1 **The expected effect of deleterious mutations on within-host adaptation of**

2 **pathogens**

3

4

5 Judith M. Fonville[a,b,c#]

6

7 Centre for Pathogen Evolution, Department of Zoology, University of Cambridge,

8 UK[a]; WHO Collaborating Center for Modeling, Evolution, and Control of

9 Emerging Infectious Diseases, UK[b]; Department of Viroscience, Erasmus MC,

10 The Netherlands[c]

11

12 Running Head: Expected effect of deleterious mutations on adaptation

13

14 #Address correspondence to Dr. Judith M Fonville, jmf77@cam.ac.uk.

15

16 **Word count:**   Abstract: 237

17   Text introduction-acknowledgements: 6855

18
19

22

23

ABSTRACT

Adaptation is a common theme in both pathogen emergence, for example in zoonotic cross-species transmission, and pathogen control, where adaptation might limit the effect of the immune response and antiviral treatment. When such evolution requires deleterious intermediate mutations, fitness ridges and valleys arise in the pathogen's fitness landscape. The effect of deleterious intermediate mutations on within-host pathogen adaptation is examined with deterministic calculations, appropriate for pathogens replicating in large populations with high error rates. The effect of deleterious intermediates on pathogen adaptation is smaller than their name might suggest: when two mutations are required, and each individual single mutation is fully deleterious, the pathogen can jump across the fitness valley by obtaining two mutations at once, leading to a proportion of adapted mutant that is 20-fold lower than for the situation where all mutants are neutral. The negative effects of deleterious intermediates are typically substantially smaller, and outweighed, by fitness advantages of the adapted mutant. Moreover, requiring a specific mutation order has a substantially smaller effect on pathogen adaptation than the effect of all intermediates being deleterious. These results can be rationalized when calculating the number of routes of mutation available to the pathogen, providing a simple approach to estimate the effect of deleterious mutations. The calculations discussed here are applicable when assessing the effect of deleterious mutations on the within-host adaptation of pathogens, for example in the context of zoonotic emergence, antigenic escape, and drug resistance.

47 **IMPORTANCE**

48 Adaptation is critical for pathogens after zoonotic transmission into a new host

49 species, or to achieve antigenic immune escape and drug resistance. Using a

50 deterministic approach, the effects of deleterious intermediate mutations on

51 pathogen adaptation are calculated whilst avoiding commonly made

52 simplifications that do not apply to large pathogen populations replicating with

53 high mutations rates. Perhaps unexpectedly, pathogen adaptation does not halt

54 when the intermediate mutations are fully deleterious. Negative effects of

55 deleterious mutations are substantially outweighed by fitness gains of adaptation.

56 To gain an understanding of the effect of deleterious mutations on pathogen

57 adaptation, a simple approach is introduced that counts the number of routes

58 available to the pathogen with and without deleterious intermediate mutations.

59 This methodology enables a straightforward calculation of the proportion of the

60 pathogen population that will cross a fitness valley or traverse a fitness ridge,

61 without reverting to more complicated mathematical models.

62

INTRODUCTION

The fitness landscape of a pathogen is likely to have a rugged shape and consist of multiple optima. Reductions in fitness occur when underlying combinations of genetic mutations lead to an unfit or deleterious phenotype, creating depressions in the fitness landscape. One phenomenon causing sharp peaks and troughs in the fitness landscape is sign epistasis, where a beneficial adaptation involves a combination of individually deleterious mutations (1–5). In the case where intermediate mutations are less fit than the wild type and adapted virus, a fitness valley is created – a barrier of disadvantageous mutations hampering the access to other landscape regions (4, 6). If there is a specific order in which mutations can occur without compromising the fitness, for example where compensatory or obligatory co-mutations can remove the deleterious effect of another mutation, the landscape contains a fitness ridge. Such fitness valleys and ridges are commonplace in virology, as will be illustrated with examples drawn from the influenza field.

During zoonotic overspill infections of an avian influenza virus into humans, pressure exists for the pathogen to adapt to this possible new host (7–9). The virus was fit in its original host, and needs to be fit in the new host, but this adaptation process might require deleterious intermediate mutations. The need for adaptation of a zoonotic pathogen is illustrated by the requirement of a combination of mutations in avian A/H5N1 virus for airborne transmission

86 between mammals (10, 11). Interestingly, two of the mutations that were found

87 necessary to confer airborne transmissibility, polymerase basic protein 2 (PB2)

88 E627K and polymerase basic protein 1 (PB1) H99Y (11, 12), increased the

89 fitness of the adapted virus if both mutations occurred together, as inferred from

90 substantially larger plaque sizes than the wild type, yet each individual mutation

91 decreased the fitness compared to the wild type virus (12). Similarly, Imai *et al.*

92 showed that the receptor-binding mutations N224K and Q226L in the

93 hemagglutinin gene (HA), required for an airborne transmissible phenotype of

94 A/H5N1, reduced the stability of HA, but could be compensated for by mutation

95 T318I in the HA stalk, which restored protein stability (10). Although the

96 stabilizing mutation was not essential for virus survival, it did substantially

97 increase viral fitness.

98

99 Another example of deleterious intermediate mutations is escape from pre-

100 existing host immunity through fitness-decreasing mutations for antigenically

101 variable pathogens (13, 14). For example, the altered receptor-binding avidity

102 and lower replication resulting from the antigenic escape mutation HA K165E in

103 A/Puerto Rico/8/1934(H1N1) could be compensated for by mutations in HA or

104 the neuraminidase (NA) (15, 16), and stabilizing mutations were required to

105 occur prior to the introduction of immune-escape mutations in influenza A/H3N2

106 virus (17). Similarly, there are numerous examples where antiviral-resistance

107 conferring mutations come at a fitness cost for the virus, that can be

108 compensated for by other mutations: several neuraminidase substitutions can

109   occur and have occurred as either permissive or compensatory mutations to

110   counteract the adverse fitness effects of the oseltamivir-resistance mutation NA

111   H275Y in influenza A/H1N1 virus (18–20); and similarly the I222V NA mutation in

112   A/H3N2 partially restored the viral fitness-decreasing oseltamivir-resistance

113   mutation NA E119V (21).

114

115   The name "deleterious mutation" may suggest that the existence of such

116   mutations is unlikely, and thus the expectation that the crossing of a fitness valley

117   comprised of individually deleterious mutations is difficult, if not impossible.

118   Indeed, when evolution is described as an adaptive walk or directed evolution,

119   adaptation consists of a series of incrementally neutral or beneficial mutations,

120   and thus the crossing of the fitness valley would be technically impossible (22–

121   26). Also the possibility of obtaining several mutations at once, to "jump across" a

122   fitness valley, is not considered in some theoretical models (27–29). A

123   methodological framework frequently used to study pathogen evolution making

124   such assumptions is the "strong-selection-weak-mutation" (SSWM)

125   approximation (30, 31). Models using the SSWM assumption describe the

126   evolutionary trajectory of a population where selective sweeps cause the

127   sequential fixation of advantageous mutations, whilst deleterious or neutral

128   mutations are disregarded (32–34).

129

130   Here, we demonstrate how pathogens replicating in large population sizes and

131   with high error rates, such as RNA viruses, can cross fitness valleys, based on a

132   simple, and appropriate, deterministic model of within-host pathogen evolution.

133   Instead of following the evolution of a pathogen population toward fixation of

134   certain mutations, as is for example done in SSWM models, we calculate the

135   probability of a randomly drawn virion from the within-host virus population after

136   initial infection with a single genotype to have obtained a set of mutations after a

137   given number of replication rounds. This probability, when multiplied with the

138   pathogen population size, gives the expected number of virions with this specific

139   set of mutations. In other words, the probability is directly related to the

140   proportion of viruses in the total within-host population with this set of mutations.

