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Combining mathematics and empirical data to predict emergence of RNA viruses that differ in reservoir use

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RNA viruses may be particularly capable of contributing to the increasing biomedical problem of infectious disease emergence. Empirical studies and epidemiological models are informative for the understanding of evolutionary processes that promote pathogen emergence, but rarely are these approaches combined in the same study. Here, we used an epidemiology model containing observations of pathogen productivity in reservoirs, as a means to predict which pathogens should be most prone to emerge in a primary host such as humans. We employed as a model system a collection of vesicular stomatitis virus populations that had previously diverged in host-use strategy: specialists, directly selected generalists and indirectly selected (fortuitous) generalists. Using data from experiments where these viral strategists were challenged to grow on unencountered novel hosts *in vitro*, logistic growth models determined that the directly selected generalist viruses tended to grow best on model reservoirs. Furthermore, when we used the growth data to estimate average reproductive rate across secondary reservoirs, we showed that the combined approach could be used to estimate relative success of the differing virus strategists when encountering a primary host. Our study suggests that synergistic approaches combining epidemiological modelling with empirical data from experimental evolution may be useful for developing efforts to predict which types of pathogens pose the greatest probability of emerging in the future.

Keywords: epidemiology; evolutionary ecology; infectious disease; niche breadth; vesicular stomatitis virus

1. INTRODUCTION

Despite advances in public health surveillance, emerging virus pathogens continue to cause major and minor epidemics in human populations, exemplified by human immunodeficiency viruses I and II, H5N1 (avian flu) and H1N1 (swine flu) strains of influenza A virus and SARS coronavirus (Griffin *et al.* 1988; Guan *et al.* 2003; Webby & Webster 2003). The factors promoting emergence are difficult to resolve, because of various underlying societal and biological causes. Easy long-distance travel and dense population groups foster epidemic spread of emerging diseases in humans (Schrag & Wiener 1995; Cleaveland *et al.* 2007; Pulliam 2008; Woolhouse 2008); when these factors are coupled with poverty and poor clinical acumen in the developing world, emerging pathogens can severely impact human populations least prepared to counter them (Weiss & McMichael 2004; Basu & Galvani 2007; Morens *et al.* 2008). Biological features that may influence emergence potential of a virus include its immunogenicity, genetic content (RNA versus DNA) and genome architecture, especially propensity to undergo genetic mixis (recombination, reassortment; Cleaveland *et al.* 2001; Woolhouse & Gowtage-Sequeria 2005; Pulliam & Dushoff 2009). A fundamental goal in disease research is to resolve how these myriad factors impact emergence probability, towards better predicting future emergence events.

Emergence is one example of a biological population attempting to successfully invade a new environment (host shift), or extend its current environment (range expansion). Generalized ability to use new resources (i.e. hosts) should impact this success. Evolutionary ecology suggests that invasibility should be influenced by historical selection for evolved resource-use strategies of niche generalism versus specialism (Kassen 2002; Caley & Munday 2003; Jasmin & Kassen 2007). The niche constitutes biotic and abiotic environments allowing survival and reproduction. Generalists have a relatively broad niche, whereas specialists grow and/or reproduce under fewer conditions (narrow niche). Theory predicts that the extent of environmental variability should determine whether populations tend to become dominated by specialists versus generalists (Levins 1968; Lynch & Gabriel 1987; Whitlock 1996; see reviews by Wilson & Yoshimura 1994; Kassen 2002). Adaptive generalization is favoured when the population faces ecological change which is complete, deterministic

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and spans multiple generations. In contrast, a constant environment is assumed to favour evolved specialists, which perform strongly in the current environment at the expense of reduced performance under other circumstances (Levins 1968; Futuyma & Moreno 1988; Palaima 2007). However, generalists may also evolve in a constant environment via indirect effects of selection; traits that improve fitness in the current environment may also increase performance elsewhere, owing to a correlated response to selection.

High mutation rates, short generation times and disease importance cause RNA viruses to be efficient and relevant models for examining experimental evolution of host use (Novella 2003; Dennehy 2009). Vesicular stomatitis virus (VSV) is a negative-sense ssRNA *Vesiculovirus* in the family *Rhabdoviridae*. Because VSV is an arthropod-borne virus capable of naturally infecting various mammals and insect species, it is possible to experimentally evolve VSV *in vitro* under different host-use regimes (Elena *et al.* 1996; Novella *et al.* 1999; Turner & Elena 2000; Remold *et al.* 2008), and to examine the consequences of this evolution for future emergence (Turner *et al.* in press).

In a previous study (see §2 for details), we experimentally evolved VSV populations for 100 generations (25 passages) on (i) constant hosts derived from human cervical cancer cells (HeLa), (ii) constant hosts derived from dog kidney cells (MDCK) and (iii) variable hosts comprised of alternating passages (HeLa/MDCK) (Turner & Elena 2000; see also Remold *et al.* 2008). By examining patterns of fitness improvement on the selected hosts and the original host (BHK cells), we determined that constant selection on HeLa cells led to specialization: improvement on the selected host and decreased performance on other hosts. In contrast, alternating-host selection promoted evolved generalism, where viruses improved on both hosts used in selection. Last, constant selection on Madin–Darby canine kidney cells (MDCK) led to improvement on the selected host, as well as correlated ('fortuitous') improvement on the original host. We recently categorized the VSV populations as specialists, directly selected generalists and indirectly selected generalists, and determined that direct selection for generalism fostered emergence on laboratory 'challenge hosts' unencountered during selection (Turner *et al.* in press).

While these laboratory experiments are tractable and useful for studying pathogen emergence, they do not match the complexities of public health realities. Thus, our current goal was to achieve greater relevance by re-examining the emergence data in our VSV system, in light of an epidemiological model for pathogen spread. Mathematical models have emphasized how evolution impacts emergence probability, especially the effects of mutation rate and basic reproduction number (R_0; number of secondary cases caused by a primary infection) (Antia *et al.* 2003). One approach is to use mathematical branching processes to estimate emergence potential; initial R_0 and mutation rate for a pathogen are used to calculate its probability of invading a population of susceptible hosts. Even if $R_0 < 1.0$, small changes in R_0 approaching unity may significantly improve the probability for a pathogen to adaptively emerge in a susceptible host population, because this affords the possibility for further adaptive mutations that improve growth in the new host. Of course, various other environmental and genetic factors (too numerous to list) may influence emergence success. One intriguing factor is the relative ability of pathogens to infect few versus many reservoir species (e.g. alternate hosts and/or vectors) (Reluga *et al.* 2007). All else being equal, a pathogen that grows in multiple reservoirs may gain an emergence advantage relative to a pathogen that cannot (figure 1). The general reason is that a pathogen infecting many reservoirs can, on average, better ecologically persist away from a population of susceptible primary hosts, which should create more opportunities for infectious contacts (introductions) that increase emergence probability. Although the prediction is straightforward, we are unaware of any study that has combined empirical and mathematical approaches to examine this idea.

