, including immune assessments of wild bats and factorial experiments, will be critical to mechanistically understand the causal effect of spring food shortages, bat residency outside their winter range, dietary reliance on non-native plants and other energetic stressors (e.g. pregnancy) on subsequent winter pulses of HeV shedding. Such work would also provide foundational insights into how bats control zoonotic viruses more broadly, given likely effects of stressors on immunity and tolerance (Banerjee et al., 2020; Subudhi et al., 2019).

Pathogen pressure has been suggested to be a critical determinant of spillover risk in recipient hosts (Plowright et al., 2017; Washburne et al., 2019), but quantifying the spatial and temporal distribution of shedding in ways that facilitate downstream analyses has remained challenging. Large studies with spatially replicated longitudinal sampling (Becker, Crowley, et al., 2019), extensive metadata collection and carefully designed sampling frameworks (Plowright et al., 2019) are needed but are logistically costly. Furthermore, most under-roost sampling methods, such as those used here, can obscure inference about viral load and do not map directly to infection prevalence in hosts themselves (Giles et al., 2021). Here, we propose the area under annual shedding curves (i.e. AUC) as a meaningful metric to summarise pathogen output into the environment. AUC has previously been used to summarise the cumulative pathogen output from within-host dynamics (e.g. *Mycoplasma agassizii* intensity across individual tortoises; Aiello et al., 2019) and number of infected individuals (e.g. fungal infections across *Daphnia* populations; Penczykowski et al., 2014; Shocket et al., 2018), which capture host infectiousness and local epidemic size, respectively, rather than the explicit infectious dose available to recipient hosts. For the latter, epidemic size has in turn been linked to local ecological conditions, such as lake temperature and host community composition (Penczykowski et al., 2014; Shocket et al., 2018); however, such metrics have not been applied to replicate reservoir host populations to identify predictors of pathogen pressure. In one case study of a zoonotic pathogen reservoir, data on avian influenza virus prevalence in ducks were used to derive AUC across three regions of North America, but inference on its underlying ecological drivers was limited by few replicates (Lisovski et al., 2017). However, we caution that our approximation of pathogen pressure relied on only urine pool prevalence, which may not equate to the explicit quantity of pathogen shed into the environment (Plowright et al., 2017). Future studies that combine optimised under-roost sampling designs that better map urine pool prevalence to infection prevalence (e.g. smaller sheets but in greater and random stratified

deployment around a roost; Giles et al., 2021) paired with quantitative viral intensity data could generate AUC estimates that provide a more direct measure of pathogen pressure for downstream prediction.

In our analyses, AUC was heterogeneous and varied with roost ecology in ways that differed from seasonal GAMMs. Whereas the timing and amplitude of shedding was driven both by roost type and prior food shortages, AUC was predominantly driven by roost type. This contrast could relate to the tension between a short but intense shedding pulse and a smouldering shedding dynamic with lower intensity. Short but intense shedding pulses observed after food shortages could produce similar AUC to the lower amplitude shedding that occurred year-round in roosts without acute food scarcity. Such results emphasise how shedding AUC can offer distinct insights into infection dynamics relative to shedding seasonality alone.

High pathogen pressure should theoretically have a pronounced effect on shaping the force of infection and the ultimate probability of spillover, given the hierarchical nature of cross-species transmission (Plowright et al., 2017; Washburne et al., 2019). However, empirical support relating pathogen shedding from reservoir hosts to disease cases in recipient hosts has been restricted by the rarity of spillover in many systems, such that evidence is mostly from temporal overlap between shedding events and outbreaks or broad-scale, regional analyses of reservoir infection and human disease data. For example, Marburg virus spillover to humans coincide with seasonal birth pulses of Egyptian fruit bats, when host shedding is most pronounced (Amman et al., 2012), and the density of bank voles seropositive for Puumala virus can also explain regional human incidence of nephropathia epidemica (Tersago et al., 2011).

The high frequency of HeV spillovers compared to many other spillover systems provides a tractable system for linking pathogen pressure and cross-species transmission (Plowright & Hudson, 2021). Our analyses show that annual AUC predicts observed spillovers across broad, regional scales. As a caveat, no spillovers were recorded within typical nightly foraging ranges (i.e. 25 km) of the roosts monitored in our study region during the period of HeV surveillance (Eby, 1991; Giles et al., 2018). We assume that the ecological conditions experienced by unsampled roosts in closer proximity to spillover events may be similar to those of our sampled roosts up to 500 km. We observed strong associations between AUC and spillover across all spatial scales relevant to flying fox mobility, although nonlinear least squares models identified the strongest relative support for saturating relationships at larger spatial scales. This implies that other factors might temper the contribution of high AUC to spillover risk. Importantly, our approximation of pathogen pressure is the cumulative result of a series of processes in the spillover hierarchy including the distribution