141

142   This probability of a virion to have a set of mutations is highly relevant, because

143   increased proportions are likely to correspond to increased probability of spread

144   of such mutants. When the bottleneck of transmission is narrow, for example in

145   the case where a single virion is transmitted to the next host, the probability

146   describes the likelihood that infection of the next host will begin with the adapted

147   virus. Alternatively, if the bottleneck is wide, the expected proportion of adapted

148   virus at the start of the infection of the next host can be calculated and used to

149   estimate the chances of further adaptation.

150

151   In this manuscript, we calculate the effects of deleterious mutations, fitness

152   valleys and fitness ridges on within-host pathogen evolution using a

153   straightforward deterministic model (35, 36). Such a deterministic probability

154   calculation is appropriate for studying the dynamics and evolution of large

155 populations with asexual reproduction at a high mutation rate, such as most RNA

156 viruses, because stochastic effects play a limited role. The proportion of the

157 adapted mutant is calculated for varying valley depths (i.e. the fitness of the

158 deleterious mutation) and breadths (i.e. number of deleterious mutations). Finally,

159 we also describe the probability of traversing a fitness ridge, for varying numbers

160 of mutations that need to be acquired in order.

161

METHODS

The within-host population dynamics of virus mutants were calculated as deterministic probabilities, based on the methodology described previously (35, 36). In this calculation, the errors made by the virus polymerase are represented by an error rate, and form the source of introduction of mutations, but the approach can equally be used for non-viral pathogens, where mutations are introduced through another mechanism. The probability of accumulating mutations and the within-host evolutionary dynamics of the virus population are explored as a function of the fitness of the wild type, intermediate and adapted mutants.

Calculating virus populations

A virus type j is a virus with a particular set of mutations. The probability of each virus type (N_j) after a replication round is given by the sum of contributions from each type in the previous replication rounds:

eq. 1 $\qquad N_j(t) = \sum_i [N_i(t-1)\mu_{ij}]$

Where μ_{ij} is the probability of type i mutating to type j, and each type contributes exactly its expected value. If the mutation rate μ_{ij} is low, the main contribution to the proportion of the population that is N_j at time t will be from the proportion of the population that was N_j at time t-1, and a smaller contribution from virus type

185   N_i at time *t-1* that mutated into type N_j. The probability of mutation μ is calculated

186   as follows:

187

188   **eq. 2** $\mu_{ij} = \prod_{\{m=0\}}(1-r) \prod_{\{m=1\}} r$

189

190   Where *r* is the polymerase error rate. Thus, μ_{ij} is the product of the probabilities

191   of non-mutation (1-*r*) for the set {*m=0*}, i.e. positions for which no mutation is

192   required, and of the probability for mutation (*r*) for the set of positions that need to

193   mutate {*m=1*}.

194

195   **Accounting for fitness values**

196   The deleterious and beneficial selection values were incorporated by adjusting

197   the "progeny" of each virion to express the fitness disadvantage or advantage in

198   each genome replication step, prior to the start of the next generation. The

199   starting population (generation zero) consists only of zero-mutant, the starting

200   virus. After the first replication round (*equation 1*), the population of each type N_i

201   is multiplied by its relative fitness f_i, and the population is normalized (such that $\sum_i$

202   N_{i_adj} = 1) through division by the sum of the fitness-weighted prevalence all

203   types:

204

205   **eq. 3** $N_{i\_adj}(t) = \dfrac{N_i(t)*f_i}{\sum_i[N_i(t)*f_i]}$

206

207 The N_{i_adj} represent the populations at the start of the next genome replication

208 step, and are used as N_i in *equation 1* in the multiplication with the mutation

209 matrix. When calculating the effect of deleterious mutations the fitness *f* is varied,

210 and a "fully deleterious mutant" has a fitness of *f* = 0, which causes relative

211 increases in the probability of the other virus types in the total virus population.

212

213 Unless otherwise noted, the fitness of the wild type (i.e. starting) virus, and the

214 final type with the full set of mutations of interest, the "adapted virus", is neutral: *f*

215 = 1.

216

217 Because this model normalizes the virus population via *equation 3*, and accounts

218 for back-mutations in *equation* 2, the results are slightly different from the short-

219 hand formula introduced in *equation 4* in the results section. For this reason,

220 calculating the number of routes (see below) is a fast and informative approach

221 to very closely approximate the probability of a certain set of mutations, but is not

222 analytically identical to the modeling results.

223

224 **Stochastic model runs**

225 In addition to the deterministic modeling results above, a set of stochastic

226 discrete-time multi-type branching process simulations were run, see also

227 Russell *et al*. (35). The starting population of a single virion expanded

228 exponentially with a branching factor of 32 (leading to 10^3 virions produced per

229 infected cell, after the two genome replication steps), until the population size

230   exceeded 10^{10} virions, from which point onwards the branching factor was set to

231   1. For each genome replication step, the expected number of each mutant type

232   was determined with a Poisson distributed random variable with the expectation

233   value based on the mutation matrix shown in *equation 2* and the number of

234   virions of each mutant type existing before the replication step, as was done for

235   *equation 1*. This number was then multiplied by the relative fitness of each type,

236   and rounded to the nearest integer, prior to starting the next genome replication

237   step. We performed 10,000 stochastic runs for each of the 101 settings of fitness

238   of the deleterious intermediate mutants (between 0 and 1 in steps of 0.01). The

239   intensity of the shade of the pale red and blue colors is calculated based on the

240   $\log_2$ of the number simulation runs that have the resulting proportion of double

241   mutants for each fitness setting; the average proportion across the 10,000 runs

242   per fitness setting is indicated with the line connecting the circles.

243

244   **Determining the route**

245   We introduce the terminology "through singles" to mean the process by which the

246   two mutations are acquired through separate single mutations occurring in

247   distinct replication rounds, and "through doubles" to mean to process where two

248   mutations are achieved by mutating both sites in a single replication round. We

249   investigated the probability of a double mutant to occur through doubles by

250   setting the μ for single mutations to zero in the mutation matrix. The through

251   singles probability is calculated as the difference between the probability when all

252   routes are allowed, and the through doubles probability.

253

254   Similarly, to calculate how often the required set of mutations was achieved

255   through a specific order, the fitness of any non-order mutant was set to zero. The

256   difference between the probability calculated if any order is allowed and the

257   probability when only a specific order is available determines the probability of

258   non-order mutation routes.

259

260   The fraction of available routes is calculated as the number of available routes

261   given the constraints divided by the number of original routes.

262

263   **Parameter choice**

264   The mutation rate is parameterized by the current best estimate for the influenza

265   virus polymerase error rate ($r = 1{\times}10^{-5}$ mutations per site, per genome replication

266   (37, 38)), and can trivially be adjusted for other mutation rates – indeed all results

267   in the manuscript are *not* specific to influenza virus, or viruses in general, but to

268   all large populations where mutations occur.

269

270   A "replication round" in this manuscript refers to any step in which RNA is

271   synthesized, because in each round of replication polymerase errors can be

272   introduced. For influenza viruses, where vRNA is replicated into cRNA and then

273   cRNA is copied into vRNA, there are (at least) two replication rounds per cell

274   cycle. Results are shown after 20 viral replication rounds, which corresponds to

275   five days of influenza virus infection (where each replication round lasts around 6

276   hours, and virions exit the cell after 12 hours), but again, the number of

277   replication rounds can be varied in the equations above.

278 **RESULTS**

279 **Adaptation depends on the fitness of the deleterious intermediate**

280 **mutations**

281

282 When all mutations are neutral, a simple probabilistic calculation of mutation

283 accumulation closely approximates the probability that any randomly drawn virion

284 from the within-host virus population in an individual initially infected with a single

285 genotype, would have mutated the m sites of interest over time (the number of

286 replication rounds, t):

287

288 **eq. 4** $p(m,t) = t^m \, r^m$

289

290 This equation multiplies the probability of getting m mutations (based on the

291 polymerase error rate r), r^m, with the number of combinatorial options to acquire

292 these m mutations over t generations (t^m), see also Russell *et al.* (35) and

293 Gokhale *et al.* (39).

294

295 The probability that a given virion will have mutated m = 2 sites after t = 20

296 replication rounds with a polymerase error rate r = 1×10^{-5} is approximated by

297 *equation 4* as 4×10^{-8}. Naturally, this probability of observing both sites mutated

298 will be less if either of the individual mutations is deleterious. If both individual

299 mutations are deleterious, the pathogen will have to get across a fitness valley.