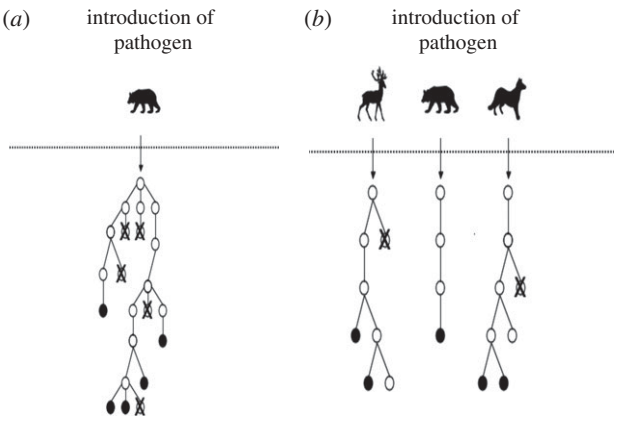

Figure 1. Hypothesized effect of reservoir growth on pathogen emergence potential. (*a*) A pathogen with high basic reproduction number (R_0) can effectively emerge when singly introduced into a host population, because it transmits quickly between individuals. (*b*) In contrast, a pathogen with relatively low R_0 can also easily emerge if it tends to infect multiple animal reservoirs; the pathogen's high reservoir reproductive rate (R_a) increases emergence probability because it affords multiple introductions into the host population. Open circle, introduced strain; filled circle, evolved, more infectious strain; cross, dead-end infection (no further transmission).

Here, we explore how direct selection for host-use (directly selected generalism) impacts the probability of RNA virus emergence from an epidemiology standpoint. In particular, we are concerned with the evolved capacity for an RNA virus to thrive in multiple reservoirs, and how this trait influences invasion success compared with viruses that evolved to specialize on a single host, or that were indirectly selected for broad host-use. Using lab-evolved VSV populations (Turner & Elena 2000), we re-examined growth data on unselected challenge hosts (model reservoirs) for virus strains categorized as specialists, directly selected generalists and indirectly selected generalists (Turner *et al.* in press). We then applied these results to an epidemiological model for emergence potential in a primary host population. Our study shows that

in vitro data in a model system such as VSV may be usefully combined with mathematical modelling to generate predictions of relative emergence success among pathogens differing in secondary-host growth.

2. MATERIALS AND METHODS

(a) *Cells*

We obtained cells from the American Type Culture Collection Centre (Manassas, VA, USA) four mammal-derived cell types described as permissive for infection by the VSV Indiana serotype: NCTC Clone 929 (ATCC no. CCL-1) derived from mouse connective tissue; BS-C-1 (ATCC no. CCL-26) derived from African green monkey kidney tissue; C6 (ATCC no. CCL-107) derived from rat glioma; PK(15) (ATCC no. CCL-33) derived from pig kidney tissue. Hereafter, these cell types are referred to as mouse, monkey, rat and pig, respectively. Monolayers were grown in Dulbecco modified Eagle's minimum essential medium with 5 per cent heat-inactivated newborn bovine calf serum, except C6 cells that were grown in Ham's F12K medium with 82.5 per cent horse serum and 15 per cent heat-inactivated newborn bovine calf serum. Cells were grown to 10^5 cells cm^{-2} in 48-well plastic plates, 37°C incubation, 95 per cent humidity and 5 per cent CO_2 atmosphere (Turner *et al.* in press).

(b) *Viruses*

Viruses were from a previous study where VSV populations were evolved for 100 generations (25 passages, 48-h per passage) (Turner & Elena 2000; Remold *et al.* 2008). Baby hamster kidney cells (BHK; kindly provided by J. J. Holland) are the typical lab host for growing VSV. An ancestral population of VSV Indiana serotype was used to found four lineages (M1–M4) evolved on MDCK (European Collection of Cell Cultures no. 85011435), four (H1–H4) on human carcinoma cells (HeLa; ATCC no. CCL-2), and four (A1–A4) via alternating host passages. These three treatments led to differing evolved host-use strategies: specialists (HeLa evolved), indirectly selected generalists (MDCK evolved) and directly selected generalists (alternating-host evolved) (Turner & Elena 2000; Remold *et al.* 2008; Turner *et al.* in press). A recent experimental evolution study suggested that viruses evolved in these treatments may experience selection under incongruent fitness landscapes, rather than fitness selection that trades off across host environments (Smith-Tsurkan *et al.* in press). Regardless, these differing interpretations do not affect the conclusions drawn in the current study.

(c) *Population growth assays*

As described earlier (Turner *et al.* in press), each evolved VSV population was grown on four novel hosts, unknown to be previously encountered: mouse, monkey, rat and pig. Monolayers were infected at multiplicity of infection (ratio of viruses to cells) of 0.01, and supernatants were obtained at 24 and 48 h post-infection and stored at −80°C for later use. We determined that 48 h was sufficient for each test population to achieve the maximum titre on a challenge host, because the host monolayer was either destroyed by infection or the cells were detached from the surface, preventing further infection. Three replicate assays on all four novel hosts were performed for each virus population in a single temporal block. Viral titres, measured in plaque forming units (pfu) were estimated using plaque assays, in which dilutions of virus samples were plated on BHK cells under standard culture conditions, as outlined by Turner *et al.* (in press). Although fitness of evolved viruses differed on BHK cells (Turner & Elena 2000), we determined that BHK cells remained an ideal host for enumerating plaques (i.e. this host did not bias against titre estimates for any of the VSV populations; Turner *et al.* in press). After 24 h incubation, media and agarose were removed and plates were stained with crystal violet to visualize plaques. All populations were assayed in a single block, and a second block with a lower range of dilutions was performed for H2 and H3 whose titres were too low to generate accurate estimates under the original dilution conditions.