and density of reservoir hosts, the prevalence and intensity of infection in those hosts and pathogen shedding into the environment (Plowright et al., 2017). Downstream factors within the relevant spatial scales of bat movements, such as the survival of HeV in the environment and the density, exposure, and susceptibility of horses, may moderate effects of high AUC on spillover risk (Childs et al., 2019; Washburne et al., 2019). For example, high temperature decreases HeV environmental persistence (Fogarty et al., 2008). Weak density-dependent transmission in this and other bat systems, as given by lack of scaling between colony size and urine pool prevalence (Figure S5), could also affect this saturating pattern (Streicker et al., 2012). Short but high amplitude pulses of virus shedding, such as those seen in years following acute food shortages, may also provide a higher dose of HeV to horses (e.g. if infection requires a threshold dose), resulting in equal spillover risks from roosts and years with moderate and high AUC (Lunn et al., 2019; Plowright et al., 2017).

Because summarising the spatiotemporal intensity of infection both provided distinct insights into ecological drivers of pathogen shedding and predicted spillover at broad regional scales, we suggest quantifying AUC would be particularly tractable and useful in systems where recipient host exposure occurs following pathogen release into the environment. Such systems include but are not limited to bat filoviruses and coronaviruses, avian influenza viruses, and helminths, protozoa and faecal-oral bacteria of various wildlife (Amman et al., 2012; Becker et al., 2018; Breban et al., 2009; Ezenwa, 2004; Joffrin et al., 2022). Careful sampling designs for these environmentally shed pathogens, such as optimised under-roost sampling methods for bats, could facilitate estimating pathogen pressure and, in turn, spillover risks (Giles et al., 2021).

In conclusion, we show that the ecological conditions experienced by bat reservoir hosts shape the timing, magnitude and cumulative intensity of zoonotic virus shedding in ways that subsequently predict observed spillover events. Importantly, such inferences were only possible by integrating both spatiotemporal data on infection with long-term studies of host ecology. Long-term, spatially replicated studies of reservoir host ecology are essential for linking environmental change and zoonotic pathogen dynamics. Despite a long-standing recognition of spatial and temporal scale in ecological research (Levin, 1992), replication across both axes remains challenging, particularly over relevant time intervals (Estes et al., 2018). Spatiotemporal sampling is especially critical in the study of infectious disease (Becker, Crowley, et al., 2019; Plowright et al., 2019), because pathogen shedding and transmission are both inherently spatial and temporal processes (Grenfell et al., 2001; Lunn et al., 2021). Connecting such data with changing ecology of wildlife further requires studies of abiotic and biotic correlates and host behaviour

and demography at similar or biologically meaningful spatial and temporal scales (Becker, Washburne, et al., 2019; Lunn et al., 2021; Ostfeld et al., 2006). Here, the ecological conditions that predict HeV shedding from flying foxes were derived from data collected over approximately 25 years and from diverse sources (Eby & Law, 2008; Eby et al., 1999; Lunn et al., 2021) (Eby et al., in review). Although collecting this kind of ecological data will accordingly present logistical difficulties, the growth of national research networks, global community consortiums and remote sensing, among other large-scale efforts, should facilitate similar approaches to link spatiotemporal data on both host ecology and infection (Dietze et al., 2018). Combining such data with infection time series greater than the three years of shedding data included here would afford stronger inference. Such careful attention to the ecology of reservoir hosts through long-term monitoring could facilitate improved early detection and pre-emptive management of zoonotic spillover, notably in the context of ongoing land use and climatic changes that can affect bat ecology.

AUTHOR CONTRIBUTIONS
D.J.B., P.E., A.J.P. and R.K.P. designed the study; P.E., A.J.P., W.M. and R.K.P. collected, collated, and managed data; D.J.B. analysed data; and D.J.B. wrote the manuscript with feedback from co-authors.

PEER REVIEW
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DATA AVAILABILITY STATEMENT
All HeV shedding data and flying fox abundance data used in this analysis are freely available from the Queensland government (CC BY 4.0; https://www.data.qld.gov.au/dataset/hev-infection-flying-foxes-easternaustralia). Original ecological covariate data and HeV spillover data (provided to the nearest town for anonymity) presented in Eby et al. (2022a) are available from the Cornell University eCommons Digital Repository: https://doi.org/10.7298/3dbp-t721 (Eby et al., 2022b), https://doi.org/10.7298/kdht-sp38 (Eby et al., 2022c), and https://doi.org/10.7298/tb5p-dr98 (Eby et al., 2022d). Those data subset to the flying fox roosts analyzed here are provided in Table 1 and Table S4, respectively. These datasets, along with R code for estimating AUC for each annual time series per roost, are also available in the Dryad Digital Repository: https://doi.org/10.5061/dryad.0k6djhb28 (Becker et al., 2022).

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