300 The fitness of each single deleterious mutant determines the likelihood of the

301 virion to cross this fitness valley. A fully deleterious mutation has a relative fitness

302 of 0, which means that no progeny is made from these virions at all, whilst for a

303 relative fitness of 0.5 half as much progeny descends from these virions

304 compared to virions with a relative fitness of 1.

305

306 *Figure 1A* explores how the probability of a pathogen to cross a fitness valley

307 depends on the deleterious effect of the intermediate mutations. In this scenario,

308 each individual mutation is equally deleterious, and the wild type (starting) and

309 the virus with the two required mutations (the "adapted virus") have neutral

310 fitness. The blue line shows the deterministic probability of a virion to be a double

311 mutant as a function of the fitness of the intermediate mutants. In the neutral

312 scenario without any fitness valley, where the fitness of each intermediate mutant

313 is 1, the probability of the double mutant after 20 replication rounds is, as

314 approximated above, 4×10^{-8}. As the relative fitness of each intermediate mutant

315 decreases toward zero (fully deleterious), the probability that any random virion

316 in the virus mixture is a double mutant decreases to 2×10^{-9}. Note that, despite

317 the two intermediate mutants being fully deleterious, the probability of the double

318 mutant is only twenty-fold lower than without the fully deleterious fitness valley.

319 The pale region is composed of 10,000 stochastic model simulations for each of

320 the 101 different settings of fitness f. The average of these runs, indicated by the

321 connected circles, is somewhat lower than the deterministic calculations, most

322 visibly for intermediate values of deleterious fitness. These stochastic simulations

323   highlight that even though stochastic effects may play a role, double mutations

324   do occur regularly, even when the intermediate mutants are fully deleterious.

325

326   Such double mutants can arise when both mutations were acquired

327   simultaneously in a single replication round, the "through doubles" mechanism.

328   The purple line in *Figure 1B* shows how much this mechanism of acquiring both

329   mutations at once contributes toward the likelihood of a virion being a double

330   mutant. Note that this probability is not affected by the relative fitness: because

331   the deleterious intermediates were never formed when two mutations were

332   obtained at once, the virions avoid having to incur the designated fitness cost.

333   The fitness valley is not crossed, but the virus "jumps" over it. The cyan line

334   describes the alternative "through singles" mechanism where the two single

335   mutations were obtained in distinct replications rounds – the situation in which

336   the virions did incur the deleterious cost of the intermediate, and actually crossed

337   through the valley. In *Figure 1B*, it can be seen that this contribution depends

338   strongly on the fitness of the intermediate single mutants. If the intermediate

339   mutants are neutral, or have a high relative fitness, the through singles

340   mechanism is the main contributor toward the probability of acquiring a double

341   mutant (right hand side of *Figure 1B*). However, when the intermediate single

342   mutants are highly deleterious the main contributor to the probability of a double

343   mutant is the through doubles mechanism.

344

345 Returning to *Figure 1A*, it appears that the through doubles mechanism is less

346 sensitive to stochastic variations than the through singles mechanism, and the

347 deviation between the deterministic model and stochastic results is largest for

348 intermediate values of deleterious fitness. Here, the non-negligible deleterious

349 cost causes stochastic loss of single mutants before the second mutation occurs.

350 Such stochastic losses are less prominent for fit intermediates ($f = 1$) or identical

351 or similar to the losses calculated in the deterministic model for highly deleterious

352 intermediates.

353

354 **An intuitive understanding: counting the number of "routes"**

355 Although the "through doubles" and "through singles" mechanisms in *Figure 1*

356 both require two polymerase errors, the probability of which is r^2, the relative

357 contribution of through singles to obtain two mutations is larger than through

358 doubles at $f = 1$. This phenomenon can be understood by considering "the

359 number of routes". The through doubles route can happen once in each

360 replication round, and thus in t different ways (here 20). However, to get two

361 single mutations, there can be e.g. single mutations in two subsequent rounds

362 (for which there are 19*2 options – the factor of two accounts for which of the

363 mutations is first), or single mutations in two replication rounds separated by a

364 replication round without mutation (for which there are 18*2 options), and so on,

365 until there is one single mutation in round 1 and one in round 20 (for which there

366 are 2 options only). The sum of these possibilities is 380 routes, which when

367 combined with the 20 routes of through doubles, corresponds to 20^2 ways to

368   obtain two mutations in twenty replication rounds, i.e. the factor t^m in *equation 4*.

369   Although the term t^m is nothing more than a combinatorial factor, it was found

370   that explicitly analyzing the number of routes represented by this term is useful

371   for reasoning about the expected effects of varying fitness valley shapes.

372

373   If the single mutants are fully deleterious, the effective number of routes to obtain

374   a double mutant through singles is 0, because a double mutant can never arise

375   from a single mutant if single mutants do not have progeny. In this situation, only

376   the through doubles mechanism is possible, and thus 20 out of the original 400

377   routes remain, causing a reduction in the probability of a double mutant by a

378   factor 0.05 (the probability decreased from 4×10^{-8} to 2×10^{-9}).

379

380   A general description to calculate the effective number of routes to obtain a

381   double mutant through singles for any relative fitness *f* of the single mutations

382   can be given as well: if single mutations happen in successive replication rounds

383   (*delay* = 1), the fitness cost is incurred once, if they are separated by one

384   replication round (*delay* = 2), the fitness cost is incurred twice, whilst if the single

385   mutations are 19 generations apart (*delay* = 19), the fitness cost *f* is incurred 19

386   times. In total, the effective contribution to the number of routes through singles

387   weighted by the incurred deleterious cost is given by:

388

389   **eq. 5** Effective number of routes $= \sum_{delay=1}^{delay=(t-1)}[2 * (t - delay) * f^{delay}]$

390

391   Where *t* is the number of replication rounds, as before, and *delay* is the time

392   lapse in replication rounds between the two single mutations (for *t* = 20

393   replication rounds, the maximum *delay* is 19). The factor of two reflects the fact

394   that the single mutations can be acquired in two different orders; the term (*t* -

395   *delay*) represents the number of options for any given delay (19 for a delay of 1,

396   18 for a delay of 2, etc.); while f^{delay} is the penalty term for the incurred fitness

397   cost over *delay* rounds of replication.

398

399   *Figure 2* illustrates how the number of effective routes is composed of the

400   contribution of the through doubles mechanism (in grey), and the different single-

401   single mutation routes. If the relative fitness of the intermediate mutants is 0, the

402   through doubles mechanism is the only contributor to the number of routes, as

403   was seen in *Figure 1*. Again, the effective number of routes for the through

404   doubles mechanism is independent of the fitness of the deleterious single

405   mutants, as any deleterious fitness cost is not incurred. *Figure 2* also

406   demonstrates that the effective number of routes of two single mutations

407   separated by many replication rounds (e.g. *delay* = 19, in pink) is substantially

408   smaller than the effective number of routes for two subsequent single mutations

409   (*delay* = 1, in red). The reason for this is twofold: first, if there are 20 replication

410   rounds, there are 19*2 routes to generate two single mutations 1 generation

411   apart, whilst there are only 1*2 routes to obtain two single mutations 19

412   generations apart. Second, any deleterious effect of the single mutants is

413   incurred for more replication rounds if the delay between the two single mutations

414 is longer, and thus the contribution of these single-single routes with longer

415 delays decreases even more as the intermediate mutants become more

416 deleterious.