(d) *Calculation of animal reservoir productivity, R_a*

The virus populations grow according to a logistic function, in which the population sizes at times t, $n(t)$, initially increase exponentially at a growth rate R_a, then the growth subsides to a carrying capacity, K:

$$\frac{\mathrm{d}n(t)}{\mathrm{d}t} = R_a n(t)\left(1 - \frac{n(t)}{K}\right). \tag{2.1}$$

Integrating this logistic growth function and solving for R_a indicates that the animal reservoir reproductive number can be estimated directly from the steady-state carrying capacity and estimates of virus population sizes at different times during the growth phase:

$$R_a = \frac{1}{t}\left[\frac{(K/n(t)) - 1}{(K/n(0)) - 1}\right]. \tag{2.2}$$

(e) *Probability of emergence derivation*

To derive the probability of emergence for a viral population from its estimate of R_a, we use the simplest and most generalizable form of the branching process to describe the likelihood of an initial virus population propagating and successfully avoiding stochastic extinction in a host population. The simple formulation assumes that the number of offspring produced by a given virus is Poisson distributed with mean R, the reproductive rate in a population of susceptible hosts (Antia *et al.* 2003). The probability of emergence is 1.0 minus the probability of extinction (P_{loss}), which is equal to the probability that j offspring are produced by each virus, times the probability of ultimate extinction given that j offspring are produced, summed across all j (Otto & Day 2007):

$$P_{\mathrm{loss}} = \sum_{j=0}^{\infty} P(\text{ultimate extinction}|j\text{ offspring produced}) \times P(j\text{ offspring produced}). \tag{2.3}$$

Given the Poisson assumption, equation (2.3) simplifies to:

$$P_{\text{loss}} = \sum_{j=0}^{\infty} P_{\text{loss}}^{j} e^{-R} \frac{R^{j}}{j!} = e^{-R} \sum_{j=0}^{\infty} \frac{(P_{\text{loss}}R)^{j}}{j!}. \quad (2.4)$$

Using a Taylor series approximation as in the following

$$\sum_{j=0}^{\infty} \frac{x^{j}}{j!} = e^{x}, \quad (2.5)$$

it follows that

$$\sum_{j=0}^{\infty} \frac{(P_{\text{loss}}R)^{j}}{j!} = e^{P_{\text{loss}}R}. \quad (2.6)$$

Therefore, equation (2.4) can be expressed as

$$P_{\text{loss}} = e^{-R} e^{P_{\text{loss}}R} = e^{-(1-P_{\text{loss}})R}. \quad (2.7)$$

Hence, the probability of emergence is:

$$P_{\text{emergence}} = 1 - P_{\text{loss}} = 1 - e^{-(1-P_{\text{loss}})R}. \quad (2.8)$$

(f) *Epsilon, €, a modifier of number of introductions*

Epsilon, €, is a parameter created to modify the probability of emergence by influencing the number of introductions for a pathogen into a population of susceptible hosts. The number of introductions (primary cases) is shown to influence the size of an outbreak (Woolhouse & Antia 2008). The number of introductions similarly modifies the probability of emergence through the coefficient:

$$€ \times (P_{\text{emergence}}) = P_{\text{introductions}}, \quad (2.9)$$

with $P_{\text{introductions}}$ being the calculated probability of emergence ($P_{\text{emergence}}$) considering the factors that increase the average number of infectious introductions into a population of susceptible hosts. Classically, this has been described as 'number of primary cases' or 'number of introductions.' In the current study, we expand these definitions to consider the biological effectors of €, as determined through empirical study of a model RNA virus pathogen. The epsilon value for a VSV population was proportional to its R_a value, a quantitative proxy for general ability to sustain infection in reservoirs. Viruses that effectively reproduce in reservoirs are more likely to infectiously 'spill over' into the primary host, thus entering a susceptible host population. We calculate a grand mean epsilon value for each of the three categories (groups) of evolved host-use strategies, based on each population's R_a relative to the mean R_a for its group. Populations that have high R_a values relative to the mean will have high epsilon values, and those with R_a values lower than the grand mean will have R_a values less than 1.0.

The epsilon value is a coefficient for emergence probability, with both mathematical and biological implications: mathematically, epsilon has no influence on R_0, the parameter used to calculate P_{loss} in a branching process model. Biologically, this translates to the epsilon value modifying the probability of emergence without influencing aspects of how a pathogen is transmitted between individuals comprising a population of susceptible hosts.

3. RESULTS

This study combines empirical and mathematical approaches to compare and contrast the probability of emergence for a collection of virus populations in the model system VSV. The populations are categorized according to their evolved host-use strategy: specialists, directly selected generalists and indirectly selected generalists. We used growth data for the VSV populations on challenge hosts (model reservoirs) to estimate the reservoir reproductive rate, R_a, for each population, and for the host-use categories. We then used this combined approach to gauge €, the relative emergence potential for the viruses.

(a) *Growth assays on novel hosts*

This study used 12 VSV populations previously evolved for 100 generations in constant (HeLa or MDCK) and temporally variable (alternating HeLa/MDCK) host environments (Turner & Elena 2000; see also Remold *et al.* 2008). We re-examined the growth of these viruses on four novel host cell types derived from different mammal sources (mouse, monkey, pig and rat; Turner *et al.* in press). These 'challenge hosts' included tissue types different from the original host (BHK) and hosts used in the prior experimental evolution (HeLa, MDCK) (see §2 for details). The growth assays were performed with a threefold replication and involved estimates of virus titre at 24 and 48 h; the 48 h data were used in a previous study (Turner *et al.* in press) to address hypotheses different from those explored here. The 288 virus titre samples (12 test viruses × 2 time points × 4 host types × threefold replication) were generated in two experimental blocks, and samples were stored as cell-free virus supernatants at −80°C for later analysis.