417

418 *Table 1* shows that the total number of routes increases (t^m) as more mutations

419 are required, listing the results for two to seven mutations required. It also shows

420 the fraction of routes remaining when all intermediate mutants are fully

421 deleterious. In the case where five mutations are required, for example, this

422 means that all individual and combined intermediates (and thus all single, double,

423 triple and quadruple mutants) are deleterious. Because all intermediate mutants

424 are fully deleterious, all mutations have to be acquired at once, for which there

425 are t options: so t out of t^m routes remain. Although there were initially many

426 routes to acquire 5 mutations (20^5), only 20 remain.

427

428 When comparing the fraction of available routes for the situation where 5

429 mutations are required, 6.3×10^{-6}, with the fraction when 2 mutations are required,

430 5.0×10^{-2}, it is clear that the fraction of the available number of routes decreases

431 greatly as the number of intermediate deleterious mutations increases. Note, in

432 addition to a larger number of deleterious intermediate mutants slowing down the

433 viral adaptation, there is also the increased difficulty of acquiring more mutations

434 in the first place (which is given by r^m).

435

436 When a set of mutations is required of which only some are deleterious, the ratio

437 of the effective number of routes compared to the total number of routes when

438 that subset of mutations was not deleterious is the same as the fraction of routes

439 available for the number of deleterious mutations. As an example, consider the

440 situation where 5 mutations are required, and two of the mutations need to be

441 acquired as a double. When none of the 5 mutations are deleterious, there are

442 20^5 routes (t^m). When the two mutations are individually fully deleterious, the

443 second mutation of the double pair needs to occur simultaneously with the first

444 mutation of the pair. As a result, the timing of mutation, for which there are 20

445 options if there are 20 replication rounds, needs only to be established for 4

446 mutations, as the timing of the last mutation needs to be identical to the timing of

447 the other mutation in the pair. Hence, when two of the five mutations need to be

448 acquired as a double, there are 20^4 routes left, and the fraction $20^4/20^5$ is 0.05,

449 see 2 mutations required in *Table 1*.

450

451 **The effect of deleterious intermediates is outweighed by the fitness**

452 **advantage of adaptation**

453 In the previous calculations, we studied situations where the fully adapted mutant

454 had neutral fitness, and the number of available routes could directly be used

455 when calculating the probability of a virion being a fully adapted mutant. Next, we

456 investigate whether the deleterious cost of an intermediate mutant can be

457 outweighed by the fitness gain that would be obtained upon achieving the full set

458 of mutations, for example as a result of obtaining a certain beneficial phenotype

459   such as antigenic escape or increased replication. In *Figure* 3, the probability of

460   any virion being a double mutant after t = 20 replication rounds is indicated by

461   color, as a function of the relative fitness advantage f of the double mutant,

462   varied from 1 to 4, and the relative fitness of the deleterious single mutants,

463   varied from 0 to 1.

464

465   *Figure 3* demonstrates that the probability of a random virion having obtained two

466   mutations after 20 replication rounds varied relatively little with the fitness of the

467   deleterious intermediates (along the x-axis): for example, the maximum change

468   in the neutral scenario (see Figure 1) was 20-fold, which corresponds to 1.3 units

469   on a $\log_{10}$ axis. In contrast, the fitness gain of the double mutant causes changes

470   across 9 orders of magnitude, and this fitness gain therefore appears to be the

471   main determinant of the probability of a double mutant. The mechanism behind

472   these observations is that, in contrast to the deleterious cost, which is often

473   incurred only briefly, or avoided altogether by obtaining both mutations at once,

474   the fitness benefit of the double mutant is incurred in every single replication

475   round once it has arisen, hence exponentially increasing its presence in the

476   pathogen population. For fitness f = 4 of the double mutant, the minimum

477   probability of a virion to be a double mutant, across all fitnesses of the single

478   mutants, was 0.99998.

479

480 **Adaptation via fitness ridges: compensating mutations imposing**

481 **order**

482 Requiring mutations to occur in a specific order is a special case of deleterious

483 mutations: imagine the scenario in which two mutations are required, whereby

484 one single mutation α compensates for or removes the deleterious effect of the

485 other single mutation β (both the single intermediate α and the double mutant $\alpha\beta$

486 have neutral fitness). To understand the effect of such imposed order on the

487 probability of obtaining a certain mutant, again the number of effective routes

488 calculation is helpful.

489

490 In the scenario where the double mutant and mutation α are neutral, while

491 mutation of only site β is fully deleterious, the two ways toward the double mutant

492 are to either get both mutations simultaneously, or to obtain the non-deleterious,

493 compensating mutation α before mutating the site β. As explained above, there

494 are 20 routes out of 400 to obtain both mutations at once, and half of the

495 remaining 380 routes will have had the compensating mutation α prior to

496 mutation of site β: in total 210 out of the 400 routes remain. Even though

497 mutation β was fully deleterious, 52.5% of the routes are still available, thus

498 incurring only a 2-fold reduction in the total proportion of double mutant.

499

500 *Table 2* shows the number of allowed routes when all mutations need to be

501 obtained in order, for situations where two to seven mutations are required. As

502 more order is required, the reduction in fraction of allowed routes increases: for

503   stringent ordering of five mutations, only 1.3% of the routes remain, which is less

504   than the 52.5% for requiring order of two mutations.

505

506   Because imposing order does not necessarily require multiple mutations to occur

507   at once, the fraction of available routes is substantially larger in the situation

508   where order is required than for the situation where all intermediate mutants were

509   deleterious. For example, when requiring five mutations, 1.3% of the routes

510   remain if requiring specific ordering of these five mutations, whereas only

511   0.00063% of the routes remained when all intermediates were deleterious (see

512   *Table 1*). Thus, using available fitness ridges is always, and often considerably,

513   easier than jumping across or crossing a fitness valley.

514

515   The red line in *Figure 4A* also shows that the effect of requiring order on the

516   probability of a virion obtaining two mutations is relatively small, even if the non-

517   ordered single mutant is fully deleterious (compare $f = 0$ and $f = 1$), especially

518   when compared to the situation where both single mutants were deleterious (blue

519   line). The pale region is composed of 10,000 stochastic model simulations for the

520   fitness ridge, in red, and fitness valley, in blue. The average of the stochastic

521   runs, shown as circles, again indicate how traversing via a fitness ridge is

522   substantially more likely than jumping or crossing a fitness valley. Moreover,

523   stochastic effects play virtually no role for the outcome of a virion that can travel

524   via a fitness ridge, as the results are very similar to the deterministic model.

525

526   *Figure 4B* shows the contribution of the three different mechanisms that could

527   lead to a double mutant. First, the virus could follow the imposed order and travel

528   via two subsequent mutations along the fitness ridge. Second, the virus could

529   simultaneously mutate both sites and jump across the surrounding fitness valley.

530   Both of these mechanisms do not violate the imposed order, and their

531   contributions in *Figure 4B* are independent of the fitness of the deleterious single

532   mutant. Third, the virus could disobey the imposed order and obtain the non-

533   ordered single mutation first, which will incur the deleterious cost for a certain

534   time. The contribution of this latter mechanism depends on the fitness of the non-

535   ordered deleterious single mutant, and becomes zero when $f = 0$. As f decreases,

536   the ridge in the fitness valley becomes the main mechanism toward obtaining the

537   set of mutations. In general, following the imposed order and travelling via the

538   fitness ridge becomes more attractive as the fitness valley deepens and widens,

539   as non-ordered single intermediates become even less viable, and obtaining

540   simultaneous mutations even less likely.

541

DISCUSSION

Using probabilistic calculations on within-host genetic evolution, we found that the effect of a fitness valley of deleterious intermediate mutations on adaptation is much smaller than might be expected, and that the effect of requiring a specific order for mutations to occur is even smaller. In coinfected individuals, mechanisms such as reassortment (if the mutations of interest are on separate genes) and recombination are additionally affecting the ability of viruses to overcome fitness valleys, processes that are not currently included in the model. Instead, we calculated, based on within-host evolution of a single starting virus genotype infecting an individual, the probability of any virion getting a set of host-adaptation mutations. This probability is directly related to the expected proportion of adapted mutant in the total population. The equations and calculations presented in this work can be used in any situation with fitness valleys and ridges where the deterministic assumptions are fulfilled, and the population reproduces asexually. As a result, this method can aid the study on the effects of deleterious mutations in a wide range of pathogens, including, for example, tuberculosis and HIV (5, 40, 41).

The methodology of counting the number of routes is a straightforward approach to calculate the effect of deleterious intermediate mutants, and understand the ways by which pathogen populations traverse fitness valleys and ridges. For example, in the situation where a virus requires two mutations that are each

565   individually fully deleterious, the evolution is not halted, as this trap is avoided by

566   acquiring multiple mutations at once. In addition, if viruses need to follow a

567   specific order of mutation, the out-of-order intermediate mutants can be

568   described as fully deleterious. If only a handful of mutations need to be acquired

569   in order, the influence on the adaptation of the virus is minimal, because the

570   compensating mutations will occur beforehand without much difficulty: many

571   routes are still available. Indeed, a key finding of this research is that fitness

572   disadvantages of intermediate mutants sometimes have a great effect on the

573   proportion of adapted mutant, but only when a large number of intermediate

574   mutants are deleterious, and their fitness cost is large.

575

576   Although some studies recognize the importance of deleterious intermediates

577   and the crossing of fitness valleys to the overall adaptive evolution of pathogens

578   (27, 29, 42, 43), and the possibility of multiple simultaneous mutations to

579   overcome such fitness valleys (44), various other models assume a strong-

580   selection-weak-mutation paradigm (32–34, 45), ignoring any adaptive trajectories

581   that require the crossing of a fitness valley. Such assumptions might be

582   appropriate for small population sizes, or pathogens with low mutation rates (5,

583   30). However, for a pathogen with a large population size and high mutation rate,

584   these SSWM assumptions are substantially violated. For influenza virus for

585   example, the mutation rate is around 1 mutation per 10^5 nucleotides, per round of

586   genome replication ($r = 10^{-5}$) (37, 38), and the population size P easily exceeds

587 10^{10} virions in a single host, hence the SSWM conditions $4rP \ll 1$ or

588 $rP \ll 1/\ln(Ps)$, where s is the fitness increase, are not fulfilled (30, 46).

589

590 It should be noted though, that the assumptions of the deterministic

591 approximation are violated in parts of *Tables 1* and *2* (which showed the

592 numbers of routes for scenarios where up to 7 mutations are required). When the

593 inverse of the error rate to the power of the number of mutations required ($1/r^m$) is

594 larger than or comparable to the population size, stochastic variations may

595 become relevant. This was seen in *Figure 1A*, where for a population size around

596 10^{10} the two mutations were not acquired as readily as the deterministic model

597 would have suggested. The smaller the population size is in comparison to ($1/r^m$),

598 the higher the likelihood that stochastic effects decrease the expected proportion

599 observed in the pathogen population. Especially when large numbers of

600 mutations are required, the expected number of times the adapted mutant occurs

601 will be small, if not zero, when taking account of the population size. Moreover,

602 stochastic death of rare intermediate or fully adapted mutants will further affect

603 the observed proportions. In a single host, one can multiply the effective virus

604 population size, say 10^{10}, with the probability of interest, e.g. 6.65×10^{-7} for a

605 random virion being a double mutant when the intermediate single mutants were

606 fully deleterious, the starting mutant neutral and the double mutant has a fitness f

607 = 1.5, to get the expected number of virions with the mutations of interest, here

608 6650. In the context of transmission, where frequently small populations

609 consisting of less than a handful of virions are estimated to start new infections

610 for e.g. influenza virus, HIV and hepatitis C (47–50), the probability of any virion

611 being a mutant of interest is informative for epidemiological studies and risk

612 assessment. In the biologically implausible case (but just to clarify) that each

613 virion has the same chance of being transmitted and starting the next infection, if

614 only a single virion starts the next infection, the chance that the next host is

615 infected with only the adapted virus is identical to the proportion of this adapted

616 virus in the donor host.

617

618 Interestingly, evolutionary models have also been used to improve the

619 understanding of the developmental stages and processes in cancer, and to

620 increase the efficacy of treatment regimes (51). In the evolution of a cancerous

621 cell, there is often a fitness valley to be crossed before the cell is able to progress

622 to expansive, uncontrolled growth (51). As with many other evolutionary models,

623 models for cancer evolution are focused on population-level adaptation. The

624 cancer literature describes two main mechanisms for the population-level

625 crossing of the fitness valley: sequential fixation, whereby the full cancerous cell

626 population acquires one mutation, and only after fixation of the first mutation, the

627 second mutation becomes fixated; and stochastic tunneling, whereby the second

628 mutation establishes prior to fixation of the first mutation (52, 53). Stochastic

629 tunneling describes the probability of fixation on a population-level, and allows for

630 sequential but not necessarily simultaneous mutations, in contrast, the

631 deterministic calculations above describe the probability of obtaining multiple

632 mutations simultaneously by any single unit (cell or pathogen or virion), and can

633 be converted to an expected proportion in the population, but does not equate to

634 fixation.

635

636 An advantage of the deterministic calculations used here is that this approach

637 can easily be adjusted to encompass more complicated schemes of required

638 mutations and associated fitnesses. It is, for example, not limited to the

639 investigation of effects of deleterious intermediates, but can also be used when

640 individual mutations are neutral or beneficial, and the combined mutation is

641 synergistic, for example mutations at positions 138 and 229 in the non-structural

642 protein 1 (NS1) (54) and 147, 339 and 588 in PB2 (55) of influenza A/H5N1 virus

643 affecting virulence.

644

645 The implementation can be easily changed to model other situations, for example

646 i) where a mutation has a fitness effect when in the vRNA, but not when

647 occurring in the cRNA, as those molecules are not transcribed into mRNA and

648 translated into protein; or ii) to encompass delayed phenotypes of mutations (56,

649 57) whereby deleterious or advantageous fitness effects are not fully observed as

650 the respective proteins are only generated in a meaningful amount at a later time.

651 Such mechanisms might alter the likelihood of deleterious and adaptive

652 mutations occurring, for example by a deleterious mutation arising as non-

653 deleterious in the cRNA, and the compensatory mutation arising in the next

654 replication round, such that deleterious vRNA is never formed and both

655 mutations were effectively neutral.

656

657   In the stochastic model, the branching factor governs population growth, and as

658   a result of the founder effect this leads to mutations arising earlier in time

659   achieving higher proportions; an effect that can be seen as a banded simulation

660   runs, for example in Figure 4A, where the top band shows that there are fewer

661   instances (lower red intensity) but higher proportions of double mutants for

662   mutants arising early. Again, the implementation of this model can be adjusted

663   such that the branching factor varies in both steps of replication to match the

664   specific parameters for the virus of interest.

665

666   The calculations enable both estimating the likelihood of crossing fitness valleys,

667   as well as the probability of passing a narrow fitness ridge. The work presented

668   here on assessing the effect of fitness ridges and required order is relevant, for

669   example, in modeling antibiotic resistance (26), and pyrimethamine resistance of

670   malaria (58). The equations can easily incorporate variable mutation rates (59),

671   which may be useful to investigate different polymerase error rates of influenza

672   virus (60), and to account for varying replication fidelity of HIV reverse

673   transcriptase along different positions in the genome (61). This feature is also

674   important in the evolutionary modeling of cancer, where disease progression

675   often involves the acquisition of decreased genetic stability, and thus an altered

676   mutation rate (62).