The growth data were used to calculate the mean $\log_{10}$ titre for each population on each challenge host at 24 and 48 h; these results are listed in table 1. The results at both time points were highly consistent for each alternating-host evolved population across the four challenge hosts, and among these populations within their treatment group (directly selected generalists). In contrast, we noted that at both time points MDCK-evolved population M1 grew worse than its counterparts (indirectly selected generalists), and that at 48 h HeLa-evolved populations H2 and H3 were less productive than the other viruses in their group (specialists) (table 1). Overall, these patterns were consistent with previous findings that directly selected generalists as a group showed statistically less variance in growth across challenge hosts at 48 h, compared with the other two groups of host-use strategists (Turner *et al.* in press). In addition, the observed titres at 24 and 48 h for each population were typically (though not always) less than the grand mean $\log_{10}$ viral productivity on the host(s) used in

Table 1. Estimates of virus titres (pfu ml^{-1}) on four challenge hosts (model reservoirs) for vesicular stomatitis virus (VSV) populations previously evolved for 100 generations in constant versus variable host environments (Turner & Elena 2000). Each virus group contains four independently evolved lineages: M (MDCK evolved) populations are indirectly selected generalists, H (HeLa evolved) populations are specialists, and A (alternating-host evolved) populations are directly selected generalists (Turner *et al.* in press). Data are $\log_{10}$ mean titres and s.d. at 24 and 48 h, for three replicate measures per population in challenge assays on cells derived from monkey, pig, rat or mouse cells (see §2 for details on challenge hosts).

population	challenge	$\log_{10}$ growth 24 h (s.d.)	$\log_{10}$ growth 48 h (s.d.)
indirect generalist M1	monkey	2.80 (0.272)	5.65 (0.079)
indirect generalist M2	monkey	6.38 (0.049)	6.74 (0.065)
indirect generalist M3	monkey	6.37 (0.042)	6.69 (0.015)
indirect generalist M4	monkey	6.31 (0.032)	6.64 (0.129)
indirect generalist M1	pig	3.02 (0.251)	5.69 (0.029)
indirect generalist M2	pig	5.24 (0.074)	6.65 (0.081)
indirect generalist M3	pig	5.19 (0.131)	6.61 (0.112)
indirect generalist M4	pig	5.19 (0.165)	6.53 (0.223)
indirect generalist M1	mouse	3.54 (0.230)	5.58 (0.049)
indirect generalist M2	mouse	6.69 (0.097)	7.54 (0.088)
indirect generalist M3	mouse	6.72 (0.109)	7.51 (0.129)
indirect generalist M4	mouse	6.75 (0.113)	7.49 (0.142)
indirect generalist M1	rat	2.62 (0.246)	5.50 (0.048)
indirect generalist M2	rat	5.15 (0.097)	5.49 (0.051)
indirect generalist M3	rat	5.13 (0.128)	5.53 (0.052)
indirect generalist M4	rat	5.17 (0.074)	5.54 (0.022)
specialist H1	monkey	3.07 (0.293)	5.29 (0.056)
specialist H2	monkey	3.81 (0.108)	3.36 (0.204)
specialist H3	monkey	3.79 (0.072)	2.58 (0.273)
specialist H4	monkey	2.63 (0.056)	5.31 (0.096)
specialist H1	pig	3.21 (0.178)	5.71 (0.034)
specialist H2	pig	3.57 (0.153)	2.97 (0.267)
specialist H3	pig	3.31 (0.093)	2.63 (0.306)
specialist H4	pig	3.14 (0.126)	5.78 (0.033)
specialist H1	mouse	3.75 (0.209)	5.34 (0.074)
specialist H2	mouse	3.76 (0.121)	3.20 (0.152)
specialist H3	mouse	3.76 (0.099)	2.62 (0.246)
specialist H4	mouse	3.30 (0.086)	5.40 (0.036)
specialist H1	rat	3.39 (0.119)	5.67 (0.009)
specialist H2	rat	2.49 (0.199)	2.97 (0.247)
specialist H3	rat	3.26 (0.134)	2.53 (0.404)
specialist H4	rat	2.76 (0.140)	5.57 (0.229)
direct generalist A1	monkey	5.49 (0.038)	6.77 (0.011)
direct generalist A2	monkey	5.54 (0.029)	6.78 (0.023)
direct generalist A3	monkey	5.54 (0.022)	6.79 (0.004)
direct generalist A4	monkey	5.55 (0.087)	6.77 (0.129)
direct generalist A1	pig	5.39 (0.038)	6.56 (0.037)
direct generalist A2	pig	5.47 (0.022)	6.54 (0.062)
direct generalist A3	pig	5.51 (0.040)	6.52 (0.051)
direct generalist A4	pig	5.48 (0.074)	6.42 (0.318)
direct generalist A1	mouse	6.37 (0.058)	6.59 (0.022)
direct generalist A2	mouse	6.38 (0.072)	6.60 (0.046)
direct generalist A3	mouse	6.39 (0.038)	6.56 (0.036)
direct generalist A4	mouse	6.39 (0.206)	6.49 (0.014)
direct generalist A1	rat	5.45 (0.040)	6.55 (0.019)
direct generalist A2	rat	5.44 (0.074)	6.55 (0.043)
direct generalist A3	rat	5.29 (0.045)	6.52 (0.091)
direct generalist A4	rat	5.33 (0.064)	6.46 (0.056)

selection: HeLa-evolved populations on HeLa, 24 h: 7.51 ± 0.17 s.d., 48 h: 7.58 ± 0.07 s.d.; MDCK-evolved populations on MDCK, 24 h: 7.43 ± 0.13 s.d., 48 h: 7.11 ± 0.11 s.d.; alternating-evolved populations on HeLa, 24 h: 7.42 ± 0.03 s.d., 48 h: 7.04 ± 0.11 s.d.; alternating-evolved populations on MDCK, 24 h: 7.52 ± 0.13 s.d., 48 h: 7.45 ± 0.61 s.d. (Turner *et al.* in press). For these reasons, the challenge hosts could be considered model reservoirs, where virus growth was often numerically less than that shown on the selected host(s) (cf. table 1).