677

678 Counting the number of routes is also a method that could be applied to

679 determine the multiplicity of drug therapy, as the acquisition of drug-resistant

680 mutations might be avoided by a treatment regime shaping the fitness valley

681 deep and wide enough to prevent the pathogen from crossing, an approach that

682 has been described with combination therapy for example in influenza,

683 tuberculosis and HIV treatment (61, 63–65). In the context of drug therapy,

684 Ribeiro *et al.* already noted that for totally defective intermediate HIV mutants, all

685 higher order strains have to be produced directly from wild-type, i.e. only allowing

686 routes where all mutations are acquired at once; they also described that for

687 smaller selective disadvantages, a k+1 mutant is most likely produced from a k-

688 point mutant, i.e. a qualitative phrasing of our quantitation of the contribution to

689 the number of routes from obtaining two subsequent mutations for less-

690 deleterious intermediates (66).

691

692 The introduction of fitness valleys can also be exploited as a mitigation strategy

693 for infectious diseases. One could, for example, design vaccines that require a

694 pathogen to obtain destabilizing mutations to enable immune escape. Models of

695 pathogen evolution can help to establish whether such approaches will

696 completely stall adaptation of the pathogen, or with what likelihood the created

697 fitness valleys would be crossed. Moreover, such approaches could also be used

698 to explore alternative routes as a result of epistatic interactions that might allow

699 deleterious mutations to occur if acquired in the right order (15, 17, 20).

700

701   The successful and efficient invasion of zoonoses into the human population is

702   often thought to be constrained by the existence of deleterious mutations on the

703   path to adaptation. Therefore, calculations on the effects of fitness valleys are of

704   critical importance in pandemic risk assessment of emerging pathogens (8, 33,

705   35, 44, 67, 68), and in addition to inform the cost-benefit analyses of gain-of-

706   function experiments and dual-use research of concern.

707

708   In summary, the ability to calculate the effect of deleterious mutations and order,

709   and to understand the results with the description of the number of available

710   routes, helps to assess the expected impact of fitness valleys and ridges in

711   pathogen evolution, with applications in drug resistance, immune escape, and

712   zoonotic risks assessments.

713 **ACKNOWLEDGEMENTS**

724

REFERENCES

1. **Whitlock MC, Phillips PC, Moore FB-G, Tonsor SJ**. 1995. Multiple fitness peaks and epistasis. Annu Rev Ecol Syst **26**:601–629.

2. **Maisnier-Patin S, Andersson DI**. 2004. Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. Res Microbiol **155**:360–369.

3. **Wilke CO, Lenski RE, Adami C**. 2003. Compensatory mutations cause excess of antagonistic epistasis in RNA secondary structure folding. BMC Evol Biol **3**:3.

4. **Poelwijk FJ, Kiviet DJ, Weinreich DM, Tans SJ**. 2007. Empirical fitness landscapes reveal accessible evolutionary paths. Nature **445**:383–386.

5. **da Silva J, Coetzer M, Nedellec R, Pastore C, Mosier DE**. 2010. Fitness epistasis and constraints on adaptation in a human immunodeficiency virus type 1 protein region. Genetics **185**:293–303.

6. **de Visser JAGM, Krug J**. 2014. Empirical fitness landscapes and the predictability of evolution. Nat Rev Genet **15**:480–490.

7. **Paulson JC, de Vries RP**. 2013. H5N1 receptor specificity as a factor in pandemic risk. Virus Res **178**:99–113.

743   8.   **Kuiken T, Holmes EC, McCauley J, Rimmelzwaan GF, Williams CS,**
744   **Grenfell BT**. 2006. Host species barriers to influenza virus infections.
745   Science **312**:394–397.

746   9.   **Webby R, Hoffmann E, Webster R**. 2004. Molecular constraints to
747   interspecies transmission of viral pathogens. Nat Med **10**:S77–S81.

748   10.   **Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, Shinya K, Zhong G,**
749   **Hanson A, Katsura H, Watanabe S, Li C, Kawakami E, Yamada S, Kiso**
750   **M, Suzuki Y, Maher EA, Neumann G, Kawaoka Y**. 2012. Experimental
751   adaptation of an influenza H5 HA confers respiratory droplet transmission
752   to a reassortant H5 HA/H1N1 virus in ferrets. Nature **486**:420–428.

753   11.   **Herfst S, Schrauwen EJA, Linster M, Chutinimitkul S, de Wit E,**
754   **Munster VJ, Sorrell EM, Bestebroer TM, Burke DF, Smith DJ,**
755   **Rimmelzwaan GF, Osterhaus ADME, Fouchier RAM**. 2012. Airborne
756   transmission of influenza A/H5N1 virus between errets. Science **336**:1534–
757   1541.

758   12.   **Linster M, van Boheemen S, de Graaf M, Schrauwen EJA, Lexmond P,**
759   **Mänz B, Bestebroer TM, Baumann J, van Riel D, Rimmelzwaan GF,**
760   **Osterhaus ADME, Matrosovich M, Fouchier RAM, Herfst S**. 2014.
761   Identification, characterization, and natural selection of mutations driving
762   airborne transmission of A/H5N1 virus. Cell **157**:329–339.

763   13.   **Kryazhimskiy S, Dushoff J, Bazykin GA, Plotkin JB**. 2011. Prevalence
764   of epistasis in the evolution of influenza A surface proteins. PLoS Genet
765   **7**:e1001301.

766   14.   **Das SR, Hensley SE, David A, Schmidt L, Gibbs JS, Puigbò P, Ince
767   WL, Bennink JR, Yewdell JW**. 2011. Fitness costs limit influenza A virus
768   hemagglutinin glycosylation as an immune evasion strategy. Proc Natl
769   Acad Sci U S A **108**:E1417–E1422.

770   15.   **Myers JL, Wetzel KS, Linderman SL, Li Y, Sullivan CB, Hensley SE**.
771   2013. Compensatory hemagglutinin mutations alter antigenic properties of
772   influenza viruses. J Virol **87**:11168–11172.

773   16.   **Hensley SE, Das SR, Gibbs JS, Bailey AL, Schmidt LM, Bennink JR,
774   Yewdell JW**. 2011. Influenza A virus hemagglutinin antibody escape
775   promotes neuraminidase antigenic variation and drug resistance. PLoS
776   One **6**:e15190.

777   17.   **Gong LI, Suchard MA, Bloom JD**. 2013. Stability-mediated epistasis
778   constrains the evolution of an influenza protein. Elife **2**:e00631.

779   18.   **Duan S, Govorkova EA, Bahl J, Zaraket H, Baranovich T, Seiler P,
780   Prevost K, Webster RG, Webby RJ**. 2014. Epistatic interactions between
781   neuraminidase mutations facilitated the emergence of the oseltamivir-
782   resistant H1N1 influenza viruses. Nat Commun **5**:5029.

783   19.   **Bloom JD, Gong LI, Baltimore D**. 2010. Permissive secondary mutations

784   enable the evolution of influenza oseltamivir resistance. Science

785   **328**:1272–1275.

786   20.   **Butler J, Hooper KA, Petrie S, Lee R, Maurer-Stroh S, Reh L,**

787   **Guarnaccia T, Baas C, Xue L, Vitesnik S, Leang S-K, McVernon J,**

788   **Kelso A, Barr IG, McCaw JM, Bloom JD, Hurt AC**. 2014. Estimating the

789   fitness advantage conferred by permissive neuraminidase mutations in

790   recent oseltamivir-resistant A(H1N1)pdm09 influenza viruses. PLoS

791   Pathog **10**:e1004065.

792   21.   **Simon P, Holder BP, Bouhy X, Abed Y, Beauchemin CAA, Boivin G**.

793   2011. The I222V neuraminidase mutation has a compensatory role in

794   replication of an oseltamivir-resistant influenza virus A/H3N2 E119V

795   mutant. J Clin Microbiol **49**:715–717.