Using the minimum possible set of three data points (0, 24, 48 h), titres on the challenge hosts were used to estimate logistic growth curves for each population on each host (data not shown). Figure 2 depicts these results averaged across all four challenge hosts, where growth curves are based on grand mean $\log_{10}$ titres for the groups of populations in each host-use

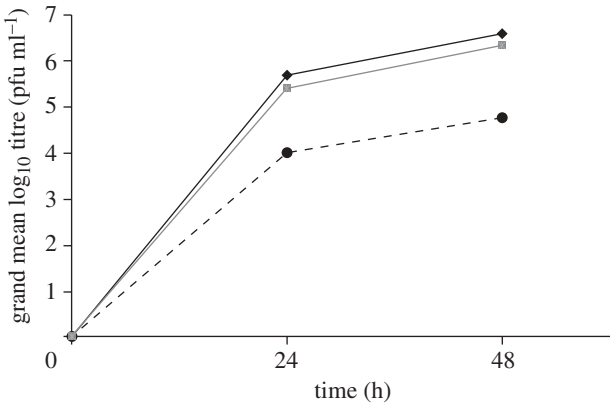

Figure 2. Viruses directly selected for host-use generalization grow best on challenge hosts. The logarithmic growth curves are generated from grand mean $\log_{10}$ titres of four vesicular stomatitis virus (VSV) populations per group, measured on a collection of four novel challenge hosts (animal reservoirs) *in vitro* (see text for details). Filled diamond with continuous lines, direct generalist; filled circle with dashed lines, specialist; filled square with continuous lines, indirect generalist.

category. Although this summary obscures the detailed data listed in table 1, it is useful for comparing general trends in the dataset among the three groups of evolved VSV populations. Clearly, the trend shows that, on average, viruses evolved on alternating hosts (directly selected generalists) grew slightly better than the populations evolved strictly on MDCK (indirectly selected generalists), indicating that both direct and indirect selection for generalization promoted novel host emergence. In contrast, the summarized data strongly suggested that viruses that specialized on HeLa cells were generally disadvantaged when grown on novel hosts (figure 2).

(b) *Animal reservoir reproductive rate, R_a*

Using the grand mean $\log_{10}$ titres of growth estimates, R_a values were calculated for each group of VSV host-use strategists using equation (2.2). Analyses are shown in table 2. We conducted a one-way ANOVA to compare these values among the three groups of host-use strategists. Results showed that the specialist (HeLa evolved) viruses had an R_a value that was substantially lower than the values for the directly selected (alternating-host-evolved) and indirectly selected (MDCK evolved) generalists. ANOVA showed a statistically significant effect of host-use category on the grand mean R_a across challenge hosts (table 2). This outcome also held when R_a comparisons were analysed on each host separately (data not shown).

Clearly, the data in table 1 and figure 2 suggested that this ANOVA result was probably influenced by the relatively poor performance of the specialist group. Therefore, we conducted separate pair-wise comparisons between the groups of directly and indirectly selected generalist viruses. Averaged across all four hosts, the two generalist groups of viruses were close in grand mean R_a values, showing a marginally significant difference ($p = 0.083$). Figure 3 depicts these differences in grand mean values of R_a, according to the host-use strategy category and the challenge host type. We then conducted two-tailed *t*-tests comparing the directly and indirectly selected generalists on each host type. R_a values of the two generalist types did not differ significantly on monkey hosts, and were marginally different on rat hosts (table 2). On pig and mouse hosts, however, the directly selected generalists showed an R_a value significantly higher than that of the indirectly selected generalists (table 2). We concluded from these analyses that the selected generalist viruses tended to be advantaged when growing on unencountered hosts, because they were never relatively disadvantaged on any challenge host, and performed better than their counterparts on two of the hosts. These results were consistent with earlier analyses of the 48-h data alone (Turner *et al.* in press); viruses directly selected for generalism showed relatively higher or equivalent host growth, lower among-population variance in host growth, and lower population variance in growth across hosts.

Table 2. Results of mixed model ANOVA showing that reservoir reproductive rates (R_a) of VSV groups (specialists, directly selected generalists, indirectly selected generalists) differ when analysed across all challenge hosts, and on each host separately. Results of pair-wise comparisons between direct and indirect generalists for R_a values estimated on each host separately.

effect	d.f. $_{\text{num., denom.}}$	F	p
(*a*) ANOVA			
VSV group (all hosts)	2, 116	43.06	<0.0001
VSV group (mouse)	2, 26	24.02	<0.0001
VSV group (monkey)	2, 27	13.57	<0.0001
VSV group (rat)	2, 27	23.01	<0.0001
VSV group (pig)	2, 27	34.39	< 0.0001

(*b*) pair-wise comparison: *t*-test (2 tailed, homoscedastic)

comparison	p
direct generalist versus indirect generalist (mouse)	0.04
direct generalist versus indirect generalist (monkey)	0.24
direct generalist versus indirect generalist (pig)	0.002
direct generalist versus indirect generalist (rat)	0.07

(c) *Probability of emergence*

On the basis of the general trends observed in the above analyses, we used the data to gauge how evolution of VSV host-use strategy (degree and type of generalism) influenced emergence probability in a population of susceptible primary hosts. To do so, we used ratios of reservoir reproductive rates (R_a values) in the different challenge hosts (model animal reservoirs) to determine an associated epsilon value (table 3). We modelled the probability of a virus emerging in a population of susceptible hosts over a range of basic reproductive (R_o) values to

Table 3. Estimates of epsilon (€) values for VSV groups on each challenge host (model reservoir). Viruses directly selected for generalization tended to show the highest values for this model parameter, increasing their emergence potential by improving the odds of an infective contact between animal reservoir and human-host population.

evolved host-use strategy	mouse	monkey	pig	rat
specialist	0.705	0.776	0.833	0.831
indirect generalist	1.07	1.15	1.02	1.05
direct generalist	1.22	1.07	1.14	1.12

examine how the differing virus types would succeed as pathogens emerging in a hypothetical host population such as humans.

Results showed that the directly selected and indirectly selected generalists were generally advantaged in emergence probability relative to viruses that specialized on HeLa cells when considering growth on collected (figure 4*a*) and individual reservoirs (figure 4*b–e*), provided that R_o was high enough that viruses could effectively spread in the host population. In addition, we detected that in most cases directly selected generalists were superior in the emergence probability at intermediate values of R_o; the lone exception occurred when R_a was based on growth in monkey reservoirs, where indirectly selected generalists fared best at intermediate R_o values (figure 4*b*). We noted that very high values of R_o always caused the two types of generalists to converge in emergence probability, because their typically robust growth in reservoirs afforded maximum rate of spread in the primary host, under conditions where specialist viruses never achieved the maximum (figure 4*a–e*). We concluded that efficient growth in reservoirs caused generalist viruses to be favoured in the emergence probability, and that VSV populations directly selected for the generalization were most often the favoured type of host-use strategy.