796   22.   **Maynard Smith J**. 1970. Natural selection and the concept of a protein

797   space. Nature **225**:563–564.

798   23.   **Orr HA**. 2005. The genetic theory of adaptation: a brief history. Nat Rev

799   Genet **6**:119–127.

800   24.   **Romero PA, Arnold FH**. 2009. Exploring protein fitness landscapes by

801   directed evolution. Nat Rev Mol Cell Biol **10**:866–876.

802   25.   **Orr HA**. 1998. The population genetics of adaptation: the distribution of
803   factors fixed during adaptive evolution. Evolution (N Y) **52**:935–949.

804   26.   **Weinreich DM, Delaney NF, DePristo MA, Hartl DL**. 2006. Darwinian
805   evolution can follow only very few mutational paths to fitter proteins.
806   Science **312**:111–114.

807   27.   **Covert AW, Lenski RE, Wilke CO, Ofria C**. 2013. Experiments on the role
808   of deleterious mutations as stepping stones in adaptive evolution. Proc Natl
809   Acad Sci U S A **110**:E3171–3178.

810   28.   **Gerrish PJ, Lenski RE**. 1998. The fate of competing beneficial mutations
811   in an asexual population. Genetica **102-103**:127–144.

812   29.   **Cowperthwaite MC, Bull JJ, Meyers LA**. 2006. From bad to good: fitness
813   reversals and the ascent of deleterious mutations. PLoS Comput Biol
814   **2**:1292–1300.

815   30.   **Gillespie JH**. 1983. Some properties of finite populations experiencing
816   strong selection and weak mutation. Am Nat **121**:691–708.

817   31.   **Dean AM, Thornton JW**. 2007. Mechanistic approaches to the study of
818   evolution: the functional synthesis. Nat Rev Genet **8**:675–688.

819   32.   **Franke J, Klözer A, de Visser JAGM, Krug J**. 2011. Evolutionary
820   accessibility of mutational pathways. PLoS Comput Biol **7**:e1002134.

821   33.   **Park M, Loverdo C, Schreiber SJ, Lloyd-Smith JO**. 2013. Multiple scales

822   of selection influence the evolutionary emergence of novel pathogens.

823   Philos Trans R Soc Lond B Biol Sci **368**:20120333.

824   34.   **Weinreich DM, Watson RA, Chao L**. 2005. Perspective: Sign epistasis

825   and genetic constraint on evolutionary trajectories. Evolution (N Y)

826   **59**:1165–1174.

827   35.   **Russell CA, Fonville JM, Brown AEX, Burke DF, Smith DL, James SL,**

828   **Herfst S, van Boheemen S, Linster M, Schrauwen EJ, Katzelnick L,**

829   **Mosterín A, Kuiken T, Maher E, Neumann G, Osterhaus ADME,**

830   **Kawaoka Y, Fouchier RAM, Smith DJ**. 2012. The potential for respiratory

831   droplet-transmissible A/H5N1 influenza virus to evolve in a mammalian

832   host. Science **336**:1541–1547.

833   36.   **Fonville JM, Burke DF, Lewis NS, Katzelnick LC, Russell CA**. 2013.

834   Quantifying the fitness advantage of polymerase substitutions in influenza

835   A/H7N9 viruses during adaptation to humans. PLoS One **8**:e76047.

836   37.   **Sanjuán R, Nebot MR, Chirico N, Mansky LM, Belshaw R**. 2010. Viral

837   mutation rates. J Virol **84**:9733–9748.

838   38.   **Drake JW**. 1993. Rates of spontaneous mutation among RNA viruses.

839   Proc Natl Acad Sci U S A **90**:4171–4175.

840   39.   **Gokhale CS, Iwasa Y, Nowak MA, Traulsen A**. 2009. The pace of
841   evolution across fitness valleys. J Theor Biol **259**:613–620.

842   40.   **Gagneux S, Long CD, Small PM, Van T, Schoolnik GK, Bohannan**
843   **BJM**. 2006. The competitive cost of antibiotic resistance in Mycobacterium
844   tuberculosis. Science **312**:1944–1946.

845   41.   **Coffin JM**. 1995. HIV population dynamics in vivo: implications for genetic
846   variation, pathogenesis, and therapy. Science **267**:483–489.

847   42.   **Weissman DB, Desai MM, Fisher DS, Feldman MW**. 2009. The rate at
848   which asexual populations cross fitness valleys. Theor Popul Biol **75**:286–
849   300.

850   43.   **Loverdo C, Lloyd-Smith JO**. 2013. Evolutionary invasion and escape in
851   the presence of deleterious mutations. PLoS One **8**:e68179.

852   44.   **Alexander HK, Day T**. 2010. Risk factors for the evolutionary emergence
853   of pathogens. J R Soc Interface **7**:1455–1474.

854   45.   **Weinreich DM, Chao L**. 2005. Rapid evolutionary escape by large
855   populations from local fitness peaks is likely in nature. Evolution (N Y)
856   **59**:1175–1182.

857   46.   **Desai MM, Fisher DS**. 2007. Beneficial mutation-selection balance and the
858   effect of linkage on positive selection. Genetics **176**:1759–1798.

859   47.   **Varble A, Albrecht RA, Backes S, Crumiller M, Bouvier NM, Sachs D,**
860   **Garcia-Sastre A, TenOever BR**. 2014. Influenza A virus transmission
861   bottlenecks are defined by infection route and recipient host. Cell Host
862   Microbe **16**:691–700.

863   48.   **Wilker PR, Dinis JM, Starrett G, Imai M, Hatta M, Nelson CW,**
864   **O'Connor DH, Hughes AL, Neumann G, Kawaoka Y, Friedrich TC**.
865   2013. Selection on haemagglutinin imposes a bottleneck during
866   mammalian transmission of reassortant H5N1 influenza viruses. Nat
867   Commun **4**:2636.

868   49.   **Wang GP, Sherrill-Mix S a, Chang K-M, Quince C, Bushman FD**. 2010.
869   Hepatitis C virus transmission bottlenecks analyzed by deep sequencing. J
870   Virol **84**:6218–6228.

871   50.   **Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT,**
872   **Salazar MG, Sun C, Grayson T, Wang S, Li H, Wei X, Jiang C,**
873   **Kirchherr JL, Gao F, Anderson JA, Ping L-H, Swanstrom R, Tomaras**
874   **GD, Blattner WA, Goepfert PA, Kilby JM, Saag MS, Delwart EL, Busch**
875   **MP, Cohen MS, Montefiori DC, Haynes BF, Gaschen B, Athreya GS,**
876   **Lee HY, Wood N, Seoighe C, Perelson AS, Bhattacharya T, Korber BT,**
877   **Hahn BH, Shaw GM**. 2008. Identification and characterization of
878   transmitted and early founder virus envelopes in primary HIV-1 infection.
879   Proc Natl Acad Sci U S A **105**:7552–7557.

880 51. **Merlo LMF, Pepper JW, Reid BJ, Maley CC**. 2006. Cancer as an evolutionary and ecological process. Nat Rev Cancer **6**:924–935.

882 52. **Iwasa Y, Michor F, Nowak MA**. 2004. Stochastic tunnels in evolutionary dynamics. Genetics **166**:1571–1579.

884 53. **Komarova NL, Sengupta A, Nowak MA**. 2003. Mutation-selection networks of cancer initiation: tumor suppressor genes and chromosomal instability. J Theor Biol **223**:433–450.

887 54. **Fan S, Macken CA, Li C, Ozawa M, Goto H, Iswahyudi NFN, Nidom CA, Chen H, Neumann G, Kawaoka Y**. 2013. Synergistic effect of the PDZ and p85β-binding domains of the NS1 protein on virulence of an avian H5N1 influenza A virus. J Virol **87**:4861–4871.

891 55. **Fan S, Hatta M, Kim JH, Halfmann P, Imai M, Macken CA, Le MQ, Nguyen T, Neumann G, Kawaoka Y**. 2014. Novel residues in avian influenza virus PB2 protein affect virulence in mammalian hosts. Nat Commun **5**:5021.

895 56. **Valcárcel J, Ortín J**. 1989. Phenotypic hiding: the carryover of mutations in RNA viruses as shown by detection of mar mutants in influenza virus. J Virol **63**:4107–4109.

898 57. **Wilke CO, Novella IS**. 2003. Phenotypic mixing and hiding may contribute to memory in viral quasispecies. BMC Microbiol **3**:11.

900 58. **Lozovsky ER, Chookajorn T, Brown KM, Imwong M, Shaw PJ,**
901 **Kamchonwongpaisan S, Neafsey DE, Weinreich DM, Hartl DL**. 2009.
902 Stepwise acquisition of pyrimethamine resistance in the malaria parasite.
903 Proc Natl Acad Sci U S A **106**:12025–12030.

904 59. **Mansky LM, Cunningham KS**. 2000. Virus mutators and antimutators:
905 roles in evolution, pathogenesis and emergence. Trends Genet **16**:512–
906 517.

907 60. **Suárez P, Valcárcel J, Ortín J**. 1992. Heterogeneity of the mutation rates
908 of influenza A viruses: isolation of mutator mutants. J Virol **66**:2491–2494.

909 61. **Ribeiro RM, Bonhoeffer S, Nowak MA**. 1998. The frequency of resistant
910 mutant virus before antiviral therapy. AIDS **12**:461–465.

911 62. **Loeb LA**. 1991. Mutator phenotype may be required for multistage
912 carcinogenesis. Cancer Res **51**:3075–3079.

913 63. **Schrag SJ, Perrot V, Levin BR**. 1997. Adaptation to the fitness costs of
914 antibiotic resistance in Escherichia coli. Proc R Soc London Ser B
915 **264**:1287–1291.

916 64. **Perelson AS, Rong L, Hayden FG**. 2012. Combination antiviral therapy
917 for influenza: predictions from modeling of human infections. J Infect Dis
918 **205**:1642–1645.

919   65.   **Müller B, Borrell S, Rose G, Gagneux S**. 2013. The heterogeneous

920   evolution of multidrug-resistant Mycobacterium tuberculosis. Trends Genet

921   **29**:160–169.

922   66.   **Ribeiro RM, Bonhoeffer S**. 1999. A stochastic model for primary HIV

923   infection: optimal timing of therapy. AIDS **13**:351–357.

924   67.   **Pepin KM, Lass S, Pulliam JRC, Read AF, Lloyd-Smith JO**. 2010.

925   Identifying genetic markers of adaptation for surveillance of viral host

926   jumps. Nat Rev Microbiol **8**:802–813.

927   68.   **Holmes EC**. 2013. What can we predict about viral evolution and

928   emergence? Curr Opin Virol **3**:180–184.

929

930 **FIGURES:**

931 **Figure 1: A)** The blue line shows the deterministic probability of any virion being

932 a double mutant ($\log_{10}$) as a function of the relative fitness of the intermediate

933 single mutants (a relative fitness of 0 means the single mutants are fully

934 deleterious). The probability is shown after $t = 20$ replication rounds, in the

935 situation where the starting (wild type) virus and the adapted (double) mutant are

936 neutral (relative fitness = 1). The pale shades of blue indicate the results of

937 10,000 stochastic simulations for each of the 101 settings of the relative fitness f.

938 The circles indicate the average of the stochastic runs for each fitness setting. **B)**

939 The probability of any virion being a double mutant is split into a mechanism

940 where two mutations were acquired in a single replication round ("through

941 doubles"); and a mechanism where the two single mutations occurred in distinct

942 replication rounds ("through singles").

943

944 **Figure 2:** The effective number of routes to a double mutant is shown as a

945 function of the relative fitness of the intermediate single mutants (a relative

946 fitness $f = 0$ means the single mutants are fully deleterious). The results are

947 shown after $t = 20$ replication rounds, in the situation where the starting (wild

948 type) virus and the adapted (double) mutant are neutral ($f = 1$). The colors

949 illustrate the relative contributions of a double mutation at once (grey, delay = 0),

950 and the single-single mutation routes, with an increasing delay of 1 until 19

951   replication rounds between the mutation events represented by the gradient

952   shown in the colorbar.

953

954   **Figure 3:** The probability of any virion being a double mutant ($\log_{10}$) is shown in

955   color as a function of the relative fitness of the deleterious intermediate single

956   mutants (x-axis) and the relative fitness of the double, i.e. host-adapted, mutant

957   (y-axis). The probability is shown after $t = 20$ replication rounds, and the starting

958   (wild type) virus is neutral ($f = 1$).

959

960   **Figure 4: A)** The deterministic probability of any virion being a double mutant

961   ($\log_{10}$) is shown as a function of the relative fitness of the deleterious

962   intermediate single mutant(s). The graph shows the situation when both single

963   mutants are deleterious ("fitness valley", see the blue line in *Figure 1*), or when

964   only the non-ordered single mutant is deleterious ("fitness ridge"). The probability

965   is shown after $t = 20$ replication rounds, the starting (wild type) virus, ordered

966   single mutant (for the fitness ridge) and double mutant are neutral ($f = 1$). The

967   pale shades of blue and red indicate the results of 10,000 stochastic simulations

968   for each of the 101 settings of the relative fitness f for the fitness valley, and

969   fitness ridge, respectively. The circles indicate the average of the stochastic runs

970   for each fitness setting. **B)** The probability of double mutant for the fitness ridge

971   (red line) is divided into the contribution toward this probability by the mechanism

972   where two mutations were acquired in a single replication round ("through

973   doubles"); as two single mutations in distinct replication rounds in order ("through

974   ordered singles"); and as two single mutations in distinct replication rounds

975   occurring in the incorrect order, where the deleterious single mutation is obtained

976   first and incurs the fitness cost ("through deleterious singles").

977 **TABLES**

978

	# mutations required					
	2	3	4	5	6	7
Effective # of routes	20	20	20	20	20	20
Total routes	400	8×10^3	1.6×10^5	3.2×10^6	6.4×10^7	1.28×10^9
Fraction available	5.0×10^{-2}	2.5×10^{-3}	1.3×10^{-4}	6.3×10^{-6}	3.1×10^{-7}	1.6×10^{-8}

979

980 **Table 1:** The effective number of routes if all intermediate mutants are fully

981 deleterious, the number of available routes if all intermediate mutants were viable

982 (total routes), and the fraction of routes available (Effective # of routes/Total

983 routes). The results are shown for two to seven mutations required, for t = 20

984 replication rounds. The starting (wild type) virus and the adapted (double) mutant

985 are neutral (f = 1), and all possible intermediates are fully deleterious (f = 0).

	# mutations required					
	2	3	4	5	6	7
Allowed routes	210	1540	8855	42504	177100	657800
Total routes	400	8×10^{3}	1.6×10^{5}	3.2×10^{6}	6.4×10^{7}	1.28×10^{9}
Fraction available	0.525	0.193	0.055	0.013	0.003	5.14×10^{-4}

988

989 **Table 2:** The number of allowed routes if complete and strict order is required for

990 all mutations, the number of available routes if all intermediate mutants were

991 viable (total routes), and the fraction of routes available, for situations where two

992 to seven mutations are required. As an example, for 4 mutations required, this

993 would mean that mutation A has to occur prior to or simultaneously with mutation

994 B; mutation B has to occur prior to or simultaneously with mutation C; and

995 mutation C has to occur prior to or simultaneously with mutation D. The results

996 are shown for $t = 20$ replication rounds, the starting (wild type) virus and each

997 ordered mutant are neutral ($f = 1$), and all possible non-ordered intermediate

998 mutants are fully deleterious ($f = 0$).

999