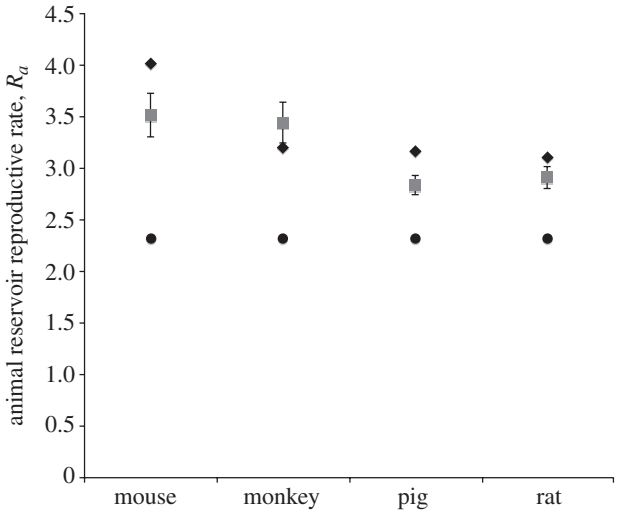

Figure 3. Viruses directly selected for host-use generalization show higher or equivalent animal reservoir reproductive rates (R_a) on challenge hosts. Symbols are grand means (± s.e.) of four populations per group; error bars too small to be distinguished are omitted for clarity. Filled circle, specialist; filled square, indirect generalist; filled diamond, direct generalist.

4. DISCUSSION

Emergence describes greater spread of an infectious disease agent through a new introduction into a host population (host shift), as well as an increased incidence of a pathogen in an existing host (host expansion). Previous studies have used empirical or modelling approaches to explore factors influencing the emergence probability, including pathogen evolution, life history and transmission opportunity (e.g. Antia *et al.* 2003; André & Day 2005; Dennehy *et al.* 2006). A pathogen variant may emerge owing to its altered characteristics in response to intervention, such as evolved resistance to drug therapy (Cleaveland *et al.* 2001; Woolhouse *et al.* 2005; Pulliam 2008). More generally, emergence is assumed to involve pathogen improvement in adaptive traits promoting an infectious spread, such as greater host encounter or increased within-host growth (Pulliam 2008). The current study combined empirical data from a model system with a theoretical model to better understand how the RNA virus growth in secondary-(reservoir) hosts influences the emergence probability in a population of susceptible primary hosts. In particular, the combined approach addressed the assumed positive impact of evolved generalism on emergence; this strategy should afford greater transmission opportunity between infected reservoirs and susceptible primary hosts, thus increasing the likelihood of emergence.

We previously examined host-use divergence in VSV populations evolved for 100 generations on constant versus variable hosts (Turner & Elena 2000; Remold *et al.* 2008). Turner *et al.* (in press) categorized the evolved VSV strains as host-use specialists (HeLa evolved), directly selected generalists (alternating-host evolved) and indirectly selected 'fortuitous' generalists (MDCK evolved) based on their relative abilities to grow on human-derived HeLa cells, dog-derived MDCK cells and hamster-derived BHK (original host) cells. These VSV populations were challenged to grow on each of four novel hosts (monkey, mouse, pig, rat) and the resulting 48-h titres determined that the direct selection for host breadth more often fostered emergence; viruses directly selected for generalism showed relatively higher or equivalent host growth, lower among-population variance in host growth and lower population variance in growth across hosts (Turner *et al.* in press). Here, we presented titre results at 24 h, and applied the 24- and 48-h data to a mathematical model examining how reservoir growth of virus types would influence their emergence probability on a primary host.

We showed that at both time points, the directly selected generalists usually (but not always) presented higher mean growth ($\log_{10}$ titres) on challenge hosts, compared with the indirectly selected generalists. In all cases, the specialist viruses showed relatively poor growth on the novel hosts. Because the VSV populations tended to grow worse on the challenge hosts than on the hosts used in prior selection (HeLa and/or MDCK cells), our data suggested that the challenge hosts could be considered suitable model reservoirs.

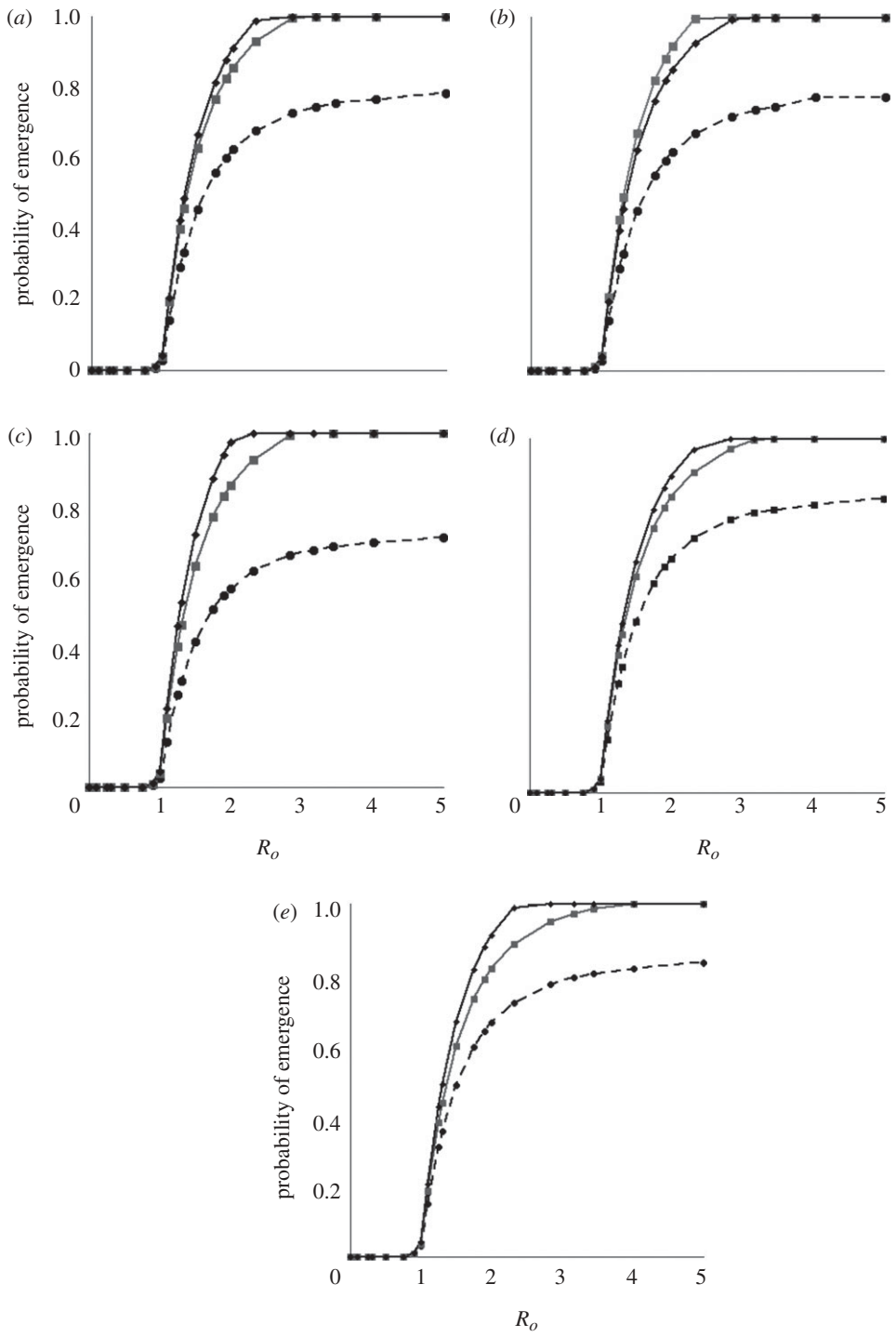

Figure 4. (*a–e*) Relative probabilities of emergence on a primary host for groups of VSV populations that differ in reservoir growth. Empirical data on challenge hosts (reservoirs) were combined with a mathematical model to generate $P_{\text{emergence}}$ values associated with evolved host-use strategies. Model results reflect data for virus growth across (*a*) all challenge hosts, (*b*) monkey, (*c*) mouse, (*d*) rat and (*e*) pig. In the majority of cases, the typically high reservoir reproductive rates (R_a) of directly selected generalists are predicted to afford a relative emergence advantage, especially at intermediate values of basic reproductive rate (R_0) on the primary host. The observed differences in emergence probabilities are defined by relative epsilon (€) values in our model (see text for details). Filled circle with dashed lines, specialist; filled square with continuous line, indirect generalist; filled diamond with continuous line, direct generalist.

We then used the input inoculum (time 0 h) and observed titres (24 and 48 h) as a minimum dataset for generating logistic growth curves for each population and a general growth estimate for each virus group. These data also produced estimates of virus reproductive rates in the individual and collected reservoirs, R_a. We determined that direct selection for generalism produced a significant advantage in R_a on two hosts (mouse, pig), marginally significant advantage on one host (rat) and no disadvantage on the remaining host (monkey). These data echo our earlier observations that directly selected generalists possess the greatest emergence potential, when considering only 48-h titres (Turner *et al.* in press).

In our mathematical model, we investigated how the niche breadth of a pathogen such as an RNA virus could be previously shaped by evolution, thereby influencing its probability to successfully emerge via an increased capacity to be infectiously introduced from one or more reservoirs. By applying the *in vitro* data to our model, we observed a greater tendency for directly selected generalists to establish a foothold in the reservoirs, making their spillover into a primary host more likely, assuming that ecological conditions would allow such infectious encounters to readily occur. The results emphasize that some ecological influences on emergence probability may extend beyond the strict pathogen ability to reproduce within a population of susceptible primary hosts (R_0); rather, emergence may be fostered by the average number of introductions into a population of primary hosts from reservoir species.

Although, we demonstrated how results from an empirical model system could be combined with a simple mathematical model to contrast the emergence potential of pathogen types, we acknowledge that many aspects of the *in vitro* system do not match the realities of host–parasite interactions in nature. However, it is crucial to also emphasize that this match is not generally the goal of experimental evolution research (Garland & Rose 2009). Rather, models such as VSV and tissue culture cells are highly useful for examining the evolutionary potential of RNA viruses in selected and novel environments, harnessing a well-characterized biological system (e.g. Elena *et al.* 1996, 2001, 2002; Novella *et al.* 1999; Turner & Elena 2000; Remold *et al.* 2008; Alto & Turner 2010; Turner *et al.* in press). One unavoidable limitation of this system is use of cells immortalized for growth in tissue culture, as the cell biology of these 'domesticated' tissues differs from that of their original source. Thus, the challenge hosts were drawn from distinct species (mouse, monkey, pig, rat) and tissue types (connective tissue, kidney tissue, glioma). But it is unknown whether these particular model reservoirs should be considered more (or less) alike than those of their distinct sources would suggest. Essentially, these hosts were obtained at random from the cell types commercially available for infection by VSV Indiana serotype (Turner *et al.* in press), and we see no reason why these hosts should favourably bias growth of the directly selected generalist strains of VSV in our study. In addition, we note that exponential growth of viruses in challenge hosts was equated with virus establishment in reservoirs; thus, the virus growth rate in culture was used to mimic pathogen growth in a reservoir population of susceptible individuals without possibility for immunity. We believe that our study demonstrates the potential power of linking a simple mathematical model to empirical results in a tractable biological system, and our hope is that this combined approach motivates future such studies involving conditions more realistic in host–pathogen interactions.

Below, we discuss our findings in relation to natural factors that can influence pathogen introductions into primary host populations, and to the evolution of virus strategies promoting such introductions.

(a) *Ecological factors influencing pathogen introduction into host populations*

In addition to pathogen niche breadth, which was the focus of our study, various other factors can influence the likelihood that a pathogen is introduced into a population of susceptible primary hosts. For brevity, we will discuss a subset of two such factors, and limit our consideration to the biomedical problem of pathogen emergence in human populations.

One important factor influencing pathogen introductions is the frequency with which infected host individuals enter (i.e. migrate to) a population of susceptible human hosts. This phenomenon is relevant in cases where a pathogen circulates in a localized population, and then has the opportunity to spread when infected individuals enter a larger population of susceptibles in which the epidemic may take off. One example is movement of infected individuals from prison confinement into the general human population. Following a prison epidemic, an infected former prisoner (migrant) can enter a population of susceptible hosts, potentially leading to a community-level epidemic (Stuckler *et al.* 2008). We have used RNA viruses as models for examining effects of migration (rates of transmission) on sustainability of pathogens, under circumstances of emergence (Dennehy *et al.* 2006), and under conditions where the migration is strictly unidirectional (J. Dennehy & P. Turner 2010, unpublished data) as in the case of infected former prisoners. VSV has been used to model pathogen migration occurring between individuals of the same host species, and it was observed that virus adaptation was positively correlated with migration rate (Miralles *et al.* 1999). However, when VSV was permitted to evolve with the migration on heterogeneous hosts (i.e. three different host-cell types), adaptation was negatively correlated with migration rate (Cuevas *et al.* 2003). These results suggest that migration may be beneficial for pathogen adaptation when movement occurs between individuals of the same host type, but migration may inhibit adaptation when selection occurring on host types is incongruent (see also Duffy *et al.* 2007). Further studies of RNA virus evolution under differing migration levels are warranted in order to draw general conclusions.

One other important factor influencing pathogen introductions in humans is the anthropophilia of secondary-host species which act as reservoirs. The anthropophilia of an animal reservoir defines how closely the habitat of the reservoir resembles (or allows proximity to) that of susceptible human hosts. This proximity could be determined either by actions of the human host such as animal agriculture, or adaptations of the vector that facilitate survival in human dominated habitats. In either case, increased anthropophilia should greatly increase the probability of a spillover event, as implicated in the pandemic of H1N1 influenza virus and in the cat-scratch disease caused by the intracellular bacterium *Bartonella* (Colford & Newman 1994; Groseth *et al.* 2007).

We note that various factors promoting pathogen introductions may not be mutually exclusive, and thus could serve to simultaneously and synergistically

influence host–pathogen interactions. However, actual outbreaks may be predominantly influenced by one factor over others. For example, a pathogen might have an increased probability of invading a population of human hosts because it infects multiple reservoirs (niche breadth), and because a subset of one or more of these reservoir hosts are specifically anthropophilic. We therefore emphasize that myriad factors may increase the epsilon factor described in the current study, and niche breadth provides but one example.

(b) *Virus strategies promoting host introductions*

There are several biological characteristics of viruses that might play a role in their emergence potential. In general, an emerging virus must be able to effectively reproduce, avoid host/reservoir immunity and maintain stable infection levels. But different virus strategies may evolve depending on which aspects of virus ecology present the most important challenges for shaping virus characteristics. For a pathogen with strong emergence potential owing to high reproductive rates across hosts, the viral replication rate and traits harnessing the host for this purpose are perhaps the most likely characters modified by natural selection that influence emergence. Our current and previous studies emphasize that indirect (correlated) selection for these traits in RNA viruses has nearly the same potential for promoting emergence as when the traits are directly moulded through selection (Turner & Elena 2000; Turner *et al.* in press).

For a pathogen that owes its emergence propensity to an increase in the average number of introductions, we might expect that it is equipped to sustain a low level of virulence in its animal reservoir(s), even if this pathogen causes aggressive illness in the primary hosts where it can emerge. In order to maintain avirulence in animal reservoirs, this pathogen must establish a low level of infection or otherwise hide from the immune system so that it is not efficiently eradicated by the reservoir's immune system. Thus, one might expect that a virus adept at surviving in a large number of reservoirs may have evolved to 'trick' the reservoir host to avert immune attack. In the case of VSV, the Matrix protein (M) provides many functions and is purported to play a role in suppressing the innate immune system (Ahmed *et al.* 2003; Stojdl *et al.* 2003). Insofar as this protein is important for maintaining virus productivity across varied hosts, evolved changes in the protein should also affect low levels of infection when a virus is replicating in a secondary reservoir. However, the we note that wild-type VSV is already a virus capable of infecting various hosts, and perhaps laboratory selection on a single host would cause the virus to change in terms of generalized virus/cell interactions through drift (i.e. erosion of niche breadth because selection is not acting to maintain the trait). Thus, the VSV M protein that is especially useful for virus/cell interactions and promoting virus productivity may or may not de-evolve even when the virus is selected on only a single host type in tissue culture. In fact, we observed that the directly selected generalist (alternating-host-evolved) viruses underwent fewer M protein substitutions than their counterparts selected on constant hosts (Remold *et al.* 2008). However, this idea is highly speculative as experiments confirming functional significance of the evolved substitutions are still currently underway in our laboratory.

5. CONCLUSION

We explored a potential connection between pathogen evolutionary ecology (evolved host history) and disease emergence in the context of an RNA virus; emergence potential increases through the evolved ability to infect a greater variety of hosts, thereby increasing an average number of primary-host introductions.

Our study suggests that the defining characteristic of emergence success in a biomedically important pathogen is not necessarily its ability to spread between humans. Rather, the important criterion may be how often the pathogen is introduced into human hosts from other sources. This factor is understandably gaining attention in public health, as awareness should aid efforts in determining which pathogens pose the greatest threats to emerge in human populations (Woolhouse & Gowtage-Sequeria 2005; Greger 2007; Reluga *et al.* 2007).

If a pathogen owes its high probability of emergence to a large number of introductions from reservoirs, then prevention efforts should consider the high probability of such introductions and focus on ways to minimize contacts with certain pathogen-infected species. Consequently, virus vaccine strategies that target herd immunity may be most appropriate in preventing the initiation of an epidemic caused by a virus that resides in reservoirs, because the introduction of such a virus into humans may be ecologically inevitable. In addition, reservoir management and behavioural modification to limit potential infectious interactions between animal reservoirs and humans might effectively decrease the probability of emergence. Such efforts might include discouraging human handling of certain species, animal culling, vaccination of reservoir animals and other measures. The decision to undertake some measures and not others is the product of several factors, many having to do with the political and economic limitations of modern societies. However, such limitations do not trump the need to further understand the evolutionary ecology of emerging pathogens, and our study suggests that knowledge of evolved host-use strategies may be used to predict the emergence potential and contribute to the need for developing evolutionary biology as a more predictive science (Ogbunugafor in press).

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